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ISOLATION AND USE OF EXPERIMENTAL PHAGES FOR TYPING *BACILLUS CEREUS*

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Abstract

Attempts to isolate phages for typing *Bacillus cereus* were made. Ten phages were isolated from the lysogenic strains and the primary cultures of *B. cereus* isolates, and used for typing of 858 strains isolated from foods, spices, animals, soils, and food poisoning incidents. A hundred and twelve (13.1%) of the 858 strains were typable at either routine test dilution (RTD) or 100 × RTD, and could be differentiated into 43 phage patterns.

Introduction

Bacillus cereus is a gram-positive and endospore-forming bacillus. This organism is widely distributed in soils and waters, and commonly considered to be a harmless saprophyte under most circumstances. As far back as 1906, *B. cereus* has been recognized as an etiologic agent of food poisoning outbreaks[2]. There are two distinct forms of gastroenteritis characterized either by diarrhea and abdominal pain 8 to 16 hours after ingestion of the contaminated food or by nausea and vomiting with an incubation of <1 to 5 hours[11]. A variety of foods have been involved in the diarrheal type syndrome, including soups, cooked meat, poultry and vegetables, dessert dishes and sauces. In contrast, the vomiting type syndrome has been associated almost exclusively with the consumption of cooked rice. In addition to food poisoning incidents, *B. cereus* has been implicated in various human infections [1,3,4,7,8] such as septicemia, pneumonia, and meningitis, and bovine mastitis[5]. Thus, much attentions are given to this organism at the present days.

Methods for subdividing pathogenic organisms are necessary for the epidemiologic, epizootiologic, or ecologic studies. With regard to *B. cereus*, serotyping[9,10] and biotyping[6] have been examined for that purpose. However, no efficient method has been established. This paper deals with the isolation and use of experimental phages for typing *B. cereus* to take a preliminary step toward the establishment of a phage typing scheme for subdividing *B. cereus*.

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Materials and Methods

Bacterial strains

A total of 858 strains of *B. cereus* isolated from foods, spices, animals, soils and food poisoning incidents were used. All strains were stocked at room temperature in nutrient agar slant cultures consisting of 10 g of beef extract (Mikuni Chemical Co., Tokyo), 10 g of polypeptone (Daigo Nutritive Chemical Co., Osaka), 2 g of NaCl, 15 g of powdered agar (Nissui Seiyaku, Tokyo) and 1,000 *ml* of distilled water, at pH 7.2.

Induction of phages

The induction of phages was done as follows. Namely, 0.5 *ml* of an overnight culture of *B. cereus* in nutrient broth was inoculated into 4.5 *ml* of nutrient broth containing 0.5 *μg/ml* of mitomycin C (Kyowa Hakko Co., Tokyo). After incubation for 4 hours at 30°C, the culture was centrifuged 5,000×g for 15 minutes. The supernatant fluid was examined for the presence of free phages.

Detection of lysogenic strains

Lysogenic strains were detected by plaque formation on agar plate. Overnight nutrient

broth culture of an indicator strains was seeded on a nutrient agar plate. Then the supernatant fluid to be tested was spotted on the plate. The plate was incubated for 18 hours at 30°C. The presence of phages was shown by appearance of lysis at the site of spotted culture.

Isolation of phages

The supernatant fluid containing phages was spread over an agar plate with a susceptible strain and incubated for 18 hours at 30°C. When plaques were formed, an isolated plaque was fished and transferred into 0.5 *ml* of nutrient broth to serve for the next passage. After 7 passages, plaques were harvested with 4.5 *ml* of nutrient broth. The broth was then centrifuged at 5,000×g for 15 minutes. The supernatant fluid was filtrated through a membrane filter (450 nm, Millipore Corporation, Bedford, Ma.), giving the filtrate as a phage suspension. The phage was serially propagated until a satisfactory titer of 1,000 times of routine test dilution (RTD) or more. Phages were stored at 4°C. Isolation of free phages from the primary cultures of *B.cereus* isolates was attempted in addition. Overnight nutrient broth culture of a sample which was used for the isolation of *B. cereus* was centrifuged at 1,000×g for 30 minutes. The supernatant fluid was examined for the presence of phages. The detection and isolation of phages were done as described above.

Phage typing

Phage typing was performed at RTD. Strains untypable at RTD were retested at 100×RTD. Technical procedures were as follows. Nutrient broth culture of a strain to be tested was incubated for 18 hours at 30°C, and 0.2 *ml* of the broth was seeded on a nutrient agar plate. A drop (0.02 *ml*) of each phage was then applied to the seeded plate. The plate was incubated for 18 hours at 30°C. Lytic reactions were recorded according to the following criteria : confluent lysis, CL; more than 50 plaques to near-confluent lysis, ++; 20 to 50 plaques, +; less than 20 plaques, ±; inhibition, 0; no reaction, −. Strains showing strong lysis, ++ and CL were regarded as typable.

Results

Incidence of lysogenic strains

Seventy-two strains isolated from foods and spices were examined for lysogenicity. As indicator strains, 98 strains isolated from foods, spices, and soils, including the 72 strains to be tested for lysogenicity, were used. In this way, a total of 6,984 (72 × 97) combinations were made. Of the 72 strains, 21 (29.2 %) were proved to be phage carriers. Fourteen (14.3%) of the 98 indicator strains were shown to be susceptible to any of the phages released from the carriers. Strains showing the highest propagating ability to each phage derived from the lysogenic strains were selected as the propagating strains.

Isolation of free phages from the primary cultures of *B. cercus* isolates

Nutrient broth cultures of 414 samples of foods and spices, which were used for the isolation of *B. cereus*, were examined for the presence of free phages. Fifty-three strains isolated from foods, spices, animals, soils, and food poisoning incidents were used as indicator strains. Of the 414 specimens, 44 (10.6%) provided phages showing plaques with 4 indicator strains. The propagating strains were selected from the viewpoint of the phages' propagation ability.

Selection of typing phages

From the 65 phages isolated from the primary cultures of *B. cereus* and the lysogenic strains, 10 phages were selected by judging their activity range, stability, propagation ability and degree of lysis. The 10 phages were designated C1-10, and arranged for phage typing. Lytic spectrums of the 10 phages against their propagating strains are shown in Table 1.

Phage typing of *B. cereus*

A total of 858 strains of *B. cereus* were subjected to typing with the experimental phages (Table 2). A hundred and twelve (13.1%) of the 858 strains were typable at either RTD or 100×RTD, and could be differentiated into 43 phage patterns. Of the typable strains, 44 (39.3 %) were typed with one or more of the phages at RTD, and 68 (60.7%) at 100 × RTD. The remaining 746 strains were resistant to lysis due to the experimental phages at RTD

Table 1. Lytic spectrums of experimental *B. cereus* phages

Propagating strain*1	Phage									
	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
B1	5*2	5	5	5	5	5	5	5	5	—
B5	—	—	—	—	5	4	4	3	—	—
B6	—	—	—	—	—	5	4	2	4	—
B7	—	—	—	—	—	5	5	5	5	—
B8	—	—	—	—	—	5	5	5	5	—
B9	—	—	—	—	—	—	—	—	5	—
B10	—	—	—	—	—	—	—	—	—	5

*1 The propagating strain of phages C1-4 is B1. The other propagating strains correspond with the number of the phages.

*2 5 = titer equal to that on propagating strain ; 4 = titer 10^1 to 10^2 times less than that on propagating strain ; 3 = titer 10^3 to 10^4 times less than that on propagating strain ; 2 = titer 10^5 to 10^6 times less than that on propagating strain ; 1 = weak reaction with undiluted phage ; — = no reaction.

or 100×RTD.

Discussion

For subdividing *B. cereus*, two methods have been examined, serotyping and biotyping. TAYLOR and GILBERT[9] developed a provisional serotyping scheme based on the strain specificity of the flagellar (H) antigen. They reported that 132 (96.4%) of the 137 strains isolated from food poisoning incidents and 28 (42.2%) of the 66 strains isolated from uncooked rice were typable with sera of the 18 H serotypes. TERAYAMA *et al.*[10] found that 276 (22.5%) of the 667 strains isolated from foods were typable with the 18 typing sera of TAYLOR and GILBERT, and added 12 H serotypes with the result that 276 (41.4%) of the 667 strains were typable. Serotyping is the most prevalent method for classifying *B. cereus* at the present time. However, this method has some disadvantages, *e.g.* many typing sera are needed, the procedure for preparation of the antigen is complicated and a number of strains remain untypable. On the other hand, KOZASA *et al.*[6] proposed a biotyping scheme based on the 12 biotypes according to the 9 biological characteristics. However, efficacy of the biotyping scheme has not been evaluated. The authors performed the biotyping of the 858 strains used in the present study, and found that 479

Table 2. Phage typing of *B. cereus* with experimental phages

Phage pattern	No. of strains
C9	21*1 (16)*2
C2	13 (8)
C6	10 (9)
C2/C6	8 (8)
C1/C2/C3	6
C6/C8	4 (3)
C6/C7/C8/C9	3
C4	3 (1)
C2/C6/C8	3 (3)
C1/C2	3 (3)
C1/C2/C3/C6/C7	2
C5/C6/C7/C8	2
C6/C7	2
C1/C2/C6	2 (1)
C7	2 (1)
C1/C2/C3/C4/C5/C6/C7/C8/C9, C1/C2/C7, C1/C2/C3/C6/C7/C8, C1/C2/C3/C7, C1/C2/C4/C6, C1/C2/C6/C7, C2/C6/C7, C2/C7/C8, C6/C7/C8, C5/C8, C6/C9, C7/C9, C10	13
C1/C2/C4/C6/C7/C8, C1/C2/C4/C6/C7, C1/C2/C6/C7/C8, C1/C2/C3/C6, C1/C2/C3/C9, C1/C2/C4/C8, C1/C2/C8, C1/C2/C5/C8, C1/C2/C6/C8, C2/C6/C9, C4/C6/C8/C9, C2/C5, C2/C8, C4/C9, C8	15 (15)
Untypable	746

*1 Number of strains typable at RTD of 100×RTD.

*2 Number of strains typable only at 100×RTD shown in parentheses.

(55.9%) strains were untypable and could be differentiated into 61 patterns (data are not shown).

Phage typing is one of the prevalent methods employed for a detailed characterization of pathogenic bacteria such as *Staphylococcus* and *Salmonella*. Attempts to establish a phage typing of *B. cereus* have been seldom made. In the present study, the authors isolated the phages from the lysogenic strains and the primary cultures of *B. cereus* isolates, and used them experimentally in the phage typing of *B. cereus* isolated from various specimens. The typability of the isolates were considerably low on account that the lytic range of the experimental phages seemed to be highly limited. Phages that would have more broad lytic ranges, such as phage W and phage 201[2], are needed for the development of phage typing of *B. cereus*. In any case, it is anticipated that the experimental phages and the typing method employed in the present study will be of value in the establishment of phage typing of *B. cereus*.

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セレウス菌ファージの分離とファージ型別

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要　約

セレウス菌（*Bacillus cereus*）ファージの分離を溶原菌および分離菌の初代培養の培養上清について試みた。その結果，10株のファージが分離された。これらのファージを用いて，食品，香辛料，動物，土壌および食中毒由来の計858株の型別を行ったところ，112株（13.1%）が型別され，43種のファージパターンに区分された。