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Whole Genome Sequence Analysis of *Lactiplantibacillus plantarum* Bacteriophage P2

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Abstract

Phage P2 was isolated from failed fermentation broth carried out by *Lactiplantibacillus plantarum* IMAU10120. A previous study in our laboratory showed that this phage belonged to the *Siphoviridae* family. In this study, this phage's genomic characteristics were analyzed using whole-genome sequencing. It was revealed that phage P2 was 77.9 kb in length and had 39.28% G + C content. Its genome included 96 coding sequences (CDS) and two tRNA genes involved in the function of the structure, DNA replication, packaging, and regulation. Phage P2 had higher host specificity; many tested strains were not infected. Cell wall adsorption experiments showed that the adsorption receptor component of phage P2 might be a part of the cell wall peptidoglycan. This research might enrich the knowledge about genomic information of lactobacillus phages and provide some primary data to establish phage control measures.

K e y w o r d s: *Lactiplantibacillus plantarum* phage P2, genome characteristics, functional genes, host specificity, adsorption receptor

Introduction

Phages are ubiquitous in dairy environments, including fermentation vats, pipelines, and air (Ma et al. 2015). In 1983, Trevors et al. (1983) successfully isolated *Lactiplantibacillus plantarum* bacteriophage from meat products for the first time. Since then, more and more researchers have successfully isolated other *L. plantarum* phages. As reported, lytic phages can effectively inhibit the growth of pathogenic and spoilage bacteria and eventually reduce the loss of food products (Salmond and Fineran 2015). Similarly, phages can also eliminate multi-drug-resistant pathogens, providing new options for treating drug-resistant bacterial diseases worldwide (Kortright et al. 2019). However, in the fermentation industry, due to the ability of virulent phages to rapidly lyse the cells of bacterial strains, it may cause massive death of culture strains in a short time, which might increase fermentation time, resulting in lower viscosity values, poor organoleptic properties of fermentation products, and finally express a negative impact on the quality and value of final products and eventually economic losses (Ofir and Sorek 2018; Jamal et al. 2019; Mancini et al. 2021; White et al. 2022).

As the most abundant living entities on the planet, bacteriophages are known to heavily influence the ecology and evolution of their hosts (Grose et al. 2014). However, there are still huge gaps in our understanding of phages and their life cycles. So far, the complete genomic information of 118 *Lactobacillus* phages is publicly available; 21 of them are *L. plantarum* phages. Therefore, it is necessary to isolate more *Lactobacillus*

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phages and elucidate their genomic sequences to accumulate enough phage reserves to prevent and control fermentation hazards caused by phage infection, laying the foundation for the development of agricultural and industrial biotechnology in the future.

In 2019, we isolated *L. plantarum* phage P2 from abnormal fermentation broth (the broth culture of a slowly fermenting *L. plantarum* IMAU10120), and the morphological features showed that this phage belonged to the *Siphoviridae* family (Chen et al. 2019). As we know, most of the *Lactobacillus* phages are highly host-specific (Korniienko et al. 2022). This study presented the complete genome sequence of *L. plantarum* phage P2 and compared it with other *L. plantarum* phages. This study will further the knowledge about genomic information of lactobacillus phages and provide some primary data to establish phage control measures.

Experimental

Materials and Methods

Bacterial strain, phage amplification and culture conditions. The host strain, *L. plantarum* IMAU10120, was cultured in de Man, Regosa, and Sharpe broth (MRS) at 37°C and stored at 4°C after continuous subculturing for three days. For phage amplification, MRS was supplemented with 10 mM $CaCl_2$. Phage stocks were prepared as previously described and stored as lysates at 4°C (Neviani et al. 1992).

Host range of phage. Fifty-seven *L. plantarum* strains were assayed for the host range of phage P2 using the double-layer plate method (Korniienko et al. 2022). The experiment was repeated three times and three parallel samples were taken each time. The tested strains are listed in Table SI.

Cell wall preparation. The extraction method of *L. plantarum* cell wall was carried out according to the methods described by Quiberoni et al. (2000). *L. plantarum* was cultured to $OD_{600} \approx 0.5$ and centrifuged at $3{,}000 \times g$ for 10 min. Afterward, the supernatant was removed and washed twice with 0.1 mol/l phosphate buffer (pH 6.8), followed by centrifugation at $3{,}000 \times g$ for 10 min. Then the precipitates were suspended in a phosphate buffer by adding glass beads (0.1–0.15 mm diameter) at 1:1 (vol/ vol) and thoroughly mixed. The mixture was vortexed for 30 s, followed by an ice bath for 30 s; the total time was 45 min. The cell disruption was observed by optic microscopy and the spread plate counting method (Leach and Stahl 1983).

The precipitate was beaten with glass beads repeatedly four times (4°C, 2 h) and the supernatant was by centrifugation at $12{,}000 \times g$ for 15 min. Afterwards, the precipitate was resuspended in TRIS-HCl (pH 7.5) and treated with DNase (0.1 mg/ml) and RNase (0.15 mg/ml) for 30 min at 37°C. The cell walls were collected by centrifugation at $12{,}000 \times g$ for 15 min. Finally, it was washed by five successive resuspensions in 10 mmol/l phosphate buffer (pH = 6.8). After centrifugation, the purified cell wall was stored at –20°C.

Cell wall adsorption. According to the adsorption method described by Quiberoni and Reinheimer (1998), 100 µl cell wall was mixed with 100 µl phage lysate (10^6 PFU/ml) in MRS-Ca broth and incubated at 37°C for 30 min. The mixtures were then centrifuged at $12{,}000 \times g$ for 5 min, and the number of unadsorbed phages in the supernatant was counted by the double-layer method, and the adsorption rate was calculated (Yasin and Mustafa 2002).

Phage adsorption to cell wall after chemical and enzymatic treatments. The prepared cell wall was treated with SDS (0.1%) (BioFroxx, Germany), lysozyme (50 U/ml) (Tiangen Biotech(Beijing) Co., Ltd., P.R. China), proteinase K (0.1 mg/ml) (Tiangen Biotech(Beijing) Co., Ltd., P.R. China) at 37°C for 30 min, and trichloroacetic acid (TCA) (5%) (Tianjin Xinbote Biotech Co., Ltd., P.R. China) at 100°C for 15 min. The treated cell wall was washed with phosphate buffer for five times and then centrifuged at $12{,}000 \times g$ for 5 min. A hundred microliters of the treated cell wall were mixed with an equal volume of phage lysate, placed at 37°C for 30 min for adsorption, and centrifuged at $12{,}000 \times g$ for 5 min. The number of unadsorbed phages in the supernatant was used to determine the adsorption rate; untreated cell walls were used as a control. As previously described, the formula for calculating the adsorption rate is as follows:

$$adsorpton\ rate = \left(1 - \frac{N_1}{N_2}\right) \times 100\% \quad (1)$$

N_1 – the number of unadsorbed phage in the supernatant (PFU/ml), N_2 – the initial titer of phage P2 lysate (PFU/ml).

Phage DNA preparation. The phage lysate was added to the host bacteria culture medium to $OD_{600} \approx 0.5$ for propagation to obtain a high concentration of phage lysate. The obtained lysate was centrifuged at $8{,}000 \times g$ for 5 min to remove cell debris. The supernatant was filtered through a filter membrane, and then 1ml lysate was pipetted into a 2 ml Eppendorf tube. Phage DNA was obtained by phenol-chloroform-isoamyl alcohol extraction (Mastura et al. 2017). Briefly, the filtrate was treated with DNase I and RNase A (1 µg/ml) and incubated at 37°C for 1 h. Then EDTA (0.5 M) was added, followed by proteinase K (10 mg/ml) and SDS (10%). The mixture was incubated at 37°C for 30 min and removed quickly to cool it on ice. Next, an equal volume of phenol-chloroform-isoa-

myl alcohol (25:24:1) was added, mixed gently until a white emulsion appeared, and centrifuged at 8,000 × *g* for 10 min to collect the supernatant. Subsequently, the supernatant was mixed well with isopropanol and kept at –20°C for 30 min. After that, the DNA pellet was washed with 75% ethanol and centrifuged at 12,000 × *g* for 10 min. Finally, all DNA pellets were suspended in 20 µl TE and stored at –20°C. The DNA concentration and integrity were assessed by agarose gel electrophoresis and Nanodrop spectrophotometer (Gene Company Limited, USA).

Genome sequencing and bioinformatic analysis. High-quality DNA was used to construct the library. Whole-genome sequencing was performed on the Illumina Hiseq4000 platform (the Asbios (Tianjin, China) Technology Co., Ltd.) with pair-end read sizes of 150 bp. The raw reads were quality checked with FastQC and trimmed with FASTX-Toolkit. On average, Illumina PE reads 1 and reads 2 had > 90% and > 75% of bases with a quality score of at least 30 (Q30), respectively. The Flye program was used for assembly (Bzikadze and Pevzner 2020), and the parameters (–g 50,000, other parameters were default). The data were first filtered to 50 Mb (random extraction) before assembly so that even if there was a host sequence, the host sequence could not be assembled, and the depth was too low for the host sequence. After the assembly was completed, the Flye program gave information about which loops were formed. For small genomes like bacteriophages, Flye can generally be assembled at one time, with only one contig. Our result was a 77.9 kb sequence, and Flye gave the information about the non-repetitive loops (Kolmogorov 2019). Gene prediction of *L. plantarum* phage P2 was obtained using GeneMark 3.25 (Tang et al. 2014). Comparative analysis of *L. plantarum* phage P2 with other known sequences of *Lactobacillus* phages was performed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide sequences of 20 *L. plantarum* phages (including P2) were aligned by ClustalW (Luo et al. 2012). The genome sequence has been submitted to the GenBank database (https://www.ncbi.nlm.nih.gov) and is publicly available with the accession number KY381600.1.

Results and Discussion

Host range of phage P2. Among all the 57 tested strains, *L. plantarum* phage P2 was only infectious to *L. plantarum* IMAU10140, *L. plantarum* IMAU10372, *L. plantarum* IMAU10942, and *L. plantarum* IMAU11029. All these strains were isolated from the fermented milk of a cow (Table SI), which indicated that phage P2 expressed high host specificity. Capra et al. (2006) reported that *Lactobacillus paracasei* phage ϕPL-1 and *Lactobacillus casei* ϕJ-1 shared similar host spectra, were able to infect seven out of 16 strains of *L. paracasei,* and two out of six strains of *L. casei*. Moreover, Zago et al. (2013) also reported that *L. plantarum* phage ϕK9 was able to infect 14 out of 49 strains of *L. plantarum*. Compared to the above phages, the host range of phage P2 was relatively narrow.

Table I
The adsorption rate of phage P2 on cell wall after chemical and enzyme treatment.

Treatment	Phage P2 adsorption (%) (mean ± S.D.)
none (control)	97.26 ± 1.21[a]
1% SDS (30 min, 37°C)	98.26 ± 4.08[a]
50 U/ml lysozyme (30 min, 37°C)	75.78 ± 3.01[b]
0.1 mg/ml proteinase K (30 min, 37°C)	97.80 ± 1.81[a]
5% TCA (15 min, 100°C)	57.14 ± 6.25[c]

[a, b, c] – average values in the same column with different letters indicate significant differences ($p < 0.05$)

Phage adsorption on treated cell walls. The cell walls of *L. plantarum* IMAU10120 were treated with different chemicals and enzymes. SDS treatment can remove membrane-bound proteins or change the conformation of proteins, and proteinase K can hydrolyze peptide bonds (Binetti et al. 2002). From Table I, we can see that SDS and proteinase K treatments did not significantly reduce the adsorption rate of this phage. However, the adsorption rate of phage P2 to the cell wall decreased significantly after lysozyme and TCA treatment ($p < 0.05$). Lysozyme, an alkaline enzyme, can hydrolyze sticky polysaccharides in cell wall. It breaks the adsorption receptor of phage by breaking the β-1,4 glycosidic bond among peptidoglycan (Khalil et al. 2007). TCA can destroy polymers linked to peptidoglycan in the cell wall, such as polysaccharides. In this study, after treatment of lysozyme and TCA, the adsorption rate of phage P2 was decreased by 75.78% and 57.14%, respectively. Therefore, we inferred that the adsorption receptor of phage P2 might be a part of the cell wall peptidoglycan, consistent with previous studies (Binetti et al.2002; Quiberoni et al. 2004).

Genome analysis of *L. plantarum* Phage P2. Genome sequence analysis revealed that the genome of *L. plantarum* phage P2 was 77.9 kb in length with 39.28% G + C content. A total of 96 coding sequences (CDSs) and two tRNAs were predicted, of which 59 were in the positive strand, and 37 were in the negative strand (Fig. 1). Thirty-seven coding sequences were annotated to known functions (Table II). Two tRNAs were encoded in the phage P2 genome, suggesting that the phage may depend on its own tRNA after entering the host. Similar to our results, Lu et al. (2020) found that *Shigella flexneri* phage SGF2 encodes the

Table II
Predicted function genes of *L. plantarum* P2.

CDS	Strand	Predicted function	Function
CDS12	+	terminase small subunit	packaging
CDS14	+	terminase large subunit	
CDS15	+	portal protein	
CDS16	+	prohead protease	structure
CDS17	+	major capsid protein	
CDS18	+	putative tail protein	
CDS20	+	head-tail joining protein	
CDS21	+	head-tail adaptor	
CDS22	+	tail protein	
CDS23	+	major tail protein	
CDS25	+	tape measure protein	
CDS26	+	distal tail protein	
CDS27	+	baseplate protein tail-like protein	
CDS28	+	tail fiber protein	
CDS57	–	membrane protein	
CDS35	+	integrase	host interaction
CDS59	–	ATP/GTP- binding protein	regulation
CDS48	–	PemK family transcriptional regulator	
CDS83	+	putative DNA binding protein	
CDS37	–	DNA polymerase	DNA replication
CDS58	–	DNA polymerase	
CDS72	+	DNA helicase	
CDS73	+	DNA primase	
CDS74	+	single-stranded-DNA-specific exonuclease	
CDS1	–	HNH endonuclease	
CDS3	–	HNH endonuclease	
CDS11	+	HNH endonuclease	
CDS38	–	HNH endonuclease	
CDS41	–	HNH endonuclease	
CDS45	–	HNH endonuclease	
CDS56	–	HNH endonuclease	
CDS65	–	HNH homing endonuclease	
tRNA	+	tRNA-Pro	
tRNA	+	tRNA-Gly	
CDS44	–	extracellular transglycosylase	additional function
CDS69	+	deoxynucleoside kinase	
CDS96		thymidine kinase	

tRNA gene in its genome. The tRNA gene might also be involved in phage protein synthesis and help phage SGF2 adapt to the specific host.

From Table II, the tail structure of bacteriophage P2 consisted of four proteins, including tail protein (CDS22), major tail protein (CDS23), distal tail protein (CDS26), and tail fiber protein (CDS28). Tail protein is considered the conduit for genome delivery; tail fiber protein can accurately recognize and bind to the host surface receptors (Yoichi et al. 2005). Major tail protein and distal tail protein are considered critical components of the phage tail module (Pell et al. 2009). Moreover, head-tail joining protein (CDS20) and head-tail adaptor protein (CDS21) are required for assembling phages' heads and tails during the last step of morphogenesis (Maxwell et al. 2002).

Terminase is one of the main components of the DNA packaging module, including large and small subunits. In general, the large and small subunits of terminase are adjacent. The small subunit (CDS12) is mainly

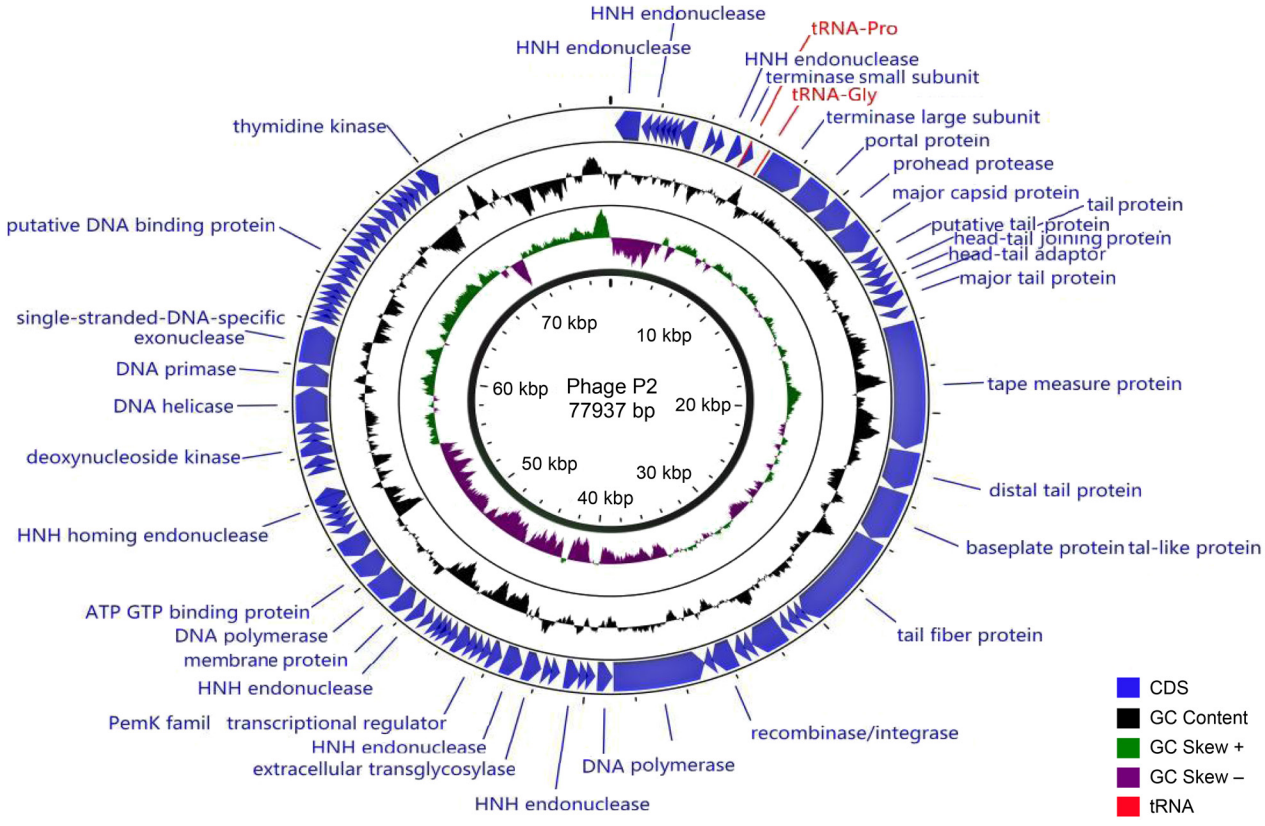

Fig. 1. Circular representation of the *Lactiplantibacillus plantarum* phage P2 genome. The innermost circle indicates the GC skew on the positive and negative strand (green and purple). The second circle indicates the GC content (black). The outer circle indicates predicted CDS located on the positive and negative DNA strand (lavender). Red indicates tRNA coding genes.

involved in recognition of the phage genome, the large subunit (CDS14) is responsible for ATP-driven DNA translocation, and the small subunit interacts with the large subunit and initiates packaging (Gherlan 2022). In addition, portal protein (CDS15) encoded by phage P2 is also included in DNA packaging modules as a phage tail attachment site.

Proteins associated with DNA replication, such as DNA helicase (CDS72), DNA polymerase (CDS37 and CDS58), HNH endonuclease (CDS1, CDS3, CDS11, CDS38, CDS41, CDS45, CDS56, CDS65) were also found in the phage P2 genome. DNA helicase can be responsible for unwinding DNA double strands to prevent supercoiling of the DNA double helix (Lee et al. 2006). DNA polymerase maintains the normal replication of DNA duplexes (Cao et al. 2019). DNA replication requires exonuclease activity. The protein sequences of HNH endonuclease are highly conserved. These HNH endonucleases are important in reproduction and infection as assembly machines in the phage life cycle (Moodley et al. 2012).

This phage also encoded its own transcription regulator, and peek family transcription regulator (CDS48) was found in the genome of phage P2, indicating that this gene might have played a role in the transcription of phage P2.

Interestingly, integrase (CDS35) gene was found in the genome of phage P2. In our previous studies on its biological characteristics, phage P2 exhibited lytic properties and expressed a large burst size. However, the presence of the integrase gene suggests that phage P2 might have its own lytic/lysogenic determination mechanism. Similar to our studies, Briggiler Marcó et al. (2012) isolated *L. plantarum* virulent phage 8014-B2 from anaerobic sewage sludge, and found it has the integrase gene. In 2016, Jaomanjaka et al. (2016) isolated virulent phage ϕOE33PA that infected *Oenococcus oeni* from red wine. It contained an integrase gene in its genomes, suggesting that it may have evolved from a lysogenic ancestor. According to previous research, we speculate that bacteriophage P2 may have evolved from a lysogenic ancestor. It requires further research to confirm.

Phylogeny analysis. Genome sequences of other 19 *L. plantarum* phages, obtained from the NCBI database (Table SII), were used to compare with that of *L. plantarum* phage P2 (Fig. 2). The phylogenetic tree showed that 20 *L. plantarum* phages were dispersed into three clades, and their distribution was source dependent. For example, Clade2 was mainly derived from organic waste samples, Clade3 was mainly obtained from *L. plantarum* isolated in food.

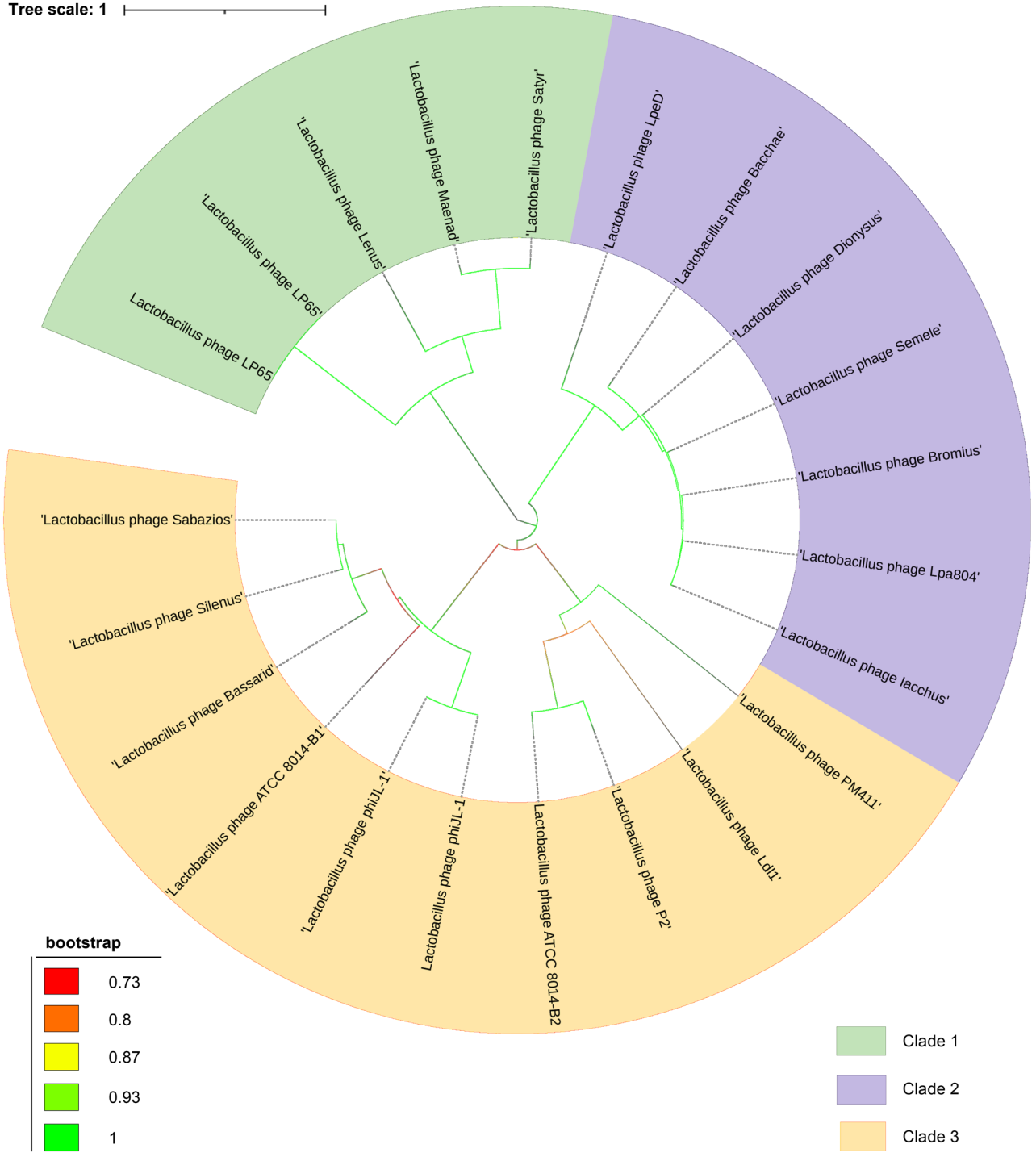

Fig. 2. Comparative phylogenetic analysis. Comparative phylogenetic analysis of nucleotide sequences was aligned by ClustalW and performed using the neighbor-joining method in MEGA5.2. Numbers associated with each branch represent bootstrap values.

Phage P2 was closely related to 8014-B2 (Fig. 2). Average nucleotide identity (ANI) analysis based on the poxvirus genome shows that 98% ANI threshold can be used for virus species rank (Deng et al. 2022). The ANI value between phage B2 and P2 was 77.08%, suggesting that their genome sequences were different. In addition, phage P2 and B2 were isolated from different sources, such as from abnormal fermentation broth and anaerobic sewage, respectively. Phage 8014-B2 infected only *L. plantarum* ATCC 8014 and PLN in all tested strains and had a different host range from P2. It could be speculated that phage P2 is a new member of the *Siphoviridae* family.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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