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Characterization and genome analysis of the *Bacillus cereus*-infecting bacteriophages BPS10C and BPS13

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Abstract Due to the emergence of antibiotic-resistant strains, bacteriophages are considered to be an alternative approach for the control of pathogens. In this study, the bacteriophages BPS10C and BPS13 were isolated and characterized to investigate their ability to control food-borne pathogenic *Bacillus cereus*. Phage BPS13 exhibited slightly higher host lysis activity compared with phage BPS10C. In addition, phage BPS13 exhibited greater stability under various pH and temperature conditions. To extend our knowledge of the lysis of *B. cereus* by these phages, their genomes were completely sequenced and analyzed, revealing that these phage genomes encode endolysin and two tail lysins, which are likely involved in host lysis and invasion mechanisms, respectively. These lysis-related proteins may increase the bactericidal activities of these phages, suggesting that they may be good candidates for the potential control of *B. cereus*.

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Bacteriophages are bacteria-specific viruses that can lyse the host bacteria [3]. Due to their host lysis activity, bacteriophages have been used for the control of specific bacteria in research and various other applications. Currently, bacteriophages are being studied to determine their potential as novel biocontrol agents against food-borne pathogens and antibiotic-resistant strains [4]. These approaches are quite beneficial because phages target specific pathogens without affecting other beneficial bacteria in foods or in humans [13], as supported by the US Food and Drug Administration (FDA) approval of bacteriophage applications as food additives [2, 11]. Therefore, the development of novel biocontrol agents using bacteriophages has recently been spotlighted.

Bacillus cereus is a Gram-positive food-borne pathogen that is frequently found in fresh vegetables and fruits grown in soil. This pathogen produces enterotoxins and cytotoxins that cause diarrhea and vomiting [1, 8]. Antibiotics are not usually recommended because of the potential for development of bacterial resistance to β-lactam antibiotics [6, 14]. Therefore, the use of bacteriophage is an excellent alternative approach for the development of biocontrol agents for the control of this pathogen. Recently, a few *B. cereus*-targeting virulent phage genomes, such as BCP78, PBC1, Bc431v3, and B4, were completely sequenced and analyzed to extend our understanding of host-phage interaction and infection mechanisms [5, 7, 9, 10].

To further develop this novel type of biocontrol agents, phages BPS10C and BPS13 were isolated in this study

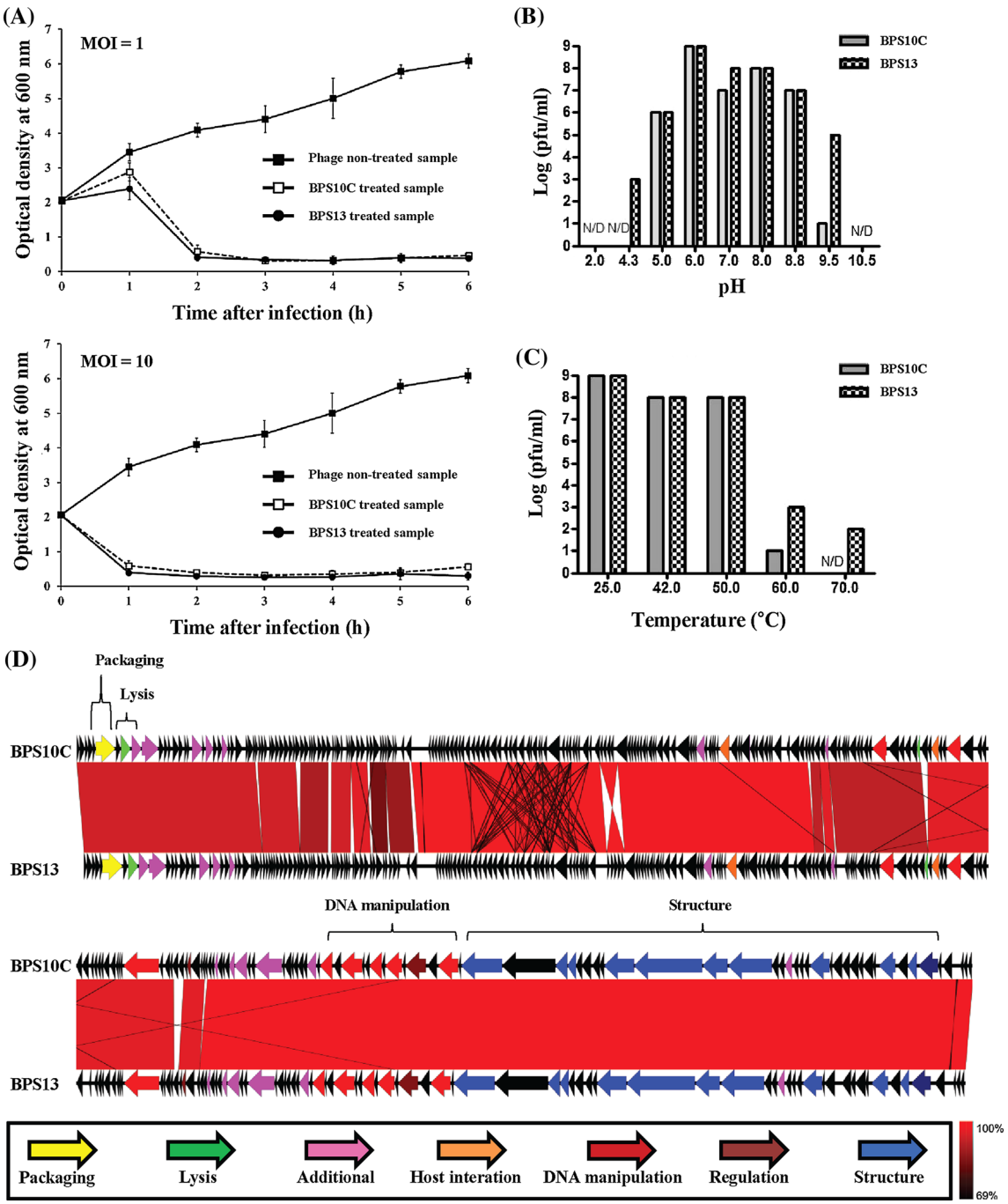

Fig. 1 **A**. Bacterial challenge assay of phages BPS10C and BPS13 against *B. cereus* ATCC 10876 at two different multiplicities of infection (MOI), 1.0 and 10. These graphs indicate optical density (OD) at 600 nm of samples collected every hour. Each strain was infected with phages BPS10C and BPS13 when $OD_{600\ nm}$ was 2.0. Uninfected sample, filled square; BPS10C-infected sample, open square; BPS13-infected sample, filled circle. **B**. pH stability test of phages BPS10C and BPS13. The stability test was performed under various pH conditions. Each phage (final concentration 10^9 PFU/ml) was added to pH-adjusted SM buffer (from pH 2.0 to pH 10.5), and the phage suspensions were incubated at 37 °C for 24 h. N/D, not detected. **C**. Temperature stability of phages BPS10C and BPS13. The stability test was performed under various temperature conditions. Each phage (final concentration 10^9 PFU/ml) was added to SM buffer and incubated at 20, 42, 50, 60, and 70 °C for 1 h. N/D, not detected. **D**. Comparative genome map of bacteriophages BPS10C (above) and BPS13 (bottom). The similarities between the two genomes at the DNA level were determined using the Easyfig program and are represented by gradations in color from red (100 % similarity) to black (69 % similarity)

Table 1 Functional groupings of the predicted ORFs in bacteriophages BPS10C and BPS13

Functional group	Predicted function	Locus_tag	
		BPS10C	BPS13
Structure	Adsorption-associated tail protein	BPS10C_237	BPS13_0236
	Baseplate J protein	BPS10C_239	BPS13_0238
	Baseplate protein	BPS10C_240	BPS13_0239
	Minor structural protein	BPS10C_245	BPS13_0244
	Putative tail fiber	BPS10C_246	BPS13_0245
	Tail lysin 1	BPS10C_247	BPS13_0246
	Tail lysin 2	BPS10C_248	BPS13_0247
	Tail sheath protein	BPS10C_255	BPS13_0254
	Major capsid protein	BPS10C_263	BPS13_0261
Packaging	Terminase large subunit	BPS10C_006	BPS13_0006
	Portal protein	BPS10C_266	BPS13_0264
Host lysis	Endolysin	BPS10C_008	BPS13_0008
	Putative holin	BPS10C_172	BPS13_0174
DNA manipulation	DNA helicase 1	BPS10C_231	BPS13_0230
	DNA helicase 2	BPS10C_235	BPS13_0234
	Primase	BPS10C_224	BPS13_0223
	Possible DNA polymerase	BPS10C_167	BPS13_0169
	Putative DNA polymerase	BPS10C_193	BPS13_0194
	Exonuclease	BPS10C_226	BPS13_0226
	Recombination/repair protein	BPS10C_178	BPS13_0179
Host interaction	RNA polymerase sigma factor	BPS10C_136	BPS13_0137
	Integration host factor	BPS10C_175	BPS13_0176
Regulation	Transcriptional regulator	BPS10C_233	BPS13_0232
Additional function	Ribose-phosphate pyrophosphokinase	BPS10C_009	BPS13_0009
	Nicotinate phosphoribosyltransferase	BPS10C_010	BPS13_0010
	Thymidylate synthase	BPS10C_018	BPS13_0018
	Dephospho-CoA kinase	BPS10C_020	BPS13_0020
	Dihydrofolate reductase	BPS10C_023	BPS13_0023
	Metal-dependent hydrolase	BPS10C_130	BPS13_0131
	MazG nucleotide pyrophosphohydrolase	BPS10C_156	BPS13_0157
	Thioredoxin	BPS10C_207	BPS13_0206
	Flavodoxin	BPS10C_211	BPS13_0210
	Ribonucleoside-diphosphate reductase subunit alpha	BPS10C_215	BPS13_0214
	Ribonucleoside-diphosphate reductase subunit beta	BPS10C_212	BPS13_0211
	Deoxyuridine 5′-triphosphate nucleotidohydrolase	BPS10C_222	BPS13_0221

from food waste samples using *B. cereus* ATCC 10876 as a host strain due to the high lytic activities of these phages against *B. cereus* (see Supplementary methods). An analysis of these phages using an energy-filtered transmission electron microscope (EF-TEM) was conducted as described previously [15]. These phages are quite similar to each other and have heads and contractile tails, which suggests that they belong to the family *Myoviridae* (Fig. S1). The diameters of the heads and tails were approximately 79.9 nm and 18.7 nm, respectively, and the non-contracted and contracted tail lengths were approximately 193.5 nm and 177 nm, respectively (Fig. S1). In addition, analysis of their host range revealed that these phages can inhibit *B. cereus, B. thuringiensis,* and *B. mycoides* but cannot inhibit *Listeria monocytogenes, Staphylococcus aureus*, and *S. epidermis*, which indicates their host specificity at the genus level (Table S1).

To understand the inhibitory effect of phages BPS10C and BPS13 against *B. cereus*, a bacterial challenge test was conducted in liquid culture (see Supplementary methods). Interestingly, the initial inhibition of *B. cereus* growth by phage BPS13 was slightly higher than that of the BPS10C phage at a multiplicity of infection (MOI) of 1 (Fig. 1a). However, at an MOI of 10, the extent of inhibition of the

host strain by BPS13 and BPS10C was similar (Fig. 1a). This result suggests that phage BPS13 may exhibit slightly higher host inhibition ability at the initial inhibition step against *B. cereus* compared with phage BPS10C. For successful application against *B. cereus*, BPS10C and BPS13 must be virulent phages. To confirm this, we randomly picked potential colonies from the center of ten BPS10C and ten BPS13 plaques, and the infection abilities of phages BPS10C and BPS13 against these colonies were verified using a standard dotting assay. However, we did not find any resistant colonies against phages BPS10C and BPS13, suggesting that these phages are not lysogenic (data not shown). In addition, a phage stability test revealed that phage BPS13 is more stable at a wider range of pH and temperature conditions than BPS10C, suggesting that phage BPS13 is a better candidate than phage BPS10C for *B. cereus* inhibition applications (Fig. 1b, c).

Phage BPS10C contains a 159,590-bp DNA genome with a G+C content of 38.74 % and 271 ORFs, whereas the genome of phage BPS13 consists of 158,305 bp with a G+C content of 38.75 % and 268 ORFs. Neither of these genomes contains a tRNA gene. The functional ORFs of these two phages were classified into seven groups: structure, packaging, host lysis, DNA manipulation, host interaction, regulation, and additional functions (Fig. 1d). The functionally classified genes in each group are listed in Table 1.

Interestingly, these two phages have two tail lysins (*BPS10C_247* and *BPS10C_248* in phage BPS10C and *BPS13_0246* and *BPS13_0247* in phage BPS13) within the structure group, which are most likely involved in the additional host lytic activity against *B. cereus* (Table 1). A previous report showed that the endolysin of phage BPS13 (*LysBPS13*, *BPS13_0008*) exhibits effective lytic activity and remarkable thermostability in the presence of glycerol, which suggests that LysBPS13 has high potential as a new biocontrol agent [12]. Due to the high similarity of the endolysin of phage BPS10C (*BPS10C_008*) to *LysBPS13*, phage BPS10C is predicted to have a host lysis mechanism against *B. cereus* that is similar to that of phage BPS13. In addition, the ORFs encoding holins (*BPS10C_172* and *BPS13_0174*) are located far from the ORFs encoding endolysins in these two phage genomes, which is different from the results obtained with other general phages. Thus, this finding suggests that their functions as holin proteins are not clearly understood and need to be confirmed experimentally. Although tail lysins may be associated with endolysin for host lysis, their inhibitory mechanisms should be confirmed experimentally.

Analysis of the DNA manipulation group showed that these phages may replicate their own DNA with help from host DNA replication proteins. Interestingly, each phage genome has two transcription sigma factors (*BPS10C_136* and *BPS10C_175* in phage BPS10C *BPS13_0137* and *BPS13_0176* in phage BPS13) in the host interaction group, which suggests that these phages may have transcription regulation mechanisms that are different from those of the host (Table 1). Analysis of these two phage genomes revealed the presence of host lysis proteins and the absence of toxin-associated genes, which indicates their potential usefulness as a novel biocontrol agent for the control of pathogenic *B. cereus*.

Nucleotide sequence accession numbers

The complete genome sequences of the *B. cereus*-infecting phages BPS10C and BPS13 are available in the GenBank database under the accession numbers KC430106 and JN654439, respectively.

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