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Genomic analysis of WCP30 Phage of *Weissella cibaria* for Dairy Fermented Foods

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Abstract

In this study, we report the morphogenetic analysis and genome sequence of a new WCP30 phage of *Weissella cibaria*, isolated from a fermented food. Based on its morphology, as observed by transmission electron microscopy, WCP30 phage belongs to the family *Siphoviridae*. Genomic analysis of WCP30 phage showed that it had a 33,697-bp double-stranded DNA genome with 41.2% G+C content. Bioinformatics analysis of the genome revealed 35 open reading frames. A BLASTN search showed that WCP30 phage had low sequence similarity compared to other phages infecting lactic acid bacteria. This is the first report of the morphological features and complete genome sequence of WCP30 phage, which may be useful for controlling the fermentation of dairy foods.

Keywords *W. cibaria*, phage, genome, dairy products, fermentation

Introduction

The genus *Weissella*, which was proposed in 1993, includes species previously classified as *Leuconostoc* and *Lactobacillus* (Collins *et al.*, 1993). Currently, there are 21 valid *Weissella* spp., and strains in this genus have recently received attention for their potential use as probiotics (Fessard and Remize, 2017; Fusco *et al.*, 2015). *Weissella* spp. are widely found in diverse environments as well as in various fermented foods (dairy fermented products, fermented meat and fish based products, cereal based fermented food and vegetables and fruits fermented foods). They are heterofermentative lactic acid bacteria, producing CO₂ from carbohydrate metabolism and lactic and acetic acids as the major end products of glucose metabolism (Björkroth *et al.*, 2014). *Weissella cibaria* is a good starter for fermentation as it produces less lactic acid than homofermentative lactic acid bacteria, and some *W. cibaria* strains have been shown to produce high levels of exopolysaccharides, which have desirable texturizing properties (Carr *et al.*, 2002; Fessard and Remize, 2017). Phages that infect lactic acid bacteria are a concern in the fermented food industry, especially in the production of fermented dairy foods, because phage contamination can reduce product quality (Garneau and Moineau, 2011; Kot *et al.*, 2014). Phages are viruses that only infect bacteria, and they are the most abundant microorganisms on the earth; thus, they are widespread and are found on many foods (Hendrix, 2003). However, phages are also used in various

biotechnology applications, including phage display, bacterial detection, biofilm degradation, and pathogen biocontrol (Greer, 2005; Hudson *et al.*, 2005). Due to the host specificity of phages, they are ideal agents for controlling bacterial contamination of foods. Phage-based applications, such as phage therapy (Sulakvelidze *et al.*, 2001), have been used to control pathogens in livestock, for sanitation, specifically the decontamination of carcasses and other raw products (Martinez *et al.*, 2008; Modi *et al.*, 2001), and for the control of lactic acid bacteria in the fermentation industry (Liu *et al.*, 2015). In this study, we report the morphogenetic and genome sequence analyses of a WCP30 phage, which was isolated from a fermented food and may be useful for controlling the fermentation in dairy products.

Materials and Methods

Isolation of phage for *W. cibaria*

All reagents and media were purchased from Sigma-Aldrich (USA) and BD BBL (USA), respectively. To isolate phage that infect *W. cibaria*, various fermented food samples were collected from a local market in Korea. *W. cibaria* KCTC3807 was used as the host strain and was grown in Lactobacilli MRS broth or agar supplemented with 10 mM CaCl₂ (LBC) at 37°C overnight. To isolate *W. cibaria* phage, the samples were analyzed by plaque assay with the aforementioned host strain. Briefly, sample (5 g) was mixed with *W. cibaria* (7-8 Log CFU/mL) and incubated at 37°C for 24 h. Then, the culture was centrifuged, and the supernatant was filtered through a 0.22 µm membrane syringe filter. The filtrate was tested for the presence of *W. cibaria*-infecting phage by a plaque assay using a double agar overlay. A plaque was picked, and the phage was eluted with SM buffer (100 mM NaCl, 8 mM MgSO₄•7H₂O, and 50 mM Tris-Cl [pH 7.5]), and a plaque was picked from a fresh plate for plaque purification (Sambrook and Russel, 2001).

Morphology and phage DNA extraction

To confirm the morphological characteristics, purified phage particles were negatively stained with 2% aqueous uranyl acetate (pH 4.5) on a carbon-coated grid and examined by transmission electron microscopy. Phage DNA was collected from polyethylene glycol precipitated phage particles by method (Manfioletti and Schneider, 1988) with some modifications. DNase I (10 µg/mL) and RNase A (20 µg/mL) were added to phage lysate, respectively.

After incubation at room temperature for 15 min, 0.5 M EDTA (pH 8) and proteinase K (1 mg/mL) were added, followed by incubation at 65°C for 30 min. After incubation, the nucleic acid was extracted with phenol-chloroform-isoamyl alcohol. The nucleic acid was precipitated with ethanol, and resuspended in sterile distilled water. Phage DNA was stored at -80°C.

Genome sequence analysis

The genome sequence was determined by ultra-high throughput GS FLX sequencing (average coverage, 20-fold redundancy). Then, the obtained nucleotide sequence was compared to sequences in GenBank by BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>). The open reading frames (ORFs) were identified with ORF Finder at the National Center for Bioinformatics (<http://www.ncbi.nlm.nih.gov/gorf.html>). The molecular weights and isoelectric points of the predicted proteins were calculated with the Compute pI/Mw program (http://www.expasy.ch/tools/pi_tool.html). The promoters and tRNAs were identified with promoter hunter (<http://www.phisite.org>) and tRNAscan-SE (<http://lowelab.ucsc.edu/tRNAscan-SE/>), respectively.

Nucleotide sequence accession number

The complete genome sequence of WCP30 phage was deposited at GenBank under accession number NC_031101.1

Results and Discussion

WCP30 phage was isolated from a fermented food and

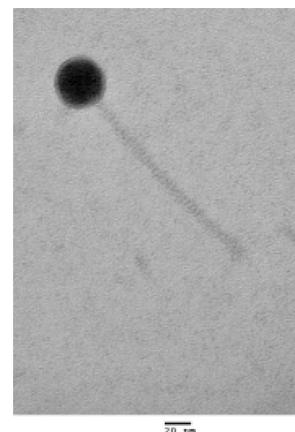


Fig. 1. Electron micrograph showing morphology of WCP30 phage. The scale bar in the lower center represents 20 nm.

purified by plaque assay. To evaluate the morphology, WCP30 phage was observed by transmission electron microscopy (Fig. 1). According to the morphological analysis, WCP30 phage had a long non-contractile tail and an icosahedral head, thus it belonged to the family *Siphoviridae* in the order *Caudovirales*. Morphologically, phages can be tailed, polyhedral, filamentous, or pleomorphic; most of them contain a double-stranded DNA genome (Ackermann, 2003). The WCP30 phage genome was 33,697 bp in length, with 41.2% G+C content. The genome sequence were searched against GenBank by using

BLASTN, and the results showed that WCP30 phage did not shared sequence of all phage for lactic acid bacteria. Bioinformatic analysis of the WCP30 phage genome revealed 35 putative ORFs. Of these, 20 ORFs had matches in GenBank with annotated functions. Comparison of the WCP30 phage sequence to sequences in GenBank showed that it was composed of the typical basic functional modules of phages, including replication, DNA packaging, structure/morphogenesis, and lysis modules (Table 1 and Fig. 2).

In the structure/morphogenesis module, the predicted

Table 1. Database matches of the functional ORFs of WCP30 phage

ORF	Start	End	BLAST protein		pI/MW	Link
			Identity	Positives		
1	511	1362	145/276 (53%)	187/276 (67%)	4.94 / 30214.42	ref YP_223889.1 Major head protein [<i>Lactobacillus virus phiJL1</i>]
3	1375	1920	71/162 (44%)	106/162 (65%)	4.64 / 19692.97	ref YP_223888.1 Putative scaffold protein [<i>Lactobacillus virus phiJL1</i>]
4	2139	2951	130/264 (49%)	186/264 (70%)	6.85 / 30977.99	ref WP_089940223.1 Phage head morphogenesis protein [<i>Leuconostocaceae bacterium R-53105</i>]
5	2092	4470	270/517 (52%)	365/517 (70%)	4.82 / 60326.49	ref WP_089940220.1 Phage portal protein [<i>Leuconostocaceae bacterium R-53105</i>]
6	4736	6208	137/262 (52%)	158/262 (60%)	5.62 / 53218.19	ref CCJ66388.1 Structural protein [<i>Leuconostoc pseudomesenteroides</i> 4882]
7	6272	7555	271/423 (64%)	337/423 (79%)	7.76 / 48966.74	ref WP_047975578.1 PBSX family phage terminase large subunit [<i>Fructobacillus</i> sp. EFB-N1]
8	7539	7886	54/118 (46%)	75/118 (63%)	7.88 / 13615.48	ref WP_089135350.1 Small terminase subunit [<i>Lactobacillus murinus</i>]
9	8004	8756	99/266 (37%)	150/266 (56%)	4.59 / 28244.49	ref EEY34199.1 DNA (cytosine-5-)-methyltransferase [<i>Leptotrichia goodfellowii</i> F0264]
15	12756	13604	65/251 (26%)	104/251 (41%)	5.10 / 32827.08	ref CUO01482.1 Replicative DNA helicase [<i>Bacteroides xylanisolvens</i>]
16	14565	14999	70/147 (48%)	90/147 (61%)	4.69 / 16084.19	ref WP_006860165.1 Deoxyuridine 5'-triphosphate nucleotidohydro- lase yncF [<i>Marvinbryantia formatexigens</i>]
18	16046	16990	126/296 (43%)	177/296 (59%)	9.29 / 35113.29	ref WP_061992536.1 SGNH/GDSL hydrolase family protein [<i>Fructobacillus ficulneus</i>]
19	17892	18332	106/163 (65%)	124/163 (76%)	4.79 / 16232.85	ref WP_056973096.1 Single-stranded DNA-binding protein [<i>Weissella confusa</i>]
20	18525	19184	85/166 (51%)	112/166 (67%)	6.10 / 24926.17	ref ALM63598.1 SAK protein [<i>Lactococcus</i> phage 936 group phage Phi129]
24	20011	20388	58/84 (69%)	71/84 (84%)	6.28 / 14610.55	ref KMK52910.1 YopX protein [<i>Fructobacillus</i> sp. EFB-N1]
25	20638	21390	170/245 (69%)	209/245 (85%)	9.40 / 27889.91	ref KRN24617.1 Nicotinamide mononucleotide transporter [<i>Weissella confusa</i>]
26	22250	23185	186/313 (59%)	223/313 (71%)	5.45 / 33644.49	ref WP_012305270.1 N-acetylmuramoyl-L-alanine amidase [<i>Leuconostoc citreum</i>]
27	23187	23555	19/81 (23%)	43/81 (53%)	9.52 / 13725.08	ref WP_065868596.1 Holin [<i>Streptococcus mutans</i>]
29	27482	28276	121/267 (45%)	165/267 (61%)	5.06 / 29409.12	ref WP_002828426.1 Tail protein [<i>Weissella paramesenteroides</i>]
30	28317	31364	649/960 (68%)	752/960 (78%)	9.38 / 107498.44	ref WP_063083150.1 Phage tail tape measure protein [<i>Weissella cibaria</i>]
33	32237	32740	52/161 (32%)	87/161 (54%)	4.32 / 18626.39	ref WP_010692146.1 Phage major tail protein, TP901-1 family [<i>Fructobacillus fructosus</i>]

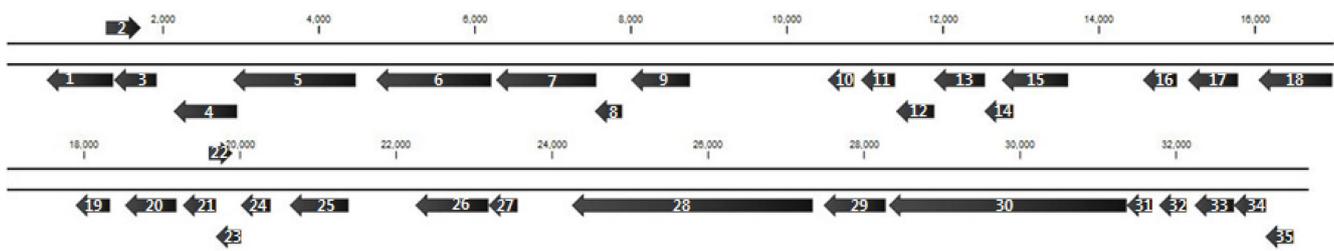


Fig. 2. Schematic representation of the dsDNA genome of the WCP30 phage. Structure/morphogenesis: ORF1, ORF3, ORF4, ORF29, ORF33, ORF30; DNA-packaging: ORF7, ORF8, ORF5; Lysis: ORF18, ORF27, ORF26; Replication: ORF9, ORF15, ORF16, ORF19, ORF20, ORF25, ORF24.

protein encoded by ORF1 was identified as the major capsid protein, as it showed similarity to the major head protein of *Lactobacillus* phage phiJL1. The ORF3 protein showed 44% similarity to a putative scaffolding protein, which is required for the correct assembly of coat protein and the incorporation of minor proteins. The ORF4 protein was identified as a head morphogenesis protein, as it showed 49% similarity to that of a phage infecting *Leuconostocaceae* bacterium R-53105. The ORF29 protein was identified as a tail protein, as it showed 45% similarity to a tail protein of *W. paramesenteroides*. The ORF33 protein was homologous (32% identity) to the major tail protein of *Fructobacillus fructosus*. The ORF30 protein, which was the longest ORF of WCP30 phage, showed sequence similarity to a tape measure protein (TMP) repeat in *W. cibaria* (68% identity). In a phage, the TMP determines the length of the tail by functioning as a template during tail assembly. Another structure/morphogenesis-related protein was encoded by ORF6. Within the DNA-packaging cluster, the proteins encoded by ORF7 and ORF8 were identified as a terminase and a small terminase subunit, respectively. The ORF5 protein showed sequence similarity (52% identity) with the phage portal protein from *Leuconostocaceae* bacterium R-53105. Together with the portal protein, the terminase, which is a protein complex composed of a large and a small subunit, drives DNA packaging (Moore and Prevelige, 2002). Within the lysis module, the ORF18 protein showed 43% similarity to the SGNH/GDSL hydrolase family protein of *Fructobacillus ficulneus*. ORF26 was predicted to encode a protein that is nearly identical to the N-acetylmuramoyl-L-alanine amidase from *Leuconostoc citreum*, and the ORF27 protein was identified as a putative holin, as it showed 23% similarity to a holin in a phage of *Streptococcus mutans*. Holins typically generate a lesion in the bacterial cytoplasmic membrane through which the endo-

lysin passes (Gründling *et al.*, 2001). In the replication module, ORF9 was predicted to encode a DNA (cytosine-5)-methyltransferase, as it showed 37% similarity to a DNA (cytosine-5)-methyltransferase of *Leptotrichia goodfellowii* F0264. ORF15 protein showed 26% similarity to the replicative DNA helicase of *Bacteroides xyloisolvens*, and ORF16 was predicted to encode a protein with 48% identity to the deoxyuridine 5'-triphosphate nucleotidohydrolase *yncF* of *Marvinbryantia formatexigens*. ORF 19 is predicted to encode a protein with 65% identity to a single-stranded DNA-binding protein of *W. confusa*, and ORF20 is predicted to encode a protein that is 51% similar to the SAK protein of the *Lactococcus* phage 936 group phage Phi129. The predicted ORF24 protein showed 69% identity to YopX of *Fructobacillus* sp. EFB-N1, and ORF 25 was predicted to encode a product that is homologous (69% identity) to the nicotinamide mononucleotide transporter of *W. confusa*. Nicotinamide mononucleotide transporter is integral membrane proteins that are involved in transport of nicotinamide mononucleotide (Fusco *et al.*, 1990).

In conclusion, we analyzed the morphology and genome sequence of WCP30 phage, which was isolated from a fermented food. The genome sequence of this newly isolated WCP30 phage of *W. cibaria* was different from that of other phages that infect lactic acid bacteria. Further study is needed to determine the relationship between WCP30 phage and *W. cibaria*, especially when used as a starter for the fermentation of dairy products.

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