Clostridium sporogenes Isolates and their Relationship to *C. botulinum* based on Deoxyribonucleic Acid Reassociation

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(Received 30 November 1976)

SUMMARY

Sixty-two isolates of *Clostridium sporogenes* from canned foods were examined for cultural properties, heat resistance and DNA-DNA homology to *Clostridium botulinum* type A190. Sporulation was observed in most of 21 umbonate and rhizoidal colony-forming strains (colony-type I strains), but not in most of the 41 strains with convex and circular or crenate colonies with a mat to semi-glossy surface (colony-type II strains). More than half of the latter strains showed much higher heat resistance than the rhizoidal colony-forming strains. The DNA isolated from colony-type II strains was 81 % or more homologous to *C. botulinum* A190 DNA, forming duplexes which had thermostabilities similar to homologous duplexes of strain A190 DNA. Colony-type I strains differed from *C. botulinum* by 30 to 40 % DNA homology and the DNA duplexes formed between these strains and strain A190 showed $\Delta T_{m(e)}$ values of 7.0 °C when compared with the $T_{m(e)}$ of homologous DNA duplexes of strain A190.

INTRODUCTION

The majority of clostridia isolated from canned foods are identified as *Clostridium sporogenes* (Aschehoug & Jansen, 1950; Kato *et al.*, 1975; Smith, 1975). Some strains of *C. sporogenes* and *C. botulinum* type A could not be differentiated in DNA-DNA reassociation assays (Lee & Riemann, 1970; Wu, Riemann & Lee, 1972). In view of the uncertain taxonomy of *C. sporogenes*, we have examined isolates from canned foods for cultural properties, heat resistance and sporulating ability, and have estimated the degree of binding between DNA preparations from representative strains and thymidine-labelled DNA from strains of *C. botulinum* and *C. sporogenes*.

METHODS

Bacterial strains. Sixty-two isolates from canned foods (from N. Matsuda, Research Laboratories, Canner's Association of Japan, Yokohama, Japan) were identified (Kato *et al.*, 1975) as *Clostridium sporogenes* using cultural, biochemical and gas chromatography data (Holdeman & Moore, 1975), and designated J-1 to J-62. *Clostridium sporogenes* strain PA3679 was also supplied by N. Matsuda. A laboratory strain of *C. botulinum* type A190 was used as a reference strain for the DNA reassociation assays; this is the representative strain of *C. botulinum* type A most frequently used in Japan (Iida, 1964; Kiritani *et al.*, 1973; Takumi & Kawata, 1970).

Colonial properties. These were observed on 10 % (v/v) blood agar supplemented with

2 % (w/v) glucose (BG agar) and on brain heart infusion agar (BHI agar; Nissan, Tokyo, Japan).

Determination of sporulation frequency. A loopful of an overnight culture at 37 °C in cooked-meat broth was streaked on to plates of BHI agar and peptone agar [2 % (w/v) proteose peptone no. 2 (Difco), 0.5 % (w/v) NaCl and 1.5 % (w/v) agar], and incubated anaerobically under hydrogen at 37 °C for 48 h. Colonies were smeared, stained by the method of Wirtz (1908), and at least 1000 cells and spores were counted; the sporulation frequency was defined as the ratio of the number of spores to the total count. When no spores were observed in any of 10 microscopic fields, the sporulation frequency was recorded as 'not detectable'.

Heat resistance test. Ten ml of BHI supplemented with 0.05 % (w/v) L-cysteine.HCl was inoculated with 0.5 ml of a 24 h cooked-meat broth culture and incubated at 37 °C for 48 h. The heat resistance was then determined qualitatively or quantitatively. In the qualitative test, cultures were heated at 75 °C for 15 min or 100 °C for 1, 2 or 3 h. Samples (1 ml) of the heated cultures were transferred to 10 ml BHI broth supplemented with 0.05 % L-cysteine.HCl, 0.1 % agar, 1 % glucose and 1 % soluble starch (all w/v) and incubated at 37 °C for 48 h. In the quantitative test, viable counts were determined using pour plates of BHI supplemented with 1 % glucose and 1 % soluble starch, incubated at 37 °C for 48 h under hydrogen. The same procedure was used to determine the heat-resistant spore population after heating the culture as described above.

Preparation of DNA. The organisms were grown at 37 °C for 4 to 6 h in medium containing (% w/v): proteose peptone (Daigo, Osaka, Japan), 1.0; polipeptone (Daigo), 1.0; yeast extract (Difco), 1.5; glucose, 1.0; fructose, 0.1; L-cysteine.HCl, 0.05; and mineral salts (Holdeman & Moore, 1975). For the preparation of labelled DNA from *C. sporogenes* strain J-53, 10 µCi of [³H]thymidine (Japan Isotope Association, Tokyo, Japan) was added to 100 ml of medium containing (% w/v): trypticase (BBL), 3.0; glucose, 1.0; L-cysteine.HCl, 0.05; rongalite (Wako, Osaka, Japan), 0.03; mineral salts (Holdeman & Moore, 1975) and vitamins (Cummins & Johnson, 1971). Rongalite (sodium thioglycollate) was added to reinforce the reducing ability of cysteine. For the preparation of labelled DNA from *C. botulinum* type A190, this medium was supplemented with 0.3 % (w/v) BHI. In all cases penicillin G (50 µg 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, suspended in 0.15 M-NaCl solution containing 0.01 M-EDTA at pH 8.0, heated at 60 °C for 20 min, then treated with 1 mg pronase ml⁻¹ (Kaken, Tokyo, Japan) and 1 mg lysozyme ml⁻¹ (Seikagaku, Tokyo, Japan) for 10 to 15 min at 37 °C, and lysed with 3 % (w/v) sodium lauryl sulphate. DNA was extracted from this lysate by the method of Marmur (1961).

Tests for DNA-DNA homology and the thermostability of DNA duplexes. These were performed as described previously (Nakamura *et al.*, 1974).

RESULTS

Only 21 of the 62 isolates showed the umbonate and rhizoidal ('Medusa head') colonies characteristic of *C. sporogenes* (Fig. 1*a*). On both BG agar and BHI agar these colonies were so firmly attached to the surface of the agar that they could not easily be removed with a loop. In contrast, the remaining 41 strains, which gave convex or crenate colonies with a mat to semi-glossy surface, were easily detached (Fig. 1*b*). The former colonies were designated colony-type I, and the latter colony-type II. The colonial properties of the

Fig. 1. Colonies on blood-glucose agar. (a) Colony-type I (strain J-53); (b) colony-type II (strain J-62).

Table 1. *Sporulation and heat resistance of the two colony-types of C. sporogenes*

Colony-type	Colonial properties*	No. of strains tested	No. of strains showing: Spores†	Resistance to heating at:		
				75 °C for 15 min		
				100 °C for 1 h	100 °C for 2 h	
I	Rhizoidal or 'Medusa head'	21	18	21	9	0
II	Circular to crenate, mat to semi-glossy	41	8	32	28	26

* See Fig. 1.

† Spores were detected by microscopic examination.

isolates remained stable, with the exception of strains J-5 and J-10 which gave both colony types.

The colonial characters seemed to be correlated with sporulation (Table 1). Sporulation was consistently observed in most colony-type I strains but not in most colony-type II strains; among the latter, sporulation was detected in only eight strains. However, the heat resistance tests gave unexpected results. All 21 colony-type I strains, most of which sporulated abundantly, resisted heating at 75 °C for 15 min, but only nine resisted heating at 100 °C for 1 h and none resisted heating at 100 °C for 2 h. In contrast, 28 of the 41 colony-type II strains, most of which did not show detectable sporulation, resisted heating at 100 °C for 1 h, and all but two of these also resisted heating at 100 °C for 2 h. However, nine of the 41 colony-type II strains did not resist heating even at 75 °C for 15 min. To analyse these unexpected results, five strains selected randomly from each of the colony-type groups were examined using the quantitative test. The results confirmed our qualitative findings (Table 2).

DNA-DNA homology tests

DNA was extracted from eight randomly selected isolates of colony-type II and seven of colony-type I and tested against labelled reference DNA (Table 3). Colony-type II strains

Table 2. *Sporulation and heat resistance of strains of C. sporogenes*

Colony-type and strain	Sporulation (%) on:		$10^{-8} \times$ Total viable counts ml ⁻¹	No. of cells ml ⁻¹ resistant to heating at:		
	Peptone agar	BHI agar		75 °C for 15 min ($\times 10^{-4}$)	100 °C for 1 h	100 °C for 3 h
Type I						
J-1	2.3	51.0	4.1	650	0	0
J-2	33.0	17.0	3.3	790	0	0
J-5	5.8	9.0	6.2	2700	0	0
J-6	3.4	54.5	5.7	110	0	0
J-10	87.5	83.3	3.0	1300	0	0
Type II						
J-12	—	—	0.59	0	0	0
J-14	—	—	1.8	26	16	0
J-17	—	—	1.7	0.17	55	18
J-52	—	—	0.53	0.61	960	39
J-57	—	—	2.2	1.30	6900	49

—, No spores detected.

Table 3. *Reassociation of DNA samples from C. sporogenes isolates with DNA from the reference strains C. botulinum A190 and C. sporogenes J-53*

The extent of binding between DNA from the test isolates and labelled DNA from reference strains is expressed as a percentage relative to the extent of binding between labelled and unlabelled DNA from the reference strain. Specific activities of the labelled DNA from strains A190 and J-53 were 15000 and 1300 c.p.m. μg^{-1} respectively. $\Delta T_{m(e)}$ is the difference between the temperatures at which there is 50% dissociation of homologous and heterologous DNA duplexes.

DNA source	Labelled DNA source				Sporu- lation*	Resistance to heating at:	
	Strain A190		Strain J-53			75 °C for 15 min	100 °C for 1 h
	Relative DNA bound (%)	$\Delta T_{m(e)}$ (°C)	Relative DNA bound (%)	$\Delta T_{m(e)}$ (°C)			
<i>C. botulinum</i> type A190	100	0	67	5.5	—	+	—
<i>C. sporogenes</i> Colony-type II							
J-62	91	1.0	66		—	+	+
J-12	91	0.5	69		—	+	—
J-17	87	1.5	69		—	+	+
J-20	86		67		—	+	+
J-60	86		74		—	—	—
J-36	85	1.0	68		—	+	+
PA3679	83	1.0	68		—	+	+
J-19	81		66		+	+	+
J-21	81	1.4	68	5.3	+	+	+
Colony-type I							
J-53	70	7.0	100	0	+	+	—
J-2	72		100		+	+	—
J-6	72		100		+	+	—
J-10	72		100		+	+	—
J-32	71		100		—	+	—
J-59	73		92		+	+	—
J-1	66	7.0	89	0	+	+	—

* —, No spores detected; +, spores detected.

Fig. 2. Thermal elution profiles of homologous and heterologous DNA duplexes formed with strain A190 DNA. (○) Homologous DNA duplexes of strain A190; (●) heterologous DNA duplexes between strains J-36 and A190; (□) heterologous DNA duplexes between strains J-53 and A190.

shared 81 % or more DNA with *C. botulinum* type A190 reference DNA but showed a smaller degree of nucleotide sequence homology (66 to 74 %) with the *C. sporogenes* J-53 reference system. The well-known strain PA3679, classified in the colony-type II group, was 83 % homologous to *C. botulinum* in the DNA-DNA homology test (Table 3). The colony-type I strains showed less DNA homology (66 to 73 %) with DNA from the reference strain of *C. botulinum* type A190 but had a high degree of homology (89 to 100 %) with DNA from the *C. sporogenes* J-53 reference system.

Further tests were performed to determine the thermostability of the duplexes formed between DNA from *C. botulinum* type A190 or *C. sporogenes* J-53 and DNA from colony-type I or II strains of *C. sporogenes*. The temperatures ($T_{m(e)}$) at which 50 % of the DNA duplexes between colony-type II strains and *C. botulinum* A190 dissociated were similar to that of homologous DNA duplexes of *C. botulinum* A190 ($\Delta T_{m(e)}$ 0.5 to 1.4). $T_{m(e)}$ values for the DNA duplexes between colony-type I strains and *C. botulinum* A190 were 7 °C lower than those for homologous DNA duplexes of *C. botulinum* A190. These genetic data again indicated that colony-type II strains were homologous to *C. botulinum* type A, and that colony-type I strains were close to but distinct from *C. botulinum* type A190. Figure 2 depicts representative thermal elution profiles of homologous and heterologous DNA duplexes of *C. botulinum* type A190.

DISCUSSION

Colony-type II strains of *C. sporogenes* were non-sporulating and some had highly heat-resistant cells and some had no heat resistance. We have frequently found similar characteristics among substrains of a given strain: thus when substrains obtained after plating out a heat-resistant strain were tested for their heat resistance, some were not resistant to heating to 75 °C for 15 min and the rest were resistant even to 100 °C for 3 h. We have previously

demonstrated an inverse correlation between sporulation and heat resistance in *C. perfringens* strains that had been exposed to high temperatures (Nakamura & Nishida, 1974). Since the isolates used in this study were recovered from canned foods preheated to high temperatures, the loss of heat resistance or selection of highly heat-resistant strains with few spores is probably due to this preheating.

Strains with few heat-resistant spores were more autolytic than colony-type I strains (unpublished). Anema & Geers (1973) found that when there was severe lysis of vegetative cells the resulting spores were highly heat-resistant. This might help to elucidate why there should be an inverse correlation between the extent of sporulation and the degree of heat resistance.

Several investigators have demonstrated that some strains of *C. sporogenes* cannot be differentiated from proteolytic *C. botulinum* using phenetic and genetic data (Kiritani *et al.*, 1973; Lee & Riemann, 1970; Wu *et al.*, 1972). In the present study colony-type II strains of *C. sporogenes* were homologous to *C. botulinum* strain type A190 in DNA-DNA reassociation assays, and colony-type I strains of *C. sporogenes* differed from *C. botulinum* only by 30 to 40 % homology. Lee & Riemann (1970) and Wu *et al.* (1972) considered *C. sporogenes* strains showing nucleotide sequences homologous to *C. botulinum* to be *C. sporogenes* on the basis that toxigenicity is the most important discriminant for the taxonomy of pathogenic clostridia (Oakley, 1962). However, it remains to be shown whether or not *C. sporogenes* strains homologous to *C. botulinum* are non-toxigenic strains of *C. botulinum*.

Two strains, J-5 and J-10, gave colonies of types I and II, although they had shown only types I colonies on first isolation. Attempts to 'purify' individual colonies proved unsuccessful for each colony type. The two colony types of strain J-10 were subjected to three serial isolations and then cultivated and their DNA was extracted. The colony-type I and II substrains of strain J-10 were 100 % homologous to *C. sporogenes* J-53, an established colony-type II strain, but differed from *C. botulinum* type A190 by approximately 30 % homology.

Several investigators (Bulloch *et al.*, 1919; Meyer & Lang, 1926; Prévot, Turpin & Kaiser, 1967) have described strains similar to the present colony-type II strains of *C. sporogenes*; some of these had been established as distinct species and others as varieties of *C. sporogenes*. The present study clarifies the taxonomic position of these aberrant forms of *C. sporogenes*.

We wish to thank Dr N. Matsuda for supplying the *C. sporogenes* strains.

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