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Original article

Characterization of *Staphylococcus epidermidis* phage vB_SepS_SEP9 – a unique member of the *Siphoviridae* family

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Abstract

Relatively few phages (<10) of coagulase negative staphylococci (CoNS) have been described. *Staphylococcus epidermidis* phage vB_SepS_SEP9 is a siphovirus with a unique morphology as a staphylococcal phage, possessing a very long tail. Its genome is unique and unrelated to any phage genomes deposited in public databases. It appears to encode a nonfunctional integrase. Due to the not having a recognizable lysogeny module, the phage is unable lysogenize. The genome comprises 129 coding sequences (CDS), 46 of which have an assigned function and 59 are unique. Its unique morphology and genome led to the proposal of the establishment of a new *Siphoviridae* genus named "Sep9likevirus".

Keywords: *Staphylococcus epidermidis*; Bacteriophages; *Siphoviridae*; Genomics

1. Introduction

Many *Staphylococcus* species are associated with nosocomial infections, endocarditis, sepsis, pneumonia and other diseases, being now considered a threat to public health [1,2]. The interest in *Staphylococcus* phages has increased significantly mostly due to the increasing resistance of this bacterium to antibiotics [3].

Phages can be characterized by many taxonomical criteria, while the nomenclature of phage taxa is regulated by the International Committee on the Taxonomy of Viruses (ICTV) [4]. Staphylococcal phages have been classified into distinct groups, or clusters, based on virion characteristics (dimensions, particle weight, buoyant density) and DNA (molecular weight, G + C %, genome sequences) [5–8].

The reduction in sequencing costs has caused an increasing number of phage genomes to be deposited in online databases, presently over 1400 (http://www.ebi.ac.uk/genomes/phage.html). Over 80 of these genomes are of staphylococcal phages, 90% of which are viruses infecting *Staphylococcus aureus*. Recently, 46 fully sequenced *Siphoviridae* genomes of staphylococcal phages were analyzed, according to nucleotide and protein sequences and phage morphology. This analysis resulted in the proposal of the genera "3alikeviruses," "77likeviruses" and "Phietalikeviruses" [9]. In addition to siphoviral staphylococcal phages, there are four sequenced phages of the *Podoviridae* family, all of which belong to the 44AHJD-like genus of the *Picovirinae* subfamily, and while the majority of the 13 sequenced *Myoviridae* phages, belong to the *Twortlikevirus* genus of the *Spounaviridae* subfamily there

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are a few that still remain unclassified (http://www.ebi.ac.uk/genomes/phage.html).

To date, only a few genomes from phages for coagulase negative staphylococci (CoNS) of the following species have been sequenced and characterized: six for *Staphylococcus epidermidis* [10–13], two for *Staphylococcus hominis* [5] and one for *Staphylococcus capitis* [5]. Of these CoNS phage genomes, one *S. epidermidis* phage is from the *Myoviridae* family [13], while all others belong to the *Siphoviridae* family.

The current interest in finding new *S. epidermidis* phages is due to the implication of this organism as the second major cause of bloodstream infections [14]. Previously, we isolated five different *S. epidermidis* phages and fully characterized one of them, the broad-host-range *Twortlikevirus* phage SEP1 [13]. Phage vB_SepS_SEP9 (SEP9) also has a broad lytic spectrum but produces completely different plaques. This prompted the investigation of its morphology and genome, which showed unique features.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Forty-six staphylococcal strains belonging to four different species were used in this study, namely 38 clinical isolates of *S. epidermidis* and two *Staphylococcus haemolyticus*, four *S. aureus* collection strains and three *Staphylococcus intermedius* veterinary isolates (Table 1). All strains were grown in Tryptic Soy Broth (TSB, Oxoid), on Tryptic Soy Agar (TSA, Oxoid) or in TSA soft agar overlays (0.4% agar) at 37 °C.

2.2. Phage propagation

Phage SEP9 was isolated from raw effluents of a wastewater treatment plant (ETAR de Frossos, Braga, Portugal) [13]. Phage particles were amplified in solid media as described elsewhere [13], using the double agar layer method with *S. epidermidis* M129 as the host. Phages were spread on the host bacterial lawns using paper strips. After overnight incubation at 37 °C and observation of lysis, 4 ml SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris/HCl (pH 7.5), 0.002% (w/v) gelatin were added to each plate. After 24 h agitation at 120 RPM (PSU-10i Orbital Shaker (BIOSAN, Riga, Latvia)) at 4 °C, the homogenized top-agar layer was collected, centrifuged (10 min, 10,000 × g, 4 °C) and filtered as described before. Chloroform (10 ml) was added to each 50 ml of phage suspension and samples were stored at 4 °C.

SEP9 was titrated essentially as described elsewhere [15]. Samples were serially diluted in SM buffer, after that 100 µl of each phage dilution and 100 µl of host bacterium were mixed with 3 ml of a soft agar top-layer. PFU (plaque forming unit) were counted after 18 h incubation at 37 °C.

2.3. Electron microscopy

Transmission electron microscopy was performed as described by Melo et al. [13]. Briefly, phage particles were collected after centrifugation (1 h, 25000 × g, 4 °C) (Beckman J2-21 centrifuge with a JA-18.1 rotor). Using the same conditions the pellet was washed twice in tap water. Phages were deposited on copper grids with carbon-coated Formvar films, stained with 2% uranyl acetate (pH 4.0) and observed using a Philips EM 300 electron microscope [16]. Magnification was monitored with T4 phage tails.

Table 1
SEP9 host range and EOP on different staphylococci.

Strain	Infectivity	EOP[a]
S. epidermidis M129	+	High
S. epidermidis DEN110	+	High
S. epidermidis FJ6	+	High
S. epidermidis ICE21	+	High
S. epidermidis ICE9	+	High
S. epidermidis IE186	+	High
S. epidermidis IE75	+	High
S. epidermidis ITL34	+	High
S. epidermidis JI6	+	High
S. epidermidis URU23	+	High
S. epidermidis 9142	+	Moderate
S. epidermidis DEN19	+	Moderate
S. epidermidis TAW113	+	Low
S. epidermidis 1457	+	LFW
S. epidermidis COB20	+	LFW
S. epidermidis DEN116	+	LFW
S. epidermidis DEN120	+	LFW
S. epidermidis DEN185	+	LFW
S. epidermidis GRE26	+	LFW
S. epidermidis ICE120	+	LFW
S. epidermidis ICE192	+	LFW
S. epidermidis ICE24	+	LFW
S. epidermidis LE7	+	LFW
S. epidermidis MCO150	+	LFW
S. epidermidis MEX37	+	LFW
S. epidermidis PE9	+	LFW
S. epidermidis PLN64	+	LFW
S. epidermidis RP62A	+	LFW
S epidermidis CV45	–	–
S. epidermidis COB17	–	–
S. epidermidis DEN69	–	–
S. epidermidis DEN94	–	–
S. epidermidis HUR51	–	–
S. epidermidis ICE102	–	–
S. epidermidis ICE21	–	–
S. epidermidis ICE5	–	–
S. epidermidis IE214	–	–
S. epidermidis MEX60	–	–
S. intermedius 4363	–	–
S. intermedius 4837	–	–
S. intermedius 4878	–	–
S. haemolyticus IE246	–	–
S. haemolyticus M176	–	–
S. aureus CECT 86	–	–
S. aureus CECT 239	–	–
S. aureus CECT 976	–	–

[a] The EOP was recorded as high, moderate, low and lysis from without (LFW) representing 10, 0.1–1, 0.1%, and no individual plaques formed, respectively.

2.4. Host range and efficiency of plating (EOP)

Host range and EOP of phage SEP9 were determined by dropping 5 µl aliquots from a 10-fold dilution series of the

phage, starting at 10^9 PFU ml^{-1}, on double-layer agar plates seeded with the 47 staphylococcal strains to be tested. Plaques were counted after 24 h of incubation at 37 °C. The EOP was calculated by comparing the numbers of PFU on phage-susceptible strains to PFU obtained with *S. epidermidis* M129.

2.5. One-step growth curve

One-step growth analysis of SEP9 was performed as described previously [13]. Briefly, a mid-exponential phase culture at an OD_{600} of 0.5 was centrifuged (5 min, 7000 × g, 4 °C) and resuspended in 10 ml of fresh TSB to attain an OD_{600} of 1.0. SEP9 was added to this suspension to a MOI of 0.005. After incubation for 5 min at 37 °C and 120 RPM to allow for adsorption, the culture was centrifuged and the pellet resuspended in fresh warm TSB. Samples were taken every 5 min over a period of 30 min and then every 10 min until 1 h post-infection and titrated.

2.6. Nature of the phage

To determine whether SEP9 is virulent or temperate, putative lysogens were isolated as described elsewhere with some modifications [17]. Briefly, the host strain was grown in broth overnight being further diluted 1/1,000,000 fold into 20 tubes of broth and regrown to late log phase. Phage was added to an MOI of 10 to the culture, allowed to attach for 15 min and plated out. One colony was selected per plate, streaked out 3 times and then tested for: (a) insensitivity for superinfecting phage, (b) spontaneous production of phage, and (c) PCR.

A primer pair was designed to amplify 537 bp of the major capsid protein of SEP9 (SEP9-mcp). Amplification products were generated by PCR, using KAPA Taq PCR Kit (Kapa Biosystems, Boston, MA, USA). The reaction was performed in 25 µl, containing 1 × KAPA Taq Buffer, containing a final concentration of 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer (SEP9-mcp-F 5′ – AAGCAGGTTTCGTTGGAGAA 3′; SEP9-mcp-R5′ – CACCAATGTTGCCAAAGATG 3′), 1U KAPA Taq DNA Polymerase and 25 ng of DNA template using the following steps: denaturation at 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 30 s; annealing at 50 °C for 30 s and elongation at 72 °C for 45 s, ending with an additional elongation step of 10 min at 72 °C. The amplified products were analyzed by electrophoresis on a 1.5% agarose gel.

2.7. Genome sequencing and analysis

SEP9 total DNA was extracted as described before [13]. Briefly, before DNA extraction, the concentrated crude lysate was treated with 0.016% (v/v) L1 buffer [300 mM NaCl, 100 mM Tris/HCl (pH 7.5), 10 mM EDTA, 0.2 mg BSA ml^{-1}, 20 mg RNase A ml^{-1} (Sigma), 6 mg DNase I ml^{-1} (Sigma)] for 2 h at 37 °C. After a thermal inactivation of the enzymes for 15 min at 70 °C, 50 µg proteinase K ml^{-1}, 20 mM EDTA and 1% SDS were added and proteins were digested for 18 h at 56 °C. SEP9 was extracted with one volume phenol:-chloroform:isoamyl alcohol solution (25:24:1, v/v). After repeating the previous step, an equal volume of chloroform was added. The supernatant was precipitated on ice with isopropanol 3 M sodium acetate (pH 4.6), air-dried and it was resuspended in nuclease-free water (Cleaver Scientific, Rugby, UK). Genome sequencing was performed on a 454 sequencing platform (Plate-forme d'Analyses Génomiques at Laval University, Québec, Canada) to 50-fold coverage. Sequence data was assembled using SeqMan NGen4 software (DNASTAR, Madison, WI, USA).

The genome of this virus was autoannotated, using MyRAST [18]. The presence of non-annotated CDSs, along with genes in which the initiation codon was miscalled, were checked manually using Geneious 6.1.6 (Biomatters, San Francisco, CA, USA). BLASTX was used to examine for potential frameshifts [19] and BLASTP were used to search for homologous proteins [20], with an *E* value threshold of $<1 \times 10^{-5}$. Protein motif search was HHpred using Pfam, InterProScan, and COG with an *E* value threshold of $<1 \times 10^{-5}$ and at least 80% query coverage [21]. The presence of transmembrane domains was checked using TMHMM [22] and Phobius [23] and were annotated when both tools were in concordance. Protein molecular weight (Mw) and isoelectric point (pI) were determined using ExPASy Compute pI/Mw [24]. The search of putative tRNA encoding genes was done using ARAGORN [25] and tRNAscan-SE [26]. Fragments 100 bp upstream of each predicted ORF were extracted and MEME [27] was used to search for putative promoter regions that were further manually verified. Rho-independent terminators were predicted using ARNold [28] and the energy was calculated using Mfold [29].

2.8. Nucleotide sequence accession number

The genome sequence of *Staphylococcus* phage vB_SepS_SEP9 was deposited in the GenBank database under the accession number KF929199.

3. Results and discussion

3.1. SEP9 isolation and morphology

S. epidermidis phage SEP9 was isolated previously [13] and is herein characterized. On 0.4% agar plates, SEP9 formed 0.9 mm clear plaques surrounded by an increasingly turbid halo indicative of the presence of depolymerase activity [30]. This phage was designated vB_SepS_SEP9, in accordance with the recommendations proposed by Kropinski et al. [31]. Electron microscopy showed a siphovirus with a head of 64 nm in diameter and a very long flexible tail of 375 × 10 nm, conspicuous transverse striations and a six-sided star-like baseplate (Fig. 1). Heads were icosahedra, as shown by the observation of both hexagonal and pentagonal capsids. In addition to normal heads, we observed occasional small heads of about 55 nm in diameter. A similar phenomenon was also reported for coliphage P1, which produces heads of three

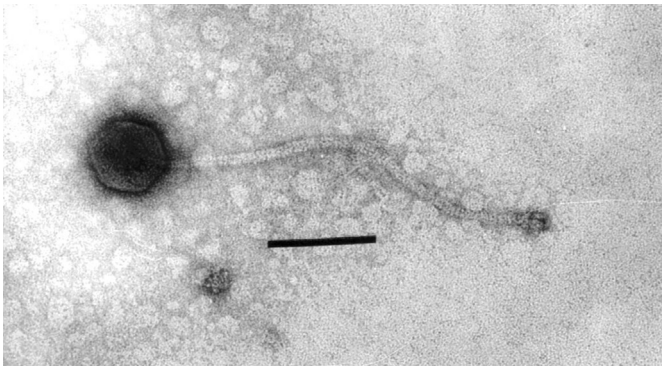

Fig. 1. Transmission electron micrographs of *S. epidermidis* phage vB_SepS_SEP9. The bar indicates 100 nm.

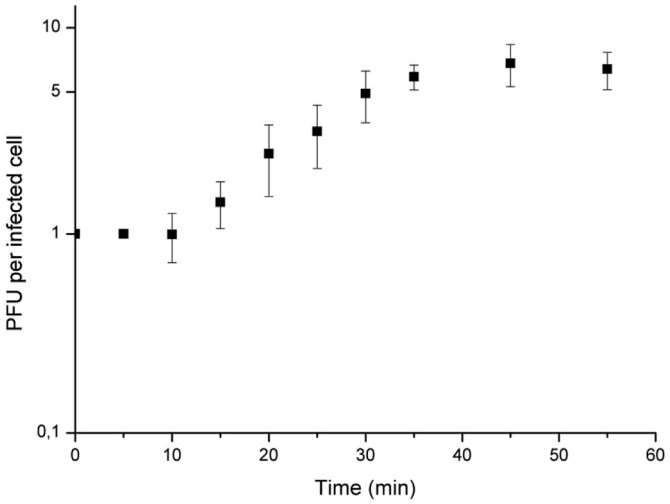

Fig. 2. One-step growth curve of phage vB_SepS_SEP9 in *S. epidermidis* M129 at 37 °C. Shown are the PFU per infected cell.

different diameters [32]. It may represent a new species within the *Siphoviridae* family.

3.2. Host range and efficiency of plating (EOP)

A collection of forty-six staphylococcal strains was used to determine SEP9 host range and EOP (Table 1). The *S. epidermidis* strains tested were isolated from diverse types of infection in different countries [13]. SEP9 was specific to *S. epidermidis* and infected 74% of the strains tested, which is considerably higher than infection rates of around 38% observed in other *S. epidermidis* phages [33]. SEP9 is able to propagate in 14 clinical strains, though with different EOPs (high, moderate and low). Similar to phage SEP1, no correlation was observed between lytic activity and the infection and geographical origin of the strains [13]. Furthermore, in contrast with other staphylococcal Twortlikeviruses [34], SEP9 did not lyse the other staphylococcal species tested.

3.3. One-step growth curve

To examine the infection parameters of SEP9, one-step growth curve experiments were performed. SEP9 has a latent period of approximately 10 min and a burst size of approximately 6.39 ± 0.47 PFU after 30 min at 37 °C (Fig. 2). The short latent period and the small burst size are in accordance with other *S. epidermidis* phages [33,13]. The small burst size observed may be related to SEP9's short lysis time [35].

3.4. Genomic properties

SEP9 possesses a linear dsDNA genome of 92,417 bp with a G + C content of 29.6%. This G + C content is significantly lower than in other *S. epidermidis* phages (~32%) [36] and lower than in 46 other staphylococcal phages (33–38%) [9].

This phage displays 129 putative CDSs (coding sequences) that are tightly packed and occupy ~89% of the genome (Fig. 3). Of these CDSs, 46 have an assigned function and 59 are unique (Table S1). The majority of the CDSs present an ATG start codon (89%), while seven start with TTG, five with GTG and two with ATT. A single tRNA gene, specifying a prolyl-tRNA, was discovered in SEP9. A BLASTN search revealed that no identical phages were found in the database. SEP9 genome was compared with its most homologous phage, vB_SepiS-phiIPLA7 and as visible on Fig. 3, there are few homologous regions throughout their genomes. The results showed 79% of identity with a query of 3%, essentially on the region of tail-associated proteins.

Despite the uniqueness of SEP9, this phage possesses the same modular genomic architecture as the majority of dsDNA phages [37]. Consequently, the packaging, structure/morphogenesis, host lysis and replication/regulation modules are present and well defined throughout the genome. MEME analysis revealed seven putative promoters (consensus: TTGACA(N17)TATAaT) while ARNold/MFOLD revealed eight rho-independent terminators, reinforcing the modular arrangement and suggesting a modular control of gene expression.

The phylogeny of the large subunit terminase (TerL) has been used to predict the packaging strategy used by bacteriophages [38]. SEP9 TerL homologs, identified using BLASTP, are preferentially found in prophage proteins in *Bacillus*, *Kurthia*, *Lactobacillus*, *Oribacterium*, and *Veillonella* strains. Restricting the search to "Viruses (taxid: 10239)" resulted in hits among the *Bacillus*, *Enterococcus*, *Geobacillus*, *Lactobacillus*, *Leuconostoc* and *Staphylococcus* phages. A phylogenetic tree constructed using the "one click" mode at *phylogeny.fr* [39], showed that SEP9 TerL is closely related to TerL proteins of *Lactobacillus delbrueckii* lytic phages (Fig. S1). *Lactobacillus* phages c5 and LL-Ku are members of the *Siphoviridae* and their genomes possess 11-bp 3′ overhang cohesive ends (LL-Ku, 5′-TAACACCCGAA-3′; c5, 5′-AAACACCCGAA-3′) [40]. A similar site (CAACACCCGAA) was found between residues 85982 and 85992, 6480 upstream of *terL*, which we suspect corresponds to the true left end of the *Staphylococcus* phage SEP9 genome. The

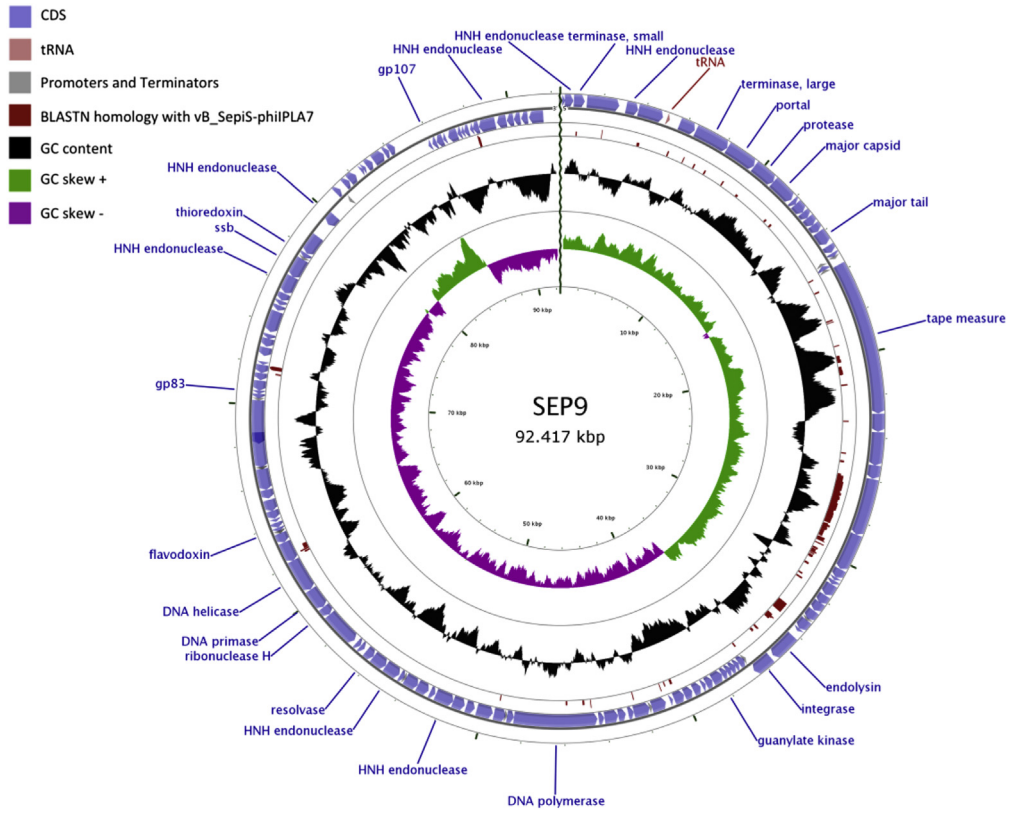

Fig. 3. Circular view of SEP9 genome and BLASTN comparison with vB_SepiS-phiIPLA7. The outer ring represents SEP9 CDSs. At red are represented the homologies with vB_SepiS-phiIPLA7. The GC content appears in the black ring and the green ring is GC skew+ and the pink ring is the GC skew−. Some important genes are highlighted. It should be clearly stated when a figure must be published in color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

genome was reopened at this point to render it collinear with that of the *Lactobacillus* phages. Furthermore, the presence of a prohead protease is an additional argument that favors the hypothesis that SEP9 is a cos-type phage.

In the morphogenesis module, the major capsid protein displays high sequence identity with *Lactobacillus* phage ATCC 8014-B2, while the major tail protein resembles that of *Listeria* phage B025. Both phages are also members of the *Siphoviridae* family. A correlation between the size of the gene encoding the tail-tape measure protein (TMP) and the size of the virion tail has been described [41]. This might explain the very large size of SEP9 tail (375 nm), since the SEP9 gene encoding the TMP is 2395 aa. This protein, like that of other staphylococcal siphoviruses [9] contains an N-terminal lytic transglycosylase SLT domain and a C-terminal peptidase_M23. SEP9 encodes a head-tail connector and head-tail adapter are described to be involved in DNA packaging being localized inside the head shell, where the tail attaches [42].

SEP9 also encodes a virion-associated hydrolase with a N-terminal endopeptidase domain (gp16) and a pre-neck appendage protein (gp18) with a pectin-lyase like domain that could be involved in the degradation of extracellular polymers [11]. The presence of depolymerases may help the efficacy of some phages degrading biofilm matrix [11]. In the tail morphogenesis module, two proteins, gp15 and gp19 were predicted to be involved in the receptor binding. Remarkably, these two proteins are homologous to receptor-binding proteins of other CoNS phages [10,5].

SEP9 lysis cassette is comprised of holin and endolysin. Although both proteins are similar to marine *Staphylococcus saprophyticus* phage φRS7 (NC_022914) lysis proteins, in SEP9 they are separated by two undefined genes (27 and *28*). The endolysin contains an N-terminal CHAP endopeptidase domain and a SH3 peptidoglycan-binding domain in the C-terminus separated by an N-acetylmuramoyl-L-alanyl-amidase domain. This structure is typical of *Staphylococcus* phage endolysins [43]. Furthermore, two transmembrane domains were predicted in the holin, and this protein can be classified as being part of the lactococcal φLC3 family [44] similar to *Staphylococcus* phage ϕ11 [45].

Although encoding an integrase (gp30), SEP9 does not appear to possess a complete set of genes required for lysogeny. It lacks a repressor of the CI type, which is normally present in other staphylococcal phages [46]. The fact that there are no similar sequences on the more than 80 staphylococcal genomes deposited in databases might suggest that the phage is not temperate. Furthermore, our experiments show that SEP9 is unable to lysogenize, as the 20 selected colonies were sensitive to phage, no phage was detected in their supernatants and the major capsid protein of SEP9 was not detected by PCR on the isolated colonies (data not shown). Results showed that of the 66 putative proteins of SEP9 homologous to other

phages, 40 are homologous to virulent phages (61%), while 26 are homologous to temperate phages (39%), which enlightens the mosaic structure of staphylococcal phages [6].

The replication module differs from that of other staphylococcal phages. SEP9 has a DNA polymerase that is similar to the replicase of *Bacillus* phage SPBc2. Other replication proteins, namely a repair exonuclease (gp66), DNA primase (gp68) and an exonuclease (gp128) are also similar to that *Bacillus* phage. SEP9 encodes two DNA methyltransferases (gp58 and gp59) that showed homology to a *Campylobacter* phage CP220 protein and to *Twortlikevirus Staphylococcus* phage SA11 respectively. Ribonuclease H (gp67) is a protein that plays a role in mRNA processing. While homologs of this protein are somewhat common in siphoviruses of Proteobacteria, this is the first example in *Siphoviridae* of Firmicutes. Finally, the ribonucleotide-reductase beta subunit (gp78) is homologous to that of *Listeria* phage A511 (*Myoviridae*) and the ribonucleotide-reductase alpha subunit (gp81) is homologous to those of *Bacillus* siphoviruses Slash and Staley.

Interestingly, a response regulator (gp98) was predicted after the detection of a HTH_LUXR domain, which is part of transcriptional regulators of LuxR family [47]. Regulators with this motif have been reported previously in other virulent phages genomes [48], however, using BLASTP no homologies were detected between gp98 and other phage proteins.

Overall, seven ORFs coding for HNH endonucleases and two intron-associated endonucleases were annotated in SEP9 genome. These proteins are commonly observed in phages of the genus *Twortlikevirus* [34], but are rare in staphylococcal *Siphoviridae* phages. Nevertheless, they were observed in phages 85 and X2 [49], and are stated to be involved in insertion or transposon elements on phage genomes [50]. Furthermore, using both BLASTP and HHPred no virulence genes, of bacterial origin, were detected, which is important to select good candidates for phage therapy [51].

3.5. Concluding remarks

A novel virulent *S. epidermidis* phage SEP9 was characterized. This phage has a broad lytic spectrum and a short life cycle. Its morphology is different from those of the already defined genera "77likevirus", "3alikevirus" and "Phietalikevirus" of staphylococcal siphoviruses. Its linear dsDNA genome has 129 CDSs, including 59 unique CDSs, and no identified virulence genes. Morphological and genomic unique characteristics of this phage led to the proposal of the creation of a new *Siphoviridae* genus named "Sep9likevirus".

Conflict of interest

There is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2014.09.012.

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