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ARTICLE

Genome Analysis of Phage SMSAP5 as Candidate of Biocontrol for *Staphylococcus aureus*

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Abstract

In this study, we reported the morphogenetic analysis and genome sequence by genomic analysis of the newly isolated staphylococcal phage SMSAP5 from soil of slaughterhouses for cattle. Based on transmission electron microscopy evident morphology, phage SMSAP5 belonged to the *Siphoviridae* family. Phage SMSAP5 had a double-stranded DNA genome with a length of 45,552 bp and 33 % G+C content. Bioinformatics analysis of the phage genome revealed 43 open reading frames. A blastn search revealed that its nucleotide sequence shared a high degree of similarity with that of the *Staphylococcus* phage tp310-2. In conclusion, this study is the first report to show the morphological features and the complete genome sequence of the phage SMSAP5 from soil of slaughterhouses for cattle.

Key words: *S. aureus*, phage, genome, slaughterhouse

Introduction

Staphylococcus aureus among the food-borne pathogens is recognized worldwide as the important bacteria due to a combination of toxin mediated virulence, invasiveness and antibiotics resistance (Chambers, 2001). The primary reservoir is on the skin and mucous membranes of animals (Jaglic *et al*., 2010; van Duijkeren *et al*., 2004), and also *S. aureus* is the main etiological agent of bovine mastitis (Fessler *et al*., 2010). Slaughter is a process with many risks for contamination of the cattle carcass with potentially *S. aureus. S. aureus* can be used as an indicator of the general hygiene, including the hygienic status of the soil, water and equipment in the slaughterhouse (Borch *et al*., 1996).

Staphylococcal food-borne diseases resulting from consumption of contaminated food are the second common cause among reported foodborne illnesses in US (Altekruse *et al*., 1997; Bunning *et al*., 1997; Holmberg and Blake, 1984; Levine *et al*., 1991). The estimated $50 million is spent each year for managing *S. aureus* poisoning in hospitals, and the costs to the dairy industry are even higher in Canada (Bradley and Teresa, 2005; Kim, 2001). The cases reported have been around 1600 in France between 1999 and 2000 (Le Loir *et al*., 2003). According to a report from MFDS (Ministry of Food and Drug Safety in Korea), *S. aureus* poisoning has increased from 363 patients in 2001 to 863 patients in 2005 in domestic (MFDS, 2005).

S. aureus produces various virulence factors, which are staphylococcal enterotoxin and toxic shock syndrome toxin I inducing superantigenic activity (Hennekinne *et al*., 2012). Especially, phage-encoded virulence factors provide *S. aureus* with various toxins such as staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin E (SEE), exfoliative toxin A, and Panton-Valentine leukocidin (Pinchuk *et al*., 2010). Through bioinformatics analysis and molecular characteristics, the relationships between staphylococcal phages and *S. aureus* in antibiotic resistance, gene transfer, pathogenesis, and evolution have been recently discussed (Skippington and Ragan, 2011). Also, staphylococcal phages have been intensively studied and have been used in a variety of practical applications such as phage therapy (Sulakvelidze *et al*., 2001), the detection and biocontrol of foodborne pathogens (Dinnes *et al*., 2007; Greer, 2005; Hudson *et al*., 2005), and bioremediation (Withey *et al*., 2005). More recently, phages

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have been exploited in an attempt to remove bacterial biofilms (Lu and Collins, 2007). In application of phage for *S. aureus*, phages are effective against *S. aureus* in contaminated foods or food-stuffs (Choi, 2009; Manoharadas; 2009; O'Flaherty *et al*., 2005; Rodriguez, 2010), and phages active against *S. aureus* have been widely studied in experimental infections in animals (Capparelli *et al*., 2007; García *et al*., 2009; Matsuzaki *et al*., 2003; Wills *et al*., 2005). In this study, we reported the morphogenetic analysis and genome sequence analysis of phage SMSAP5 for *S. aureus* isolated from soil of slaughterhouses for cattle.

Materials and Methods

Isolation of phage for *S. aureus*

To isolate the phage for *S. aureus*, soil samples collected from the Icheon slaughterhouse in Korea. Host strain of phage was *S. aureus* ATCC25923. This strain was grown in Luria-Bertani (LB) broth or agar supplemented with 10 mM $CaCl_2$ (LBC) at 37ºC overnight in a shaking incubator. To isolate phage for *S. aureus*, soil samples were analyzed by a plaque assay. Briefly, 5 g soil sample was mixed with 7-8 Log CFU/mL of *S. aureus* and incubated with shaking at 37°C for 24 h. Then, the culture was centrifuged, and the supernatant was filtered by 0.22 μm syringe membrane filter. The filtrate was tes- ted for phage by plaque assay using double overlay agar. The plaque was picked, phages eluted with SM buffer [100 mM NaCl, 8 mM $MgSO_4$•$7H_2O$, 50 mM Tris-Cl (pH 7.5)], and re-picked. Phage was propagated and purified by method of Sambrook (Sambrook and Russel, 2001).

Morphology and phage DNA extraction

To confirm the morphological characteristics, purified phage particles were negatively stained with 2% aqueous uranyl acetate (pH 4.5) on a carbon-coated grid and examined by transmission electron microscopy. Phage DNA was collected from polyethylene glycol precipitated phage particles by method of Manfioletti *et al.* (Manfioletti and Schneider, 1988) with some modifications. DNase I (10 μg/mL) and RNase A (20 μg/mL) were added to phage lysate, respectively. After incubation at room temperature for 15 min, 0.5 M EDTA (pH 8.0) and proteinase K (1 mg/mL) were added, followed by incubation at 65°C for 30 min. After incubation, the nucleic acid was extracted with a mixture of phenol-chloroform-isoamyl alcohol (25:24:1). The nucleic acid was precipitated with ethanol, and resuspended in sterile distilled water. Phage DNA was stored at -80°C.

Genome sequence analysis

The genome sequence was generated by ultra-high throughput GS FLX sequencing to 20-fold redundancy on average. The nucleotide sequences were compared with those of other genes in GenBank by the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The open reading frames (ORFs) were identified with the ORF Finder at the National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov/gorf.html). The molecular weight and isoelectric point were calculated with the Compute pI/*M*w program (http://www.expasy.ch/tools/pi_tool.html). The promoter and tRNA were identified with promoter hunter (http://www.phisite.org) and tRNAscan-SE program (http://lowelab.ucsc.edu/tRNAscan-SE/), respectively.

Nucleotide sequence accession number

The complete genome sequence of phage SMSAP5 was deposited at GenBank under accession number JQ779023.

Results and Discussion

Phage SMSAP5 was isolated and purified from soil of slaughterhouse by a plaque assay. To confirm morphology, phage SMSAP5 was confirmed by transmission electron microscopy (Fig. 1). According to morphological analysis, Phage SMSAP5 had long, non-contractile tails and icosahedral heads belonging to the *Siphoviridae* fam-

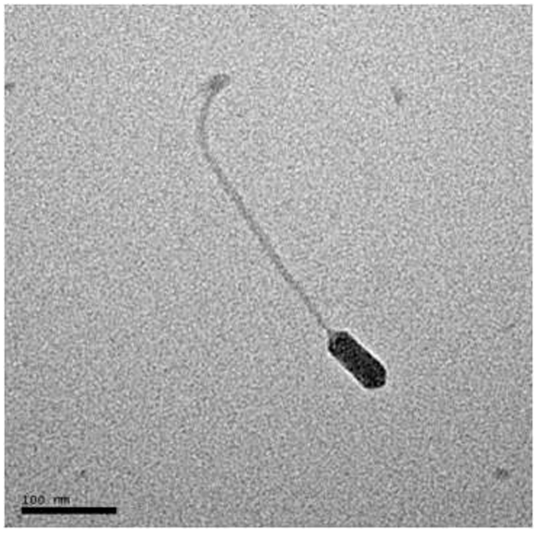

Fig. 1. Transmission electron micrograph showing morphology of phage SMSAP5. The scale bar in the lower right corner represents 100 nm.

ily in the order *Caudovirales*. Phages can be tailed, polyhedral, filamentous, or pleomorphic, and most of them contain double-stranded DNA. About 5,568 bacterial viruses have been examined by electron microscopy. At least 5,360 (96.2%) of these are tailed bacteriophages known as *Caudovirales* (Ackermann, 2007). The length of phage SMSAP5 genome was 45,552 bp with 33 % G+C contents. As the results of genome sequence were searched by blastn, the Phage SMSAP5 sequence shared a high nucleotide similarity (89%) with the bacteriophage tp310-2 (accession number: EF462198). Bioinformatic analysis of the phage SMSAP5 genome revealed 43 putative ORFs. A total of 28 ORFs were similar to gene in the GenBank with annotated function. In accordance with comparison of phage SMSAP5 sequences were attributed to composition of basic functional modules, as follows; replication, DNA packaging, morphogenesis, lysis, and lysogeny (Fig. 2).

In the structure/morphogenesis module, the predicted protein encoded by ORF28 was identified as the minor structural protein and showed similarity to the tail fiber protein of *Staphylococcus* phage phiSauS-IPLA35. This protein was found to play the most important role in host specificity and adsorption of phage to the outer membrane of a bacterial cell (Wood *et al*., 1978). ORF31 protein was the longest predicted ORF of phage SMSAP5 genome and showed similarities to the tape measure protein repeat from *S. aureus* subsp. *aureus* MRSA131. Tape measure protein determined tail length by working as a template for measuring length during tail assembly (Katsura and Hendrix, 1984). ORF34 and ORF35 encoded the products homologous to the putative minor tail protein by 100% identity. The protein encoded by ORF39 showed 100% identity to the putative capsid protein of *Staphylococcus* phage phi2958PVL. Other structural/morphogenesis-related proteins were those encoded by ORF30 and ORF40. Within the DNA-packaging cluster, the protein encoded by ORF42 and ORF43 was identified as a terminase large subunit and a terminase small subunit, respectively. Moreover, the protein encoded by ORF41 showed 100% similarity to the phage portal protein of *Staphylococcus* phage phi 12. The protein encoded by ORF23 in the lysogenic region was identified as a site-specific recombinase (accession number: EGS98875).

In the replication modules, ORF1 showed 100% identity to HNH endonuclease of *S. aureus* A9754. HNH endonucleases are small DNA binding and digestion proteins characterized by two His residues and an Asn residue (Moodley *et al*., 2012). ORF2, ORF18, and ORF32 were identified as phage regulatory proteins. ORF3 encoded a protein homologous with helicase from *Staphylococcus aureus* subsp. *aureus* EMRSA16 with 100% identity. ORF7 showed 97% identity to dUTP diphosphatase superfamily protein of *S. aureus* subsp. *aureus* ATCC BAA-39. ORF8 encoded a protein 91% similar in identity to acetyltransferase family protein from *S. aureus* subsp. *aureus* CIGC348. The putative protein of ORF11 matched a putative DNA-binding protein from *Staphylococcus* phage phi2958PVL. ORF12 showed 99 % similarity with DNA polymerase from *S. aureus* A6224. ORF22 encoded protein homologous with the Na/K ATPase from *Staphylococcus* phage phi 12 with 100% identity.

In the module for lysis, ORF24 encoded a protein identical nearly to the *N*-acetylmuramoyl-$_L$-alanine amidase from *S. aureus* subsp. *aureus* COL, and ORF25 encoded the same protein with the holin from *Staphylococcus* phage phiSLT. The function of holin might be to create a lesion in the cytoplasmic membrane through which endolysin passed (Young, 1992). The putative protein of ORF29 was identified as a endopeptidase tail family protein. This family is of prophage tail proteins that are probably acting as endopeptidases. Finally, the predicted protein of ORF4 showed similarity to the virulence-associated protein E from *S. aureus* subsp. *aureus* USA300_FPR3757 with 100% identity.

In conclusion, we analyzed morphological property and genome sequence of phage SMSAP5 isolated from soil of slaughterhouses for cattle. Newly isolated phage SMSAP5 for *S. aureus* was slightly different to genome sequence of any other phages of the members of *S. aureus*. Lately,

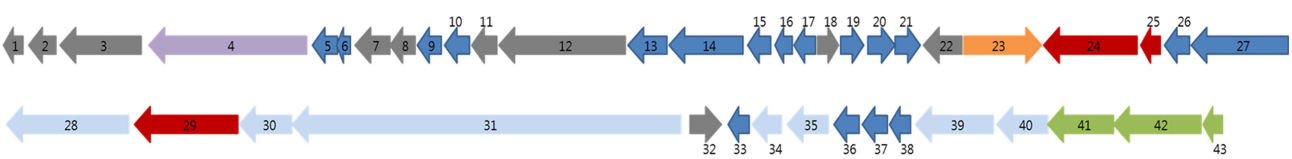

Fig. 2. Schematic representation of the dsDNA genome of the phage SMSAP5. Putative ORFs are presented as arrows, with predicted functions where available. Proposed modules are based on predicted functions. Grey, replication; purple, virulence; orange, lysogeny; red, lysis; pale blue, morphogenesis; green, DNA packaging.

phages have been intensively studied, and have been used for a variety of practical applications, such as a phage therapy, the biocontrol of food-borne pathogens, and the detection of pathogenic bacteria-like phage-typing. Therefore, phage SMSAP5 should be studied more for biocontrol of *S. aureus* in livestock industry such as dairy and meat processing industries.

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