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ISOLATION OF TOXIGENIC STRAINS OF *CLOSTRIDIUM PERFRINGENS* FROM SOIL

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ABSTRACT

YAMAGISHI, TAKAYOSHI (Kanazawa University, Kanazawa, Japan), SHOICHI ISHIDA, AND SHOKI NISHIDA. Isolation of toxigenic strains of *Clostridium perfringens* from soil. J. Bacteriol. **88**:646-652. 1964—Strains of *Clostridium perfringens* were isolated from soil specimens heated at various temperatures for different lengths of time and were toxigenically examined. When higher temperatures were used, fewer toxigenic strains were isolated. Toxigenic strains could not be obtained from soil specimens heated at 100 C for 60 min. All toxigenic strains were killed by heating at 100 C for 10 min; strains that were resistant to these conditions were found to be nontoxigenic. These findings suggest that sporulating ability is closely linked to toxigenicity.

Clostridium perfringens has been isolated from various sources, such as soil and feces (McCoy and McClung, 1938; Smith, 1955). While examining the toxigenicity of strains isolated from soil, we noticed that the higher the temperatures used in the isolation procedures, to kill nonspore-forming bacteria, the less toxigenic were the strains of *C. perfringens* that were isolated. Previous investigators have used various combinations of time and temperature to isolate *C. perfringens*, but none of them has mentioned any relationship between susceptibility to heat and toxigenicity. Our preliminary findings seemed important not only in the isolation of strains of this organism but also in indicating that physiological phenomena involved in sporulation might be associated either with the formation of toxin within the cells or with the release of toxin from the cells. In the present paper, we describe a study designed to elucidate the relation between toxigenicity and sporulation of *C. perfringens*. This study was carried out by examining 168 strains isolated by use of various heating conditions.

MATERIALS AND METHODS

Soil specimens. Samples about the size of a pea were suspended in about 8 ml of sterilized saline and emulsified. Samples (1 ml) from each emulsion were placed in small tubes (12 by 105 mm), and each batch was heated in a bath at 60, 70, 80, or 90 C for 10 min, or at 100 C for 10, 30, or 60 min. Approximately 0.5 ml of each heated suspension was transferred into chopped-meat broth and incubated for 15 to 18 hr. One loopful of each culture was streaked on Zeissler's (1930) blood-agar, and colonies resembling *C. perfringens* were successively subcultured on the same medium to make certain of the purity. Doubtful colonies were sometimes exposed aerobically in an incubator to see whether they became tinted green or brown. Colonies of *C. perfringens* always exhibited one of these two colors under these conditions. Only one strain for investigation was isolated from each specimen of soil. Identification of strains of *C. perfringens* was carried out by use of the method of Willis and Hobbs (1958). However, the strains were also examined for the biological characteristics mentioned by Sterne and van Heyningen (1958) and Smith (1955).

Medium for production of α- and μ-toxins. Peptone (3%; Poli peptone, Daigo Co., Osaka, Japan) broth was adjusted to pH 8.5, and chopped meat particles were added to a concentration of 20% (v/v). After autoclaving, the pH of the medium was usually between 6.4 and 6.8. Test tubes (17 by 165 mm), usually containing 10 ml of the medium, were used. Fructose and inoculum (1% each) were added immediately after the medium had been heated at 100 C for 20 min and cooled. Cultures were harvested after 7 to 9 hr of incubation at 35 C (Nishida, Murakami, and Yamagishi, 1962).

Medium for production of θ-toxin. Broth without meat particles with a pH value of 8.0 was found suitable for this experiment; the pres-

ence of 0.25% fructose was found to be effective for higher yields.

Estimation of α-toxin. Estimation of α-toxin was carried out in accordance with Evans' (1945) method, which involves neutralization of the α-toxin by antitoxin, with egg-yolk solution as an indicator. The results are reported in the number of units of α-antitoxin required to neutralize the lecithinolytic activity of 1 ml of culture supernatant fluid.

Antitoxin was kindly provided by Y. Nagai, Chiba Serum Institute, Chiba, Japan. This was found to contain about 300 provisional units per ml. However, the provisional unit proved to be lower in its ability to neutralize α-toxin than the unit of the standard antitoxin. Actually, one provisional unit neutralized only 57 LD_{50} for mice weighing 16 to 18 g.

Egg-yolk solution. This solution was prepared by Van Heyningen's (1941) method.

Estimation of other toxins. Quantitative estimation of μ- and θ-toxins was performed by the methods described in previous papers (Nishida et al., 1962). In the present study, however, results are shown only by stating the presence or absence of toxin in the culture.

Resistance against heating at various temperatures. The heat-resistance test was performed for strains cultivated in 10% chopped-meat broth for 24 hr and, in later experiments, 48 hr. About 1-ml volumes of each culture were distributed in small test tubes (12 by 105 mm), and each batch was heated at 70, 80, 90, or 100 C for 10 min. An 0.5-ml amount of the heated culture was immediately transferred to a tube of chopped-meat broth containing 1% lactose. The cultures were examined macroscopically for growth after 48 hr of incubation.

Quantitative estimation of heat resistance. The number of viable cells was determined before and after the heat-resistance test at 100 C for 10 min; 48-hr-old cultures were used. The number of living cells per milliliter was calculated by the most probable number method (Hoskins, 1934). The ratio of heat-resistant cells to total cells was defined as the sporulating potency of the strains tested. This definition will be presented in more detail in the following section.

RESULTS

Criteria for toxigenicity. Extremely small amounts of α-toxin can be measured by the lecithovitellin reaction. This reaction is so sharp that lecithinase can be detected far beyond the amount needed to demonstrate reactions in vivo. Preliminary experiments were carried out to determine the lecithinase units corresponding to one minimal lethal dose, or the critical boundary between lethality and nonlethality in vivo.

Eleven strains of *C. perfringens* were cultivated as described, and 0.1-ml portions of each culture supernatant fluid were injected intravenously into the tail veins of three mice. Eight culture supernatant fluids with lecithinase activity corresponding to 0.1 antitoxin units did not kill any of the mice. However, each of the three culture supernatant fluids with 0.2 α-antitoxin units killed all mice used, revealing that the critical dose corresponded to lecithinase activity of 0.2 α-antitoxin units. Considering that the toxin yield by each strain varied somewhat from one culture to another and was dependent upon the medium used, we adopted 0.4 α-antitoxin units as the critical boundary between "toxigenicity" and "nontoxigenicity." In this paper, therefore, strains producing 0.4 units or more of α-toxin are designated as toxigenic strains; those producing 0.1 units or less are designated as nontoxigenic strains. Strains producing 0.2 units per ml are not classified in either group. This assumption of a critical level was reinforced by the following experiments. Twenty strains of *C. perfringens* of differing toxigenicity were subcultured in chopped-meat broth containing 1% glucose. With strains having a toxigenicity of 0.2 units or more, toxin production was elevated as subcultures were repeated, whereas the strains with a toxigenicity of 0.1 or lower did not show any perceptible increase of toxin production during repeated subculture (Table 1).

Isolation of toxigenic strains of C. perfringens. Strains of *C. perfringens* were isolated from soil specimens heated at 60 C for 10 min, 80 C for 15 min, and 100 C for 60 min, and from unheated specimens. As shown in Table 2 (experiment a), it was found that the higher the temperatures used, the less toxigenic were the strains isolated. This finding suggested that toxigenic strains may be more sensitive to higher temperatures and, consequently, were liable to be excluded when high temperatures were used in selection procedures.

The marked difference in α-toxigenicity between the group heated at 100 C for 60 min and

TABLE 1. *Increase of α-toxigenicity by repeated subculture in chopped-meat broth containing 1% glucose*

Strain	α-Toxigenicity*		
	Before subculture	After 5th subculture	After 10th subculture
WS 043	0.1	0.1	0.1
WS 045	0.4	0.4	0.6
WS 046	1.5	1.5	1.5
WS 051	0.2	0.4	0.6
WS 053	0.3	0.5	0.8
WS 054	1.0	1.0	1.0
WS 060	<0.05	<0.05	0.05
WS 7101	0.2	0.5	0.8
WS 7102	0.05	0.05	0.05
WS 7105	0.1	0.2	0.2
WS 7110	0.2	0.3	0.3
WS 7112	0.2	0.4	0.6
WS 7114	1.0	1.0	1.0
WS 7115	0.6	0.8	1.0
WS 7121	0.05	0.05	0.05
WS 1644	<0.05	<0.05	0.05
WS 1645	0.05	0.05	0.05
WS 1650	0.05	0.05	0.05
WS 1654	<0.05	<0.05	0.05
WS 1655	0.05	0.05	0.05

* The units of α-antitoxin neutralizing 1 ml of culture filtrate.

TABLE 2. *Toxigenicity of Clostridium perfringens isolated from soil specimens heated at various temperatures*

Expt	Heat applied	No. of strains isolated	α-Toxigenicity (avg units)	No. of strains producing	
				θ toxin	μ toxin
a	Control (non-heated)	7	1.30	5/7*	2/7
	60 C for 10 min	8	1.12	5/8	5/8
	80 C for 15 min	10	0.87	5/10	5/10
	100 C for 60 min	9	0.10	0/9	2/9
b	90 C for 10 min	7	1.35 (0.79)†	4/7	6/7
	100 C for 10 min	8	1.01 (0.59)†	3/8	0/8
	100 C for 30 min	7	0.35 (0.20)†	1/7	1/7

* The denominator is the number of strains examined.

† Values corrected by the coefficient, 1.7.

the group heated at 80 C for 15 min was presumed to be due to the great difference in the method of heating applied. An experiment was undertaken, therefore, to investigate the intermediary conditions, such as 90 or 100 C for 10 min and 100 C for 30 min (experiment b). It was confirmed that strains isolated became less toxigenic as the duration and temperature of heating were increased. Toxin production in experiment b was a little higher than was anticipated from the results of experiment a. Assuming that this inconsistency was due to variations in the ability of different batches of chopped-meat broth to support α-toxin production, we determined toxin production using both batches of media and eight strains of *C. perfringens*. It was found that the toxic potency of culture fluid with the medium of experiment a was 1.7 times weaker than that obtained with the medium of experiment b. The rather high values in experiment b fell between those obtained with the culture isolated at 80 C for 15 min and that isolated after treatment at 100 C for 60 min, after they had been corrected with the factor 1.7.

Further experiments were undertaken to confirm these findings (Table 3).

Heat resistance of isolated strains. The heat resistance of bacterial spores varies considerably, depending on the circumstances in which they were formed (Murrell, 1961). Therefore, we investigated the heat resistance of various strains grown under identical conditions, after they had been grown in chopped-meat broth at 37 C for either 24 or 48 hr.

In this experiment, the four groups of strains used were obtained under different conditions of heating, such as 90 C for 10 min, 100 C for 10 min, 100 C for 30 min, and 100 C for 60 min (Table 4). Of the eight strains isolated from speci-

TABLE 3. *Toxigenicity of Clostridium perfringens isolated from soil specimens heated at various temperatures**

Heat applied	No. of strains isolated	α-Toxigenicity (avg units)
Unheated	15	0.9
100 C for 60 min	11	0.16
60 C for 10 min	21	0.6
100 C for 60 min	9	0.08
Unheated	19	0.8
70 C for 10 min	21	0.44
100 C for 60 min	14	0.07

* Values were not corrected.

mens heated at 90 C for 10 min, seven strains proved to be quite susceptible to heating, succumbing even to 80 C for 10 min. One strain of this group was resistant, however, to 100 C for 10 min. It was the only nontoxigenic strain in this group.

Strains of the second group were isolated from specimens heated at 100 C for 10 min. The results of the heat-resistance tests were similar to those obtained with the first group. Of eight strains, six were not resistant to 80 C for 10 min. One strain could resist 100 C for 10 min, and this strain was found to be nontoxigenic. Another strain, which resisted 90 C for 10 min but not 100 C for 10 min, proved to be a weak toxin producer.

The strains of the third group were similar to those of the fourth group in heat resistance and in toxigenicity. Only two strains of the fourth group were examined, because it had been found in this laboratory that nearly all strains of this group were resistant to 100 C for 10 min, under the conditions used, and were nontoxigenic. Both of the strains tested in this experiment also proved to be heat-resistant and nontoxigenic.

TABLE 4. *Heat resistance of strains isolated from soil specimens heated at various temperatures*

Heat applied	Strain isolated	α-Toxigenicity	Heat resistance* at			
			70 C	80 C	90 C	100 C
90 C for 10 min	WS 9105	3.0	+	−	−	−
	WS 9102	2.0	+	−	−	−
	WS 9104	2.0	+	−	−	−
	WS 9103	2.0	−	−	−	−
	WS 9106	1.0	−	−	−	−
	WS 9101	0.8	+	−	−	−
	WS 9108	0.4	−	−	−	−
	WS 9107	0.05	+	+	+	+
100 C for 10 min	WS 1103	3.0	−	−	−	−
	WS 1104	1.0	−	−	−	−
	WS 1106	1.0	−	−	−	−
	WS 1108	0.8	+	−	−	−
	WS 1102	0.8	+	−	−	−
	WS 1109	0.6	−	−	−	−
	WS 1107	0.4	+	+	+	−
	WS 1105	0.1	+	+	+	+
100 C for 30 min	WS 1303	1.0	+	+	+	−
	WS 1305	0.4	+	+	+	+
	WS 1306	0.4	+	+	+	+
	WS 1307	0.2	+	−	−	−
	WS 1301	0.1	+	+	+	−
	WS 1302	0.1	+	+	+	+
	WS 1304	0.1	+	+	+	+
100 C for 60 min	WS 1601	0.1	+	+	+	+
	WS 1602	0.1	+	−	+	+

* Treatment was for 10 min; + = resistant; − = nonresistant.

TABLE 5. *Heat resistance of toxigenic and nontoxigenic strains*

Expt*	α-Toxigenicity (units)	No. of strains examined	No. of strains resistant to†			
			70 C	80 C	90 C	100 C
1	6.0	2	0	0	0	0
	3.0 to 2.0	5	3	0	0	0
	1.0 to 0.4	12	7	4	4	2
	0.1 to 0.05	11	11	10	11	10
2	2.0	2	2	2	0	0
	1.0 to 0.4	29	29	25	14	0
	0.1 to 0.05	16	16	13	12	10

* In experiment 1, the heat-resistance test was performed with 24-hr chopped-meat broth cultures; in experiment 2, the test was performed with 48-hr cultures.

† Treatment was for 10 min.

Of the strains mentioned above, 84 were arranged into four groups according to toxin production (Table 5) to determine any relationship between toxigenicity and heat resistance. These findings may be summarized as follows. (i) The more toxigenic the strains, the lower the heat resistance was. The highly toxigenic strains PB 6K and S 107 were particularly susceptible to heat. (ii) Of 50 toxigenic strains, 48 were found to have lost their ability to resist heating at 100 C for 10 min; the remaining two strains which resisted this temperature for this time were only slightly toxigenic. (iii) Of 27 nontoxigenic strains, 20 were heat-resistant under the conditions used, and all the heat-resistant strains were nontoxigenic.

Quantitative analysis of heat resistance. The classification "heat-resistant" seems to be somewhat ambiguous, for a strain can be defined as heat-resistant even when it contains only one heat-resistant cell in 10^8 cells. To analyze the heat resistance more accurately, the number of spores and the total number of cells in cultures of 12 strains were determined, and the ratio was desig-

TABLE 6. *Sporulating potencies of toxigenic and nontoxigenic strains*

Strain	α-Toxigenicity (units)	No. of living cells before heating*	No. of cells surviving after heating*
WS 6106†.....	3.0	1.1×10^8	0
WS 9105†.....	2.0	7.9×10^7	0
WS 1103†.....	2.0	7.0×10^7	0
WS 6110†.....	1.0	3.3×10^7	0
WS 9103†.....	1.0	3.3×10^7	0
WS 9104†.....	1.0	2.3×10^7	0
WS 1105‡.....	0.1	3.3×10^7	2.0×10^1
WS 1302‡.....	0.1	4.0×10^6	4.0×10^1
WS 1601‡.....	0.1	7.0×10^7	3.3×10^2
WS 1304‡.....	0.1	2.7×10^7	10^0 to 10^1
WS 1602‡.....	0.1	3.3×10^7	10^0 to 10^1
WS 1301‡.....	0.1	1.7×10^7	10^0 to 10^1

* Heating at 100 C for 10 min.
† Toxigenic strains.
‡ Nontoxigenic strains.

TABLE 7. *Sporulating potencies of ten substrains of WS 1601**

Substrain no.	No. of living cells before heating (100 C, 10 min)	No. of cells surviving after heating (100 C, 10 min)
WS 1601-1.....	3.3×10^7	7.0×10^2
WS 1601-2.....	1.1×10^7	7.0×10^2
WS 1601-3.....	1.7×10^7	1.1×10^3
WS 1601-4.....	1.3×10^7	3.3×10^2
WS 1601-5.....	1.8×10^6	1.1×10^3
WS 1601-6.....	7.8×10^6	4.9×10^2
WS 1601-7.....	2.3×10^7	4.9×10^2
WS 1601-8.....	7.8×10^6	1.3×10^3
WS 1601-9.....	1.1×10^7	4.9×10^3
WS 1601-10.....	7.9×10^7	3.3×10^2

* WS 1601, the prototrophic strain, exhibited a sporulating potency of 3.3×10^2 resistant cells per 7.0×10^7 total grown cells per milliliter.

nated as the sporulating potency. The difference in heat resistance between the strains of toxigenic and nontoxigenic groups were revealed to be only at the level of 10^0 to 10^2 heat-resistant cells per milliliter (Table 6).

This seemed almost negligible compared with the total number of cells. The significance, however, was soon established by the following experiments. A strain of *C. perfringens*, WS 1601, was plated on Zeissler's plate agar, and ten substrains were established from ten colonies that were picked. These substrains were tested by the quantitative heat-resistance test previously described (Table 7). Regardless of the fact that the prototrophic strain of WS 1601 possessed only 7.0×10^2 heat-resistant cells in a total of approximately 10^7 cells, the substrains exhibited approximately the same results by the quantitative test. The same type of experiment was performed with the toxigenic strain WS 6106. Since the prototrophic strain did not contain resistant cells, none of the ten substrains displayed heat resistance.

These findings were also confirmed by the following experiment. Two strains each of toxigenic and nontoxigenic groups were plated on Zeissler's agar, and 50 substrains were established from

TABLE 8. *Heat resistance of 50 substrains of toxigenic and nontoxigenic strains*

Strain	α-Toxigenicity (units)	No. heat-resistant* of 50 tested
WS 064†.....	3.0	0
WS 9105†.....	2.0	0
WS 1601‡.....	0.05	30
WS 1645‡.....	0.05	50

* Resistant to 100 C for 10 min.

TABLE 9. *Heat resistance of each group of ten substrains of toxigenic and nontoxigenic strains*

Strain	α-Toxigenicity (units)	No. of subcultures resistant* to		
		80 C	90 C	100 C
WS 6106†.....	3.0	7	1	0
WS 6110†.....	1.0	1	0	0
WS 9103†.....	1.0	3	0	0
WS 9104†.....	1.0	0	0	0
WS 9105†.....	2.0	3	2	0
WS 1103†.....	2.0	10	7	0
WS 1105‡.....	0.1	10	10	10
WS 1301‡.....	0.05	8	5	7
WS 1302‡.....	0.1	10	8	8
WS 1304‡.....	0.1	10	10	9
WS 1601‡.....	0.1	10	10	10
WS 1602‡.....	0.1	4	5	2

* Heat treatment was for 10 min.
† Toxigenic strains.
‡ Nontoxigenic strains.

each strain. Their heat resistance was determined (Table 8). Toxigenic strains were found to give rise to heat-nonresistant substrains, whereas the nontoxigenic strains gave rise to heat-resistant substrains. In another experiment, six strains each of the toxigenic and nontoxigenic groups were plated, and ten substrains were established with each strain. Their heat resistance was determined, by use of temperatures of 80, 90, and 100 C for 10 min. None of the six toxigenic strains gave rise to resistant substrains able to resist 100 C for 10 min, whereas all of the nontoxigenic strains gave rise to resistant substrains, varying from 20 to 100% (Table 9). These findings indicate that the proportion of heat-resistant cells in a culture is a phenotypic expression of sporulating potency of a strain, and that differences in the phenotypic expression may be reliably determined when the proper conditions for cultivation are employed.

DISCUSSION

The heat-resistant nature of the bacterial spore has recently been shown to be associated with the calcium salt of dipicolinic acid accumulated in the spore (Church and Harvorson, 1959). Yoneda and Kondo (1959) demonstrated that the spore coat of *Bacillus cereus* consists of an extremely acid-fast layer of polymerized β-hydroxybutyric acid. There does not seem to be any obvious relationship between the genesis of toxin and the biosynthetic process of dipicolinic acid or polymerized β-hydroxybutyric acid, for their chemical structures are entirely different from those of toxins.

Hardwick and Foster (1952) and Foster and Perry (1954) postulated that the mechanism of sporulation consists of a depolymerization process of the bacterial body into rather small-sized protein molecules and their reorganization into the spore. This hypothesis suggests to the authors that an unbalanced metabolism of sporulation might yield toxic protein; however, the loss of heat resistance, particularly that associated with dipicolinic acid, might be independent of the genesis of the toxin, regardless of the extremely intimate relationship between toxigenicity and sporulating potency. It should be realized that α-toxigenicity is always accompanied by a loss of heat resistance. Acquisition of toxigenicity seems to be the main causative agent for the loss of heat resistance, but the existence of other minor causes should not be overlooked.

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LITERATURE CITED

CHURCH, B. D., AND H. O. HALVORSON. 1959. Dependence of the heat resistance of bacterial endospores on their dipicolinic acid content. Nature **183**:124–125.

EVANS, D. G. 1945. The in vivo production of α-toxin, θ-haemolysin and hyaluronidase by strains of *Clostridium welchii* type A, and the relationship of in vitro properties to virulence in guinea pigs. J. Pathol. Bacteriol. **57**:75–85.

FOSTER, J. W., AND J. J. PERRY. 1954. Intracellular events occurring during endotrophic sporulation in *Bacillus mycoides*. J. Bacteriol. **67**:295–302.

HARDWICK, W. A., AND J. W. FOSTER. 1952. On the nature of sporogenesis in some aerobic bacteria. J. Gen. Physiol. **35**:907–927.

HOSKINS, J. K. 1934. Most probable numbers for evalution of *Coli-Aerogenes* tests by fermentation tube method. Public Health Rept. (U.S.) **49**:393–403.

MCCOY, E., AND L. S. MCCLUNG. 1938. Serological relations among spore-forming anaerobic bacteria. Bacteriol. Rev. **2**:47–97.

MURRELL, W. G. 1961. Spore formation and germination as a microbial reaction to the environment, p. 100–150. *In* G. G. Meynell and H. Gooder [ed.], Microbial reaction to environment. Cambridge University Press, Cambridge.

NISHIDA, S., M. MURAKAMI, AND T. YAMAGISHI. 1962. Chopped-meat broth as a medium for the production of toxins by clostridia. I. Production of toxins by *Clostridium welchii*. Japan. J. Microbiol. **6**:33–40.

SMITH, L. DS. 1955. Introduction to the pathogenic anaerobes, 1st ed. The University of Chicago Press, Chicago.

STERNE, M., AND W. E. VAN HEYNINGEN. 1958. The clostridia, p. 343–351. *In* R. J. Dubos [ed.], Bacterial and mycotic infections of man, 3rd ed. J. B. Lippincott Co., Philadelphia.

VAN HEYNINGEN, W. E. 1941. The biochemistry of the gas gangrene toxins. I. Estimation of the α-toxin of *Clostridium welchii* type A. Biochem. J. **35**:1246–1256.

WILLIS, A. T., AND G. HOBBS. 1958. A medium for the identification of Clostridia producing

opalescence in egg-yolk emulsions. J. Pathol. Bacteriol. **75**:299–305.

YONEDA, M., AND M. KONDO. 1959. Studies of poly-β-hydroxybutyrate in bacterial spores. I. Existence of poly-β-hydroxybutyrate in mature spores of strains of *Bacillus cereus* and its relation to the acid-fast stainability. Biken's J. **2**:247–258.

ZEISSLER, J. 1930. Anaerobenzüchutung, p. 30. *In* W. Kolle, R. Kraus, and P. Uhlenhuth [ed.], Handbuch der pathogenen Mikroorganismen 3rd ed. Gustav Fisher, Berlin.