Isolation and characterization of two new Staphylococcus aureus bacteriophages with potential to infect distinct bacteria from bovine mastitis

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Abstract

Bovine mastitis is an important disease of dairy cows, and *Staphylococcus aureus* is the etiologic agent most prevalent among the microorganisms. Mastitis caused by *S. aureus* present low cure rate with antimicrobials treatment and low vaccines efficacy. Bacteriophages or phages have been considered as an alternative for treating this disease. This study, we isolated and characterized two new *S. aureus* phages, namely B_UFSM4 and B_UFSM5, from bovine milk of cows with mastitis. The adsorptions rates were 10–20 min for B_UFSM4 and 20–30 min for B_UFSM5. Phages activities were relatively stable at pH 3–11; however, at temperatures of 50 °C-60ºC-70ºC/60 min, the phages were completely inactivated. These viruses presented infectivity in various bacteria isolated from bovine mastitis, where the lytic activity of phages B_UFSM4 and B_UFSM5 were 34.2%(13/38) and 42.1%(16/38), respectively, including isolates from *S. aureus, Pseudomonas aeruginosa*, *Staphylococcus sciuri*, and *Rothia terrae.* The complete genomes of B_UFSM4 and B_UFSM5 have 41.396 bp and 41.829 bp, with GC-content 33.97% and 33.98%, respectively. Both phages comprise 61 putative ORFs. The viruses have double stranded DNA and linear architecture. Phylogenic similarity was observed by proteome with *Staphylococcus* prophage phiPV83 (45,536 nt), *Staphylococcus* phage CN125 (44,492 nt) and *Staphylococcus* phage JS01 (43,458 nt). Based on the morphology, the phages belong to *Siphoviridae* family, presenting icosahedral head with a long tail, *Caudovirales* order and *Biseptimavirus* genus. Thus, two *S. aureus* phages (B_UFSM4 and B_UFSM5) were isolated and characterized, and these phages can be used as therapeutic or prophylactic candidates against *S. aureus* infections in cattle mastitis.

1. Introduction

Mastitis is an important disease in dairy cows, caused by several pathogens, and results in high economic losses due reduced milk quality and production, as well as high treatment costs [1–3]. *Staphylococcus aureus* is the etiologic agent most prevalent among the microorganisms [4–6] and its main characteristic is to persist in mammary glands causing subclinical infection [7, 8]. *S. aureus* present several virulence factors, such as adhesion to epithelial cells, encapsulation, formation of microabscesses, and biofilms formation, which can difficult antimicrobial therapy, allowing the evolution to chronic infection [7–9].

Antimicrobial therapy is a major advance in the control of bovine mastitis; however, it is less effective in infection caused by *S. aureus*, particularly during the lactation stage [10–12]. Moreover, the mismanagement of antimicrobial therapies has led to increased antimicrobial resistance as observed in *S. aureus* from bovine mastitis worldwide [2, 13, 14].

Several studies have reported the development of vaccines to control bovine mastitis caused by *S. aureus*; however, these immunobiologicals have been found to be less effective [15]. Therefore, other alternatives to control of this disease include the use of silver nanoparticles, cytokines, natural compounds, and the bacteriophages [15–17].

Bacteriophages (or phages) are viruses able to infect and kill bacteria, and are considered antimicrobial agents for more than one century [18]. They may be important tools in controlling antimicrobial resistance and multiresistant bacteria [19]. Lytic phages of *S. aureus* have been isolated from bovine mastitis, and their efficiency and specificity are described [20–22]; however, some do not present lytic efficiency [23, 24] and few demonstrate a wide host range [25].

Considering the global resistance of bacteria to conventional antimicrobials and the need for new treatments for bovine mastitis caused by *S. aureus*, this study aimed to isolate and characterize bacteriophages of *S. aureus* from bovine milk of cow with mastitis, as well as to investigate the lytic efficiency of these viruses in bacteria isolated from bovine mastitis.

2. Material And Methods

2.1. Bacteriological analysis

Initially, *California Mastitis Test* (CMT) was performed to identify subclinical mastitis in cows of a dairy farm from Southern Brazil. Positive mammary glands were disinfected with cotton soaked in 70% (v/v) ethyl alcohol. Thereafter, 10–15 mL of milk samples were collected in sterile tubes and immediately transported to the laboratory under refrigeration. The samples were inoculated in 5% sheep blood and MacConkey Agar and incubated at 37 °C for 24 h. The bacterial isolates were identified by colony morphology and Gram staining. Next, colonies of Gram - positive cocci, were subjected to catalase and coagulase tests to identify coagulase positive *Staphylococcus* (CPS).

2.2. Isolation of bacteriophages

Bacteria identified as coagulase positive *Staphylococcus* (CPS) and *Staphylococcus aureus* ATCC 25923 were cultivated in 5 mL Tryptic Soy Broth (TSB) and incubated under agitation (150 rpm) overnight at 37 °C. Both were used as host bacteria for virus isolation. Thus, six CPS isolates and ATCC 25923 was mixed in the bovine milk samples, which comprised samples collected for three consecutive days from CPS mammary glands.

A milk pool (100 mL) and 50 µL of each bacterium isolate and ATCC were added in 100 mL TSB 2× and incubated at 37 °C for 24 h under agitation (160 rpm). The culture was centrifuged at 4000 rpm for 20 min and filtered through 0.22 µm syringe filter. Thereafter,100 µL of bacterial culture (in log phase) of each isolate was mixed with 5 mL de molten soft agar (TSB with 0.6 % agar), spread on TSA plates (double-layer agar plate method), and incubated at 37 °C for 18 h [26]. The plates were then screened for presence of cleared zones on bacterial lawn, which indicated the presence of lytic phages. The bacterial species of host isolate was confirmed by PCR and DNA sequencing using *16S ribosomal RNA (16S rRNA)* gene.

2.3. Phage purification, propagation and titration

After isolation, five cleared zones were randomly selected and pealed thrice on six pre-prepared TSA plates (double-layer agar) containing bacteria host for purification. For propagation, method described previously [27] with some modifications, was used. Briefly, cleared zones were streaked on various pre-prepared plates and incubated overnight at 37ºC. Afterward, salt magnesium buffer (SM; 100 mM NaCl, 8 mM MgSO4, 50 mM Tris-HCl; 2% gelatin; pH 7.5) was added in the plates and incubated at 4ºC overnight under agitation (50 rpm). Next, the solution was centrifuged at 8000×g at 4ºC for 10 min and thereafter, 0.584 g NaCl and 1 g polyethylene glycol (PEG, M.W. 8,000) was added to 10 mL of this solution. Subsequently, the solution was incubated at 4ºC under agitation (150 rpm), for 12–18 h (overnight), and then the solution was centrifuged at 8000×g at 4 °C for 10 min. Furthermore, 1 mL chloroform was added in the pellet and subjected to strong agitation for 1 min. Then, 4 mL of SM was added and centrifugated at 8000×g at 4 °C for 10 min. The supernatant was collected and filtered using syringe filter 0.22 µm and stored at 4 °C for further analyses. The phages titer was assessed using the conventional double-layer agar method previously described [26] and experiments were performed in triplicate.

2.4. Multiplicity of infection (MOI) assay

CPS isolate (bacteria host) was pre-cultivated in TSB and added to 15 mL TSB (OD_{600} 0.05), and were then incubated at 37 °C under agitation (160 rpm) until 1×10^6 colony forming units (CFU). Phage suspensions were added into the cultures at three different ratios (MOI = 0.01, 0.1 and 1). Solutions were incubated at 37 °C for 3.5 h. Thereafter, each solution was centrifuged (10.000×g at 4 °C) for 3 min. The supernatants were filtered (0.22 µm), diluted (10^{-1} to 10^{-8}) and seeded into double-layer agar containing CPS. Cell culture with CPS only were used as negative control. All assays were performed in triplicate [28].

2.5. One-step growth

This assay was performed with some modifications based in previous protocols [20, 28]. Briefly, CPS isolate (bacteria host) was cultivated (OD_{600} 0.1) and mixed in phage suspensions at MOI = 1, and then incubated at 37ºC for 30 min under agitation (160 rpm). Thereafter, it was centrifuged at 5.000×g/10 min and the pellets containing infected cells were resuspended with 2 mL of TSB, incubated at 37ºC under agitation. Next, 100 µL was collected at 10 min intervals up to 2 h and each aliquot was immediately subjected to titration by the double-layer agar assay. This experiment was performed in triplicate.

2.6. Adsorption

This experiment was based on previously protocols, [21, 29] with some modifications. CPS (bacteria host) (1×10^6 CFU) was infected with a phage's suspensions (MOI = 1) and incubated at 30ºC under agitation (160 rpm). Aliquots of 100 µL were collected at 5, 10, 15, 20, 30, and 40 min and diluted in 0.9 mL of TSB. Following centrifugation (12.000×g, 5 min), the supernatants were titrated (10^{-1} to 10^{-8}) by the double-layer agar assay. This experiment was performed in triplicate.

2.7. Thermal and pH stability

Thermal and pH stability tests were performed based on previous protocol [20] and pH stability test with adaptation. To perform the pH stability assay, the phage suspensions were added in a series of tubes containing SM buffer with different pH values (3, 5, 7, 9, and 11) and incubated overnight at 37ºC. Posteriorly, the solutions were quantified by the double-layer agar plate method. All experiments were carried out in triplicate.

2.8. Bacteriolytic activity

These tests were carried out according with previous protocol [21] and the following modifications: a suspension of CPS isolate (bacteria host) was adjusted to OD_{600} 0.05 and, thereafter the phages were added at an MOI = 1 for at 37º C for 6 h under agitation (160 rpm). In parallel, an aliquot of the same suspension, but without phage, was used as a control. The bacteriological activity of the phages was evaluated by monitoring the cell absorbance of the culture solution (OD_{600}) at 1 h intervals up to 6 h after phage infection.

2.9. Determination of host amplitude of bacteriophages

The host amplitude was performed by spot test method [30]. The following standard strains were used: *S. aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12225, *Escherichia coli* ATCC 25522, *Pseudomonas aeruginosa* ATCC 25853, *Klebsiella pneumoniae* ATCC 13853, *Enterococcus faecalis* ATCC 29212, and *Enterobacter cloacae* ATCC 13087. Additionally, isolates of CPS, coagulase negative *Staphylococcus* (CNS), *Streptococccus agalactie, Streptococccus dysgalactie, Streptococccus uberis*, and *P. aeruginosa* isolated from milk samples recovered from cattle with mastitis were used. All samples belong to Laboratory Bacteriology (LABAC) at Universidade Federal de Santa Maria (UFSM), Rio Grande of Sul State, Brazil. All bacterial isolates are registered in the Brazilian platform “Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado” (SISGEN) with the code A7085CE. Furthermore, the bacterial isolates were subjected to molecular analysis for identification by DNA sequencing of *16S ribosomal RNA (16S rRNA)* gene.

2.10. Transmission electron microscopy (TEM)

After performance purification and propagation, as previously described, the viral particles were ultra-centrifugated at 27.000 rpm at 4 °C for 4 h, at Sorvall™ WX, Ultracentrifuge series, Thermo Scientific™, rotor T-865. The pellet was dissolved in 300 µL of SM buffer. Negative staining was performed from one drop of viral suspension mixed with phosphotungstic acid 2% in grids of 100 mesh and was observed using a transmission electron microscope 120 keV, Jeol, JEM-1400, coupled with EDS microprobe of Centro de Microscopia da Zona Sul - FURG (CEME-SUL) at Universidade Federal de Rio Grande (FURG), Brazil.

2.11. Viral DNA isolation, sequencing and assembly

After, purification and propagation of phages as described above, 1.25 µL of DNAse I 100U (1/µL) and RNAse A (20 mg/mL) (Invitrogen®) were added in 1 mL phage suspension and incubated at 37ºC for 1 h. Thus, the DNA extraction protocol was performed [31], with some modifications. The viral DNAs were

dissolved in 50 µL of sterile Milli-Q water. The DNAs quality were assessed on the spectrophotometry using Picodrop™. The DNA integrity was verified by agarose gel electrophoresis. The viral DNA was stored at 4ºC.

Whole-genome sequencing was performed in Illumina MiSeq® platform, with v2 500-cycle kit (paired-end). DNA libraries were prepared with Nextera XT sample preparation kit following the manufacturer's instructions (Illumina™).

The quality of generated sequences was evaluated using FastQC tool [32]. Low-quality sequences were trimmed with the aid of FastQ Toolkit V.2.2.0. The paired-end sequence reads were assembled into contigs with SPAdes genome assembler v.3.9.0. All assemblies were confirmed by mapping reads to contigs [33] Geneious Software (version R9). Additionally, the ResFinder Server [34] and Virulence Factor Predictor [35] were used to identify in the determinant's antibiotic resistance and virulence genes on genomes.

2.12. Genomic annotation and phylogenetic analyses

Genomes annotation was carried by web serve PHASTER (Phage Search Tool Enhanced Release) [36]. In addition, when necessary, ORFs were examined to search for similarities in known sequences (*nr* database) using blastN and blastX software.

To understand the evolutionary relationships between B_UFSM4 and B_UFSM5 phages and other *Siphoviridae* species, two different approaches were applied to reconstruct the phylogenetic tree. First, we used an analysis based on the principle of phage proteomic tree reconstruction (10.1093/bioinformatics/btx157); all encoded proteins were extracted and concatenated and further used to generate a phylogenetic tree (generated by BIONJ based on the genomic distances).

Second, representative sequences from major capsid protein (MCP) and terminase gene were obtained from GenBank (October 2020) and then aligned with the sequences identified in the present study using MAFFT software [33] which was optimized for accurate global alignment (option "G-INS-i"). Alignment was used to generate maximum-likelihood (ML) phylogenetic trees with PhyML, using the best fit substitution models determined by Smart Model Selection [37]. Statistical significance analyses of tree topologies were performed with the approximate likelihood branch support test (aLRT) [38].

3. Results

3.1. Phage isolation, purification, propagation, and titration

Initially, bacteriophages were isolated from bovine milk of cattle presenting subclinical mastitis caused by CPS. Cleared zones, suggestive of phage replication were observed; only one CPS isolate (SBP 01/19 Gaivota PE), which was subjected to DNA sequencing of 16S rRNA confirming *S. aureus.* Thereafter, it was used as the host bacteria for all laboratory assays. Thus, two new lytic phages, named

Staphylococcus phage B_UFSM4 (B_UFSM4) and *Staphylococcus* phage B_UFSM5 (B_UFSM5) were isolated and characterize. After propagation, phages reached the following titers: B_UFSM4 9×10^{11} PFU/mL and B_UFSM5 22×10^{12} PFU/mL. The host bacteria and phages were registered in SISGEN platform (AAA70C4).

3.2. Determination of optimal multiplicity of infection (MOI) and one-step growth

The optimal MOI of phages was determined to be 1, because at this MOI, higher multiplication was observed, with the phage B_UFSM4 obtaining 9×10^{7} PFU/mL and B_UFSM5 $1,2\times10^{7}$ PFU/mL. Based on the MOI 1, the one - step growth curve was determined. Both phages presented a constant increase in their multiplication until 3.6×10^{7} PFU/mL and 1.7×10^{9} PFU/mL for B_UFSM4 and B_UFSM5, respectively (Fig. 1).

3.3. Adsorption, thermal and pH stability

Initial phage adsorption was detected at 10–20 min for B_UFSM4 and at 20–30 min for B_UFSM5, according to virus reduction in the supernatant (Fig. 2a). Phages activities were relatively stable at pH 3–11; however, the B_UFSM5 decreased activity at pH 11. (Fig. 2b). Phages activity remained at the same higher level when heated at 37 °C and completely disappeared when heated at 50 °C, 60ºC or 70ºC for 60 min (Fig. 2c, d).

3.4. Bacteriolytic activity

Bacteriolytic activity of both phages was evaluated by absorbance measurement (OD_{600}) of a CPS culture incubated with each phage. Bacteria culture absorbance remained stable and increased significantly after 3–4 h of incubation; however, after 5 h, the host cell growth decreased, reaching 0.03 OD_{600} at 6 h in the cultures incubated with both phages. In comparison, the control (only CPS culture) reached OD_{600} 1.250 (Fig. 3).

3.4. Determination of host amplitude

The host amplitude of phages was investigated in different species of bacterial isolates recovered from milk samples obtained from cows with mastitis (n = 38) and ATCC strains (n = 7). The phage B_UFSM4 exhibited ability to produce plaques in 13 (34.2%, 13/38) bacteria isolates and B_UFSM5 in 16 (42.1%, 16/38) isolates. Both phages did not replicate in the ATCC strains (Table 1).

Notably, the host specie (*S. aureus*) reveled 50% (3/6) and 66.6% (4/6) efficiency for B_UFSM4 and B_UFSM5, respectively. In addition, plaques were also visualized in CPS, CNS, *Staphylococcus sciuri*, *Rothia terrae*, and *P. aeruginosa*, including antimicrobial resistant and/or multiresistant isolates (Table 1 and Fig S1).

Table 1
Lytic activity of phages against bacterial isolates of bovine mastitis and standard strains.

Bacterial species	Isolates/ strains	Antimicrobial resistence	B_UFSM4	B_UFSM5
S. aureus	SB 93/16 340 PE	Gen, PolB	-	-
	MRSA 2		+	+
	MRSA 3		+	++
	SB 113/06 162 PE	PolB, Sut, Cfc, Amp, Oxa, Pen	+++	+++
	SBP 120/19 Nevasca PD		-	+
	ATCC 25923		-	-
CPS	SB 101/16	PolB	+	+
	SB 53/17 20 AD	PolB	-	-
	SBP 17/20 342 PE		-	-
	SBP 17/20 312 AD	Pen, Ero	+	+
	SBP 17/20 79 PE	Cfe	-	-
	SBP 112/12 Chata AE		+	+
	SBP 01/13 33 PD		++	+++
	SB 208/18		+++	+++
S. sciuri	SB 57/17 49 PD		+	+
S. hominis	SB 08/12	Pen, Amp	-	-
S. cromogenes	SB 47/12 252 AD		-	-
S. epidermidis	ATCC 12225		-	-
CNS	SB 99/17 70 PE		-	-
	SB 99/17 25 PE		-	-
	SB 52/17 PD	Pen, Amp	-	+
	SB 105/94 Pintada PD	Pen, Nit	+	+
	SB 90/12		-	-
	SB 57/16		-	-

Bacterial species	Isolates/ strains	Antimicrobial resistence	B_UFSM4	B_UFSM5
	SB 52/12 Baia AD		-	-
Rothia terrae	SB 39/10 008 PE		+++	+++
S. agalactie	SB 70/16		-	-
	SB 93/16		-	-
	SB 56/17	Ero	-	-
S. uberis	SB 34/17		-	-
	SB 37/17		-	-
	SBP 90/17		-	-
S. dysgalactie	SB 103/17		-	-
Pseudomonas aureuginosa	SB 120/19 Frida PD	Cfe, Amp, Pen, Oxa, Sut, Ero, Cef, PolB, Tet	+	+
	SB120/19 Frida AD		+	+
	SB 134/16	Sut, Pen, Amp, Cfe, Tet, Cef, Oxa, Neo, Ero	+	+
	ATCC 25853		-	-
Enterobacter **sp.**	SB 21/18		-	-
	SB 132/10 DM 120		-	-
	SB 121/16	Tet, Amp, Cfe, Cef, Oxa, Neo, Ero	-	-
Enterobacter cloacae	ATCC 13087		-	-
Acinetobacter **sp.**	SB 23/18		-	-
	SB 76/16		-	-
Escherichia coli	ATCC 25552		-	-
Krebsiella pneumoniae	ATCC 13853		-	-
Enterococcus faecalis	ATCC 29212		-	-

Legend: (-) without lytic activity; (+) low lytic activity; (++) moderate lytic activity; (+++) high lytic activity. Gen: Gentamycin. PolB: Polymyxin B. Sut: sulfazotrim. Pen: penicillin. Amp: ampicillin. Tet: Tetracycline.

Oxa: oxacillin. Ero: enrofloxacin. Neo: neomycin. Cfc: cefaclor. Cfe: Cephalexin. Cef: ceftiofur. Nit: nitrofurantoin.

3.5. Phage morphology

Transmission electron microscopy (TEM) revealed that both phages have an icosahedral head with a long tail (Fig. 4a, b). The tails are ± 120 nm in B_UFSM4 and ± 150 nm in B_UFSM5, and are non-contractile and longer than the head diameter. Thus, no long tail fibers and contractile sheath were observed. Based on these morphological characteristics, phages were assigned to the family *Siphoviridae* based on the classification systems [39, 40].

3.6. Genomic characterization and phylogenetic analyses

The genomes of B_UFSM4 and B_UFSM5 have a size of 41.396 bp and 41.829 bp, respectively, in a double stranded DNA and linear architecture. The GC-content of the phage B_UFSM4 and B_UFSM5 is 33.97% and 33.98%, respectively. The phages present nucleotide identity of 98.91% between them. In both phages, 61 protein-coding genes were identified (Fig. 5a, b; Table S1 and S2). Of the 61 coding ORFs, only 1 protein coded distinctly; particularly on ORF 14, the phages B_UFSM4 and B_UFSM5 encode hypothetical protein similar to *Staphylococcus* phage phiPV83 (GenBank accession no. NC_002486) and *Staphylococcus* phage 80 (GenBank accession no. NC_030652), respectively.

Fifty-four ORFs were located on the minus strand and only seven ORFs were presented on the plus strand. Both contain functional modules, such as phage structure, host lysis, lysogeny, phage DNA packaging, and replication. Four proteins were involved in phage structure, namely putative major tail protein (ORF52), putative phage head tail adapter (ORF55), tail length tape measure protein (ORF48), and capsid protein (ORF58). The host lysis proteins identified were holin (ORF42) and amidase (ORF41). Ten proteins were identified to be involved in lisogeny, DNA packaging, modification, replication, and transcription, including DNA packaging protein (ORF56), single strand DNA binding protein (ORF21), dUTP nucleotidohydrolase (ORF7), transcriptional activator RinB (ORF5), integrase (ORF38), portal protein (ORF60), repressor (ORF34), anti- repressor (ORF29), cro (ORF33), and putative restriction-modification protein (ORF40).

Nevertheless, majority of proteins verified ($n = 29$) are hypothetical proteins (47.5%); of these 16(55.2%) are similar to the *Staphylococcus* prophage phiPV83 (GenBank accession no. NC_002486). Additionally, the proteins including single strand DNA binding, cro, repressor, and integrase, also are similar to the prophage phiPV83 proteins. The putative major tail protein is similar to *S. aureus* phage JS01 protein (GenBank accession no. NC_021773), as well as four hypotheticals. One of the hypothetical proteins is similar to *Staphylococcus* phage tp310_1 (GenBank accession no. NC_009761), and also to, the proteins of capsid, portal and putative phage head tail adapter. Notably, the lysogeny proteins (holin and amidase) are similar to phage phiJB (GenBank accession no. NC_028669). Other proteins are the tail length tape measure is similar to *Staphylococcus* phage 13 (GenBank accession no.NC_004617), DNA packaging to *Staphylococcus* phage CN125 (GenBank accession no. NC_012784), anti- repressor to *Staphylococcus*

phage DW2 (GenBank accession no. NC_024391), and putative restriction-modification to *Acinetobacter* phage B1251 (GenBank accession no. NC_019541).

A proteomic phylogenetic tree was reconstructed using ViPTree (Fig. 6). Phages B_UFSM4 and B_UFSM5 are closely related *Staphylococcus* prophage phiPV83 (45,536 nt). These sequences form a sister clade with phages isolated from *Staphylococcus* species: *Staphylococcu*s phage JS01 (43,458 nt), *Staphylococcus* phage 13(42,722 nt), *Staphylococcus* phage CN125 (44,492 nt), and *Staphylococcus* phage tp310_1(42,232 nt). These phage sequences belong to family *Siphoviridae*, genus *Biseptimavirus* and order *Caudovirales.* Thus, the phages B_UFSM4 and B_UFSM5 can be considered as new species in the family *Siphoviridae.*

In addition, the phylogenetic tree was reconstructed using the MCP and Terminase coding regions (Fig S2). The phages B_UFSM4 and B_UFSM5, in both phylogenies, grouped with *Staphylococcus* phages tp310_1 and 13. In analyzes with the Terminase, both the phages, also are grouped in a sister clade with *Staphylococcus* phages phiPV83 and JS01. Therefore, the tree topologies are very similar, in the analysis of the proteome, MCP and Terminase.

4. Discussion

Staphylococcus aureus is a significant microorganism in udder infections in dairy herds, and the reported cure rates for mastitis caused by *S. aureus* vary considerably, depending on animal, microorganism and treatment factors. Other concern related to *S. aureus* is antimicrobial resistance, which contributes to lower cure rate [10].

Considering the increasing global resistance of bacteria to antimicrobials and the importance to preserve the organism's resident microbiota, the use of bacteriophages has emerged as an alternative to control bacterial infections, since bacteriophages have the potential to be used as antibacterial agents and in the cases of antibiotic failure [41, 42].

In this study, two new *S. aureus* phages (B_UFSM4 and B_UFSM5) were successfully isolated from bovine milk. These viruses belong to the family *Siphoviridae*, order *Caudovirales*, based on their genomic and morphological aspects. The viruses belonging to this family have long, non-contractile, thin tails, which are often flexible, and are built of stacked disks of six subunits. Moreover, viral heads and tails are assembled separately [43]. In addition, the phylogenetic data verified that the phages belong to the genus *Biseptimavirus*.

In the studied phages were found three tail proteins: putative major tail protein, putative phage head tail adapter and tail length tape measure. The structural proteins of the tail determine the tail length and form a channel to transmit DNA into the host cell [44].

In this research, the portal protein form a specialized machinery of order *Caudovirales* that are involved in crucial aspects of virus replication, such as virion assembly, DNA packaging and DNA delivery [45].

Moreover, the integrase protein was identified by molecular analysis. The presence of this protein indicates that both phages can insert their DNA into the host. Thus, beyond lytic cycle, temperate phages also have a lysogenic cycle, incorporate their genomes into the host chromosome (or maintain their genome extrachromosomally); these phages can be as considered natural vectors for gene transmission between bacteria, which play a pivotal role in the virulence and resistance of bacterial pathogens [46–48]. Furthermore, the protein transcriptional activator RinB, was identified. This protein belongs to the group of *S. aureus* bacteriophage proteins related to the *int* gene, responsible for integrative recombination [49]; however, no homologs of virulence transfer or lysogenic genes were found in the genomes of phages B_UFSM4 and B_UFSM5.

Notably, the proteins holin and amidase were identified in both phages, and are responsible for the lysis of bacterial cell. Amidase is principally related to lysis, and holin is involved in amidase activation [29, 50].

Besides the aforementioned genes, several hypothetical proteins were identified, but due to the insufficient database information about the functional genes of *S. aureus* phages genomes, we were unable to verify their functionality [51].

In regards to the phylogenetic analyses, the isolated phages have similarity with *Staphylococcus* prophage phiPV83 (GenBank accession no. NC_002486) and *S. aureus* phage JS01 (GenBank accession no. NC_021773). The prophage phiPV83 genome has 45,636 bp and 64 ORFs, including two extra operons, lukM-lukF-PV. And, also presents the proteins: integrase, repressor, cro, and anti-repressor, thus demonstrate lysogeny similar mechanisms, as well as replication proteins (transcriptional activator RinB and single strand DNA binding). But it presents other proteins such as Ntpase (replication protein), Cos (packaging protein) and protease (head protein). Futhermore, the identified genes lukM and lukF-PV encode the leukocidin toxin Panton-Valentine (PVL), a virulence factor found in strains of *S. aureus* [52].

S. aureus phage JS01 was isolated from the milk of cattle affected by mastitis and also presents proteins similar to putative major tail and hypothetical proteins [53]. Thus, the similarity was 45,536 nt with *Staphylococcus* prophage phiPV83 and 43,458 nt with *S. aureus* phage JS01.

It was verified that high temperature (50ºC-70ºC) and extreme pH (3–11) do not affect the bacteriophages B_UFSM4 and B_UFSM5 stability, which is similar to studies conducted with other phages [20, 54]. The bacteriological activity significantly reduced in the prokaryotic cells after 5 h of virus incubation. Similarly, other surveys showed this reduction between 3 h-4h after the virus incubation [28, 54]. These results corroborate that phage therapy appears to be a promising alternative in the treatment of certain multidrug resistant bacterial infections [55].

Previous studies demonstrated *in vitro* efficiency and specificity *S. aureus* phages against *S. aureus* isolates from bovine mastitis [22, 24, 28, 56–59]. In our research, both phages also demonstrated lytic efficiency mainly against *S. aureus* and CPS of isolates. Additionally, it was detected their wide host amplitude included *S. sciuri*, CNS, *R. terrae*, and *P. aeruginosa*.

Although most bacteriophages are highly host specific, some could infect different bacterial genera and species [60], as observed in the case *S. aureus* phages SA, SANF and SA2, which demonstrate lytic activity against *Staphylococcus chromogenes, Staphylococcus saprophyticus, Staphylococcus xylosus, S. sciuri, Staphylococcus succinus*, and *Macrococcos caseolyticus* isolates from bovine mastitis [25]. Another study demonstrated host amplitude of *P. aeruginosa* phage PA1Ø infecting Gram-positive bacteria, such as *S. aureus*, in which this ability to infect other genera is related to production of lytic enzymes of phages[61].

Additionally, the wide range of host may be explained to the ability of phage to adsorb to a host cell by different receptor-binding proteins (RBPs). RBPs have endless adaptation cycles; therefore, some phages can use various RBPs for adsorbing to the bacterial host cell [62]. In this study, lytic activity against other genera and *Staphylococcus* species was observed. Therefore, presumably these genera share similar viral protein receptors.

5. Conclusions

In this study, two new *S. aureus* bacteriophages, belonging to *Caudovirales* order, *Siphoviridae* family and *Biseptimavirus* genus, namely the phages B_ UFSM4 and B_UFSM5, were isolated from bovine milk samples. These phages reveal similarities each other; however, they are distinct from other *S. aureus* phages of the family *Siphoviridae*. Thus, our results indicate that these phages can efficiently infect bacteria isolates recovered from their host species and also present wide host amplitude, including isolates with antimicrobial resistance. In addition, both phages can be researched in the future as an alternative in the treatment of infections of the mammary gland by *S. aureus*, which is difficult to treat.

Declarations

Conflict of interest

There are no conflicts of interest.

Ethical approval

Not applicable.

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Figures

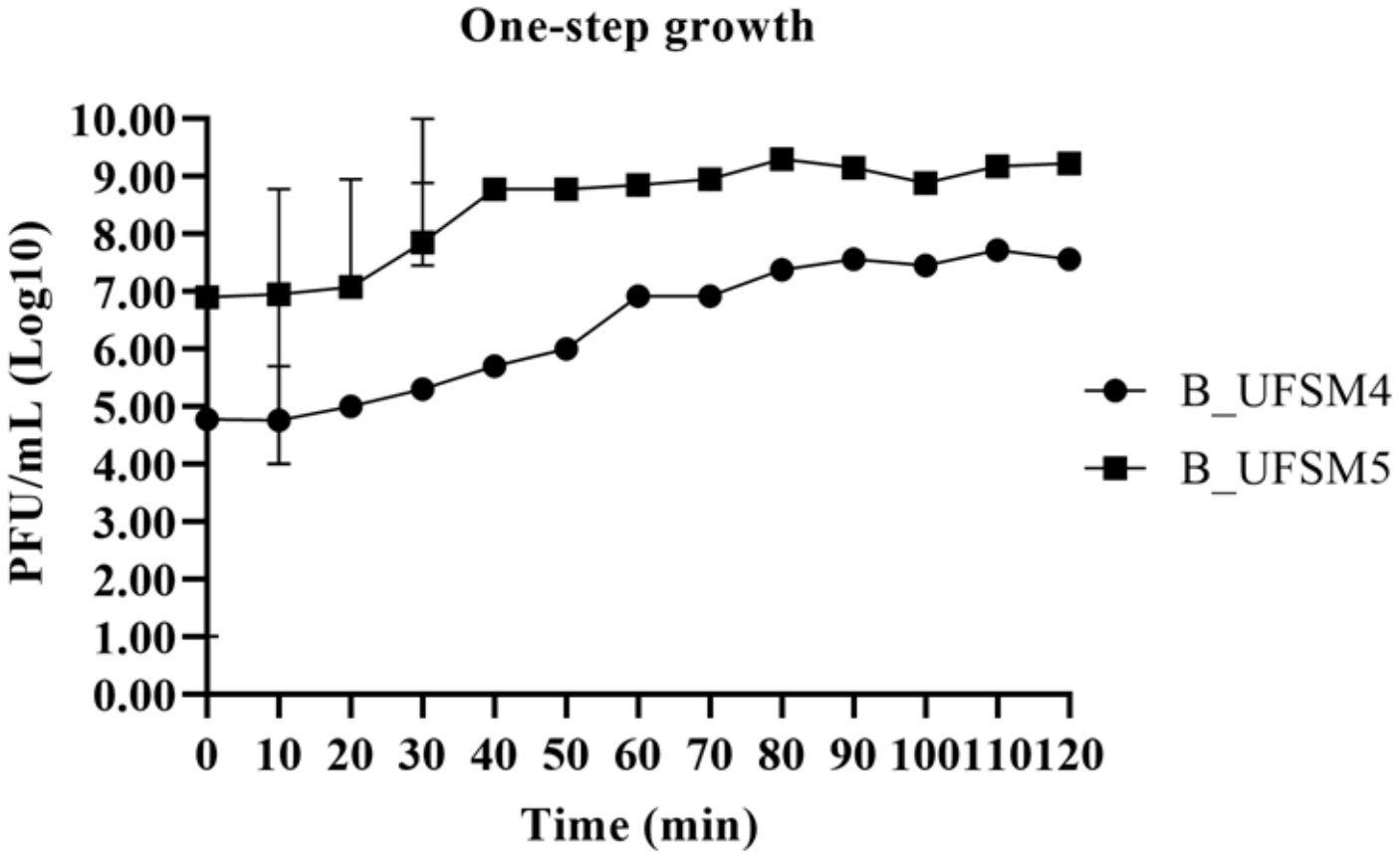

Figure 1

One-step growth curve of phages B_UFSM4 and B_UFSM5 on S. aureus (SBP 01/19 Gaivota PE) with standard deviations. The multiplication of B_UFSM4 was 7,56 PFU/mL (Log 10) and B_UFSM5 was 9,3 PFU/ mL (Log 10) after 2h growth.

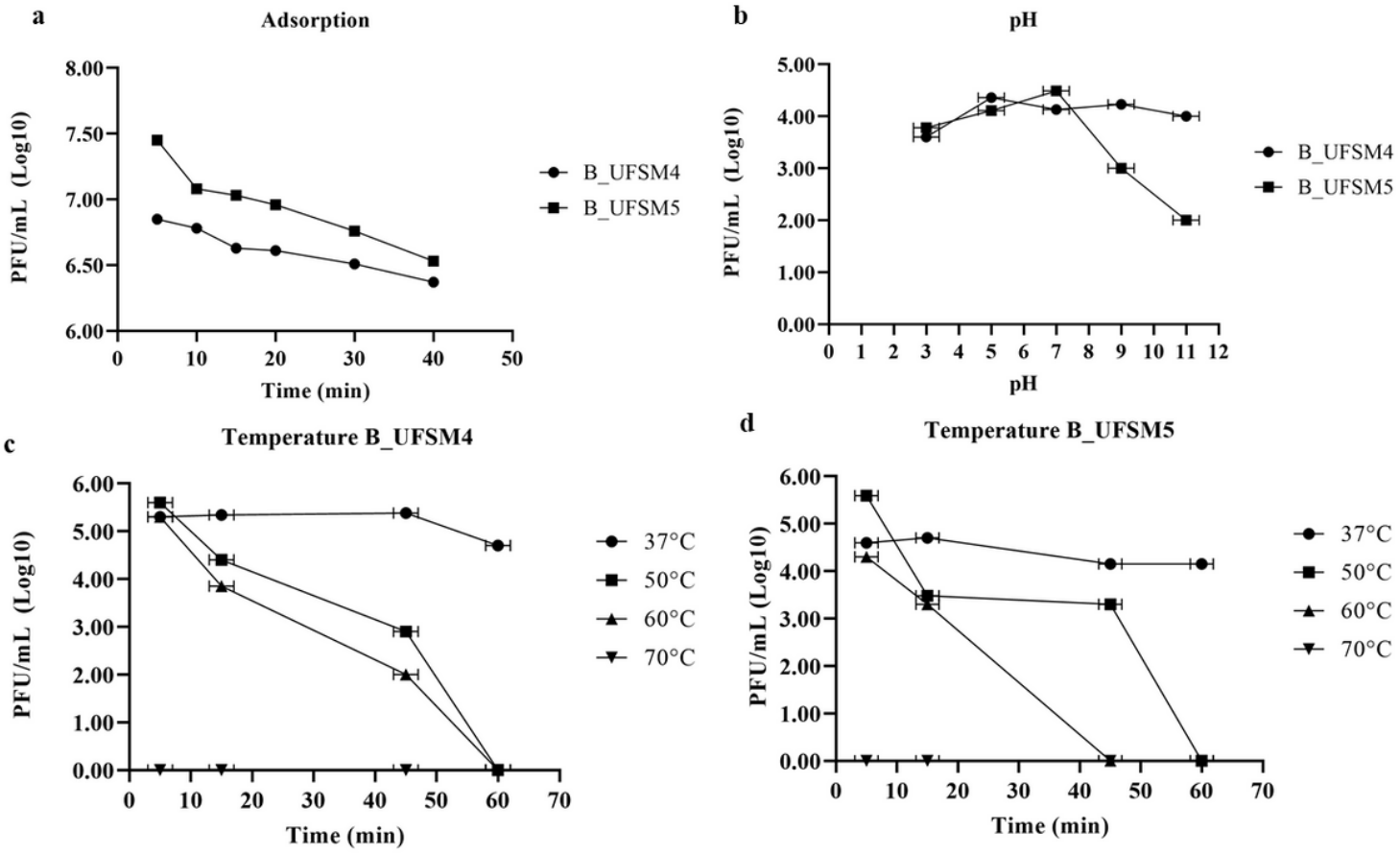

Figure 2

Adsorption (a), pH stability(B) and thermostability (c, d) of phages B_UFSM4 and B_UFSM5 on S. aureus (SBP 01/19 Gaivota PE) with standard deviations.

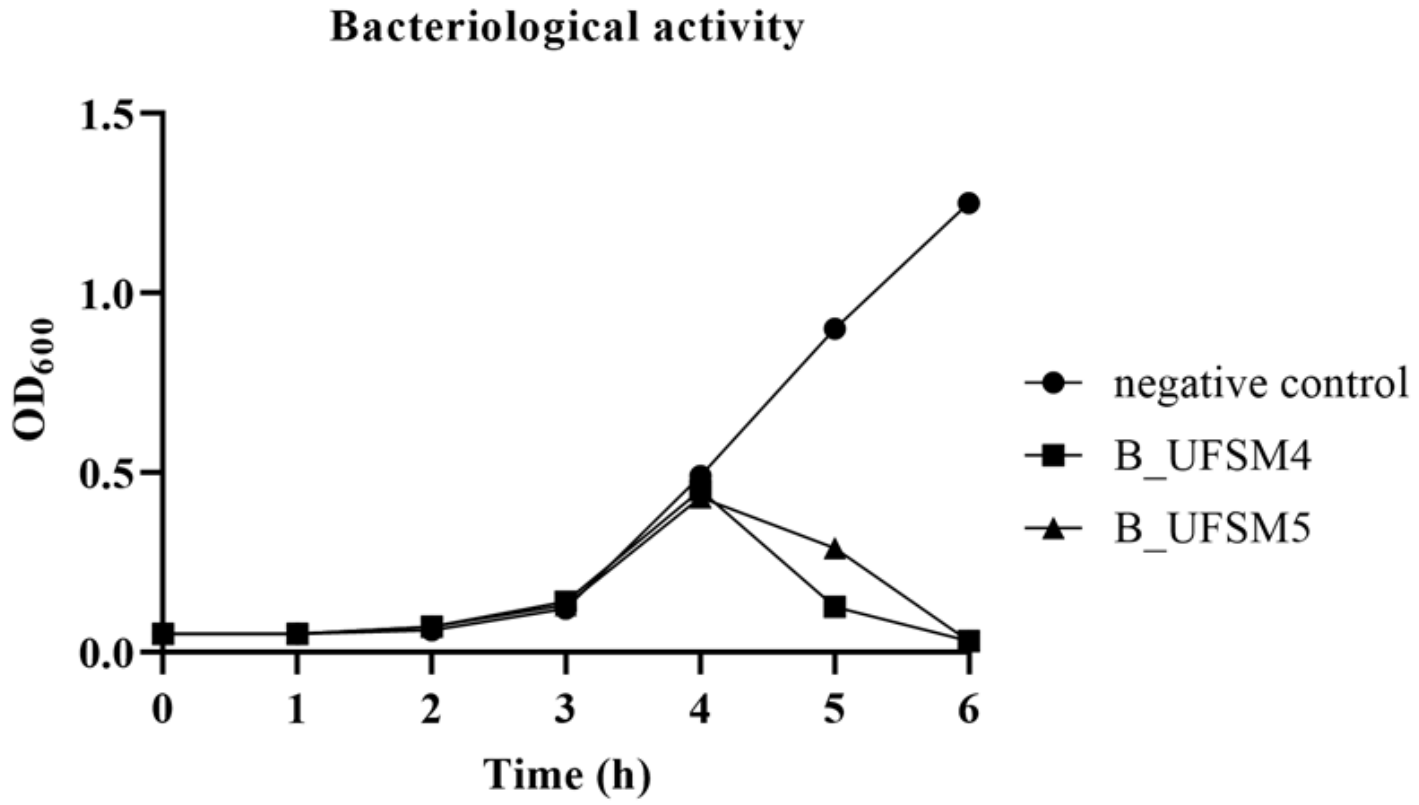

Figure 3

Bacteriolytic activity of phages B_UFSM4 and B_UFSM5 on S. aureus (SBP 01/19 Gaivota PE). After 5h incubation, both cultivations decreased to 0.030 (OD600) and the control reached 1.250 (OD600).

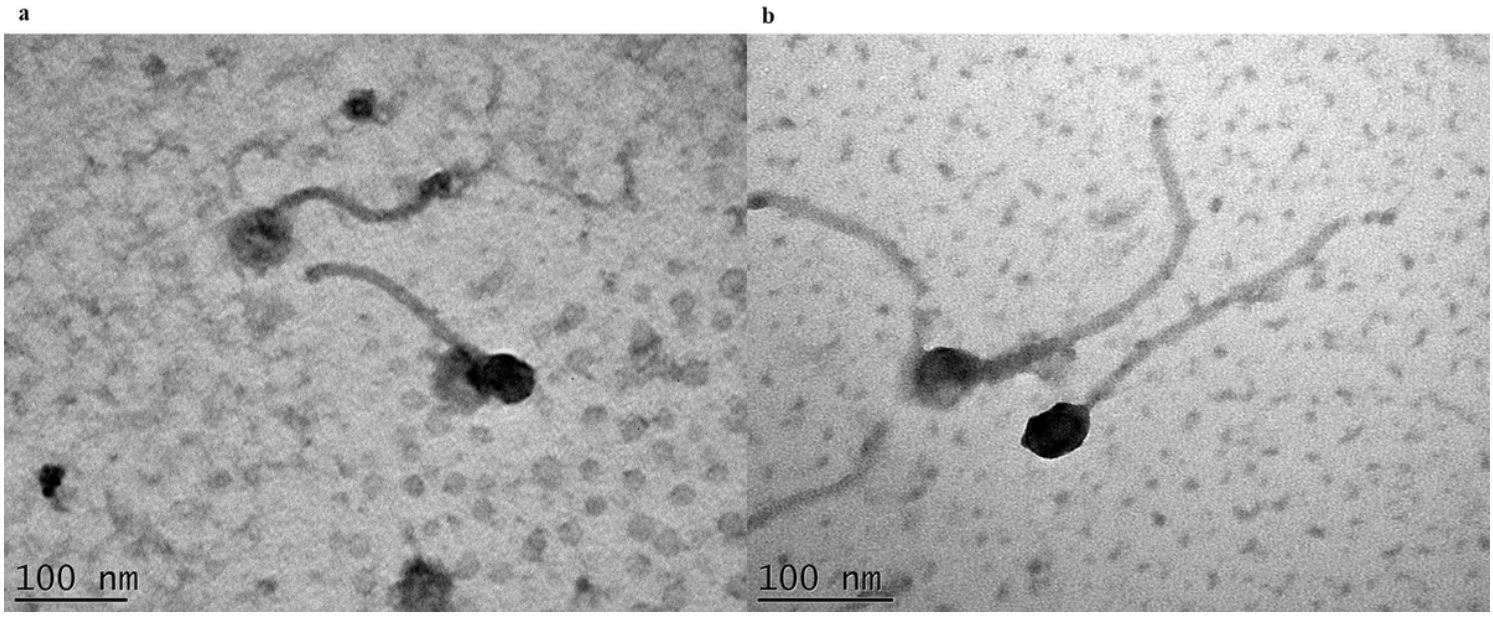

Figure 4

Transmission electron microscopy (TEM) of Staphylococcus phage B_UFSM4 (a). TEM of Staphylococcus phage B_UFSM5 (b). Bar corresponds to 100 nm.

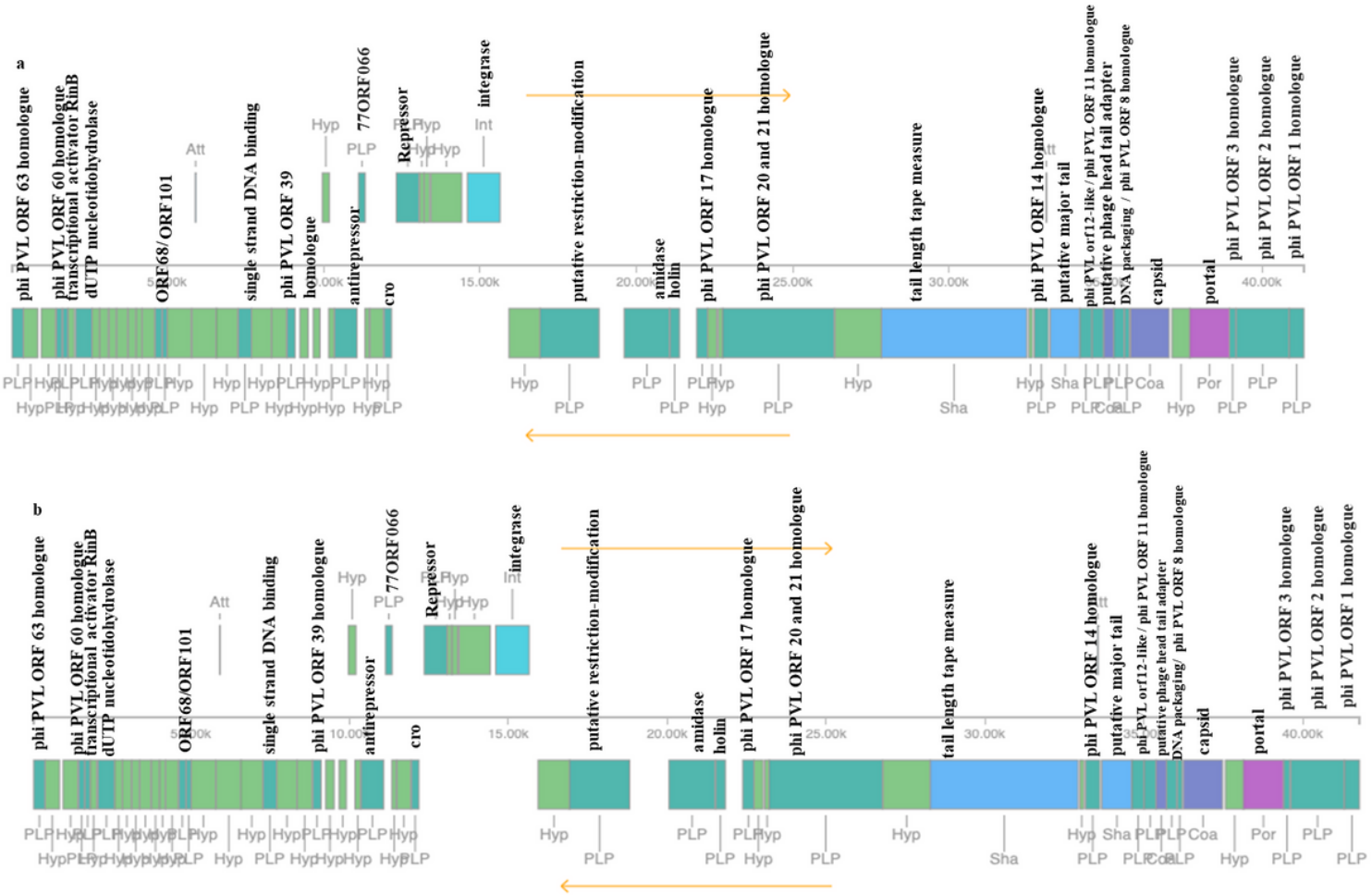

Figure 5

The graphical representation of protein-coding genes of the phage B_UFSM4 (a) and B_UFSM5 (b). The annotation of the genomes was carried by web serve PHASTER.

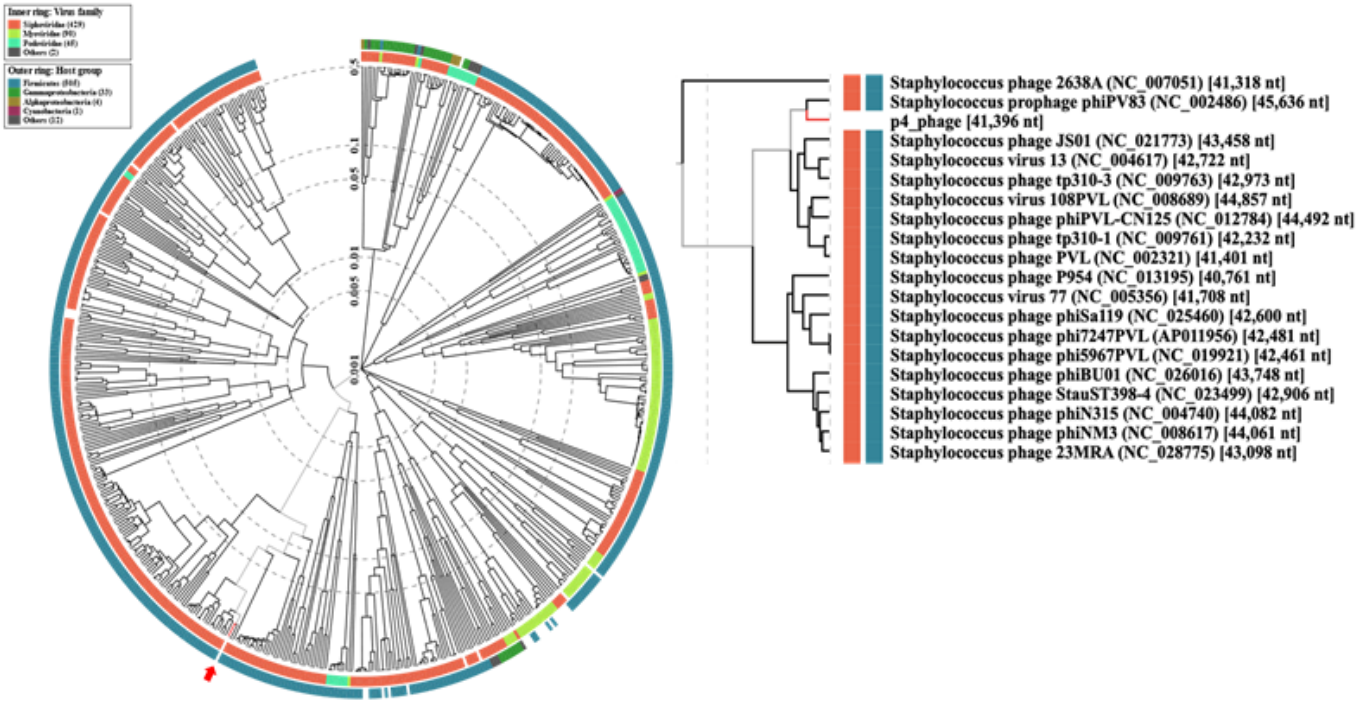

Figure 6

Graphical representation of phylogeny of the phages B_UFSM4 and B_UFSM5, using MAFFT software with alignment using PhyML - ViPTree.

Supplementary Files

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