The Relation Between the Diarrheal and Other Biological Activities of *Bacillus cereus* Involved in Food Poisoning Outbreaks

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ABSTRACT. The culture filtrates of *Bacillus cereus* strains isolated from diarrheal-type and vomiting-type food poisoning outbreaks were examined for their enterotoxigenicity, vascular permeability-enhancing (VP) activity and mouse lethality. Seven of nine diarrheal strains were starch-hydrolysis positive; all vomiting ones were negative. All diarrheal strains elicited VP reaction; the vomiting strains did not. The mouse lethal activity of the diarrheal strains paralleled the VP activity; such was not the case with the vomiting strains. The culture filtrates of four diarrheal strains among eight and none of vomiting strains caused fluid accumulation in the mouse ileal loop. The concentrated (50-fold) culture filtrates of five diarrheal strains of eight caused fluid accumulation in rabbit ileal loops; only three of 21 vomiting strains did so.—**KEY WORDS:** *Bacillus cereus*, biological activity, diarrheal toxin, food poisoning outbreak.

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Bacillus cereus causes foodborne illness of two distinct types; one is characterized by diarrhea and abdominal pain 8–12 hr after ingestion of contaminated food (diarrheal-type) and the other by nausea and vomiting with an incubation period of only 1–5 hr (vomiting-type) [3].

It appears that the two types of illness are caused by two distinct toxins, “diarrheal toxin” (enterotoxin) [13, 15, 18] and “emetic toxin” [9, 10, 16]. It has been reported that diarrheal and emetic toxins produced by *B. cereus* have such other activities as vascular permeability, mouse lethal, hemolytic, and phospholipase C activities [2, 8, 14, 16, 18]. In addition, dermal and intestinal necrotic toxins have been found [17, 18, 19].

The enterotoxigenic or emetic activity has not been well correlated to any other activities because neither enterotoxin nor emetic toxin has been purified to a satisfactorily pure state. Some investigators claimed that the permeability-enhancing, the ileal loop fluid accumulating and the mouse lethal activities are elicited by the same substance [14, 18].

This study was carried out to clarify more in detail the nature and properties of “diarrheal toxin” produced by many strains of *B. cereus*, and the correlation between the diarrheal toxicity and other biological activities.

MATERIALS AND METHODS

***B. cereus* strains:** A total of 59 strains of *B. cereus* including nine implicated in four outbreaks of diarrheal-type (referred to as diarrheal strains) and the other 50 in 21 outbreaks of vomiting-type food poisoning (referred to as vomiting strains) in Japan were used (Table 1.). The isolates from incriminated foodstuffs and clinical specimens of an outbreak showed identical biochemical properties and the same serotype [12]. So two or three strains were isolated from an outbreak of food poisoning.

For comparison, other 113 strains were isolated from such foods as powdered skim milk (35 strains), beef extract (30 strains), and cooked and uncooked rice (48 strains) which were not related to food poisoning. Of

Table 1. Outbreaks of *B. cereus* food poisoning

Outbreak No. a)	Date	City (Prefecture)	Persons at risk	Cases	Incriminated foodstuff	<i>B. cereus</i> population in incriminated food (per g)	Incubation period (hr)	Main symptom
1.	Jul. 1971	Toyonaka (Osaka)	Unknown	89	Pudding	2.0×10^6	3-7	Diarrhea
2.	Jul. 1973	Suita (Osaka)	5	5	Fried rice	6.2×10^6	2-4	Vomiting
3.	Jul. 1974	Hiraoka (Osaka)	33	12	Catered box lunch	3.0×10^7	2-12	Diarrhea
4.	Aug. 1974	Hiraoka (Osaka)	3	3	"Tempura"	4.8×10^5	2-3	Vomiting
(5.)	Aug. 1974	Nagoya (Aichi)	52	51	"Yakisoba"	1.6×10^8	1-2	Vomiting
(6.)	Oct. 1974	Tsu (Mie)	1407	194	Catered box lunch	5.7×10^6	4-10	Diarrhea
7.	Aug. 1975	Yao (Osaka)	48	24	Catered box lunch	$1.2 \times 10^{6(c)}$	1-3	Vomiting
(8.)	Sep. 1975	Sakata (Yamagata) ^{f)}	422	130(3) ^{b)}	Catered box lunch	^{d)}	2-17	Diarrhea
9.	Aug. 1977	Sakai (Osaka)	2	2(1) ^{b)}	Fried rice	1.3×10^6	0.5-1	Vomiting
10.	Jul. 1977	Neyagawa (Osaka)	6	6	Rice ball	1.6×10^7	0.5-3	Vomiting
(11.)	Sep. 1977	Osaka (Osaka)	13	9	Catered box lunch	1.2×10^7	0.5-2	Vomiting
(12.)	Sep. 1977	Fukuoka (Fukuoka)	2	2	Curry and rice	5.7×10^7	0.5-2	Vomiting
(13.)	Oct. 1977	Osaka (Osaka)	1809	211	Rice ball & "Sushi"	8.0×10^8	2-2.5	Vomiting
(14.)	Aug. 1978	Handa (Aichi)	2	2	Fried rice	^{d)}	1.5-2	Vomiting
15.	Sep. 1978	Tondabayashi (Osaka)	2	2	Fried rice	$1.9 \times 10^{4(e)}$	1.5-2	Vomiting
16.	Sep. 1978	Yao (Osaka)	1	1	Roast chicken	1.3×10^6	0.5-1	Vomiting
17.	Oct. 1978	Sakai (Osaka)	206	94	Fried rice	$2.3 \times 10^{4(e)}$	1-4	Vomiting
18.	Jul. 1979	Hiraoka (Osaka)	4	4	Fried rice	$2.1 \times 10^{5(e)}$	0.5-2	Vomiting
19.	Aug. 1979	Sakai (Osaka)	6	6	Fried rice	7.8×10^7	1-2	Vomiting
(20.)	Jun. 1981	Uzi (Kyoto)	53	35	Fried rice	1.4×10^6	1-2	Vomiting
(21.)	Jul. 1981	Maizuru (Kyoto)	4	4	Omelet and rice	^{d)}	0.5-1	Vomiting
(22.)	Jul. 1981	Kisarazu (Chiba)	338	172	Soy bean curd "Okara"	4.5×10^9	1-5	Vomiting
(23.)	Aug. 1981	Nagoya (Aichi)	61	46	Chicken and rice	$1.0 \times 10^{6(c)}$	1-2.5	Vomiting
(24.)	Aug. 1982	Soka (Saitama)	6	5	Omelet and rice	1.1×10^7	2-4	Vomiting
(25.)	Dec. 1982	Sendai (Miyagi)	2	2	Fried rice	^{d)}	1-1.5	Vomiting

a) The isolates in the outbreaks marked with circles on the outbreak numbers were provided by the respective prefectural institute of public health.

b) () Death.

c) *B. cereus* population in vomitus

d) Not determined quantitatively.

e) *B. cereus* population in feces.

f) *B. cereus*, *S. aureus* and *V. parahaemolyticus* were isolated simultaneously from the incriminated foodstuff or clinical specimens.

these strains, 53 were starch-hydrolysis-positive and the other 60 were negative.

B. cereus strains B-4ac, 4433/73, and 4810/72 (provided by Drs. J. M. Goepfert and R. J. Gilbert) were used as references.

Tests for starch hydrolysis by the strains: The test culture was streaked in a line on a nutrient agar plate supplemented with 1% soluble starch and the plate was incubated aerobically at 32°C for 24 hr. After incubation, Lugol's iodine solution was poured on the surface of the plates. The test was regarded as positive if a clear zone appeared around the streak, but as negative if such a zone appeared merely under the streak [12].

Production of B. cereus toxins: Brain heart infusion (Difco) supplemented with 0.1% glucose (BHIG) [6] was used for toxin production. A heart infusion agar slant culture was inoculated into 3 ml of BHIG medium in an L-tube of 20-ml capacity. The L-tubes were incubated for 12 hr at 32°C on a reciprocal shaker at 90 cycles/min. Then, a 1-ml portion of the seed culture was transferred to 100 ml of BHIG medium in a 500-ml shaking flask, and the flasks were incubated for 5 hr under the same conditions as for preparing the seed culture. The cultures were centrifuged at $12,000\times g$ for 20 min.

The supernatant fluid was filtered through a membrane filter (0.45 μm). A portion of the filtrate was assayed for the toxins immediately or within 48 hr of storage at 4°C. The other portion was stored at -20°C. The filtrate was concentrated by salting out at 70% saturation of ammonium sulfate. The precipitate was dissolved in distilled water, dialyzed against 0.005 M Tris-HCl buffer, pH 8.6, and then lyophilized. The lyophilized material was redissolved in distilled water to a 0.02 volume of the original one for rabbit ileal loop assay.

Assay for vascular permeability activity: Japanese albino white rabbits weighing 1.5 to 2.0 kg were used. The back of the rabbit was shaved and marked off into 25 to 32 squares.

Intradermal injection of the test sample (0.05 ml) was made into each square. Three hours after injection, 2% Evans blue (2 ml/kg body weight) was injected into the ear vein. The animals were killed in 60 min and diameters of the dark blueing zones were measured [5].

Assay for mouse lethal toxin: Doses of 0.1 to 0.4 ml of the culture filtrate were injected into the tail vein of adult mice of the ICR strain weighing 17 to 22 g. The mice were observed for 60 min.

Assay in the mouse ileal loop: Mice of the ICR strain weighing 17 to 22 g were used. They were anesthetized by *i.p.* injection of sodium pentobarbital. Segments of the ileum were tied off to form two test loops, each approximately 2.5 to 3-cm long. Intraluminal injection of 0.1 ml of a culture filtrate was made into one test loop; the other loop was injected with sterile BHIG medium as a control. The ileum was replaced into the abdominal cavity, and the incision was sutured. In 6 hr, the mice were killed and the abdominal cavities were opened immediately for measuring the fluid volume in the ligated ileal loops. The ratio of the fluid volume (ml) to the loop length (cm) was employed as a measure of the reaction [11].

Assay in the rabbit ileal loop: Japanese albino white rabbits weighing 1.3 to 1.5 kg were used. They were anesthetized by *i.v.* injection of sodium pentobarbital. Segments of the ileum were tied off to form seven loops, each approximately 7 to 8-cm long, separated from one to another with 2 cm blank loops. Intraluminal injection of 1-ml of a 50-fold concentrated sample was made into each loop. In each rabbit, one loop was injected with sterile BHIG medium as a control. The ileum was replaced into the abdominal cavity, and the incision was sutured. After their recovery from anesthesia, the rabbits were provided with only water. In 8 hr, the rabbits were killed and the abdominal cavities were opened for examinations.

The ratio of the fluid volume (ml) to the

Table 2. Starch hydrolysis of *B. cereus* strains isolated from 25 food poisoning outbreaks and various foods

Source	Number of strains tested	Starch-hydrolysis (strains)
Diarrheal type	9	Positive (7) Negative (2)
(Reference strain) ^{a)}	2	Positive (2)
Vomiting type	50	Negative (50)
(Reference strain) ^{b)}	1	Negative (1)
Various food samples ^{c)}	113	Positive (53) Negative (60)

a) *B. cereus* strain B-4ac and 4433/73 from diarrheal-type outbreaks.

b) *B. cereus* 4810/72 from a vomiting-type outbreak.

c) These included 35 samples of powdered skim milk, 30 of beef extract and 48 of cooked and uncooked rice.

loop length (cm) was employed as a measure of the reaction [13].

Assay for hemolytic activity: Nine parts of the test sample were mixed with one part of a 5% suspension of rabbit red blood cells and the mixture was incubated for 30 min at 37°C. Loss of turbidity by lysis of the red blood cells was considered to be the positive reaction. The hemolysin titer was expressed as the reciprocal of the highest dilution evidencing such clearing [8].

Assay for egg yolk turbidity factor: Equal volumes of the sample and a 20% Colbeck egg yolk solution (Difco) were mixed and the mixture was incubated for 2 hr at 37°C. The development of any turbidity was considered to be a positive reaction, and the egg yolk turbidity titer was expressed as the reciprocal of the highest dilution showing turbidity. Purified *B. cereus* phospholipase C (General Biochemical Inc., Chagrin Falls, Ohio) was used as a reference [4].

RESULTS

Of the nine diarrheal strains, seven strains isolated from three outbreaks were starch-hydrolysis positive. All vomiting strains were starch-hydrolysis negative. Among 113 strains isolated from various food samples

(including powdered skim milk, beef extract, and cooked and uncooked rice), fifty-three were starch-hydrolysis positive (Table 2.).

Culture filtrates of nine diarrheal strains strongly increased VP in the rabbit skin. The average diameters of the dark blueing zones produced by the culture filtrates ranged from 15 to 24.9 mm. Hemorrhage and edema were also seen. On the other hand, 44 vomiting strains elicited little or no characteristic VP reaction. The diameters of the blueing zones caused by the culture filtrates of these strains were less than 9.9 mm. Six strains were moderately active in VP reaction, showing blueing zones of 10–14.9 mm (Table 3.).

Culture filtrates of 53 starch-hydrolysis-positive strains from the food samples increased VP. The diameters of the blueing zones caused by the filtrates of these strains were above 5 mm. Of the 53 strains, 18 (34.0%) caused hemorrhage and edema in the center of the blueing zone. In contrast, of 60 starch-hydrolysis-negative strains, 56 produced blueing zones of less than 4.9 mm. Of these strains, only one showed a moderate VP activity with blueing zone over 10–14.9 mm. None of starch-hydrolysis-negative strains caused edema or hemorrhage (Table 3.).

The culture filtrates of three diarrheal strains were lethal to mice upon *i.v.* injection

Table 3. Vascular permeability activity of *B. cereus* strains isolated from 25 food poisoning outbreaks and various foods

Source	Number of strains tested	Number of VP-activity positive strains (%)					
		$\leq 4.9^a$	5.0–9.9	10.0–14.9	15.0–19.9	20.0–24.9	Hemorrhage
Diarrheal type	9(100)	0	0	0	4(44)	5(55)	9(100)
(Reference strain)	2					2	2
Vomiting type	50(100)	21(42)	23(46)	6(12)	0	0	0
(Reference strain)	1		1				
Various food	53(100) ^c	0	14(26)	17(32)	19(36)	3(6)	18(34)
samples ^b	60(100) ^d	56(93)	3(5)	1(2)	0	0	0

a) An average of perpendicular diameters (mm) of the dark bluing zone.

b) See the footnote of Table 2.

c) Starch-hydrolysis-positive strains.

d) Starch-hydrolysis-negative strains.

Table 4. Mouse lethal activities of *B. cereus* strains isolated from 25 food poisoning outbreaks and various foods

Source	Number of strains tested	Number of strains positive in mouse lethality ^a (%)			
		0.1 ml	0.2 ml	0.4 ml	Survival
Diarrheal type	8 ^c (100)	3(38)	5(62)	0	0
(Reference strain)	2		2		
Vomiting type	42 ^c (100)	0	13(31)	21(50)	8(19)
(Reference strain)	1		1		
Various food	53 (100) ^d	— ^f	—	44(83)	9(17)
samples ^b	60 (100) ^e	—	—	52(87)	8(13)

a) A culture filtrate at a dose of 0.1 to 0.4 ml was injected into the tail vein of each mouse.

b) See the footnote of Table 2.

c) Two strains were isolated from the same outbreak.

d) Starch-hydrolysis-positive strains.

e) Starch-hydrolysis-negative strains.

f) Not examined.

of 0.1 ml and those of the other five were lethal at a dose of 0.2 ml. The culture filtrates of 13 strains obtained from 21 vomiting-type outbreaks killed mice at a dose of 0.2 ml, those of other 21 at a dose of 0.4 ml but not at 0.1 ml, and those of the other eight failed to kill mice at 0.4 ml (Table 4.).

Of the culture filtrates of 53 starch-hydrolysis-positive strains from the food samples, 44 (83.0%) killed mice at a dose of 0.4 ml; so did 52 (86.7%) of 60 culture filtrates of

starch-hydrolysis-negative ones at the same dose (Table 4.).

Three culture filtrates of eight diarrheal strains induced mild fluid accumulation in the mouse ileal loop (0.03–0.1 ml/loop); only one showed a strongly positive reaction (≥ 0.1 ml/loop). Those of 42 vomiting strains did not cause any fluid accumulation (Table 5.).

Six culture filtrates (11.3%) of 53 starch-hydrolysis-positive strains from the food

Table 5. Fluid accumulation in the ligated mouse ileal loop by *B. cereus* strains isolated from 25 food poisoning outbreaks and various foods

Source	Number of strains tested (%)	Number of FA positive strains (%)		
		Positive		Negative
		(+) ^{a)}	(++) ^{b)}	
Diarrheal type (Reference strain)	8 ^{d)} (100) 2	3(38) 1	1(12)	4(50) 1
Vomiting type (Reference strain)	42 ^{d)} (100) 1	0	0	42(100) 1
Various food samples ^{c)}	53 ^{e)} (100)	5(9)	1(2)	47(89)
	60 ^{f)} (100)	0	0	60(100)

Duplicate 0.1 ml volumes of a culture filtrate were injected into the ligated mouse ileal loops.

a) Weekly positive (0.03–0.1 ml/loop)

b) Strongly positive (≥ 0.1 ml/loop)

c) See the footnote of Table 2.

d) Two strains were isolated from the same outbreak.

e) Starch-hydrolysis-positive strains.

f) Starch-hydrolysis-negative strains.

Table 6. Fluid accumulation in the ligated rabbit ileal loop by *B. cereus* strains isolated from 25 food poisoning outbreaks and various foods

Source	Number of strains tested (%)	Number of FA positive strains (%)			
		Positive (ml/loop) ^{a)}			Negative
		0–1.0	1.1–5.0	5.1–10.0	
Diarrheal type (Reference strain)	8 ^{c)} (100) 2	1(12) 1	3(38) 1	1(12)	3(38)
Vomiting type (Reference strain)	21 ^{d)} (100) 1	2(10)	1(5)	0	18(85) 1
Various food samples ^{b)}	10 ^{e)} (100)	2(20)	1(10)	1(10)	6(60)
	5 ^{f)} (100)	0	0	0	5(100)

Duplicate 1.0 ml volumes of a 50 fold concentrated culture filtrate were injected into the ligated rabbit ileal loops.

a) Volume of fluid (ml) per ligated loop.

b) See the footnote of Table 2.

c) Two strains were isolated from the same outbreak.

d) One strain was isolated from the same outbreak.

e) Starch-hydrolysis-positive strains.

f) Starch-hydrolysis-negative strains.

samples induced fluid accumulation; those of 60 starch-hydrolysis-negative strains did not (Table 5.).

Five culture filtrates of the eight diarrheal strains induced fluid accumulation in the rab-

bit ileal loops; one showed a strongly positive reaction (5.1–10 ml/loop). Three culture filtrates of 21 vomiting strains induced fluid accumulation (≤ 5.0 ml/loop) in the ileal loops. Those of the reference strains from

the diarrheal type induced fluid accumulation, but that from the vomiting type did not (Table 6.).

Three culture filtrates of the 10 starch-hydrolysis-positive strains from the food samples induced fluid accumulation (≤ 5.0 ml/loop) in the ileal loops and five starch-hydrolysis-negative strains did not (Table 6.).

Culture filtrates of all strains from both food poisoning and the food samples showed hemolytic and egg yolk turbidity activities up to 80 to 640-fold dilutions.

DISCUSSION

Rabbit ileal loop and monkey feeding tests have provided evidence showing that the diarrheal- and vomiting-type illnesses caused by *B. cereus* are associated with two distinct toxins [7, 9, 13, 14, 18].

It has been reported that the diarrheal toxin caused fluid accumulation in the ligated rabbit ileal loop, VP reaction in the rabbit skin and lethality of mice. However, no comparison has been made among strains isolated from diarrheal-, vomiting-type food poisoning and the various food samples in the production of the extracellular toxins.

The culture filtrates of all *B. cereus* strains isolated from diarrheal-type outbreaks of food poisoning showed high VP activities, causing blueing zones of over than 15 mm, whereas all from vomiting-type outbreaks produced blueing zones of below than 14.9 mm. There was a significant difference in the VP activity between the two types.

Turnbull *et al.* [18] classified the VP activity into five categories according to the size of the blueing zone developed in the rabbit skin. They placed the filtrates of all strains isolated from vomiting-type illness in categories 1 to 3, with blueing zones of below than 14.9 mm. Five filtrates of eight strains from diarrheal-type illness were placed into category 1 or 2, with blueing zones of less than 9.9 mm. Thus, they found no relationship

between the diarrheal and VP activities. Glatz and Goepfert [5] stated that the activity of fluid accumulation in the rabbit ileal loop (diarrheal toxin) paralleled to the VP activity. This was the case in the present experiments, namely the diarrheal activity paralleled the VP activity.

Six (12%) of 50 strains from vomiting-type outbreaks showed moderate VP activities with blueing zones of 10–14.9 mm. In *B. cereus* food poisoning of vomiting type, the symptoms are usually nausea and vomiting, sometimes with diarrhea. This may suggest that some strains involved in vomiting-type outbreaks produce also the diarrheal toxin.

We found that most diarrheal strains were starch-hydrolysis-positive, while all from the vomiting strains were negative [12]. In the VP test with the strains from the food samples, the starch-hydrolysis-positive strains always had higher activities than did the starch-hydrolysis-negative ones. Twenty-two (41.5%) of 53 starch-hydrolysis-positive strains from the food samples showed high VP activities causing blueing zones of 15.0–24.9 mm. This may suggest that these strains, possessing the same activity as those from diarrheal-type outbreaks, have the potential to cause food poisoning.

Spira and Goepfert [14], Turnbull [15] and Turnbull *et al.* [18] have suggested that diarrheal, VP and mouse lethal activities are probably elicited by the same substance. However, our experiments indicated that the diarrheal strains showed high activities of both VP and mouse lethality, while the vomiting strains generally showed very low VP activity but high mouse lethal activity. For obtaining more definite informations, purification of the mouse lethal toxin produced by a strain isolated from a vomiting-type outbreak is now under way. Biological and immunological assays indicate that the mouse lethal toxins produced by the diarrheal and vomiting strains are distinct from each other (unpub-

lished data).

Bernheimer and Grushoff [1] reported that cereolysin has the mouse lethal activity. Therefore, it seems necessary to compare the strains isolated from vomiting-type outbreaks with the cereolysin-producing strains.

In addition, fluid accumulation in mouse and rabbit ileal loops was greater with the isolates from diarrheal-type outbreaks than with those from vomiting-type outbreaks. The time required for maximum fluid accumulation was 4–6 hr.

The purification of the diarrheal toxin seems to be a prerequisite for its chemical and biological characterization, and the further work along this line is in progress.

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要 約

食中毒事例から分離したセレウス菌の下痢原性および生物活性について：品川邦汎・松坂尚典・小沼博隆¹⁾・倉田 浩¹⁾（岩手大学農学部獣医公衆衛生学教室，¹⁾国立衛生試験所衛生微生物部）——下痢型食中毒4事例および嘔吐型食中毒21事例から分離したセレウス菌(59株)の培養遠心上清について，ウサギ皮内血管透過性亢進(VP)，マウスおよびウサギ結紮腸管における液体貯留(MIL および RIL) およびマウス致死(MLT)の各活性を調べた。下痢型由来菌(下痢)株のほとんど(9株中7株)が澱粉分解陽性であるのに対し，嘔吐型由来菌(嘔吐)株は全て陰性であった。下痢株のVP活性(全て15mm以上のblueing zoneを形成)は，嘔吐株(全て14.9mm以下)より明らかに強かった。MLT活性は，下痢株のすべてがマウスを致死(0.1~0.2 ml *i.v.*)させ，嘔吐株でも80%が致死(0.2~0.4 ml *i.v.*)を示した。MIL活性は，下痢株では50%が陽性を示したが，嘔吐株では100%陰性であった。また，50倍濃縮した試料のRIL活性は，下痢株で62%が，嘔吐株では15%が陽性を示した。