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ORIGINAL ARTICLE

Characterization and complete genome sequence of a virulent bacteriophage B4 infecting food-borne pathogenic *Bacillus cereus*

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Abstract *Bacillus cereus* causes food poisoning, resulting in vomiting and diarrhea, due to production of enterotoxins. As a means of controlling this food-borne pathogen, the virulent bacteriophage B4 was isolated and characterized. Bacterial challenge assays showed that phage B4 effectively inhibited growth of members of the *B. cereus* group as well as *B. subtilis*, and growth inhibition persisted for over 20 h. One-step growth analysis also revealed the host lysis activity of phage B4, with relatively short eclipse/latent times (10/15 min) and a large burst size (>200 PFU). The complete genome of phage B4, containing a 162-kb DNA with 277 ORFs, was analyzed. The endolysin encoded by the phage B4 genome accounts for the cell lysis activity of this phage. These results suggest that phage B4 has potential as a biological agent to control *B. cereus* propagation.

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Introduction

Bacillus cereus is a food-borne pathogen that produces enterotoxins such as hemolysin BL (HBL), nonhemolytic enterotoxin (NHE), and cytotoxin K [20]. Due to these toxins, the uptake of contaminated foods can cause vomiting, diarrhea, and nausea [5, 15]. Generally, a large number of *B. cereus* cells (10^4 to 10^9 CFU per gram of contaminated food) is required for a disease outbreak [24]. However, responses to infection in humans such as diarrheal syndrome (8 to 16 h) and emetic syndrome (0.5-6 h) are relatively quick, due to toxins produced and released by *B. cereus* [8, 24]. Outbreaks of *Bacillus* species in the European Union were reported to account for up to 1.4 % of all food-borne pathogenic outbreaks in 2005 [2], and the number of *B. cereus* outbreaks has recently been increasing in developed countries, highlighting the importance of controlling *B. cereus* levels in foods [9, 17, 20].

The antibiotic resistance of *B. cereus* has been reported. Previous studies showed that *B. cereus* is generally insusceptible to penicillin-related antibiotics due to production of β-lactamase, and sometimes even to erythromycin and tetracycline [18, 31]. Therefore, an alternative bacteriophage approach has been suggested to be effective for reducing the amount of this pathogen in various foods [10]. Due to the positive potential for application of bacteriophages in foods, some bacteriophages infecting *B. cereus* have been studied and reported [3, 12, 19, 23, 32]. Two phages with highly specific host ranges, FWLBc1 and FWLBc2, were isolated and characterized to develop biocontrol agents for use in foods [23]. Bandara et al. [3] reported that divalent cations such as Ca^{2+}, Mg^{2+} or Mn^{2+} are required to enhance the host lysis activity of the bacteriophage in fermented foods. Interestingly, about 40 % of fermented foods contain *B. cereus*-infecting phages,

suggesting that *B. cereus* is prevalent in fermented foods, where bacteriophages probably inhibit its growth and consequently limit contamination [32].

To inhibit the growth of *B. cereus*, a novel bacteriophage B4 was isolated from an environmental sample and characterized using a host-range test, a bacterial challenge assay, and one-step growth curve analysis. In addition, the whole genome of phage B4 was completely sequenced and analyzed. In this report, we describe a novel potential biocontrol agent, bacteriophage B4, and provide genomic information about this phage for further applications in foods.

Materials and methods

Bacterial strains and growth conditions

B. cereus ATCC 10876 was used as an isolation and propagation host for bacteriophage B4. Bacterial strains used for the host-range test are described in Table 1. All of the bacterial strains were grown at 37 °C with shaking in Luria-Bertani (LB) broth medium (Difco, Detroit, MI, USA).

Isolation and propagation of bacteriophage B4

Mud samples from Seoul Grand Park (Gwacheon, South Korea) were used to screen for bacteriophages that infect *B. cereus*, using strain ATCC 10876 as a host. In the case of solid samples, 25 g of the sample was homogenized in 225 ml of sterile Butterfield's phosphate-buffered dilution water (0.25 M KH_2PO_4, pH 7.2) with a blender (Bac-Mixer 400; Interscience Laboratory Inc., St. Nom, France). After homogenization, 25 ml of each homogenized sample was added to 25 ml of 2× LB broth, and the mixture was incubated for 12 h at 37 °C with shaking at 220 rpm. The incubated culture was centrifuged at 9,000×*g*, 4 °C for 10 min, and the supernatant was filtered to remove bacterial cells using 0.22-μm-pore-size filters (Millipore, Billerica, MA). Ten milliliters of the filtrate was mixed with 50 ml LB broth containing 1 % overnight culture of *B. cereus* ATCC 10876 (final concentration), and the mixture was incubated at 37 °C for 12 h with shaking. After incubation, the mixed culture was centrifuged at 9,000×*g*, 4 °C for 10 min, and the supernatant was filtered again to remove bacterial cells using 0.22-μm-pore-size filters (Millipore). In order to confirm the presence of bacteriophages in the filtered supernatant, tenfold serial dilutions of the filtrate were spotted on molten 0.4 % LB soft agar containing 1 % *B. cereus* ATCC 10876 (final concentration). The plates were incubated overnight at 37 °C and monitored for formation of plaques. Each single plaque was picked with a sterile tip and eluted in 1 ml of sterilized sodium chloride-magnesium sulfate (SM) buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgSO_4 \cdot 7H_2O$). This purification step was repeated at least three times.

A culture of *B. cereus* ATCC 10876 (OD_{600} = 1.0) was infected with bacteriophages at a multiplicity of infection (MOI) of 1 and incubated at 37 °C with shaking until the culture became clear. Cell debris was removed by subsequent centrifugation at 9,000×*g* for 10 min and filtration using 0.22-μm-pore-size filters, and phage particles were precipitated by treatment with polyethylene glycol (PEG) 6,000 (Sigma, St. Louis, MO, USA). Finally, cesium chloride (CsCl) density gradient ultracentrifugation (Himac CP 100β, Hitachi, Japan) with different CsCl steps (step density = 1.3, 1.45, 1.5 and 1.7 g/ml) was carried out at 78,500×*g*, 4 °C for 2 h. Viral particles were recovered and dialyzed with stirring for 1 h at 4 °C using a Spectra/Por 4 dialysis membrane tube (Spectrum, Rancho Dominguez, CA, USA) and SM buffer.

Table 1 Host range of bacteriophage B4

Bacterial isolate	Plaque formation[a]	Source[b] or reference
Gram-positive bacteria		
Bacillus cereus ATCC 10876	CC	ATCC
Bacillus cereus ATCC 13061	C	ATCC
Bacillus cereus ATCC 14579	C	ATCC
Bacillus cereus ATCC 21768	C	ATCC
Bacillus cereus ATCC 27348	CC	ATCC
Bacillus subtilis ATCC 23857	CC	ATCC
Bacillus mycoides ATCC 6462	CC	ATCC
Bacillus thuringiensis subsp. *kurstaki* ATCC 35866	CC	ATCC
Bacillus thuringiensis ATCC 29730	CC	ATCC
Enterococcus faecalis ATCC 29212	-	ATCC
Staphylococcus aureus ATCC 29213	-	ATCC
Staphylococcus epidermis ATCC 35983	-	ATCC
Listeria monocytogenes ATCC 19114	-	ATCC
Gram-negative bacteria		
Samonella enterica serovar Typhimurium LT2	-	[26]
Salmonella enterica serovar Enteritidis ATCC 13076	-	ATCC
E. coli K-12	-	[16]
Shigella flexineri 2a strain 2457T	-	[35]

[a] CC, EOP 1 to 0.1; C, EOP 0.1 to 0.001; -, not susceptible to phage B4

[b] ATCC, American Type Culture Collection

Bacteriophage host range

One hundred microliters of each test bacterial culture in the stationary phase was added to 5 ml of the molten 0.4 % LB agar, and the mixture was overlaid onto a 1.5 % LB agar plate. Then, 10 µl of each serially diluted B4 phage suspension from 10^2 to 10^{11} PFU/ml was spotted on the overlaid plates, and these plates were incubated at 37 °C. After incubation, appropriate titers forming single plaques were selected, and the sensitivity of test bacteria to B4 phage was determined. The efficiency of plating (EOP) was calculated and determined from comparison of titers between the selected test bacterium and the host strain *B. cereus* ATCC 10876.

Transmission electron microscopy (TEM)

Diluted CsCl-purified bacteriophage B4 in SM buffer was applied to carbon-coated copper grids and negatively stained with 2 % aqueous uranyl acetate (pH 4.0) for 2 min. Electron microscopy of prepared samples was carried out using a transmission electron microscope (LIBRA 120, Carl Zeiss, Switzerland) at 80 kV. Bacteriophage B4 was identified, and the family to which it belongs was determined using the guidelines of the International Committee on Taxonomy of Viruses, based on phage morphology [13].

Bacterial challenge assay

An exponentially growing *B. cereus* ATCC 10876 culture was infected with phage B4 (MOI = 1.0) to confirm its lytic activity. After adding phages to *B. cereus* cultures ($OD_{600\ nm} = 1.0$), the optical density was monitored at 600 nm every hour, and the culture without phage infection was used as a control.

One-step growth curve

When the $OD_{600\ nm}$ of the culture of the same reference strain reached 1.0, 50 ml of the culture was harvested. B4 phage was added at an MOI of 0.01 and allowed to be adsorbed for 5 min at room temperature. The mixture was centrifuged, and the supernatant was discarded to remove the residual phage. The cell pellet was then resuspended with the same volume of fresh LB broth medium, and the resuspended culture was further incubated at 37 °C with shaking. Two sets of samples were collected every 5 min. These two sets of samples were immediately diluted and plated for phage titration. However, in order to determine the eclipse period, the second set of samples was treated with 1 % chloroform to release intracellular phages before the titration. The latent period and burst size were determined based on PFU number per cell.

Isolation and purification of bacteriophage genomic DNA

Bacteriophage genomic DNA was isolated from the phage lysate as described previously by Wilcox et al. [36]. Before purification of phage genomic DNA, the phage lysate was treated with DNase and RNaseA at 37 °C for 1 h to remove bacterial DNA and RNA, respectively. The phage lysate was then treated with lysis buffer (final concentration, 0.5 % sodium dodecyl sulfate, 20 mM EDTA and 50 µg/ml proteinase K) for 2 h at 56 °C. A standard phenol-chloroform DNA purification and ethanol precipitation were carried out [30].

Genome sequencing of bacteriophage B4 and bioinformatics analysis

Extracted B4 phage DNA was sequenced using a Genome Sequencer FLX (GS-FLX) Titanium sequencer (Roche, Mannheim, Germany) and assembled with GS *de novo* assembler software (Roche) at Macrogen Inc., South Korea. Prediction of open reading frames (ORFs) was carried out using GeneMarkS [4], Glimmer v3.02 [11] and FgenesB software (Softberry, Inc. Mount Kisco, NY) and confirmed using RBSfinder (J. Craig Venter Institue, Rockville, MD). Prediction of tRNA genes was carried out using the tRNAscan-SE program [25]. Annotation of ORFs was performed using the BLASTP [1] and InterProScan programs [37]. The complete genome sequence and its annotation data were handled and edited using Artemis14 [7]. Evolutionary phylogenetic analysis of phage B4 was conducted using MEGA5 by the neighbor-joining method [21].

Results and discussion

Isolation and characterization of bacteriophage B4

Bacteriophage B4 was isolated from a mud sample as a clear-plaque former against strain *B. cereus* ATCC 10876. TEM analysis revealed that phage B4 had an isometric head with a nonflexible and contractile tail, suggesting that it belongs to the family *Myoviridae* (Fig. 1). The diameters of the isometric head and tail were about 85 nm and 21 nm, and the non-contracted and contracted tail lengths were about 213 nm and 101 nm, respectively (Fig. 1). Morphological comparison of phage B4 and other *B. cereus* phages in the family *Myoviridae* [3, 12] showed that the head size of phage B4 is smaller than those of BCP1-1 and BCP8-2 (>95 nm) but similar to that of phage Bc431v3 (85.4 ± 3 nm). However, the tail size of phage B4 is longer than that of phage Bc431v3 (180 ± 3 nm) but similar to those of phages BCP1-1 and BCP8-2 (220 nm and 210 nm), suggesting that phage B4 has a relatively small head

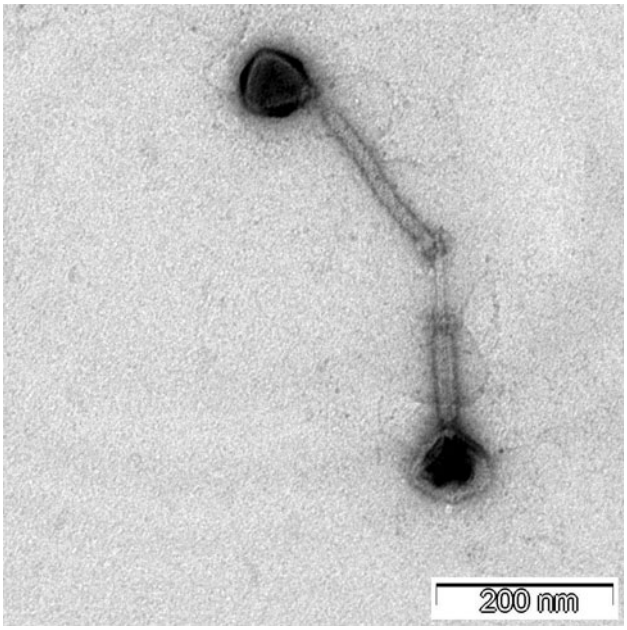

Fig. 1 Transmission electron microscopy of bacteriophage B4 revealing that it belongs to the family *Myoviridae*

and a long tail. A host range test of phage B4 revealed that phage B4 has a relatively broad inhibition range against members of the *B. cereus* group (*B. cereus, B. thuringiensis, B. mycoides*) and even *B. subtilis* (Table 1).

Bacterial challenge assay

A bacterial challenge assay performed in liquid culture showed bacterial growth inhibition by phage B4. When phage B4 was added to exponentially growing *B. cereus* ATCC 10876, a reduction in $OD_{600\ nm}$ was already observed within the first 30 min (Fig. 2). The growth inhibition activity was maintained for more than 20 h, indicating that the rate of emergence of phage-resistant bacteria is low.

One-step growth curve of bacteriophage B4

The eclipse and latent periods as well as the burst size of the B4 phage were determined by one-step growth curve analysis with *B. cereus* ATCC 10876 (Fig. 3). The eclipse and latent periods of B4 phage were 10 min and 15 min, respectively. The burst size was more than 200 plaque-forming units (PFU) per infected host cell. The short latent period with large burst size indicates host lysis activity and propagation of this phage.

Genomic analysis of bacteriophage B4

The complete genome of *B. cereus* bacteriophage B4 is 162,596 bp long with a GC content of 37.71 %. A total of

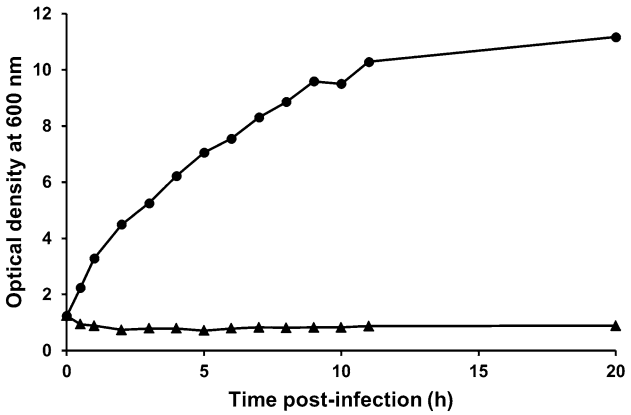

Fig. 2 Bacterial challenge assay of phage B4 against *B. cereus* ATCC 10876 at a multiplicity of infection (MOI) of 1.0. The closed circle indicates non-phage-treated *B. cereus* ATCC 10876, and the closed triangle indicates phage-treated *B. cereus* ATCC 10876

277 ORFs were identified, but no tRNA genes were detected (Fig. 4). The functional ORFs of phage B4 were categorized into eight groups, including packaging, lysis, regulation, host interaction, structure, DNA replication, RNA metabolism, and additional function, and these are listed in Table 2. The phage genome encodes all necessary phage structural proteins, including major capsid protein, structural protein, minor structural protein, portal protein, and several tail proteins (tail fiber protein, tail-lysins, tail sheath protein, putative tail protein, and baseplate proteins). In addition, this genome encodes many DNA replication proteins such as DNA polymerases, primase, helicases, exonucleases, and recombinase, implying that these proteins may collaborate with the host DNA replication proteins in replicating the phage genome. Interestingly, this genome also encodes its own putative sigma factors, including SigF-like protein (BCB4_0143) and phage RNA polymerase sigma factor (BCB4_0181). Although SigF is known to be involved in transcription of specific genes for sporulation, the protein sequence of BCB4_0143 is less than 25 % identical to those of other known *B. cereus* host SigF proteins, suggesting that its role in host sporulation cannot be deduced [12]. However, phage RNA polymerase sigma factor is relatively similar to other phage RNA polymerase sigma factors (40 % protein sequence identity to that of *Enterococcus* phage phiEF24C) but shows no homology to *B. cereus* host RNA polymerase sigma factors, suggesting that this sigma factor may play a role in transcription of phage genes. The functions of the sigma factors in these phages are not yet clearly understood, so further experiments are needed to characterize them. Furthermore, the phage genome also encodes many additional functional genes, such as metallophosphoesterase, ribonucleotide-diphosphate reductases, flavodoxin, thioredoxin, thymidylate synthase, dephospho-CoA kinase,

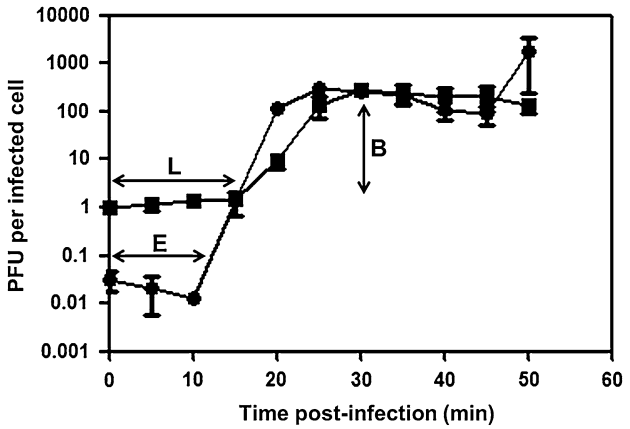

Fig. 3 One-step growth curve analysis of *B. cereus* ATCC 10876 infected by B4 phage. E, eclipse period; L, latent period; B, burst size. The closed circle indicates a chloroform-treated sample and a closed square indicates an untreated sample

and dihydrofolate. While their roles in the phage or in the host are not yet clearly understood, a few enzymes such as ribonucleotide diphosphate reductases and thymidylate synthase were previously suggested to be involved in preparation of nucleotides for DNA synthesis [27]. Additional evolutionary phylogenetic analysis of major capsid proteins (MCPs) of *Bacillus* bacteriophages revealed that phage B4 is closely related to other *Bacillus* phages such as Bastille (NC_018856), BPS13 (NC_018857) and BCP78 (NC_018860, [22]) phages in the family *Myoviridae* but quite different from those of the families *Siphoviridae* and *Podoviridae*, consistent with the previous morphological observation (Fig. 5). Furthermore, this genome does not have genes associated with toxin production and bacterial virulence, suggesting the possibility of phage application to control pathogenic *B. cereus*.

Host lysis by bacteriophage B4

The phage B4 genome encodes an endolysin (BCB4_0006) and a putative holin (BCB4_0179). While their respective genes lie unusually distant from each other in the genome [14, 28, 34], the highly lytic activity of phage B4 is most likely to be attributed to both enzymes. Interestingly, the endolysin of phage B4 (LysB4) has two conserved protein domains, PF08460 for cell wall binding and PF02557 for cell wall lysis [33]. These conserved protein domains have frequently been found in other bacteriophage endolysins against *Bacillus* and *Listeria*. Furthermore, LysB4 was experimentally characterized as an L-alanoyl-D-glutamate endopeptidase, showing optimum temperature and pH are 50 °C and 8.5, respectively [33]. The complete genome sequence of phage B4 also included two genes, BCB4_0248 and BCB4_0249, encoding putative tail lysins 1 and 2. Conserved protein analysis of these two tail lysins showed that they may function in host lysis. Tail lysin 1 has two conserved domains, cd04129 (encoding Rho2 probably related to cell wall construction) and PF00877 (encoding cell wall-associated hydrolase probably related to cell wall lysis). Tail lysin 2 also has a conserved domain, PF002901, coding for an endo-β-N-acetylglucosaminidase, which is probably involved in peptidoglycan hydrolysis. However, it is necessary to experimentally confirm that these tail lysins are really involved in host lysis. The broad

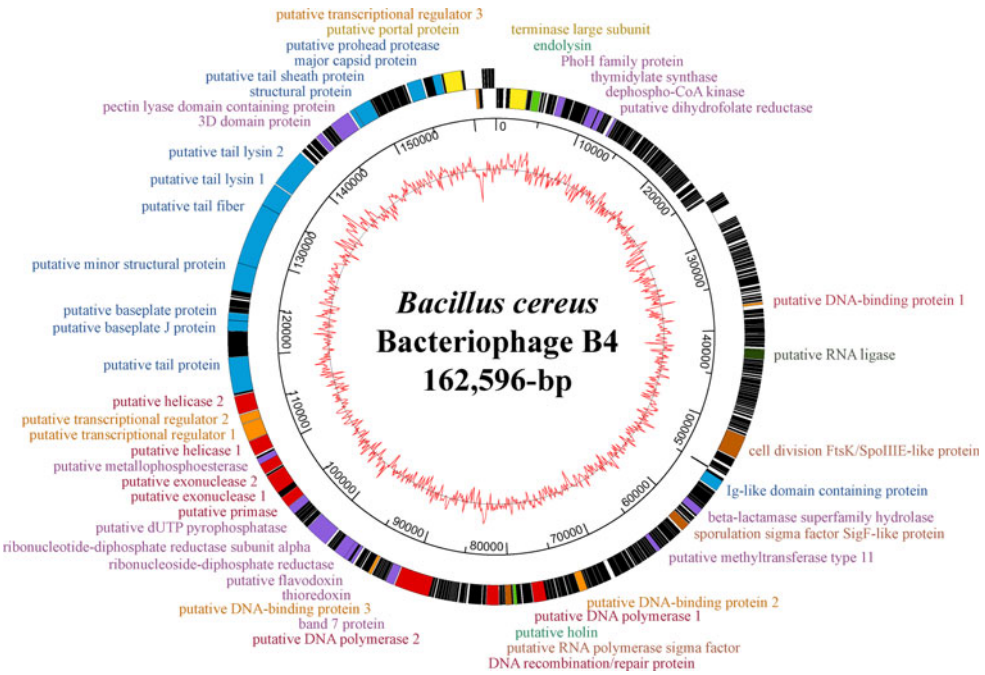

Fig. 4 Genome map of phage B4. The outer circle indicates the gene coding regions by strand. The color of each gene indicates its functional category: phage structure (blue), regulation (orange), host-phage interaction (brown), replication (red), cell lysis (green), packaging (yellow), and additional function (purple). The inner circle with a red line indicates the G+C content. The scale unit is one base pair (color figure online)

Table 2 Functional grouping of predicted ORFs in the bacteriophage B4 genome

Functional group	Predicted function	Locus_tag
Packaging	Terminase large subunit	BCB4_0004
	Putative portal protein	BCB4_0270
Lysis	Endolysin	BCB4_0006
	Putative holin	BCB4_0179
Regulation	Putative DNA-binding protein 1	BCB4_0089
	Putative DNA-binding protein 2	BCB4_0168
	Putative DNA-binding protein 3	BCB4_0208
	Putative transcriptional regulator 1	BCB4_0234
	Putative transcriptional regulator 2	BCB4_0235
	Putative transcriptional regulator 3	BCB4_0272
Host interaction	Cell division FtsK/SpoIIIE-like protein	BCB4_0127
	Sporulation sigma factor SigF-like protein	BCB4_0143
	Putative RNA polymerase sigma factor	BCB4_0181
Structure	Ig-like domain containing protein	BCB4_0133
	Putative tail protein	BCB4_0238
	Putative baseplate J protein	BCB4_0240
	Putative baseplate protein	BCB4_0241
	Putative minor structural protein	BCB4_0246
	Putative tail fiber	BCB4_0247
	Putative tail lysin 1	BCB4_0248
	Putative tail lysin 2	BCB4_0249
	Structural protein	BCB4_0258
	Putative tail sheath protein	BCB4_0259
	Putative capsid protein	BCB4_0266
	Putative prohead protease	BCB4_0268
DNA replication	Putative DNA polymerase 1	BCB4_0176
	DNA recombination/repair protein	BCB4_0184
	Putative DNA polymerase 2	BCB4_0200
	Putative primase	BCB4_0226
	Putative exonuclease 1	BCB4_0228
	Putative exonuclease 2	BCB4_0230
	Putative helicase 1	BCB4_0233
	Putative helicase 2	BCB4_0236
RNA metabolism	Putative RNA ligase	BCB4_0100
Additional function	PhoH family protein	BCB4_0011
	Thymidylate synthase	BCB4_0015
	Dephospho-CoA kinase	BCB4_0017
	Putative dihydrofolate reductase	BCB4_0021
	Beta-lactamase superfamily hydrolase	BCB4_0138
	Putative methyltransferase type 11	BCB4_0151
	Band 7 protein	BCB4_0201
	Thioredoxin	BCB4_0212
	Putative flavodoxin	BCB4_0214
	Ribonucleoside-diphosphate reductase	BCB4_0215
	Ribonucleotide-diphosphate reductase subunit alpha	BCB4_0219
	Putative dUTP pyrophosphatase	BCB4_0225
	Putative metallophosphoesterase	BCB4_0231
	3D domain protein	BCB4_0253
	Pectin lyase domain containing protein	BCB4_0257

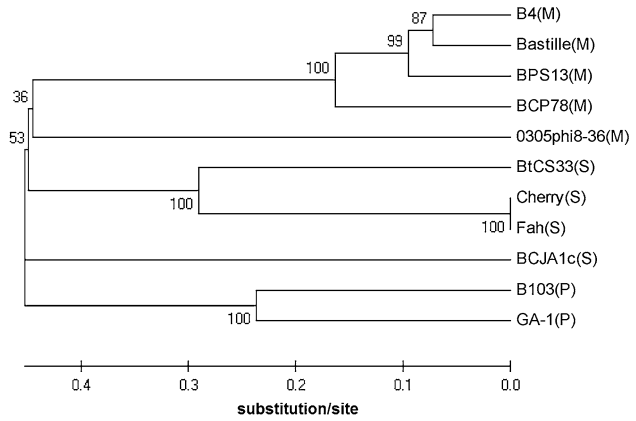

Fig. 5 Phylogenetic tree of major capsid proteins (MCPs) in *Bacillus* bacteriophages. MCPs were compared by ClustalW multiple alignments, and a phylogenetic tree was generated with the MEGA5 program using the neighbor-joining method with *P*-distance values. (M), *Myoviridae*; (S), *Siphoviridae*; (P), *Podoviridae*

host range and host lysis activity of phage B4 suggest that phage B4 can be a candidate as a novel biocontrol agent with its relatively high lysis activity against *B. cereus*.

Conclusion

Due to its toxin production and antibiotic resistance, contamination with *B. cereus* is of increasing concern in the food industry, and control methods other than classical antibiotic treatment are urgently needed. A bacteriophage-based approach has been suggested to control this kind of food-borne pathogen, and one of bacteriophage applications (ListShield^{TM} for control of *Listeria* in foods) has been approved by the FDA [6, 29]. To develop a novel biocontrol agent against *B. cereus*, bacteriophage B4 with relatively high host lysis activity was newly isolated and characterized. Subsequent complete genome sequence analysis of phage B4 revealed no genes associated with bacterial virulence or toxins and the presence of the host lysis system, suggesting that this phage may be suitable for host control. In this report, we suggest phage B4 as a possible biological candidate to control *B. cereus*.

Nucleotide sequence accession number

The complete genome sequence of *B. cereus* phage B4 is available in the GenBank database under accession number JN790865.

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