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Lytic bacteriophages as a potential alternative to control *Staphylococcus aureus*

Abstract – The objective of this work was to characterize autochthonous bacteriophages and to determine their lytic activity on *Staphylococcus aureus*. Six phages were isolated from dairy barn flush water through enrichment cultures with three *S. aureus* strains. All phages were characterized by DNA digestion by restriction enzymes and sequencing of the DNA fragment encoding endolysin. Each phage was tested against 100 *S. aureus* strains isolated from bovine mastitis and from dairy products using the lysis-plate method. The sequences of the endolysin gene were highly conserved, with nucleotide similarity higher than 99% among the isolated phages. Three domains involved in the recognition and lysis of the bacterial cell wall were identified. Two bacteriophages isolated from the dairy barns present high lytic activity on *S. aureus*, on a wide range of host strains, indicating their potential for studies on phage therapy in dairy cattle or as a biological control agent for dairy products.

Index terms: *Staphylococcus aureus*, biological control, endolysin, phage.

Bacteriófagos líticos como alternativa potencial para o controle de *Staphylococcus aureus*

Resumo – O objetivo deste trabalho foi caracterizar bacteriófagos autóctones e determinar sua atividade lítica em *Staphylococcus aureus*. Seis fagos foram isolados de água de lavagem de pisos de estábulos por meio do enriquecimento de cultura com três estirpes de *S. aureus*. Todos os fagos foram caracterizados pela digestão do DNA com enzimas de restrição e pelo sequenciamento do fragmento de DNA que codifica a endolisina. Cada fago foi testado contra 100 estirpes de *S. aureus* isoladas de casos de mastite bovina e de produtos lácteos pelo método de lise em placa. Sequências do gene de endolisina apresentaram alta conservação, com mais de 99% de similaridade a nível do nucleotídeo entre os fagos isolados. Foram identificados três domínios envolvidos no reconhecimento e na lise da parede celular bacteriana. Dois bacteriófagos isolados de estábulos apresentam alta atividade lítica em *S. aureus*, em ampla gama de estirpes, o que indica seu potencial para estudos de fagoterapia em gado leiteiro ou como agente de controle biológico para produtos lácteos.

Termos para indexação: *Staphylococcus aureus*, controle biológico, endolisina, fagos.

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Introduction

Staphylococcus aureus is a gram-positive pathogen responsible for human and animal infections. It is a frequent etiological agent of mastitis, a disease that affects dairy cattle worldwide and causes

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economic losses in the milk production chain (Boldyreva, 2014). Some *S. aureus* strains, commonly isolated from dairy products, can also produce enterotoxins, which are responsible for staphylococcal food poisoning (Donovan et al., 2006; Obeso et al., 2008; Arcuri et al., 2010). In this context, antimicrobial therapy is an important component of mastitis control in dairy production systems. However, it is dificult to eliminate *S. aureus* from the livestock, since it is moderatly susceptible to antibiotics when the infection is detected at the beginning and hard to cure in chronic infections (Barlow, 2011). Furthermore, a number of studies suggest an increasing antimicrobial resistance in *S. aureus* (Johler et al., 2011; Wang et al., 2013), complicating staphylococcal disease treatment and highlighting the need to develop novel antimicrobial agents.

Lytic bacteriophages, also known as phages, infect and lyse bacterial cells, resulting in cell death. This was a therapeutic approach developed before the industrial production of antibiotics in the 1940s (Henry & Debarbieux, 2012). Currently, research on phage therapy is being revived worldwide due to the crisis of bacterial antimicrobial resistance. Different works have reported the efficiency of phages in the control of pathogenic bacteria both in animals (Wang et al., 2017) and foods (Silva et al., 2014).

Lytic *S. aureus* phages have shown effective and comprehensive antimicrobial activity in vitro and in vivo (Donovan et al., 2006; García et al., 2009; Wang et al., 2016; Zhang et al., 2017). These phages are interesting in the context of new methods to control the pathogen since they are natural bactericidal agents, ubiquitous in nature, and may have relative low production costs. Moreover, their genomics can be exploited towards producing endolysins, which target the integrity of the host cell wall and attack specifically one of the four major bonds in the peptidoglycan (Donovan et al., 2006; Fischetti, 2010). Recombinant endolysins have been characterized for use against *S. aureus* in the treatment of bovine mastitis and in bacterial control in milk and derivatives (Obeso et al., 2008; Li & Zhang, 2014; Fan et al., 2016).

The objective of this work was to characterize autochthonous bacteriophages and to determine their lytic activity on *Staphylococcus aureus*.

Materials and Methods

Phages were isolated from 20 samples of barn flush water collected from four dairy herds located in the Zona da Mata region, in the state of Minas Gerais, Brazil (21°45'51"S, 43°20'59"W). The enrichment culture method described by Cerveny et al. (2002), with some modifications, was used for phage isolation. Each sample of barn flush water was clarified by centrifugation at 3,000 *g* for 10 min, and supernatants were filtered through 0.45-µm pore membranes. A total of 1.0 mL filtrate was added to 100 mL Brain Heart Infusion broth (DIFCO, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with a mixed culture of three *S. aureus* strains used for enrichment, being centrifuged and filtered through 0.22-µm pore membranes after incubation for 48 hours at 37ºC. The double-layer method was used for all phage detections, isolations, and purifications, as in Green & Sambrook (2012), and clear lysis zones were interpreted as a positive result. Serial dilutions were performed to obtain single phage plaques, which were propagated three times by this method to ensure the purity of the phage cultures.

The host range of the isolated phages was determined by the classical spot-test assay, according to Adams (1959). Each phage was tested three times against 100 *S. aureus* strains – 64 from the milk of cows with clinical or subclinical mastitis, 23 from bulk milk cooling tanks, and 13 from "Minas Frescal" cheese –, as well as against *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 19117, and *Streptococcus agalactiae* ATCC 12386, belonging to the bacteria collection of Embrapa Gado de Leite, located in the municipality of Juiz de Fora, in the state of Minas Gerais, Brazil. Target bacterial lawns were prepared with overnight cultures on Brain Heart Infusion agar (DIFCO, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) plates, and 10 µL purified phages (10^8 pfu mL^{-1}) were spotted. Plates were incubated at 37°C, overnight, and the appearance of clear zones around the point of application was considered as the ability to lyse that strain.

The data were analyzed by the Wilson method, used to obtain the confidence interval (CI) for the estimated proportion of strains susceptible to the isolated phages, according to Brown et al. (2001), with the confidence level set to 95%. Analyses were performed in the R

software, version 3.6.1, using the binconf function of package Hmisc (2019).

Phage DNA was isolated from purified phages by the phenol-chloroform extraction method (Green & Sambrook, 2012). Briefly, after phage propagation, bacterial debris was pelleted by centrifugation at 9,000 *g*, for 20 min, at 4°C, and the supernatant was treated with DNase I and RNase A (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) to remove any bacterial nucleic acid. The phage pellet was obtained by centrifugation at 25,000 rpm, for 2 hours, at 4°C, and, then, resuspended in SM buffer; sodium dodecyl sulfate and proteinase K were added to a final concentration of 0.5% and 50 µg mL^{-1}, respectively. Following incubation at 65°C, for 10 min, the solution was deproteinated twice by extraction with an equal volume (25:24:1). DNA was precipitated with sodium acetate and ethanol and then pelleted with a microcentrifuge. After being washed with 70% ethanol, the DNA pellet was air dried and resuspended in a Tris-EDTA buffer.

Six phages were molecularly characterized by DNA digestion with the restriction enzymes *Eco*RI, *Xba*I, *Xho*I, and *Hind*III (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) according to supplier instructions. After digestion, samples were subjected to electrophoresis in 1% (w/v) agarose, containing 1 µg mL^{-1} ethidium bromide.

The putative endolysin gene region in phage DNA was amplified by the polymerase chain reaction (PCR) using the primers previously described by Donovan et al. (2006). DNA was amplified by 45 cycles of 95ºC for 1 min, 50ºC for 1 min, and 72ºC for 1 min, with a final extension of 72ºC for 15 min. Amplification was performed in the GeneAmp PCR System 9700 (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA). The reactions were carried out in a 50 µL volume consisting of 1X PCR buffer, 1.5 mmol L^{-1} $MgCl_2$, 10 µmol L^{-1} of each dNTP, 20 pmol of each primer, 100 ng phage DNA, and 3.0 U Taq DNA polymerase (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). The PCR products were visualized by gel electrophoresis in a 1.2% agarose gel (w:v), stained with ethidium bromide, and photographed under UV light in the Eagle Eye II imaging system (Stratagene, Agilent Technologies, La Jolla, CA, USA).

Nucleotide amplicon sequencing was performed using the DYEnamic ET Terminator Cycle Sequencing Kit in the MegaBACE 1000 DNA Sequencing System (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Final sequences were assembled using the CAP3 software (Huang & Madan, 1999), with all contigs with a depth of coverage of at least three times. Searches for nucleotide and amino acid sequence alignments were carried out with nucleotide sequences and deduced gene products, using BlastN, BlastX, and BlastP (Blast, 2018). For the phylogenetic analysis, the inferred sequences were compared with sequences of other phages on the GenBank, using the MEGA, version 4, software (Tamura et al., 2007). In silico analysis, also involved searches for specific protein domains and conserved motifs with a known function were identified by comparing the endolysin amino acid sequences of the six isolated phages with those of phage phi11 – GenBank accession number AF424781 (GenBank, 2018) –, which were aligned with the DNASTAR software (DNASTAR, 2018).

Results and Discussion

Six phages, named phage 1, 2, 3, 4, 5, and 6, were isolated from the samples of barn flush water using three *S. aureus* host strains. All phages formed clear lysis plaques on the bacterial lawns of the *S. aureus* host strains (Figure 1). However, none of the phages were able to form a plaque on *E. coli* ATCC 25922, *L. monocytogenes* ATCC 19117, and *S. agalactiae* ATCC 12386.

The purified phages had variable capacity of forming lysis plaques on the 100 *S. aureus* strain lawns tested (Figure 2). Phages 2 and 4 showed high intensities of lytic activity, evidenced by the high proportions of *S. aureus* strain susceptibility, ranging from 69 to 100%; for the other phages, the proportions were all lower than 47%. There are reports of the successful use of lytic phages to control *S. aureus* in milk products (Garcia et al., 2009; Bueno et al., 2012) and in animal infections (Mishra et al., 2014; Hamza et al., 2016; Zhang et al., 2017). In the present study, phages 2 and 4 show potential to be evaluated as biocontrol agents.

Restriction profiles were similar for all phages when genomic DNA was digested with *Eco*RI, *Hind*III, and *Xho*I. Genomic digestions with the restriction enzyme *Xba*I revealed similarities among the restriction profiles

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of phages 1, 2, 3, and 6, as well as between phages 4 and 5 (Figure 3). Other authors also found similar restriction profiles among different phages isolated at distinct locations (O'Flaherty et al., 2005; Mishra et al., 2014). Despite the strong genetic relationship among the six evaluated phages, their host range and lytic activities differ, as previously mentioned. Therefore, phages 2 and 4, with wider host ranges, could be the most indicated to control *S. aureus*.

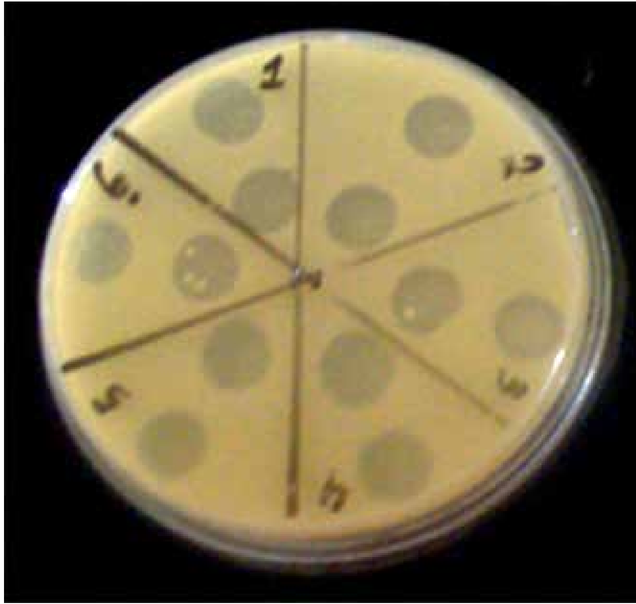

Figure 1. Lytic activity of the isolated bacteriophages against a *Staphylococcus aureus* strain isolated from a milk sample collected from a milk cooling tank. 1, phage 1; 2, phage 2; 3, phage 3; 4, phage 4; 5, phage 5; and 6, phage 6.

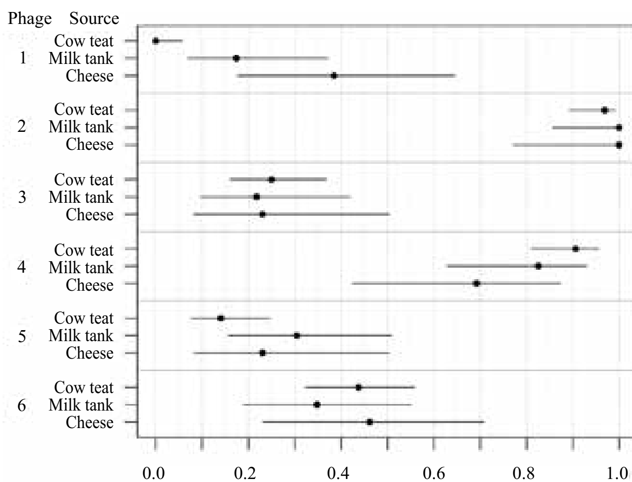

Figure 2. Proportions of *Staphylococcus aureus* strains isolated from the milk of cows with clinical or subclinical mastitis (n = 64 binomial trials for each phage type), from bulk milk cooling tank (n = 23), and from Minas Frescal cheese (n = 13), showing susceptibility to the isolated bacteriophages, with a confidence interval of 95%.

The size of the sequenced amplicons of the endolysin gene was 1,455 base pairs for all six phages. These sequences are available in the GenBank (2018), under accession numbers GU722132, GU722134, GU722133, GU722135, GU722136, and GU722131 for phages 1, 2, 3, 4, 5, and 6, respectively. The endolysin gene presented a highly conserved sequence, with a nucleotide similarity higher than 99% among the isolated phages (Table 1). In fact, the nucleotide identity between the endolysin gene sequences of phages 2

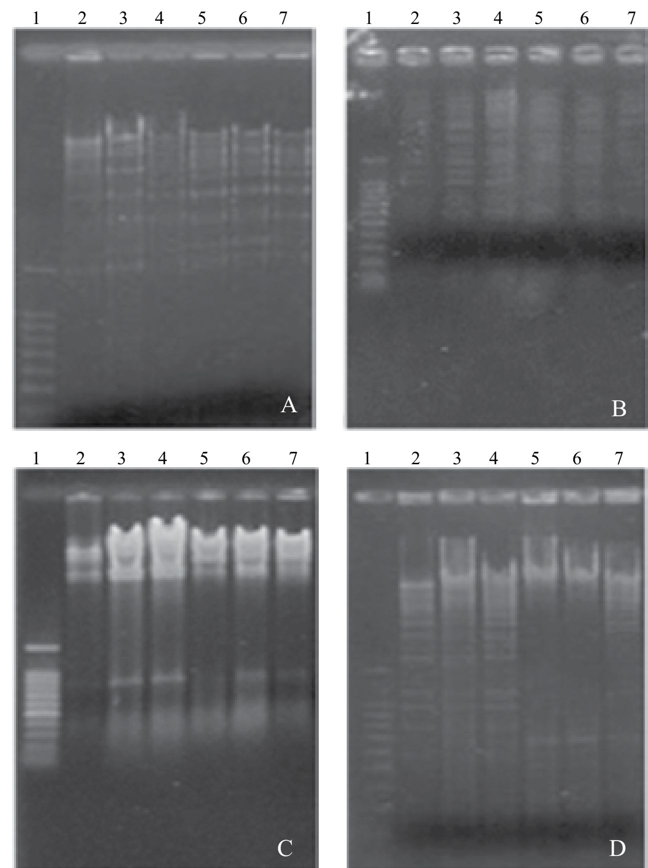

Figure 3. Genomic restriction profiles of the isolated bacteriophages. Viral DNA was extracted and digested with the *Eco*RI (A), *Hind*III (B), *Xho*I (C), and *Xba*I (D) enzymes. Lanes: 1, molecular weight using the 100bp DNA ladder (Promega Corporation, Madison, WI, USA); 2, phage 1; 3, phage 2; 4, phage 3; 5, phage 4; 6, phage 5; and 7, phage 6.

and 4, which showed the highest lytic activity, was 99.99%. However, nucleotide similarity was lower for the sequences obtained for phages 1, 3, 5, and 6. It should be noted that the endolysin gene sequences of the phages with a lower host range and lytic activity also showed higher nucleotide similarity; in this case, phages 1 and 6 had 100% nucleotide identity. The nucleotide sequence of the endolysin gene of the isolated phages presented an identity lower than 99% in relation to the other phage sequences available in the GenBank, indicating a closer relationship among the isolated phages. Similar results were found by Wu et al. (2019) when working with a phage specific to *Acinetobacter baumannii*.

The phylogenetic analysis of the nucleotide sequences of the endolysin genes showed a clustering of the isolated phages into three groups (Figure 4). Phages 1, 5, and 6 were clustered together, as well as phages 2 and 4; however, phage 3 was placed separately on a different branch. Phages 2 and 4 presented a higher phylogenetic distance in relation to the other phages, correlating to the lytic activity tests (Figure 2). The phylogenetic tree, based on the inferred amino acid sequence of the endolysin gene of the six phages and of the other phages from the GenBank, showed that the phages evaluated in the present study were clustered on the same branch, being related to phage phiMR25 (Figure 5). These phages were clustered on a separate branch of phage phi11, whose endolysin gene was the first one to be characterized, being considered the prototype (Donovan et al., 2006). Inside the isolated phage cluster, phages 1, 3, 5, and 6 were grouped in the same subgroup, reinforcing the distance among the endolysin gene of these phages and of phages 2 and 4 in the phylogenetic reconstruction based on nucleotide sequences (Figure 4). This strongly suggests that a few

Table 1. Similarity among the endolysin nucleotide sequences of the six isolated bacteriophages (phages 1 to 6) and of the other phages available in the GenBank (2018).

Phage	Nucleotide identity (%)					
	Phage 1	Phage 2	Phage 3	Phage 4	Phage 5	Phage 6
Phage 1	-					
Phage 2	99.5%	-				
Phage 3	99.7	99.6	-			
Phage 4	99.5	99.9	99.6	-		
Phage 5	99.8	99.6	99.7	99.6	-	
Phage 6	100	99.5	99.8	99.5	99.9	-
phiMR25	98.9	98.7	98.7	98.7	98.8	98.9
92	96.0	95.9	95.8	95.9	95.9	96.0
29	95.7	95.7	95.5	95.7	95.7	95.7
69	95.5	95.6	95.5	95.6	95.5	95.5
phi11	95.2	95.1	95.0	95.1	95.1	95.2
phiMR11	95.1	95.2	95.1	95.2	95.1	95.1
88	94.4	94.5	94.4	94.5	94.4	94.4
phiNM2	94.1	94.0	93.9	94.0	94.0	94.1
52A	93.8	93.9	93.7	93.9	93.7	93.8
80	93.8	93.9	93.7	93.9	93.7	93.8
85	92.7	92.7	92.7	92.7	92.6	92.7
55	92.3	92.4	92.2	92.4	92.2	92.3
phiH5	89.2	89.3	89.2	89.3	89.1	89.2
phiSauS-IPLA88	89.2	89.3	89.3	89.3	89.1	89.2
X2	85.4	85.5	85.4	85.5	85.4	85.4
37	73.8	73.8	73.7	73.8	73.7	73.8
EW	69.5	69.5	69.4	69.5	69.5	69.5

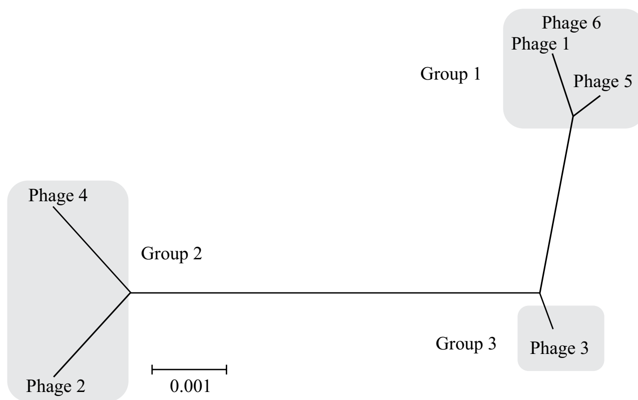

Figure 4. Phylogenetic relationships of the endolysin gene sequences of the isolated bacteriophages. The neighbor-joining tree was generated by the MEGA, version 4, software (Tamura et al., 2007). The evolutionary distances are expressed in units of the number of nucleotide substitutions per site (scale bar). The analysis was based on 1,000 bootstrap replicates, with a cutoff value of 95%.

nucleotide or amino acid variations may dramatically change host range, as observed by Wu et al. (2019). In this case, phages 2 and 4 could be the most indicated for therapeutic use.

The sequence analysis of the 481 amino acids inferred from the endolysin gene sequence of each isolated phage showed that these phage endolysins are also a modular enzyme. Three distint domains were identified on the endolysin sequences: cysteine; histidine-dependent amidohydrolase/peptidase (CHAP); amidase 2 (N-acetylmuramyl-L-alanine amidase); and SH3b, involved in bacterial cell wall recognition according to Son et al. (2018) (Figure 6 A). The endolysins expressed by the isolated phages potentially have domains for recognition and lysis of the *S. aureus* cell wall. Therefore, this modular organization containing three domains has been previously described for other

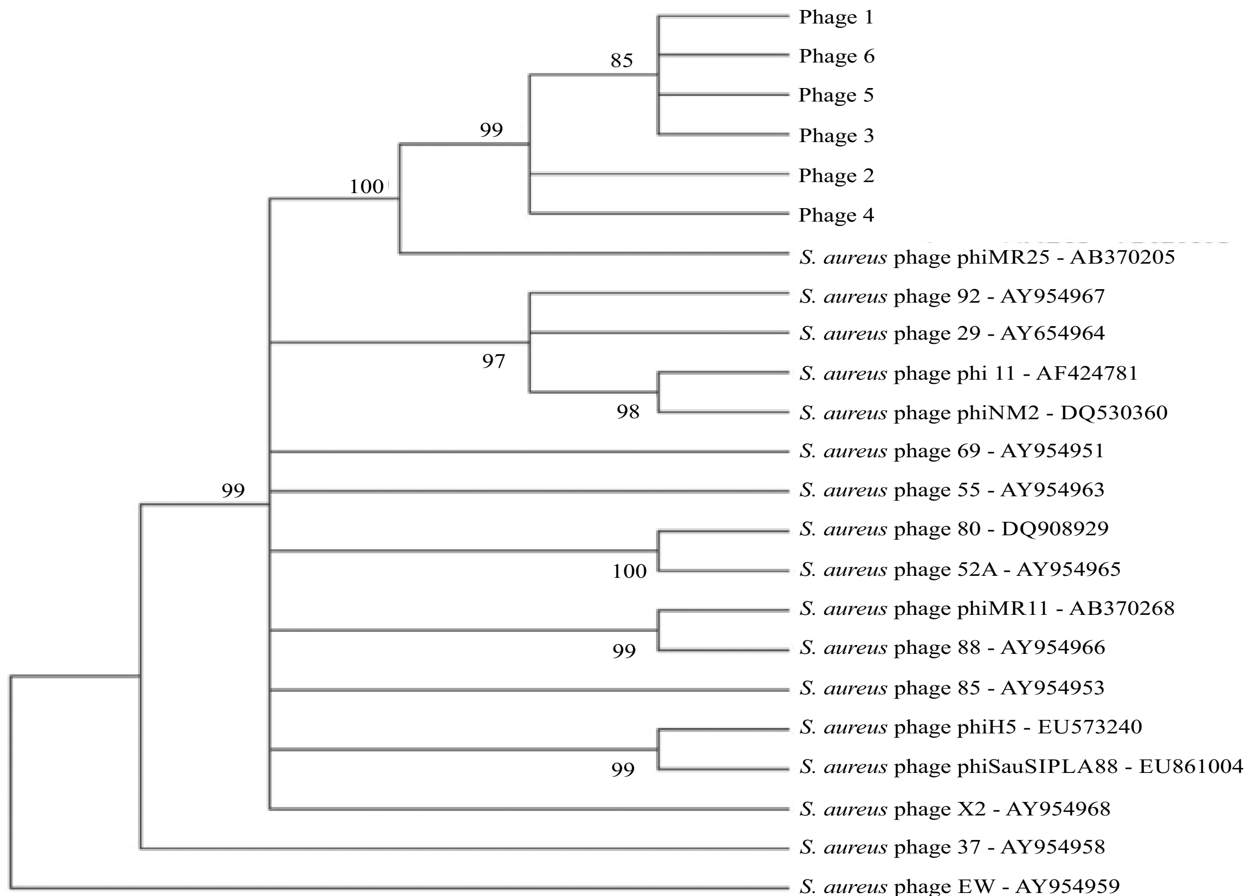

Figure 5. Bacteriophage phylogeny reconstruction based on the amino acid sequence inferred from the endolysin gene nucleotide sequence. GenBank accession numbers are given after dash (GenBank, 2018). The analysis was based on 1,000 bootstrap replicates, with a cutoff value of 95%.

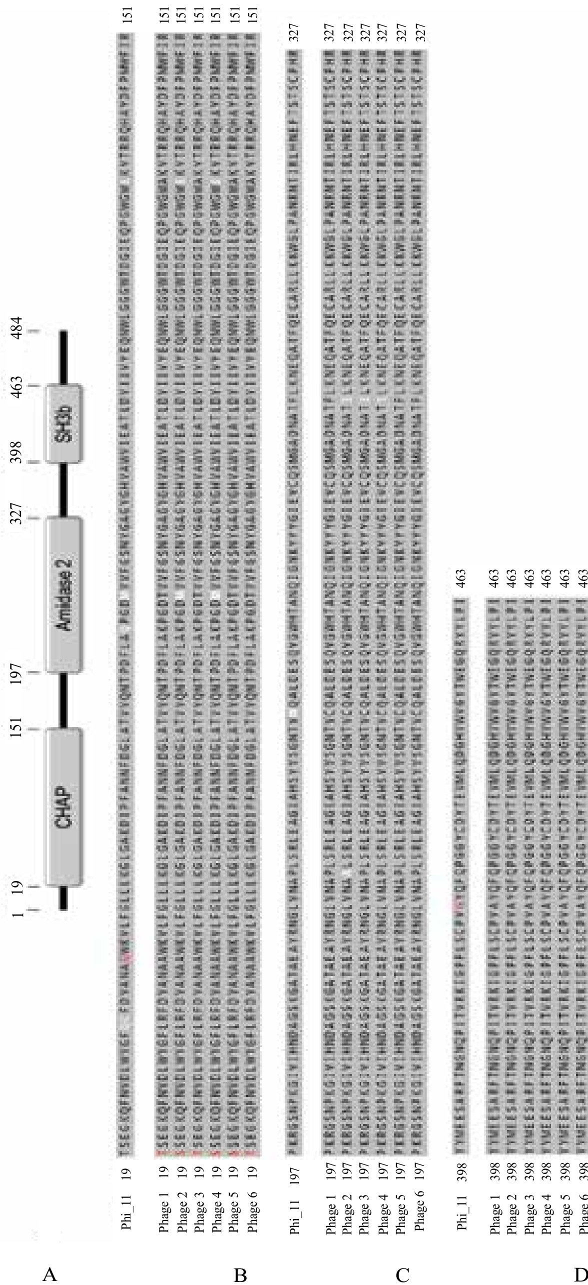

Figure 6. Amino acid sequence analysis of the endolysins of the isolated bacteriophages, showing the cysteine, histidine-dependent amidohydrolase/peptidases (CHAP), N-acetylmuramyl-L-alanine amidase (amidase 2), and SH3b (involved in the bacterial cell wall recognition) domains, identified by the Pfam, version 24.0, software (Pfam, 2018) (A); and amino acid sequence alignment of the CHAP (B), amidase 2 (C), and SH3b (D) domains of the isolated lytic phages and phage phi 11, using the DNASTAR software (DNASTAR, 2018). Identical amino acids are shaded in gray, conservative substitutions are marked in red and unrelated amino acids are in white.

S. aureus lytic phages (Donovan et al., 2006; Obeso et al., 2008). Concerning the amino acid sequence of the three domains, phages 1, 5, and 6 presented identical sequences (Figure 6 B, C and D), which were similar to that of phage 3, with only one unrelated substitution in the amidase 2 domain. The amino acid sequence of the three domains of phages 2 and 4 showed a high homology to that of phage phi11, but a lower homology to those of phages 1, 3, 5, and 6. Although the lytic activity of a phage is multifactorial, the high lytic activity presented by the endolysins of phages 2 and 4 could be related to the proximity of the prototype endolysin of phage phi11 (Donovan et al., 2006).

Three domains related to the recognition and lysis of the bacterial cell wall were identified in the present work. Further studies envolving these protein expressions and their potential use as an antimicrobiotic agent should still be conducted.

Conclusions

1. The host range of the six bacteriophages isolated from *Staphylococcus aureus* varies.

2. Phages 2 and 4 show a wide host range and potential for *S. aureus* control.

3. The endolysins of the isolated phages are similar to those of previously studied staphylococcal lytic phages.

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