

# Production of the Vacuolation Factor of *Bacillus cereus* Isolated from Vomiting-Type Food Poisoning

Kunihiro SHINAGAWA, Shoji OTAKE, Naonori MATSUSAKA, and Shunji SUGII<sup>1)</sup>

Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, Ueda, Morioka, Iwate 020 and <sup>1)</sup>Department of Serology and Immunology, School of Medical Technology, Kitasato University, Kitasato, Sagami-hara, Kanagawa 228, Japan

(Received 9 September 1991/Accepted 16 January 1992)

**ABSTRACT.** Vacuole response in HEP-2 cells was induced with culture supernatants of *Bacillus cereus* strains isolated from outbreaks of vomiting- and diarrheal-type food poisoning grown in rice flour and laboratory media. High vacuole response was obtained with culture supernatants of *B. cereus* strains isolated from vomiting-type food poisoning grown in cooked rice suspension or on a cooked rice plate, whereas no response was obtained with those of the same strains grown in brain heart infusion and trypto-soya broth media. The vacuole activity appeared only after spore formation of *B. cereus*. The activity was stable to proteolytic enzymes, heating, and exposing to pH 2.0 and 11.0. Of 124 strains isolated from *B. cereus* food poisoning that were tested, the vacuole activity was observed by 68 of 110 (61.8%) of the strains isolated from the vomiting-type food poisoning but not by all strains (14 strains) from diarrheal-type ones. Moreover, the vacuole response in the HEP-2 cells was found to be induced by 56 of 76 (73.7%) of the serotype H-1 strains isolated from vomiting-type food poisoning.—**KEY WORDS:** *Bacillus cereus*, heat-stability, toxin, vacuole response.

J. Vet. Med. Sci. 54(3): 443–446, 1992

Some strains of *Bacillus cereus* cause food poisoning characterized by either diarrhea or vomiting [1, 3, 14]. An enterotoxin (ET) produced by *B. cereus* has been demonstrated to cause diarrhea in diarrheal-type food poisoning [1, 3, 4, 9–12, 14]. Like ETs produced by *Clostridium perfringens*, enterotoxigenic *Escherichia coli*, and *Vibrio cholerae*, the ET has been assayed by vascular permeability tests and ligated intestinal loop tests using guinea pigs, rabbits, and mice [4, 9–12]. In contrast to the ET, little is known about the substance(s) responsible for the vomiting-type *B. cereus* food poisoning associated mainly with consumption of rice [9, 14]. The only assay method available for the emetic toxin is oral challenge to primates [6, 7]. More recently Hughes *et al.* [5] have demonstrated that the vacuole response in the HEP-2 cells was observed by culture supernatants of *B. cereus* strains isolated from vomiting-type food poisoning grown in rice flour and brain heart infusion broth. Szabo *et al.* [13] have most recently reported that the vacuole response in cultured cells was caused by the heat-stable toxin which was produced by *B. cereus* strains associated with illness and the emetic syndrome, but not by *B. cereus* strains isolated from foods, wounds, and unknown source. However, it is not well known whether there is any correlation between the vacuole response in the HEP-2 cells and the emetic activity of *B. cereus* culture supernatant fluids. To elucidate the correlation between these two activi-

ties, attempts were made to study production of the vacuolation factor (VF) of *B. cereus* strains isolated from outbreaks of vomiting-type food poisoning with HEP-2 cells.

## MATERIALS AND METHODS

***B. cereus* strains:** A total of 124 *B. cereus* strains, which were isolated from 48 outbreaks of food poisoning, occurring from 1973 to 1989, were used in this study. A hundred and ten strains were isolated from incriminated foods, vomitus, and feces in 34 outbreaks of vomiting-type food poisoning, whereas 14 strains were from incriminated materials in 9 outbreaks of diarrheal-type food poisoning. *B. cereus* strains No. 27 and No. 55 were isolated from a patient with vomiting-type food poisoning, Sakai, Osaka in 1977 and from fried rice involved in vomiting-type food poisoning in Nagoya in 1977, respectively. These two strains were used for production and *in vitro* stability of VF.

***Viable and spore counts of B. cereus:*** Viable counts of *B. cereus* were determined on mannitol egg-yolk polymyxin (MYP) agar. For enumeration of the *B. cereus* spores, the culture was heated at 75°C for 15 min before the viable count.

***Culture media and production of VF:*** Five different culture media such as brain heart infusion (BHI) (Difco Lab., Detroit, Michigan, U.S.A.) containing 0.1% glucose (BHIG), trypto-soya (TS) broth (Nis-

sui, Tokyo, Japan), TS agar plate, cooked rice suspension, and cooked rice plate were used for production of VF. Cooked rice suspension and cooked rice plate were prepared by suspending rice flour in water, 3.3% (w/v) for suspension and 8 or 16% (w/v) for plate, respectively; the plates were autoclaved. A 0.1 ml quantity of inoculum, prepared by incubation at 30°C for 12 hr in BHI, was transferred to 10 ml of BHIG, TS broth, or cooked rice suspension and then was incubated by shaking at 120 rpm for 35 hr at 30°C for production of VF. Bacterial cells were removed by centrifugation at  $17,000 \times g$  at 4°C for 20 min. When plate media were used for production of VF, 0.2 ml of the inoculum was placed onto the cooked rice plates and TS agar plates. The plates were incubated at 30°C for 5 days. After incubation, 3% (w/v) Taka-diaxase (Sankyo, Tokyo) was added to cultured cooked rice plate to 0.03%. The plates were then incubated at 37°C for 3 hr. The TS agar plate was frozen for 24 hr. After incubation or defrosting, culture supernatant fluids were obtained by centrifugation at  $17,000 \times g$  at 4°C for 20 min. Culture supernatant fluids were filtered through a membrane filter (0.45  $\mu\text{m}$ ). The filtrate was allowed to stand for 10 min in boiling water before the determination of the vacuole activity.

**Cell culture and determination of vacuole activity:** HEp-2 cells (human carcinoma of the larynx) [8] were provided by Dr. I. Matsumoto, Department of Microbiology, Iwate Medical College, Morioka, Iwate, Japan. According to the methods described previously [5], the cells were cultured at 37°C in Eagle's Basal Medium (BME) (Flow Lab., New York, U.S.A.) with Eagle's Salts containing 2 mM L-glutamine, 5% sodium bicarbonate, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, pH 7.8 and 10% fetal calf serum (FCS) (Flow Lab., New York, U.S.A.). For determination of the vacuole response, an aliquot of 25  $\mu\text{l}$  of 2-fold serially diluted culture supernatant fluid in saline was placed in each well of a 96-well tissue culture plate, incubated at 37°C with 100  $\mu\text{l}$  of HEp-2 cells ( $3 \times 10^5$  cells/ml) prepared in BME supplemented with 1% FCS for 37°C for 35 hr, and observed for the vacuole responses in the HEp-2 cells under light microscope. A reciprocal of the highest dilution of culture supernatant to produce vacuoles in more than 25% of the HEp-2 cells at 10 vacuoles/cell was defined as a vacuolation titer (unit/ml).

**Tests for in vitro stability at different pH values:** One ml of culture supernatant fluid with a vacuole

titer of 160 was incubated at room temperature for 2 hr in 9 ml of 0.1 M HCl-KCl buffer, pH 2.0 and 25 mM glycine-NaOH buffer, pH 11.0. After incubation, the mixture was adjusted to pH 7.4 with 0.1 N NaOH or 0.1 N HCl.

**Digestion with pepsin and trypsin:** One ml of culture supernatant fluid with a vacuole titer of 160 was incubated at 37°C for 2 hr in 9 ml of 2 mg/ml of pepsin (Sigma Chemical Co., St. Louis, U.S.A.) in 0.1 M phosphate-citrate buffer, pH 4.2 or in 9 ml of 2 mg/ml of trypsin (Sigma Chemical Co., St. Louis, U.S.A.) in 0.2 M Tris-HCl buffer, pH 8.0. After incubation, the mixture was heated in boiling water for 10 min before adjusting the pH to 7.4 with 0.1 N NaOH or 0.1 N HCl.

## RESULTS AND DISCUSSION

**Incubation period for production of VF:** To determine the incubation period for production of VF, *B. cereus* strain No. 27 was cultured in cooked rice suspension at 30°C for different periods. As shown in Fig. 1, vacuole response in HEp-2 cells was produced after incubation for longer than 10 hr (10–60 hr). The vacuole response reached a plateau after incubation for 30 hr. Spore formation began at 5 hr after incubation and reached a plateau at 12 hr. These findings suggest that production of VF may occur after spore formation as reported previously [6].

**Culture media for production of VF:** *B. cereus* strains No. 27 and No. 55 were cultured in different media and the vacuole response in the HEp-2 cells by culture supernatants of the strains grown in the different media are summarized in Table 1. The

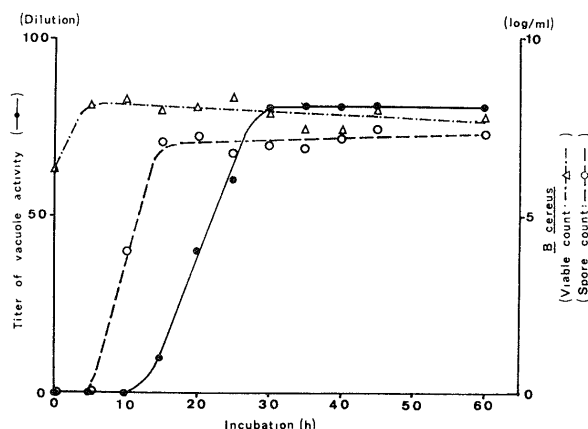


Fig. 1. Time course of vacuole factor production in cooked rice suspension at 30°C.

Table 1. Production of the vacuolation factor in culture supernatant of *B. cereus* strain No. 27 grown in various media

Medium	<i>B. cereus</i> viable count (log)/ml	<i>B. cereus</i> spore count (log)/ml	Vacuole titer (unit/ml)
Cooked rice suspension	7.9	6.7	160
Cooked rice plate (16%) <sup>a)</sup>	NT <sup>c)</sup>	NT	80
Cooked rice plate ( 8%)	NT	NT	10
BHIG broth	8.9	5.7	<10 <sup>d)</sup>
Trypto-soya broth	7.7	6.7	<10
Trypto-soya agar <sup>b)</sup>	NT	NT	<10

a) ( ): Concentration of rice flour, b) Culture condition: 30 °C, 3 days, c) NT: Not tested, d) <10: No vacuole activity was obtained with 10-fold or less diluted culture supernatant.

culture supernatants of the strains grown in cooked rice suspension showed the highest vacuole response, whereas those grown in the cooked rice plates showed slightly less response. No vacuole response was obtained by cultures grown in BHIG, TS broth, and TS agar plate although spore formation was observed in cooked rice suspension, BHIG, and TS broth (Table 1). It can be concluded from these findings that cooked rice medium is suitable for production of VF. The cooked rice medium also has been used successfully for production of the emetic toxin [6, 7] and the heat-stable toxin [13]. Although the emetic toxin or heat-stable toxin was reported to be produced by *B. cereus* grown in TS broth and TS agar media as well [6, 7], the VF was not detected in these media in the present study. On the other hand, the heat-stable toxin was produced when *B. cereus* was grown in BHI broth, BHIG broth, and milk [13]. Whether this indicates the three factors (such as emetic toxin, heat-stable

Table 2. *In vitro* stability of the vacuolation factor produced by *B. cereus* strain No. 55 to various treatments

Treatment	Vacuole titer (unit/ml) after treatment
None treatment	160
Heating at	
80°C, 10 min	160
115°C, 10 min	160
121°C, 10 min	160
Stored at	
4°C, 2 months	160
Treatment with	
pH 2.0, 2 hr	160
pH 11.0, 2 hr	160
trypsin (2 mg/ml)	160
pepsin (2 mg/ml)	160

toxin, and VF) are different or whether it is due to the use of different *B. cereus* strains or methods of incubation is not known.

*In vitro* stability of VF: The culture supernatant fluid from *B. cereus* strain No. 27 grown in cooked

Table 3. The vacuole activity of *B. cereus* strains isolated from vomiting- and diarrheal-type food poisoning

Type of food poisoning	H-serotype	Number of strains	Titer of vacuole activity (unit/ml)					
			<10 <sup>a)</sup>	10	20	40	80	160
Vomiting-type	1	76	20	5	15	26	5	5
	8	15	8	1	3	1	2	—
	14	2	2	—	—	—	—	—
	3	1	1	—	—	—	—	—
	19	1	1	—	—	—	—	—
	UT <sup>b)</sup>	15	10	—	1	1	1	2
	Total	110	42	6	19	28	8	7
Diarrheal-type	6	4	4	—	—	—	—	—
	2	2	2	—	—	—	—	—
	UT	8	8	—	—	—	—	—
	Total	14	14	0	0	0	0	0

a) <10: No vacuole activity was obtained with 10-fold or less diluted culture supernatant.

b) UT: Untypable.

rice medium was treated with proteolytic enzymes, exposed to low and high pHs, and heated to determine the stability of VF. The results are summarized in Table 2. The vacuole activity proved to be stable to all of these treatments. This corresponds favorably to the stability of the emetic toxin produced by *B. cereus* [7, 15].

*VF production of different B. cereus strains isolated from food poisoning outbreaks:* A total of 124 *B. cereus* strains isolated from food poisoning outbreaks were incubated at 30°C for 35 hr in cooked rice medium. Vacuole activity was produced by strains isolated from 27 of 39 (69.2%) outbreaks of the vomiting-type food poisoning, whereas essentially no activity was observed in the strains isolated from the 9 outbreaks of diarrheal-type food poisoning. The *B. cereus* isolated from 23, 2, and 2 of the 27 vomiting-type outbreaks belonged to H-1, H-8, and untypable serotypes, respectively.

The vacuole responses of the 124 *B. cereus* strains are summarized in Table 3. The vacuole activity was produced by 68 of 110 (61.8%) of the strains isolated from the vomiting-type food poisoning outbreaks, with 56 of 76 (73.7%) of the serotype H-1 strains producing the factor, whereas only 7 of 15 (46.7%) of the serotype H-8 strains and only 5 of 15 (33.3%) of the untypable strains produced it. However, the fact that 38.2% of the strains isolated from the vomiting-type food poisoning outbreaks did not produce the VF, raises doubts that the VF is responsible for the emetic action. This can be proved only when the VF and the emetic toxin are purified.

**ACKNOWLEDGEMENTS.** This study was in part supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan. The authors would like to express gratitude to Dr. M. S. Bergdoll, University of Wisconsin, for his valuable comments and advice in preparing the manuscript.

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