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Relationship between Heat-resistance, Dipicolinic Acid (DPA) Content and Germination Properties of *Clostridium perfringens* Type A Spores

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Abstract

The relationship between the D_{95} (decimal reduction times) -value, DPA (dipicolinic acid) content and germination properties of five strains including clinical and wild type strains of *Clostridium perfringens* type A spores were investigated in this study.

Based on the D_{95}-value of spores, the strains could be clearly classified as heat-stable, D_{95} 35.3-22.1, i.e., strains NCTC 8238 and NCTC 8239 and heat-labile, D_{95} 2.5-1.2, i.e., strains HS-13-1, S-40 and RW-18. DPA contained more than 7.5 % for the heat-stable strains, whereas the heat-labile strains had less than 6.0% of DPA in their spores. This suggested that the DPA content of spores coincided with the heat resistance of spores. Moreover, the germination properties in two different mediums were used to estimate the heat resistance of five strains of spores. The heated spores of the heat-stable strains germinated sufficiently in K-medium; however, the spores of heat-labile strains germinated scarcely in the same medium. These results shows that DPA content or germination properties had highly relationship with D_{95}-value regarding to the heat resistance of *C. perfringens* spores.

Key words: *Clostridium perfringens*, Dipicolinic acid, Germination properties, Spore

Introduction

Most cases of food-borne diseases related to *Clostridium perfringens* type A are caused by the strains which are capable of forming heat-stable spores (Hobbs et al., 1953; Yamagata et al., 1959). However, it is found that heat-labile strains are also responsible for some cases of intoxication (Hall et al., 1963; Sutton et al., 1968; Nakatsugawa et al., 1972; Kawamoto et al., 1988). Thus, the correlation between strain and its heat resistance has often been discussed in the literature (Hobbs et al., 1953; Yamagata et al., 1959; Itoh, 1972).

The heat resistance of *C. perfringens* spores is mainly determined by the number of survivors after heating the tube added with sporulating culture at 100°C for 10 or 60 min (Hobbs et al., 1953; Nakatsugawa et al., 1972).

While this semi-quantitative method is less accurate and less sufficient to evaluate the heat resistance of spores of each strain, the D-value is a quantitative procedure for evaluating the resistance of spores to heat of each strain (Labbe, 1989).

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The variations in DPA (dipicolinic acid) content (Church and Halvorson, 1959; Hashimoto et al., 1960) and the germination properties of spores (Oka et al., 1983) also provide a useful index of the heat resistance of spores. Nevertheless, very little is actually known about the relationship between the D-value, DPA content and germination properties of the spores.

In this paper, we describe the relationship between the three variables mentioned above in five clinical and wild type strains of the *C. perfringens* type A spores.

Materials and Methods

Bacterial strains

A total of five strains of *C. perfringens* type A were used in this study (Table 1). Two clinical strains, namely, NCTC 8238 (Hobbs serotype 2) and NCTC 8239 (Hobbs serotype 3), were kindly provided by the late Dr. Meiji Ito, National Institute of Infectious Diseases (former NIH, Japan), Tokyo. A clinical strain, HS-13-1 (Hobbs serotype 4), was donated by Dr. Shuji Nakatsugawa, Shizuoka Prefectural Institute of Public Health and Environmental Science. Another clinical strain, S-40, was obtained from the Food and Drug Administration, Washington D.C.; it was also associated with food-borne disease. A wild type strain, RW-18 was isolated and identified from river water in 1983 (Oka et al., 1989).

Growth medium

Fluid thioglycolate (FTG) medium was comprised of the following: 1.5% trypticase (BBL), 0.5% yeast extract (BBL), 0.05% sodium thioglycolate, 0.05% L-cystine, 0.25% sodium chloride and 0.5% glucose (pH 7.1).

Sporulating medium

Duncan and Strong (DS) medium (1968) was comprised of the following: 1.5% proteose peptone (Difco), 0.4% yeast extract (BBL), 0.1% sodium thioglycolate,

Table 1 List of 5 strains belonging to the species *Clostridium perfringens* used in this study.

Strain	Source of isolate	Thermostability	Enterotoxin production	Hobbs' serotype	Donor
NCTC 8238	Boiled Salt Beef	Heat-resistant	Positive	2	National Institute of Health, Japan
NCTC 8239	Boiled Beef	Heat-resistant	Positive	3	National Institute of Health, Japan
HS-13-1	Feces	Heat-sensitive	Negative	4	Shizuoka Prefectural Institute of Public Health and Environmental Science
S-40	Turkey	Heat-sensitive	Negative	—*	Food and Drug Administration Washington D.C.
RW-18	River Water	Heat-sensitive	Positive	—	Laboratory of Food Wholesomeness, Department of Fisheries, Hokkaido University

* non typable

0.4% soluble starch (Wako Pure Chemical Industry Ltd.) and 1.0% sodium phosphate (dibasic dodecahydrate) (pH 7.4–7.6).

Culture and sporulation conditions

All the strains were inoculated into 10 ml of cooked meat medium (Difco) and incubated at 37°C for 48 h, and the cultures were maintained at −20°C and used throughout this study as stock cultures. For spore production, several particles of stock cultures were transferred into FTG medium. The particles were first, heat shocked at 75°C for 20 min to activate the spores and to destroy the vegetative cells, and then cooled off rapidly by immersion in ice water and later incubated at 37°C for 15–16 h. This culture was subcultured into FTG medium (to 10% concentration) and incubated at 37°C for 10–12 h. The culture was added at 10% concentration to DS medium and incubated for 8–10 h at 37°C. The spores were centrifuged at 3,000×g for 15 min and subjected to ultrasonic treatment to remove the spores which still remained in their sporangia. Sonication was continued until 90 to 95% of the spores were free. Spores obtained were examined by a phase-contrast microscope to monitor breakage. Free spores were washed eight to ten times with double distilled deionized water and lyophilized and stocked until use at −70°C.

Determination of D (decimal reduction times)-value

Determination of D_{95}-value (time required for a tenfold inactivation at 95°C) was followed as described by Ando et al. (1985).

Spore suspensions in double distilled water (10 mg dry wt/ml) were heated in sealed Pyrex glass tubes (10×100 mm) deeply immersed in a thermostatically controlled ethylene glycol bath. The tubes were maintained at 95°C for different periods of time after heat activation at 75°C for 20 min. D_{95}-value was measured as colony-forming units.

Determination of dipicolinic acid (DPA; pyridine-2, 6-dicarboxylic acid) DPA concentration in spore was determined by the method of Janssen et al. (1958) with modification of Rotman and Fields (1967).

Estimation of spore germination properties

Spore germination test was followed as described by Oka et al. (1983) and Ando et al. (1985). For the identification of germination types of spores, heat-shocked spores (at 75°C for 20 min) and non-heated spores were allowed to germinate in the K-medium and A-medium, respectively. K-medium contained 50 mM KCl in 50 mM potassium phosphate buffer (pH 7.0). A-medium was composed of 1 mM L-alanine, 1 mM inosine and 40 mM $CaCl_2$ in 50 mM Tris-HCl buffer (pH 7.5). In the A-medium, germination was initiated by introducing CO_2 to the medium at the rate of 20 ml/min for 5 min before the incubation period. Incubation was carried out at 40°C for 60 min under aerobic conditions. The extent of germination was determined by measuring the decrease in O.D. (A_{540} 1 cm cuvetts) of spore suspension in germination mediums. Before incubation, spore suspensions were prepared to give a turbidity readings of 1.6 absorbance unit at 540 nm in cuvetts with a 1 cm light path.

Results and Discussion

D_{95}-value of spores

The D_{95}-values of the five strains tested in this study are presented in Fig. 1.

The D_{95}-values of the five strains under this study varied widely and ranged from 1.2 to 35.3. There is clearly genetic difference among *C. perfringens* strains regarding the heat-resistance of their spores. The D_{95}-value of strain NCTC 8238 was 35.3 and found to be the most heat-stable strain, on the other hand, RW-18 was the most heat-labile strain (D_{95}-value was 1.2).

Roberts (1968) reported that the D_{95}-value of strain NCTC 8238 was 30. Ando et al. (1985) reported that it was 43.8 in double distilled water, which was much higher than that obtained by Roberts (1968). Sunagawa et al. (1987) reported that the D_{95}-value of strain RW-18 was 1.5. The results acquired in this study were generally in agreement with those obtained by Sunagawa et al. (1987). Based on the D_{95}-value of spores, the strains could be divided into heat-stable and heat-labile strains. NCTC 8238 and NCTC 8239 were classified as heat-stable strains, whereas HS-13-1, S-40 and RW-18 were as heat-labile strains.

DPA content in spores

Many attempts have been made to demonstrate the function of DPA in spores. DPA has been considered to play the role of heat resistance (Church and Halvorson, 1959), dormancy, calcium accumulation and so on (Tochikubo et al., 1987); however, there is little agreement on the function of DPA.

In this experiment, we inquired that DPA may be related to heat resistance of the spores of *C. perfringens*.

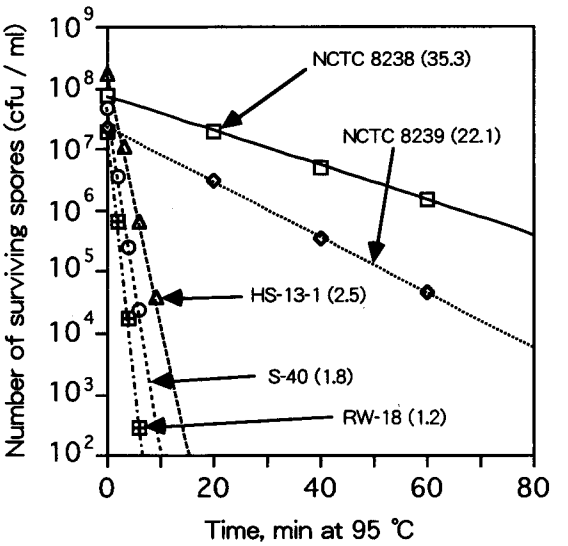

Fig. 1. Thermal survival curves (D_{95}-value) of spores of *Clostridium perfringens*. Spores were suspended in double distilled water (10 mg dry wt/ml) before measuring the D_{95}-value. Numbers in parentheses show D_{95}-value.

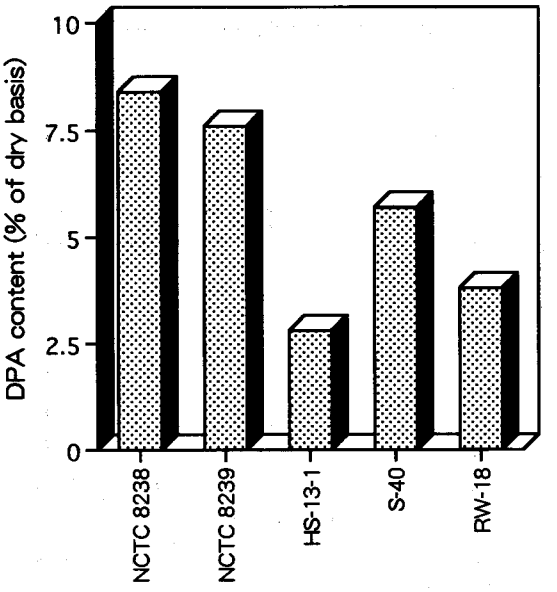

Fig. 2. DPA content in the spores of five strains of *Clostridium perfringens* type A. DPA content in spores is given by percentage in the spores (dry basis).

Fig. 2 shows the DPA content in the spores of *C. perfringens*.

Strains NCTC 8238 and NCTC 8239 (both of which are heat-stable strains) contained more than 7.5% of DPA in the spores, whereas the other three strains (which are considered to be a heat-labile group, namely, HS-13-1, S-40 and RW-18) had less than 6.0% of DPA in the spores. DPA was found to be the most abundant in strain NCTC 8238, followed by NCTC 8239, and then by S-40, RW-18 and HS-13-1. Strain HS-13-1 possessed the smallest quantity of DPA in the spores. The results shown in Fig. 1 and Fig. 2 suggest that there is a positive correlation between DPA content and D-value. DPA accounted for about 2.5-8.5% (dry basis) in *C. perfringens* spores, however, it has been found to constitute 5-10% of the dry weight of the spore in *Bacillus* spp. (Powell, 1953; Perry and Foster, 1954).

Germination properties of spores

Previous studies indicated that the germination properties in two different mediums of *C. perfringens* were able to assess the heat resistance of *C. perfringens* spores. (Oka et al., 1983; Ando et al., 1985). Here we describe the germination properties of five strains of *C. perfringens* spores (Figs. 3 and 4).

The heated spores of the heat-stable strains, NCTC 8238 and NCTC 8239 germinated sufficiently in K-medium; however, the heat-labile strains, HS-13-1, S-40 and RW-18 germinated scarcely in this medium (Fig. 3). Fig. 4 describes the germination properties of five strains in non-heated spores in A-medium. Unlike in K-medium, the heat-stable strains, NCTC 8238 and NCTC 8239 showed poor germination in A-medium.

Several observations in previous experiments indicated that the heat resistance of spores was related to DPA content (Church and Halvorson, 1959) and germination

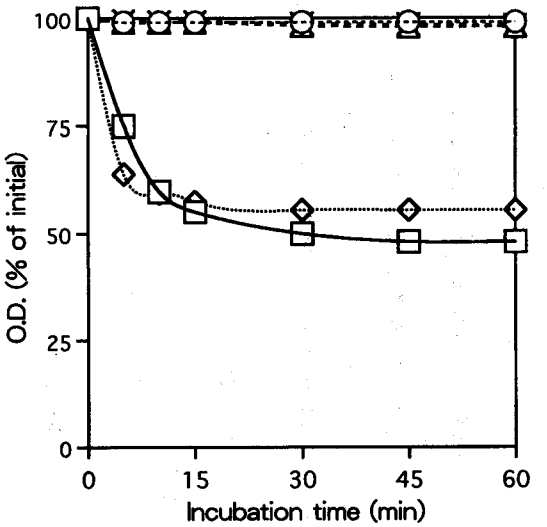

Fig. 3. Germination of heat activated spores (75°C for 20 min) of five strains of *Clostridium perfringens* type A in K-medium containing KCl (50 mM)+50 mM potassium phosphate buffer (pH 7.0).
□: NCTC 8238, ◇: NCTC 8239, ○: HS-13-1, △: S-40, ×: RW-18

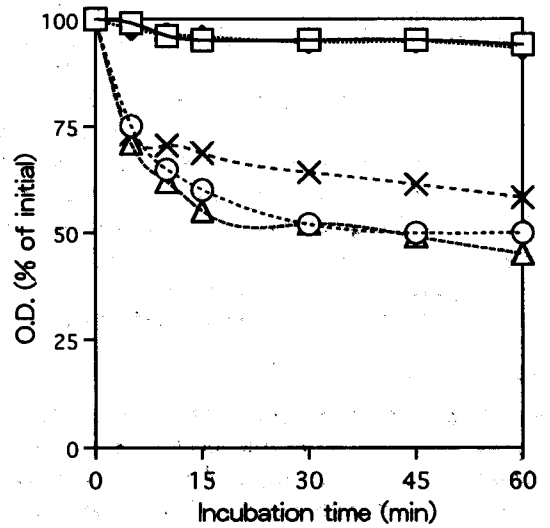

Fig. 4. Germination of non-heated spores of five strains of *Clostridium perfringens* type A in A-medium containing L-alanine (1 mM)+inosine (1 mM)+$CaCl_2$ (40 mM)+50 mM Tris-HCl buffer (pH 7.5) in the presence of CO_2.
□: NCTC 8238, ◇: NCTC 8239, ○: HS-13-1, △: S-40, ×: RW-18

properties (Oka et al., 1983; Ando et al., 1985). The D-value determination is the most accurate quantitative way to estimate the heat resistance of spores; however, it is a time-consuming and somewhat laborious technique. It concluded that one

can simply estimate the germination properties and DPA content of spores to obtain a quick measure of heat stability.

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