CHARACTERIZATION AND GENOMIC ANALYSIS OF A NOVEL BACTERIOPHAGE AGAINST METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

MEDINE ÇOTAK

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

FEBRUARY 2019

Approval of the thesis:

CHARACTERIZATION AND GENOMIC ANALYSIS OF A NOVEL BACTERIOPHAGE AGAINST METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS

submitted by **MEDINE ÇOTAK** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Biotechnology Department, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences** _____

Assoc. Prof. Dr. Can Özen
Head of Department, **Biotechnology** _____

Prof. Dr. Mahinur S. Akkaya
Supervisor, **Biotechnology, METU** _____

Prof. Dr. Zeynep Ceren Karahan
Co-Supervisor, **Medical Microbiology, Ankara University** _____

Examining Committee Members:

Assoc. Prof. Dr. Çağdaş Devrim Son
Biology, METU _____

Prof. Dr. Mahinur S. Akkaya
Biotechnology, METU _____

Assoc. Prof. Dr. Bala Gür Dedeoğlu
Biotechnology, Ankara University _____

Prof. Dr. Erdoğan Eşref Hakkı
Soil Science and Nutrition, Selçuk University _____

Dr. Mohd Kamran Khan
Soil Science and Nutrition, Selçuk University _____

Date: 05.02.2019

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Surname: Medine Çotak

Signature:

ABSTRACT

CHARACTERIZATION AND GENOMIC ANALYSIS OF A NOVEL BACTERIOPHAGE AGAINST METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS

Çotak, Medine
Doctor of Philosophy, Biotechnology
Supervisor: Prof. Dr. Mahinur S. Akkaya
Co-Supervisor: Prof. Dr. Zeynep Ceren Karahan

February 2019, 75 pages

Methicillin resistant Staphylococcus aureus (MRSA) is one of the most frequent hospital and community acquired infectious agents causing severe diseases. Bacteriophages may offer a solution to treat the bacterial infections that are not responding to classical antibiotics. In this context, a new lytic phage, named as vB_SauM-MikSA913, was isolated from sewage treatment center in Samsun (Turkey). MRSA clinical strains obtained from a local hospital were used as propagating hosts while searching for the lytic phages. Genomic analysis suggest that the isolated vB_SauM-MikSA913 belongs to the *Myoviridiae* family like the most characterized phage K. Phage vB_SauM-MikSA913 has a genome size of 134193 bp double-stranded DNA, encoding 206 open reading frames (ORFs) and 4 tRNAs. There was a high similarity between our phage to others described in the literature such as qdsa002, GH15, vB_Sau_CG, and phiSA039 with more than 90% query cover and identity. vB_SauM-MikSA913 (shortly called MikSA913) has a wide range of host and high lytic activity on MRSA strains. The bacteriolytic activity of the phage was tested over a range of multiplicity of infection (MOI) and the optimal MOI was found to be 0.001, which indicates its good lytic efficiency even at low concentration. MikSA913 was stable at a wide range of pH and temperatures. One-step growth curve

analysis showed that the eclipse and latent periods of MikSA913 was 15 min and 20 min, respectively and the burst size is 112 plaque forming units/infected cell. Bacteriophage therapy is now seriously on the table as an alternative treatment to combat with antibiotic resistance crisis. The high lytic activity, its wide host range and lack of virulence factors and antibiotic resistance genes deduced from bioinformatics analysis suggest that the phage vB_SauM-MikSA913 could be an option for treating *S.aureus* infections including MRSA infections.

Keywords: Staphylococcus aureus, Methicillin-Resistant Staphylococcus aureus, MRSA, Bacteriophage, Phage, Antibiotic Resistance, Lytic Phage, Bacteriophage Therapy

ÖZ

METHİSİLİN DİRENÇLİ STAPHYLOCOCCUS AUREUS'A KARŞI YENİ BAKTERİYOFAJ KARAKTERİZASYONU VE GENOMİK TANIMLANMASI

Çotak, Medine
Doktora, Biyoteknoloji
Tez Danışmanı: Prof. Dr. Mahinur S. Akkaya
Ortak Tez Danışmanı: Prof. Dr. Zeynep Ceren Karahan

Şubat 2019, 75 sayfa

Methisiline dirençli *Staphylococcus aureus* (MRSA), ciddi hastalıklara neden olan en yaygın hastane ve toplum kökenli bulaşıcı ajanlardan biridir. Bakteriyofajlar, klasik antibiyotiklere cevap vermeyen bakteriyel enfeksiyonları tedavi etmek için bir çözüm sunabilir. Bu bağlamda, vB_SauM-Mik913 olarak adlandırılan yeni bir litik faj, Samsun'daki (Türkiye) atık su arıtma merkezinden izole edildi. Yerel bir hastaneden elde edilen MRSA klinik suşları litik fajlar aranırken konakçı olarak kullanıldı. Genomik analiz sonuçları, izole edilmiş vB_SauM-Mik913'ün, literatürde en çok çalışılan faj K gibi *Myoviridiae* familyasına ait olduğunu göstermektedir. vB_SauM-MikSA913 genomu 134193 bp çift sarmallı DNA'ya sahiptir ve 206 ORF ve 4 tRNA'yı kodlamaktadır. Faj vB_SauM-MikSA913, MRSA suşlarında geniş konakçı aralığı vardır ve yüksek litik aktivite göstermektedir. Fajın bakteriyolitik aktivitesi, çok sayıda MOI (fajın bakteriye oranı) aralığında test edildi ve optimal MOI'nin, 0.001 olduğu bulundu; bu durum, düşük konsantrasyonda bile yüksek litik etkinliğini göstermektedir. Bakteriyofaj terapisi şimdi ciddi biçimde antibiyotik direnci kriziyle mücadele için alternatif bir tedavi olarak kabul edilmektedir. Yüksek litik aktivite, geniş spektrum aralığı ve biyoinformatik analizlerden elde edilen sonuçlara göre virülans faktörü ve antibiyotik direnç genleri eksikliği faj vB_SauM-Mik913'ün

MRSA enfeksiyonları dahil *S.aureus* enfeksiyonlarının tedavisi için bir seçenek olabileceğini düşündürmektedir.

Anahtar Kelimeler: Staphylococcus aureus, Methisilin Dirençli Staphylococcus aureus, MRSA, Bakteriyofaj, Faj, Antibiyotik Dirençliliği, Litik Faji, Faj Terapi

To my family and my love

ACKNOWLEDGEMENTS

First, I am deeply grateful to my supervisor, Prof. Dr. Mahinur S. Akkaya for giving me the endless support and sincere approach.

I express my deepest gratitude to Prof. Dr. Zeynep Ceren Karahan for being my co-supervisor.

I would like to thank to all the personnel of central clinical microbiology laboratory of Ibn-i Sina Hospital for their help during collection of clinical samples.

Finally, I would like to thank all of the committee members for their time and valuable feedbacks

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CA-MRSA	Community-associated MRSA
CBD	Cell wall Binding Domain
ccr	cassette chromosome recombinase
EAD	Enzymatically Active Domain
ICTV	International Committee on Taxonomy of Viruses
LA-MRSA	Livestock associated MRSA
LB	Luria Bertani
MOI	Multiplicity of Infection
MRSA	Methicillin resistant *Staphylococcus aureus*
NAG	N-acteylglucosamine
NAM	N-acetylmuramic acid
ORF	Open Reading Frame
PBP	Penicillin-binding protein
pfu	plaque forming unit
PRSA	Penicillin resistant *Staphylococcus aureus*
PVL	Panton-Valentine Leucocidin
SCCmec	Staphylococcal Cassette Chromosome
SM	Saline-Magnesium
VAPGH	Virion-Associated Peptidoglycan Hydrolase
VISA	Vancomycin intermediate *Staphylococcus aureus*

CHAPTER 1

INTRODUCTION

1.1. Staphylococcus aureus

Staphylococcus bacterium is an important human firstly discovered by surgeon Sir Alexander Ogston in a pus from a surgical abscess in 1881. He named it *Staphylococcus* due to its shape resemblance to grape clusters (Greek, 'staphyle' -bunch of grapes and 'kokkos'-berry). Rosenbach gave the formal name of *Stapyhylococcus aureus* in 1884. He differentiated the *Staphylococcus aureus* (Latin 'aurum'-golden) from *Staphylococcus albus* (now called as *S. epidermidis*) (Latin, 'epidermidis' -white) since *S. aureus* grows golden-yellow colonies on bacterial media (Giancarlo Licitra, 2013; Stryjewski & Corey, 2014).

S. aureus is a Gram-positive bacterium with the features of non-motility, catalase- and coagulase-positive and facultative anaerobic. *S. aureus* has a high adaptation capacity to its host and the environmental conditions and can survive in a wide range of pH and temperature (Feng et al., 2008; Le Loir, Baron, & Gautier, 2003; Schmitt, Schuler-Schmid, & Schmidt-Lorenz, 1990).

S. aureus is an opportunistic bacterium which can be both pathogen and commensal carried on healthy individuals especially in nasal cavity (Peacock, De Silva, & Lowy, 2001; Sakwinska et al., 2010; van Belkum et al., 2009; Wertheim et al., 2005). As a pathogen, *S. aureus* can infect any site of body and it is responsible for minor or lethal infections; skin and soft tissue infections, food poisoning, endocarditis, chronic osteomyletitis, penumonia, bacteremia, toxic shock syndrome, meningitis, septicemia (Bassetti et al., 2014; McGuinness, Malachowa, & DeLeo, 2017; Nickerson, West, Day, & Peacock, 2009; Todar, 2005; WHO, 2014).

S. aureus is a highly successful pathogen having a wide range of virulence factors. These virulence factors include surface proteins, toxins, enzymes promoting tissue damage and factors for evading the host immunity (Foster & Höök, 1998; Gill et al., 2005). Mobile genetic elements called pathogenicity islands and prophages are responsible for expressing the important toxins and other virulence determinants. Panton-Valentine leucocidin (PVL) is a common virulence factor carried by prophages in *S. aureus* strains (Jarraud et al., 2001; Malachowa & Deleo, 2010).

Antibiotic resistance in healthcare settings and in community is on the rise and represents a global health burden. Multi-drug resistant *S. aureus* is one of the most common cause of nosocomial infections with high rates of morbidity and mortality (Salge, Vera, Antons, & Cimiotti, 2017).

1.1.1. *S. aureus* Cell Wall

In *S. aureus* cell, the outermost layer is the polysaccharide capsule which is an important virulence factor (O'Riordan & Lee, 2004). Underneath the capsule is the cell wall and it is essential for cell integrity and host-pathogen interactions (Dmitriev, Toukach, Holst, Rietschel, & Ehlers, 2004). Peptidoglycan layer (20-40 nm) is the primary component of the cell wall comprising of alternating glycan chains NAG (N-acteylglucosamine) and NAM (N-acetylmuramic acid) cross-linked by pentaglycine bridges and stem pentapeptides (Giesbrecht, Kersten, Maidhof, & Wecke, 1998).

Teichoic acids embedded in peptidoglycan layer function as phage receptors, epitopes or as communication tools in pathogenicity with the environment (Navarre & Schneewind, 1999; Szweda et al., 2012). The overall *S. aureus* cell wall structure is shown in **Fig. 1.7**.

1.1.2. *S. aureus* Genome

S. aureus genome size is around 2.8 Mb and the genome have core and accessory regions. The core genome genome is highly conserved among *S. aureus* strains and contains essential genetic components relating to cell metabolism and replication. On the other hand, 25% of the *S. aureus* genome is the accessory part comprising of mobile genetic elements for example, prophages, pathogenicity islands, chromosomal cassettes, plasmids and transposons. These mobile genetic elements are responsible for the virulence, immune escape from host and for acquiring drug resistance (Lindsay & Holden, 2004).

1.2. Antibiotic Resistance in *S. aureus*

The antibiotic era started with the discovery of penicillin in 1940s and many bacterial pathogens including *S. aureus* were treatable with antibiotics since then (Aminov, 2010). However, many bacteria are able to adapt to changing environmental conditions including antibiotic treatment. Mobile genetic elements play significant role in acquiring resistance. *S. aureus* is also developing resistance rapidly day by day to different antibiotics introduced which is demonstrated in **Fig. 1.1** (McGuinness et al., 2017). Plasmids, transposons, bacteriophages, pathogenicity islands and staphylococcal cassette chromosomes are the kinds of mobile genetic elements in *S. aureus* conferring to the antibiotic resistance (Gill et al., 2005; Holden et al., 2004; Lindsay, 2010; McGuinness et al., 2017).

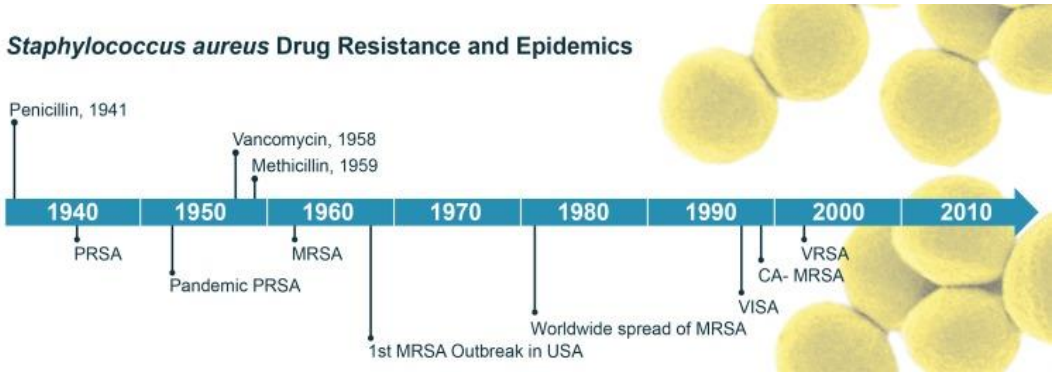

Figure 1.1 *S. aureus* drug resistance development timeline (McGuinness et al., 2017).

1.2.1. Penicillin Resistance

S. aureus infections was a highly serious disease with the mortality rate around 80% before penicillin introduction in 1940s (Lobanovska & Pilla, 2017; Lowy, 1998, 2003). Shortly after introduction of penicillin, penicillin resistant *S. aureus* (PRSA) isolates were observed in hospitals and later in community (Lowy, 1998, 2003). Penicillin resistance is due to the *blaZ* gene encoding β-lactamase (also called penicillinase) enzyme which hydrolyze β-lactam ring of penicillin rendering the antibiotic inactive (McGuinness et al., 2017; Olsen, Christensen, & Aarestrup, 2006).

1.2.2. Methicillin Resistance

Methicillin introduced in 1959 is a semi-synthetic β-lactamase resistant drug and. However, it did not take so long to gain resistance and methicillin resistant *S. aureus* (MRSA) isolates were developed rapidly. MRSA strains were first observed in UK hospital in 1961 and since then MRSA clones spread and became a global health issue (Deurenberg et al., 2007; Lowy, 2003). Methicillin resistance conferred by the *mecA* gene encoding penicillin-binding protein (PBP)2a (Gordon & Lowy, 2008). PBPs are transpeptidases that catalyze the polymerization of the glycan strand (transglycosylation) and the cross-linkage of glycan chains (transpeptidation) (Sauvage, Kerff, Terrak, Ayala, & Charlier, 2008). β-lactam antibiotics binds to the

PBPs on the cell wall and interferes with synthesis of peptidoglycan layer leading to cell death. In the presence of PBP2a, which has a low affinity for all beta-lactams including methicillin, oxacillin and third-generation cephalosporins, the cell wall synthesis continues and the cell survives (Chambers, 1997; Deurenberg et al., 2007; Ito et al., 2009). MRSA isolates are not not only resistant to methicillin but they also carry multiple resistance genes to other antibiotics (Lowy, 2003).

Staphylococcal Cassette Chromosome (SCC*mec*) is the mobile region of *S. aureus* chromosome (Ito, Okuma, Ma, Yuzawa, & Hiramatsu, 2003; Saber, Jasni, Jamaluddin, & Ibrahim, 2017). SCC*mec* consists of two fundamental components: the *mec* and *ccr* gene complexes (Zong, Peng, & Lü, 2011). Cassette chromosome recombinase (*ccr*) genes codes for recombinases those are responsible integration and excision of SCCmec into and out of chromosome (Saber et al., 2017). Thereby, SCC*mec* with its resistance elements can be transferred horizontally and vertically intraspecies and interspecies (Stojanov, Sakwinska, & Moreillon, 2013).

To date, there are eleven SCC*mec* types (I-XI) (**Fig 1.3)** identified in staphylococci having size range of 20.9 to 66.9 kb (Saber et al., 2017). SCC*mec* carries various multiple resistance genes such as for macrolides, tetracycline and these genes are carried via transposons, insertion sequences and transposons (Deurenberg et al., 2007). In addition, the antibiotic resistance genes can be found on other sites of *S. aureus* chromosome and on plasmids (Deurenberg et al., 2007).

Community-associated MRSA (CA-MRSA) causing infections in public, outside of healthcare settings, is also a serious concern worldwide (Chambers & DeLeo, 2009; Herold et al., 1998). In addition, MRSA is an important issue in food industry since livestock associated MRSA (LA-MRSA) causes infections in livestock husbandry (Cuny, Wieler, & Witte, 2015).

MRSA is a worldwide problem and in Turkey, there is a high prevalence of MRSA and the percentage of the isolates of the S. aureus that is methicillin-resistant is around 25% **(Fig 1.2)**.

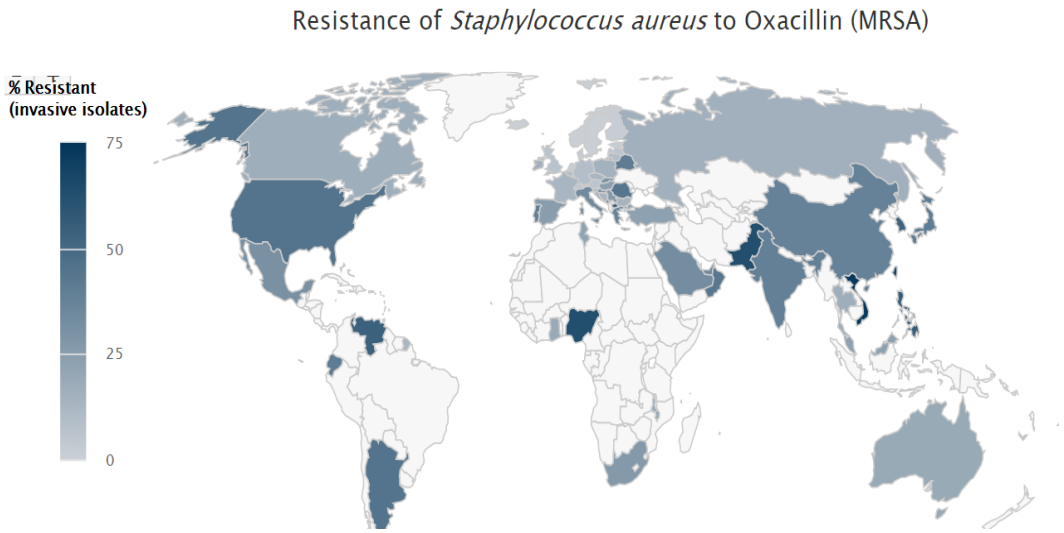

Figure 1.2 MRSA global prevalence map (The Center for Disease Dynamics Economics & Policy. Resistance Map: Antibiotic resistance. 2018).

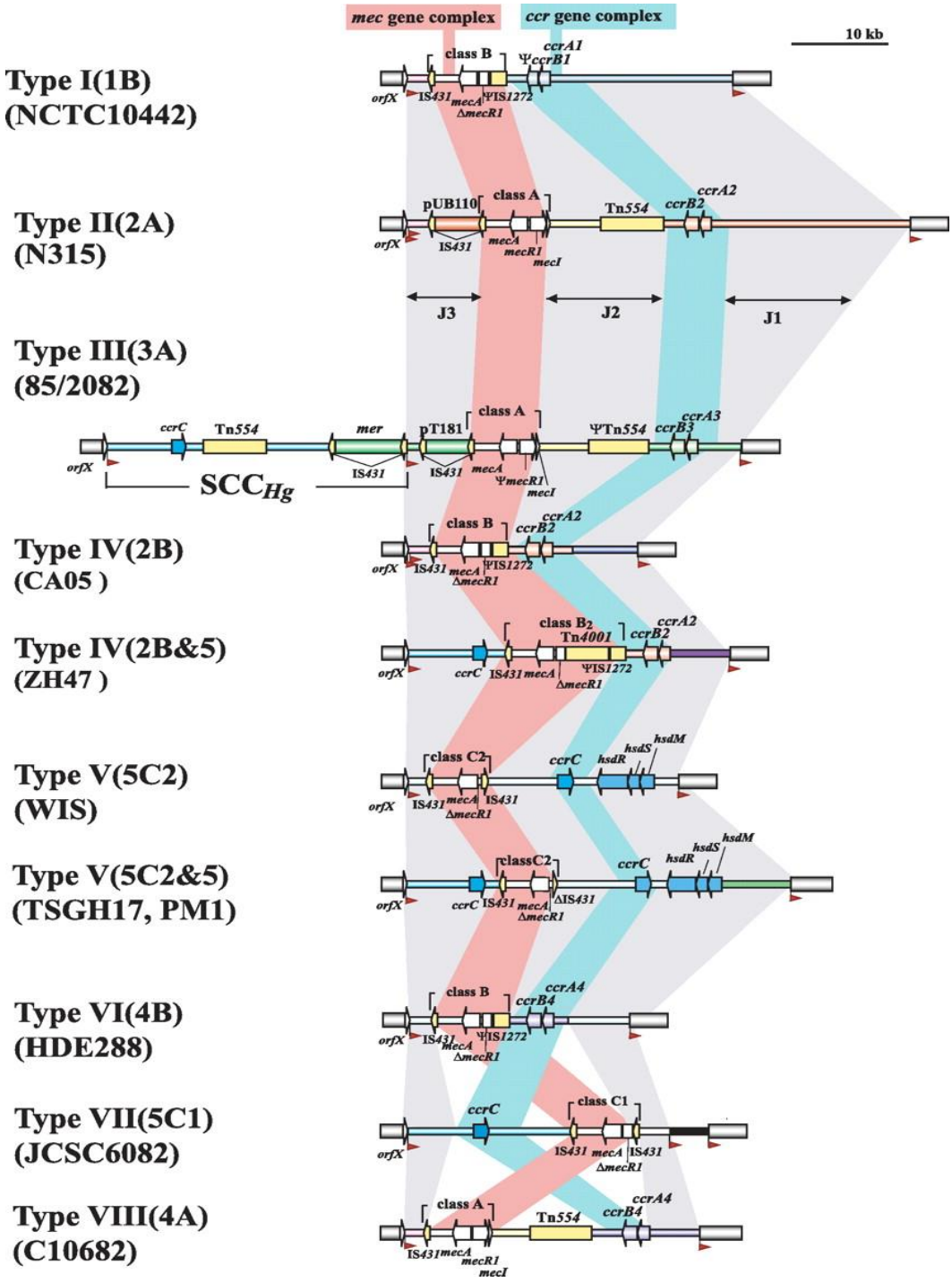

Figure 1.3 SSC*mec* types (I-VIII) identified in *S. aureus.* The key components are *mec* gene complex which is responsible for methicillin resistance and *ccr* genes needed for integration and excision of SCC*mec* (Ito et al., 2009).

1.2.3. Vancomycin Resistance

In late 1980s vancomycin was started to be used for treatment of MRSA infections. (Hiramatsu et al., 1997). *S. aureus* strains has developed two forms of vancomycin resistance mechanisms. Vancomycin intermediate *S. aureus* (VISA) strains emerged via accumulation of mutations because of vancomycin treatment on a prolonged or repeated treatments with vancomycin. These mutations are largely associated with cell wall biosynthesis and autolysis (Chen, Huang, & Chiu, 2015; Mwangi et al., 2007). On the contrary to VISA, complete *S. aureus* vancomycin resistance (MIC $\geq$ 16 μg/ml) is conferred by the *vanA* operon carried on a transposon Tn1546 which is first originated from the vancomycin resistant enterococci conjugative plasmid (Arthur, Molinas, Depardieu, & Courvalin, 1993). Vancomycin confers its anti-bacterial activity via interfering with D-Ala-D-Ala peptidoglycan precursors of newly synthesized peptidoglycan leading to inhibition of cell wall synthesis (Barna & Williams, 1984). There are two mechanisms for vanA operon-mediated vancomycin resistance; hydrolysis of normal peptidoglycan precursors (D-Ala-D-Ala) so that vancomycin cannot bind or a different modified peptidoglycan precursor (D-Ala-D lactate) synthesis which vancomycin is not able to act on (Bugg et al., 1991).

1.3. Bacteriophages

Bacteriophages (phages) are the bacterial viruses and the name was derived from words ‘bacteria’ and ‘phagein’ (Greek, to eat) (Alexander Sulakvelidze, 2011). It is estimated that there are 10^{31} phages on this planet making the phages the most abundant organisms (Weinbauer, 2004). Bacteriophages can be found in all ecosystems such as oceans, up in air, and also in our body (Comeau et al., 2008; Manrique et al., 2016; Moelling, Broecker, & Willy, 2018). Bacteriophages are important in these ecosystems, for example; they can be involved in carbon, sulfur and nitrogen cycles in the oceans (Breitbart, Bonnain, Malki, & Sawaya, 2018; Brum et al., 2015). Bacteriophages are also important subject from clinical perspective, since

they can carry disease-causing genes or the way around bacteriolytic phages can be considered as therapeutic agents of multi-resistant pathogenic bacteria.

1.3.1. Bacteriophage Classification

Bacteriophages are classified based on genome type and phage morphology by International Committee on Taxonomy of Viruses (ICTV). The genome of phages consists of RNA or DNA and can be both single-stranded and double-stranded. The genome size ranges from 3.5 kb (ssRNA phage MS2) to 500 kb (dsDNA *Bacillus* phage G) (Salmond & Fineran, 2015). Different phage morphologies are identified; tailed, polyhedral, and filamentous or pleomorphic and some have lipid or lipoprotein capsids. The most characterized, around 96% of, bacteriophages belong to the order *Caudovirales* (Latin 'cauda'-tail). *Caudovirales* phages are tailed with dsDNA genome. The families of this order are *Myoviridae* characterized by a straight contractile tail, *Podoviridae* having a short tail and *Siphoviridae* with a contractile and flexible tail **(Fig. 1.4)**.

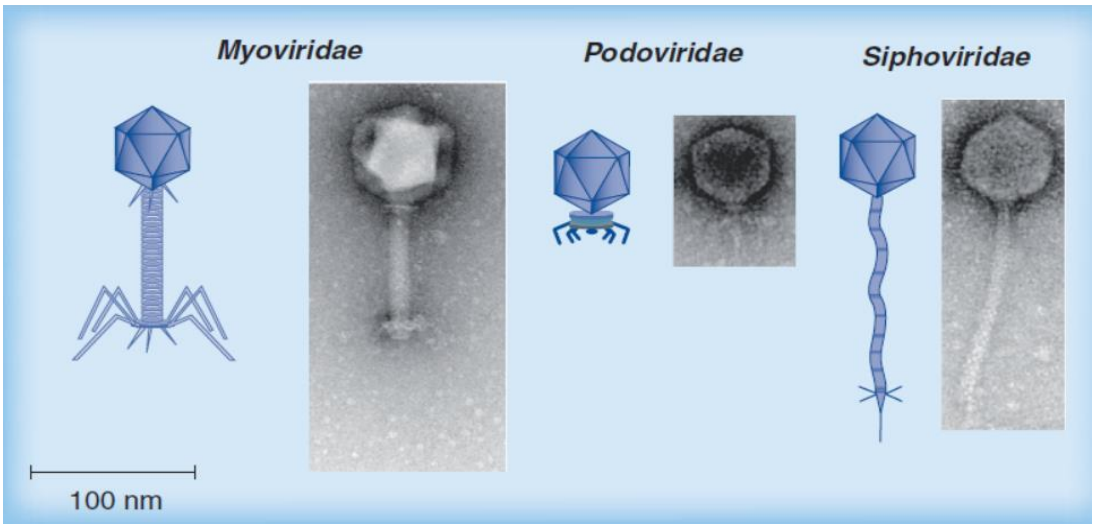

Figure 1.4 Morphology of three families of the tailed bacteriophages (*Caudovirales*) (Harper, Anderson, & Enright, 2011).

1.3.2. Bacteriophage Life Cycle

Bacteriophages first bind to the host cell receptors with its tail proteins and phage then phage genome is inserted into host cell. The fate of phage infection after adsorption vary according to the host or the phage (Abedon, 2012; Samson, Magadán, Sabri, & Moineau, 2013). There are at least four cases: 1) lytic infection: phage replicates in host and releases its progeny virions and host cell dies; 2) lysogeny: phage and host cell survives and the so called temperate phage is replicated as part of host chromosome; 3) the phage is inactivated by the host immunity systems such as restriction endonucleases (Labrie, Samson, & Moineau, 2010) and/or CRISPR-Cas systems (Jiang & Doudna, 2015) ; 4) phage and the host both dies due to abortive infection systems (Dy, Przybilski, Semeijn, Salmond, & Fineran, 2014; Nicastro, 2016).

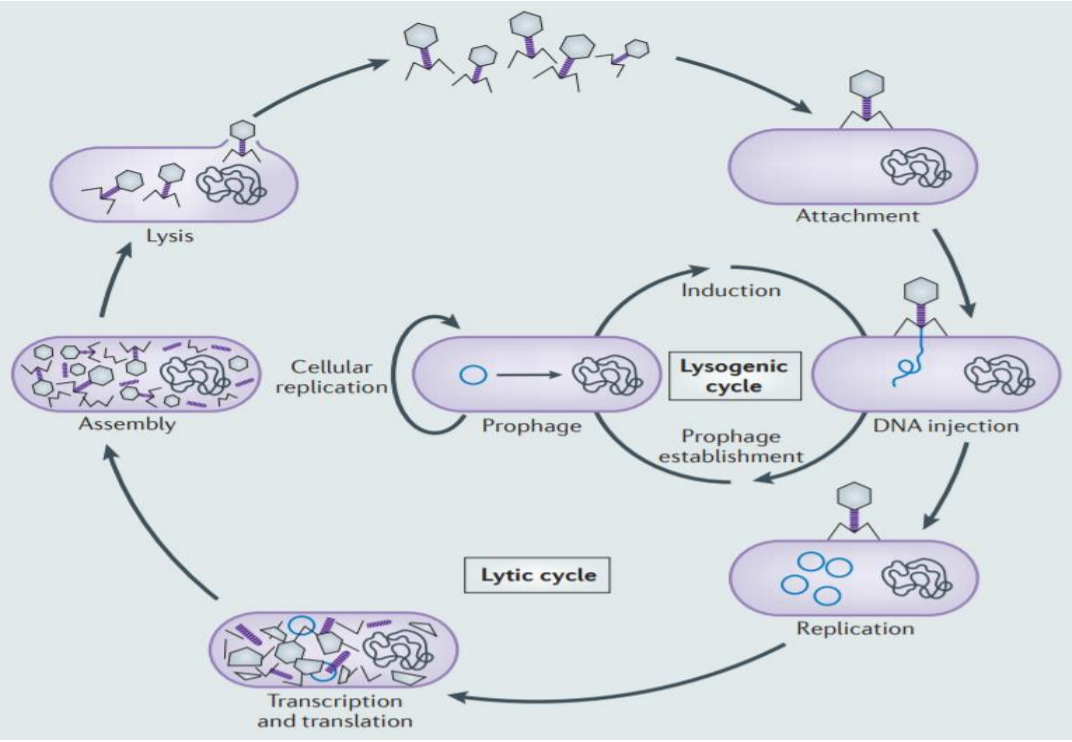

Figure 1.5 Bacteriophage life cycles: Lytic and lysogenic cycles (Salmond & Fineran, 2015).

1.3.2.1. Lytic Phages

Many phages follow lytic cycle leading to the host cell lysis and death. Lysis mechanism can be different based on the genome. Phages having single stranded genome inhibits the peptidoglycan synthesis via lysis effector. On the other hand, phages with double-stranded DNA genome cell lysis occurs upon infection and the progeny virions are released. In this case, phages adsorbed on the host cell and the genome is introduced into the host (Young, 2014). Then, the phage genes are expressed in bacterial cytoplasm and progeny phage particles are assembled in the so-called latent period. Endolysins (lysins) are the enzymes responsible for the degradation of the peptidoglycan layer resulting in osmotic cell death at the end of lytic cycle. The timing of the lysis is regulated by the 'holins' and once a certain number of virions are assembled these holins form pores opening the way to the endolysin to its substrate peptidoglycan layer (Abedon, 2012; Young, 2014).

Lytic phages and their proteins are exclusively attracting attention for the development of potential therapeutic agents against multi-drug resistant bacteria (Gutiérrez & Fernández, 2018).

1.3.2.2. Temperate Phages

Phages carried within host as a prophage are called temperate phages and this cycle is referred as lysogeny. Temperate phages often stay as prophage integrated into its host genome but external signals may induce the lytic cycle (Mardanov & Ravin, 2007). A prophage not only integrates into host genome, but also it can stably exist extra-chromosomally like plasmid. Integration into the host requires an integrase, which homologous regions in the phage and bacterial DNA leading to the site-specific recombination event (Abedon, 2012).

Interestingly, bacterial whole genomes sequenced have showed that most of the bacteria contain at least one prophage. Prophages in the bacterial genomes may

responsible for host genome evolution or pathogenicity. These phages have shown to carry genes for toxins, virulence factors, and antibiotic resistance (Kropinski & Martha, 2009).

1.4. Bacteriophage Therapy

The discovery of the phages was credited to two scientists; Frederick William Twort Felix in 1915 and d'Herelle in 1917 independently described the bacteriophages. D'Herelle and George Eliava founded Eliava Institute for Phage Therapy in Georgia, which is still active, in 1923. Phage therapy was used against open wound infections of soldiers during the Winter War between the former Soviet Union and Finland (Moelling et al., 2018). Phages were started to be visualized by the invention of electron microscopy. With the introduction of antibiotics in 1940s, antibiotics especially in western countries supersede phage therapy.

Bacteriophages are specific for their host bacteria and do not affect the mammalian cells. The application of phages has been studied as therapeutic agents to treat acute and chronic infections especially caused by the multidrug-resistant bacteria (Wittebole, De Roock, & Opal, 2014). Nowadays, the use of lytic bacteriophages and their enzymes to deal with antibiotic resistance crisis is getting renewed attraction by the researchers and also by pharmaceutical companies (Fischetti, 2008; Rodríguez-Rubio, Martínez, Donovan, Rodríguez, & García, 2013; A. Sulakvelidze & Morris, 2001). There are phages, phage cocktails and their lytic enzymes currently on the way through clinical trials.

1.5. Bacteriophage Lytic Enzymes

Lytic enzymes derived from bacteriophages can be classified as endolysins and virion-associated peptidoglycan hydrolases (VAPGHs) **(Fig. 1.6)**. Lysis cassette containing the two proteins; endolysin and holin are common in dsDNA bacteriophages.

However, some phages use host cell secretion machinery (Sec) of the host cell to release the endolysin (secretion activated endolysin). In this case, pinholin encoded by the phage provide proton motive force to activate the secreted endolysin (Catalão, Gil, Moniz-Pereira, São-José, & Pimentel, 2013). Phages infecting Gram-negative hosts have additional proteins, named spanins, that help to break outer membrane (Catalão et al., 2013).

In the beginning of the infection, phage adsorbs to the host bacterium and slightly degrades the cell wall to inject its genome into the host cytoplasm. VAPGHs, structural proteins of the phages also called tail lysins, are responsible for this cell wall degradation event.

Endolysins with the help of holin lyse the cells from within, on the other hand, VAPGHs act outside of the phage at the start of infection (Gutiérrez & Fernández, 2018). Both of these lytic systems are widely studied as antimicrobial therapeutic agents against pathogenic (Fischetti, 2008; Rodríguez-Rubio et al., 2013; A. Sulakvelidze & Morris, 2001).

The protein structure of the endolysin enzymes targeting Gram-positive and Gram-negative are different. Endolysins targeting Gram-positive bacteria evolved to have a modular design in which catalytic activity and substrate recognition are separated into two distinct functional domains called enzymatically active domains (EADs) and cell wall binding domains (CBDs). (Borysowski, Weber-Dąbrowska, & Górski, 2006; Fischetti, 2010; Loessner, 2005; Loessner, Wendlinger, & Scherer, 1995; López & García, 2004). On the other hand, outer membrane of the Gram-negative bacteria prevents the endolysin to reach its substrate peptidoglycan layer when applied exogenously. Endolysins infecting Gram-negative hosts have small-single globular domain (molecular mass between 15-20 kDa), usually without CBD module (Briers et al., 2007; Cheng, Zhang, Pflugrath, & Studier, 1994).

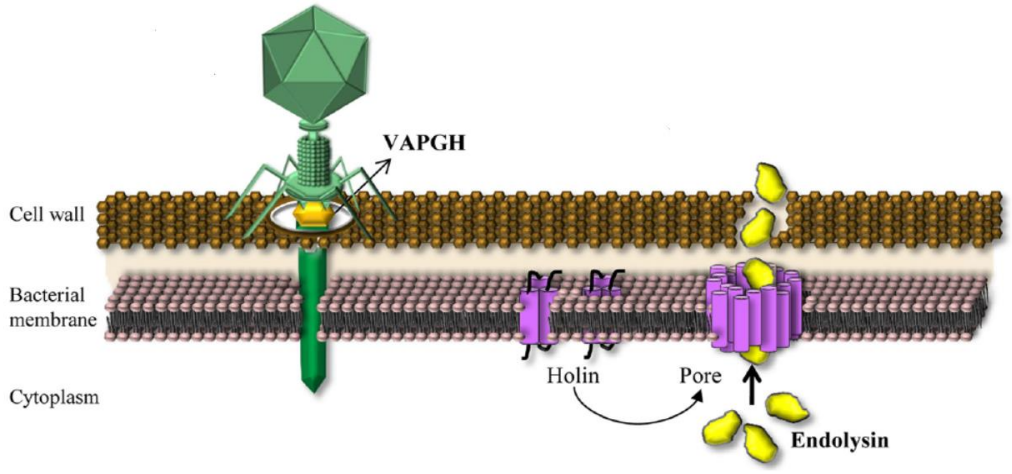

Figure 1.6 Activities of phage lytic proteins on Gram-positive cell wall. At the start of infection VAPGH proteins, tail lysins, open a gate in the cell wall by degrading the cell wall and inject the genetic material into the cytoplasm of the host bacterium. On the other side, the holins and endolysins are encoded by the phage at the end of infection to degrade the cell wall and release the phage virions. Holins polymerize to form a hole to help the endolysins to reach their substrate peptidoglycan layer. Figure modified from (Gutiérrez & Fernández, 2018).

Endolysins are antibacterial enzymes having peptidoglycan hydrolase activity. They are part of the bacteriophage life cycle; however, they can be applied exogenously as recombinant proteins. Numerous studies have shown that, endolysins expressed in suitable hosts, purified and applied as antibacterial agent with rapid killing activity.(Jun et al., 2013). Importantly, endolysins are so specific that they can target the bacteria at genus or even species level. This characteristic is important to protect normal microbiome of the human body (Hosseini, Moniri, Goli, & Kashani, 2016; O'Flaherty, Coffey, Meaney, Fitzgerald, & Ross, 2005; Schmelcher & Loessner, 2016). Additionally, bacterial strains are either slightly or develop no resistance to phage lysins (Loessner, 2005). The scenario in the case of antibiotics different since antibiotics affect both the normal flora of the body and promote resistance in the host (Fischetti 2010). Endolysin source is bacteriophages found in nature so it is a plausible idea that there is a significant diversity of endolysin proteins. Taking all together the

advantages of endolysins, they are now considered as potential antimicrobial agents to cope with multidrug resistant bacteria.

1.6. Staphylococcal Phages

In literature, all of the characterized *S. aureus* phages fall into the order of *Caudovirales;* tailed phages with an icosahedral capsid and dsDNA genome (Deghorain & Van Melderen, 2012; Xia & Wolz, 2014). Anti-staphylococcal phages can be classified into three groups: 1) ***podoviruses*** with <20kb genomes; 2) ***siphoviruses*** with around 40 kb genomes ; and 3) ***myoviruses*** with >125 kb genomes (Deghorain & Van Melderen, 2012). *Siphoviruses* are mainly temperate phages and contain virulence genes (Xia & Wolz, 2014). Unlike siphoviruses, podoviruses infecting *S.aureus* are strictly lytic but unfortunately, they are not common (Kaźmierczak, Górski, & Dabrowska, 2014). Myoviruses are also lytic phages, therefore myoviruses and podoviruses are appropriate for therapeutic usages.

1.7. Endolysins targeting *S.aureus*

S.aureus bacteriophage derived endolysins have a modular structure possessing one or more N-terminal enzymatically active domain (EAD) and a C-terminal cell wall binding domain (CBD) conferring substrate specificity (Oliveira et al., 2013). VAPGHs lack CBD but have a similar modular structure consisting of one or two catalytic domains (Donovan, Lardeo, & Foster-Frey, 2006; Obeso, Martínez, Rodríguez, & García, 2008). There are at least six enzymatically active catalytic domain types of phage endolysins, the cleavage sites of these proteins on the peptidoglycan layer are illustrated in **(Fig 1.7)**.

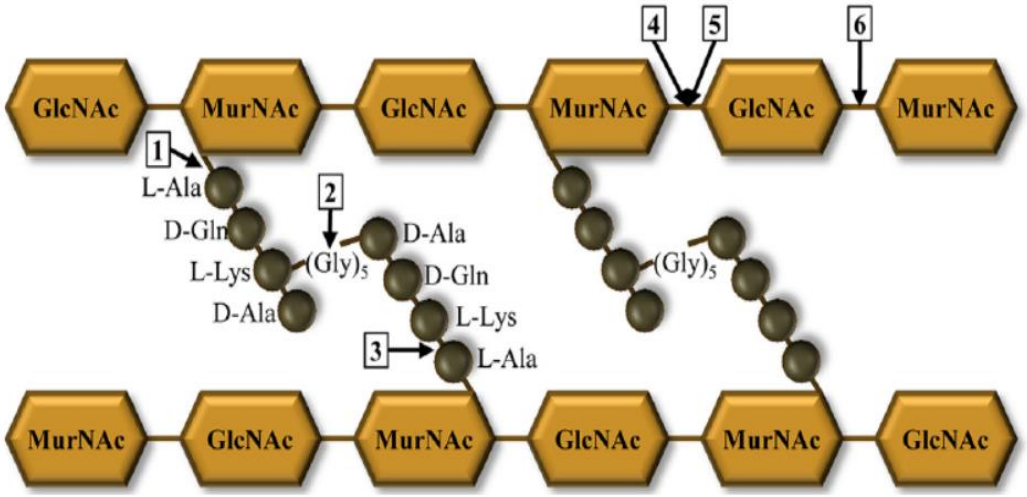

Figure 1.7 *S.aureus* peptidoglycan and the enzymatic activities of the endolysins (indicated with an arrow and a number). 1) N-Acetylmuramoyl-L alanine amidase; 2) interpeptide bridge endopeptidase; 3) L-alanoyl-D-glutamate endopeptidase; 4) N-acetyl-β-D-muramidase; 5) transglycosylase; 6) N-acetyl-β-D-glucosaminidase (modified from Gutiérrez & Fernández, 2018)

Overviewing the literature search for catalytic domains of Staphylococcal endolysins transglycosylases are found rarely. The most commonly found catalytic domains are CHAP (cysteine- and histidine-dependent amidohydrolase/peptidase), AMI-2 (amidase 2 domain) and AMI-3 (amidase 3 domain). Cell wall binding domains of Staphylococcal endolysins usually have SH3-related domains (Becker, Foster-Frey, Stodola, Anacker, & Donovan, 2009; Oliveira et al., 2013). Peptidoglycan peptide cross-bridge is the binding site of the SH3b domain (Gründling & Schneewind, 2006). The schemes of common staphylococcal endolysin and VAPGH modules are presented in **(Fig. 1.8)**.

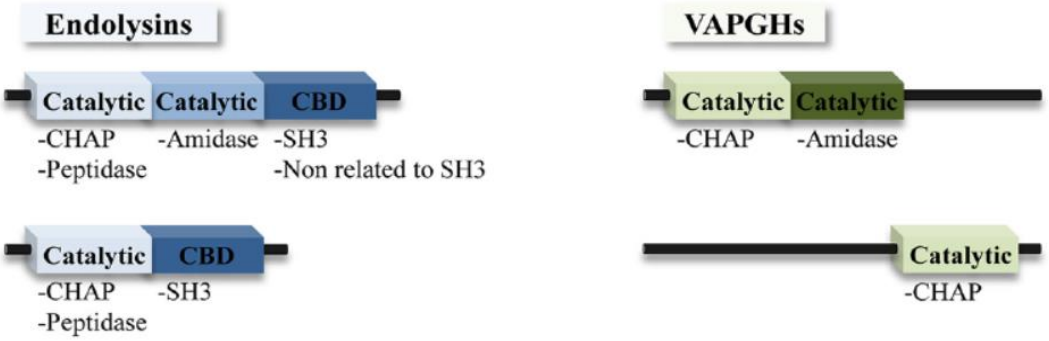

Figure 1.8 Endolysin and VAPGH domains. Schematic representation of common catalytic and CBDs in lytic enzymes targeting *S. aureus* peptidoglycan (Gutiérrez & Fernández, 2018).

Endolysins are highly effective on *S. aureus* biofilms forming on surfaces and biomedical devices (Moormeier & Bayles, 2017). Not only single endolysin proteins (SAL-2, phi11, PlyGRCS, and SAL200) but also chimeric proteins of endolysins (ClyH and ClyF) are active on staphylococcal biofilms (Fenton et al., 2013; Jun et al., 2013; Linden et al., 2014; Sass & Bierbaum, 2007; Son et al., 2010; Yang, Zhang, Wang, Yu, & Wei, 2017; Yang, Zhang, Huang, Yu, & Wei, 2014). Besides chimeric proteins, another strategy is to combine lysins with antibiotics resulting in a synergistic antimicrobial effect (Chopra, Harjai, & Chhibber, 2016; Daniel et al., 2010; Singh, Donovan, & Kumar, 2014).

The endolysins attract increased interest from pharmaceutical drug companies. There is one endolysin-based product applied topically which is developed by a Dutch biotech company, Micreos, to treat MRSA skin infections (Totté, van Doorn, & Pasmans, 2017). The company Contrafect has a drug candidate in clinical phase II formulated for the treatment of *S. aureus* bloodstream infections and endocarditis. Another endolysin drug candidate is protein SAL200 developed by Intron Biotechnology was recently passed through the clinical phase I (Jun et al., 2017). Both of these proteins are intended for intravenous administration route. Gangagen, an Indian company, have P128 (StaphTAME) lytic protein targeting nasal *S. aureus* contamination is currently undergoing phase II clinical trials.

1.8. Aim of This Study

In this study, the aim is to find new bacteriophages against local MRSA strains. We report the isolation and analysis of a *Kayvirus* genus *S. aureus* phage. Here, we show the analysis of phage MikSA913 at a genetic and proteome level. Additionally, we provide an insight into the reasons why this phage might be well suited for clinical applications by testing its lytic efficiency and host range with a broad range of human MRSA isolates and its safety at genomic level lacking of virulence factors or antibiotic resistance genes. We also present an evaluation of the biophysical parameters: pH and temperature, with intention to select optimal conditions to work with the phage MikSA913.

CHAPTER 2

MATERIALS AND METHODS

2.1. Culture media and growth conditions

The bacteria were cultured in Luria Bertani (LB) broth or on LB agar plates and incubated at 37°C. Phage propagation with host strains in liquid media were incubated at 30°C. For phage isolation, double plaque assay method was used. Double plaque assay was performed with two LB agar mediums with different concentrations: LB medium with 1.5% or 0.7% agar was used for the standard agar (top layer) and for soft agar (bottom layer), respectively. Bacteriophage enrichment assays were carried out with 10 x strength LB. LB broth was used for the storage of bacteria with 20% glycerol at -20°C.

Bacterial growth was measured by optical density at 565 nm by densitometer turbidity detector (DEN-1, Biosan) where the bacterial cell concentration of 3 x 10^8 cells/ml is approximately equal to the 1 McFarland Standard Unit.

2.2. Bacterial Strains

All the strains of MRSA and other clinical isolates were taken from a local hospital from the samples of patients (Ibn-i Sina Hospital- Ankara University). The isolates used in this study are MRSA (n=50), MSSA (n=5) , *Enterococcus faecalis* (n=2), *Staphylococcus lugdunensis* (n=2), *Klebsiella pneumoniae* (n=2) and *Bacillus subtilis* (n=1). In addition, standard strains were used: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeroginosa* ATCC 27853. All of the strains are listed for host range analysis in **Table 3.2**.

Clinical bacterial strains were identified by BD Phoenix (Becton Dickinson) automated systems. Antimicrobial susceptibilities of these isolates were tested by disk diffusion method and the results were assigned following the Clinical & Laboratory Standards Institute (CLSI) guidelines. The tested antibiotics are erythromycin, gentamicin, clindamycin, penicillin, oxacillin, cefotoxitin, vancomycin, rifampin, linezolid, teicoplanin, ciprofloxacin, quinopristi / dalfopristin, chloromphenicol, tetracycline and trimethoprim-sulfamethoxazole (Clinical and Laboratory Standards Institute, 2015). The strains that were oxacillin- and/or cefoxitin resistant were considered as methicillin resistant.

MikSA913 was isolated using a clinical MRSA strain 913 as a host and the same strain was used as a standard host strain for the phage.

2.3. Double Plaque Assay

The double agar overlay plaque assay method was used to isolate the phages and for phage tittering. In this method, phage suspensions were mixed with the host bacteria, the top agar (also called soft agar or molten top agar), and the mixture was poured onto the standard bottom agar. After enough overnight incubation at appropriate temperature, phage plaques were visualized on the bacterial lawns. The phage titration and propagation experiments were carried out with solid or liquid LB medium supplemented with 10 mM $CaCl_2$ and 10 mM $MgSO_4$. The bottom and and top agar components are listed and preparation of the layers are described as below:

Table 2.1 LB top and bottom agar compositions.

LB bottom agar (%1,5 w/v agar) 1 L	**LB top agar (0,7% w/v agar) 1 L :**
10 g peptone	10 g peptone
5 g yeast extract,	5 g yeast extract,
10 g NaCl	10 g NaCl
15 g agar	7,5 g agar
Fill up to 1 L with dH_2O. Autoclave for 15 minutes at 121°C to sterilize.	

Bottom agar:

1. LB bottom agar was prepared by adding agar (15g/L) and autoclaved.
2. The medium was let to cool down to 55-60 °C and strerile 1 M $CaCl_2$ and 1M $MgSO_4$ solutions were added to final concentration of 10 mM for each.
3. Approximately 20 ml of medium was poured onto plates (90 mm Petri dishes) and when the plates were cool enough, they were used or stored at 4°C for later usage.

Top agar:

1. LB top agar medium was prepared by adding agar (7g/L) and autoclaved.
2. 1M $CaCl_2$ and 1 M $MgSO_4$ with final concentration of 10 mM for each divalent cation were added to the medium.
3. Immediately, when the medium was still hot, the mixture was distributed into sterile test glass tubes and stored at 4°C.

For double plaque assay, soft agars stored in test tubes were melted on heating block to the tempreture of around 60-70 °C. In this step, it is important that the agar be fully in liquid form so that when poured onto plates the surface is smooth. Otherwise, the solid agar remainings which were not melted can be confusing with the results of lytic clearance points on the plaques.

2.4. Bacteriophage Isolation

The raw sewage water was collected from two waste treatment centers: at Middle East Technical University, Ankara and in Samsun and the phage enrichment procedure was followed as below:

1. Cenrifuge the sewage suspension at 9000 rpm, 10 min to remove particulates.
2. Add 45 ml of the supernatant (the clarified sewage water) and 5 ml of 10x strength LB broth containing 100 mM $MgSO_4$ and 100 $CaCl_2$ into a 500 ml Erlenmeyer flask.
3. Add 0.1 ml of 10 randomly chosen overnight grown MRSA clinical strains and incubate at 30°C with shaking at 100 rpm in a bigger size flask for enough aerobic respiration.
4. After overnight incubation, add 2,5 ml of chloroform to the flask contents and let 30 min with gentle mixing for 5-6 times at room temperature (RT).
5. Centrifuge the flask contents at 9000 rpm 10 min 4°C to discard cell debris and decant the supernatant into sterile tubes.
6. Sterilize the phage supernatant through a 0,45 μm pore size membrane.
7. Phages were isolated by double agar layer method. 0,1 ml of each overnight MRSA strain was mixed with 1,5 ml of the resultant phage supernatant (from step 6). 2.5 mL of heated (60-70 °C) soft agar was added to this mixture and the mixture was poured evenly onto the bottom agar plates. Allow the overlays harden for about 15 min under laminar flow.
8. The plates were incubated overnight at 37 °C.
9. Next day, check for the cleared zones on the plates where phage lysis occurs.
10. Plates in which bacteriophages shown with clear zones were chosen for single plaque isolation. Using sterile pipette tip, the top layer with clear zone was picked up and inoculated into 2 ml LB with 0,1 ml of corresponding host MRSA strain and incubated for 6 hours to enrich the potential phages.
11. The 2 mL enriched phage filtrate was tenfold serially diluted (1 and 10^{-1} to 10^{-9}) in 1 ml of LB broth.

12. Add 0,1 ml of overnight grown MRSA host strain to each dilution and mix with 3,5 ml of heated (60-70 °C) top agar medium and pour onto the standard agar plate with bottom agar layer and allow hardening for 15 min.
13. Incubate the plates at 37 °C overnight and the next day check for separated plaques on the dilution series.
14. The well-separated single plaque was taken with a pipette tip and suspended in LB medium and again serially diluted (1 and 10^{-1} to 10^{-4}). This single plaque isolation procedure was repeated three times to ensure single phage isolation.

2.5. Phage Propagation and Concentration

The bacteriophages isolated with single plaque isolation method as described in 2.4 were propagated with their hosts for large volume and concentrated.

1. The single plaques on the plates (section 2.4 step 14) were picked up from the plates with pipette tips and inoculated into a 500 ml Erlenmeyer flask with its host of MRSA strain (0,5 ml) in 50 ml LB broth. The mixture was incubated overnight at 30 °C with 100 rpm shaking.
2. 2,5 ml of chloroform was added to lyse the bacteria in the mixture and waited 30 min with gentle shaking for 5-6 times.
3. Centrifuge the flask contents at 9000 rpm 10 min 4 °C to get rid of cell debris and decant the supernatant into sterile tubes.
4. Filter sterilize the phage supernatant through a 0,45 um pore size membrane
5. Add 6 ml of 10% (w/v) PEG 6000 solution (containing 50% ,1M NaCl) to the phage filtrate and incubate overnight at 4 °C to precipitate phages
6. Next day, centrifuge at 15000 rpm 20 min at 4°C. Remove the supernatant and resuspend the pellet containing the phages in 1 ml Saline-Magnesium (SM) buffer and store at 4 °C. Phage lysate was supplemented with 20% glycerol and stored at -20 °C for long term.

Table 2.2 Saline-Magnesium (SM) buffer 1L

NaCl:	5,8 g
MgSO4.7H2O:	2,0g
1M Tris-HCl (pH 7.5):	50 ml
Distilled water to 1 L and autoclave 121 °C for 15 min.	

2.6. Phage Titer Calculation

The titer of concentrated single phage solution in SM buffer (Step 6 of section 2.5) was calculated as below:

1. Tenfold dilutions of concentrated phage preparation (1 and 10^{-1} to 10^{-11}) were prepared.
2. 100 µl of the selected dilution was poured into 3 ml of soft agar medium and 100 µl of overnight culture of corresponding host strain were mixed into a test tube.
3. The top agar mixture was poured onto a bottom standard agar plate, allowed to cool down for hardening, and then incubated overnight at 37 °C.
4. The plates with 30-300 plaques were counted.
5. The titer of the original phage lysate was calculated as following formula:
   Plaque forming units /ml (pfu/ml) = (Number of plaques) x 10 x (1/dilution).

2.7. Multiplicity of Infection (MOI) Assay

The ratio of phages added to host bacteria is designated as multiplicity of infection (MOI). MOI is important for further studies, in this context; wide range of values of MOI was tested to find out the optimal MOI. The stock phage MikSA913 (3×10^{11} pfu/ml) was tenfold serially diluted. MRSA-913 overnight grown bacteria was adjusted to concentration of of 3×10^{8} cfu/ml corresponding to McFarland unit of 1. Then, the bacteria was inoculated with the phage with different ratios (0.001, 0.01, 0.1, 1, 10 and 100). The phage and bacteria mixture was kept at 37 °C without shaking

allowing 15 min adsorption time. Afterwards, unadsorbed free phages were removed by centrifugation at 9000 rpm for 5 min and pellets were resuspended in LB medium. The resuspended phage samples were incubated for 6 hours at 37 °C and the phage titer was determined.

2.8. Phage Host Range Analysis (Spot Testing)

The host range of isolated phages were checked by spot testing:

1. Put the overlay mediums in test tubes (0,7% w/v, LB agar containing 10 mM $CaCl_2$ and 10 mM $MgSO_4$) onto heat block and melt to 60-70°C.
2. Add 0,1 ml tested overnight bacteria into the overlay top medium (1,5% w/v, LB agar containing 10mM $CaCl_2$ and 10 mM $MgSO_4$) and mix gently and quickly pour onto standard agar plate avoiding any crystallization of agar.
3. After hardening 15 min, spot 10 µl of each bacteriophage (around 3 x 10^7 pfu/ml per spot) onto the bacterial lawn using a new sterile pipette for each spot and incubate the plates overnight at 37 °C.
4. The next day, the clear zones on the plates were examined and the spots were classified as suggested by Kutter (Kutter, 2009). The classification scheme ranges from complete lysis (++++) to no lysis (-).

2.9. Effect of Calcium on Adsorption Kinetics

Divalent cations, especially calcium ion was shown to affect adsorption of phages to their hosts. The effect of calcium concentration on adsorption of the MikSA913 to its host cell was assessed by the method described by Chibber et al. (Chhibber, Kaur, & Kaur, 2014) with some changes. Phage MikSA913 was inoculated with host cells (3 x10^8 cfu/ml) at MOI 0,001. Phage adsorption was assessed in the presence and absence of 10 mM $CaCl_2$ and the samples containing the mixtures were incubated at 37°C with shaking at 160 rpm. 100 µl aliquots were removed at 5 min intervals and the number

of the free unadsorbed phages was determined by double plaque assay phage titration. The percentage of the unabsorbed phages in each case was shown in **Table 3.1**. From this data adsorption rate constants were calculated.

2.10. One-Step Growth Curve

The growth characteristics of the phage; burst size, latent, rise and eclipse phases of the MikSA913 were calculated using one-step growth curve method. One step growth experiment was carried out following the procedure adapted from the previous study (Casey et al., 2015).

1. Firstly, MRSA host strains were grown in 50 mL of LB to a McFarland unit of 1.0, which corresponds to approximately 3 x 10^8 cfu/ml.
2. Cells of host MRSA then harvested by 9000 rpm, 5 min and resuspended in 500 µl of LB broth.
3. 500 µl of phage suspension was added at MOI 0.0001 to the bacteria and allowed 5 min at 37°C for phage adsorption. In order to remove unadsorbed phages, the mixture was centrifuged at 9000 rpm, 5 min.
4. The final pellet was resuspended in 50 mL of LB and incubated at 37°C. Two aliquot samples (100 µl) was taken 5 min intervals for 1 hour for 1 hour and centrifugation at 9000 rpm for 1 min. Phages in the resultant supernatant was serially diluted and titered by double-layer agar plate assay. One of the samples were plated immediately without any treatment while the other samples were treated with 1% (vol/vol) chloroform to burst the host cell and release the phages inside the cell.
5. Burst size was calculated as:
   Burst size= (phage titer following burst –initial titer)/ (phage added-initial titer)

2.11. Thermal and pH stability

Thermostability of the bacteriophages were tested by the protocol as described earlier (Kwiatek et al., 2012) with some modifications. Samples of the isolated bacteriophages were incubated at various temperatures ranging from 30°C to 80 °C as shown in Fig. 3.7 and aliquots were taken after 5, 15, 30, 60, 90, and 120 minutes and double plaque assay was performed to calculate phage titer (section 2.6).

For pH stability assay, phage lysates were inoculated into LB medium in Eppendorf tubes and NaOH or HCl were used to adjust pH values ranging from 2 to 12. Following incubation at 37 °C for 1 hour, phage titer was calculated by double plaque assay (sections 2.3 and 2.6).

2.12. Phage Genomic DNA Isolation

Phage DNA was extracted from the isolated phage stock solution (3x 10^{11} pfu/ml). DNA isolation of phage particles was performed with DNA isolation kit (DNA, RNA, and Protein Purification Kit, NucleoSpin Tissue ™, Macherey-Nagel) following to the manufacturer's instructions. Phage DNA isolation was performed as described below:

1. 100 µl of phage stock solution in SM buffer was taken into Eppendorf tube and 2µg/µl of DNase I (Promega) was added and incubated at 37°C for 45 min on heating block.
2. Then, 2 u/µl DNase Stop Solution (Promega) was added and followed by incubation on heating block at 65 C° for 10 min.
3. 300 µl of T1 solution was added and vortexed. Proteinase K (50 µg/ml) (Macherey Nagel) and 400 µl B3 solution were added. Then, the mixture was vortexed briefly and incubated at 55°C for 15 min.
4. The next step is to stop the reaction by 15 min incubation at 70°C.
5. 420 µl of pure ethanol (70%) was added.

6. 700 µl of sample was loaded onto the DNA binding column and centrifuged at 8000 rpm for 1 min and flowthrough was discarded.
7. Another 700 µl of the sample was added and loaded onto the column followed by centrifugation at 8000 rpm for 1 min and discard flow through.
8. 600 µl BW buffer was addedd and centrifuged at 8000 rpm for 1 min. The flow through was discarded.
9. 600 µl B5 solution was added and again centrifuged at 8000 rpm for 1 min. The flow through was discarded.
10. 600 µl B5 solution and again centrifuge at 13000 rpm for 2 min. The flow through was discarded.
11. The column was air dried at 70 °C for 10 min on heating block.
12. For elution of the DNA, the column was put onto the new fresh Eppendorf tube and 100 µl of TE buffer was loaded onto the column and centrifuged at 3000 rpm for 1 min.
13. The eluted DNA was stored at -20°C for further experiments.

2.13. Whole Genome Sequencing

The isolated bacteriophage genome concentration was measured with spectrophotometer NanoDrop (Thermofisher) and the DNA concentration of phage MikSA913 was 9 ng/ml. Phage DNA was sequenced at a commercial local firm. For next generation sequencing, the DNA library was constructed with Nextera sample prep kit (Illumina). Paired-end sequencing was performed by Illumina MiSeq PE300 sequencer (Illumina) with the 300 nucleotide read length.

2.14. Bioinformatics Analysis

The assembled whole genome sequence was first searched by BLAST for comparative analysis to identify the phage. The prediction of open reading frames (ORFs) were identified by GeneMarkS optimized for phage genome (Besemer & Borodovsky, 2005). The putative ORFs were annotated by BLAST and structural predictions and motif searches were performed with InterPro and by the Conserved Domain database of NCBI. The putative genes and the amino acid sequences were searched by BLASTn and BLASTp databases, respectively. The nucleotide sequence was scanned in all reading frames with start codons ‘ATG and alternative start codons with a threshold of 75 nucleotide.

tRNA-encoding genes was searched with the tRNAscan-SE software (Lowe & Eddy, 1996) and ARAGORN (Laslett & Canback, 2004). Rho-independent transcription terminators was identified by ARNold (Naville, Ghuillot-Gaudeffroy, Marchais, & Gautheret, 2011). Genome was scanned for virulence factor with the virulence factor database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm).

CHAPTER 3

RESULTS

3.1. Isolation and characterization of staphylophages

In this thesis study, six phages were isolated from sewage after enrichment with MRSA strain mixtures. The purified phages are named according to their host strain number and the isolated phages are MikSA55, MikSA745, MikSA861, MikSA862, MikSA1034, and MikSA913. All of the phages have plaques with different morphology; phage MikSA745 has a halo around the plaques indicative of depolymerase activity and MikSA55 has no complete clearance but faint lytic zones (**Fig. 3.1)**. The plaques of phages MikSA861 and MikSA1304 (not shown in Figure) are very small compared to rest of the other phages isolated.

The whole genomes of MikSA913 and MikSA1034 were sequenced and according to the genomics analysis results, MikSA1034 is a prophage although there was no induction by mitomycin C or UV procedure we followed. The genome sequence of MikSA1034 have PVL coding sequence, which is an important virulence factor and integrase gene which is typical of prophages needed for integration of phages to their host. From therapeutic perspective, we excluded this phage for further analysis since our aim is to identify lytic phages for clinical purposes.

Among all of the phages, phage MikSA913 was chosen for genomic characterization and for physical characterization since it has significant lytic activity and broad host range. MikSA913 was isolated from the sewage treatment center in Samsun (Turkey) using a clinical MRSA isolate (MRSA-913) as the host for phage isolation and propagation. Clear plaques were observed wherever phage lysate was spotted onto LB agar plates covered with a bacterial lawn of MRSA-913. The plaque size is around 1-2 mm in diameter **(Fig. 3.2)**.

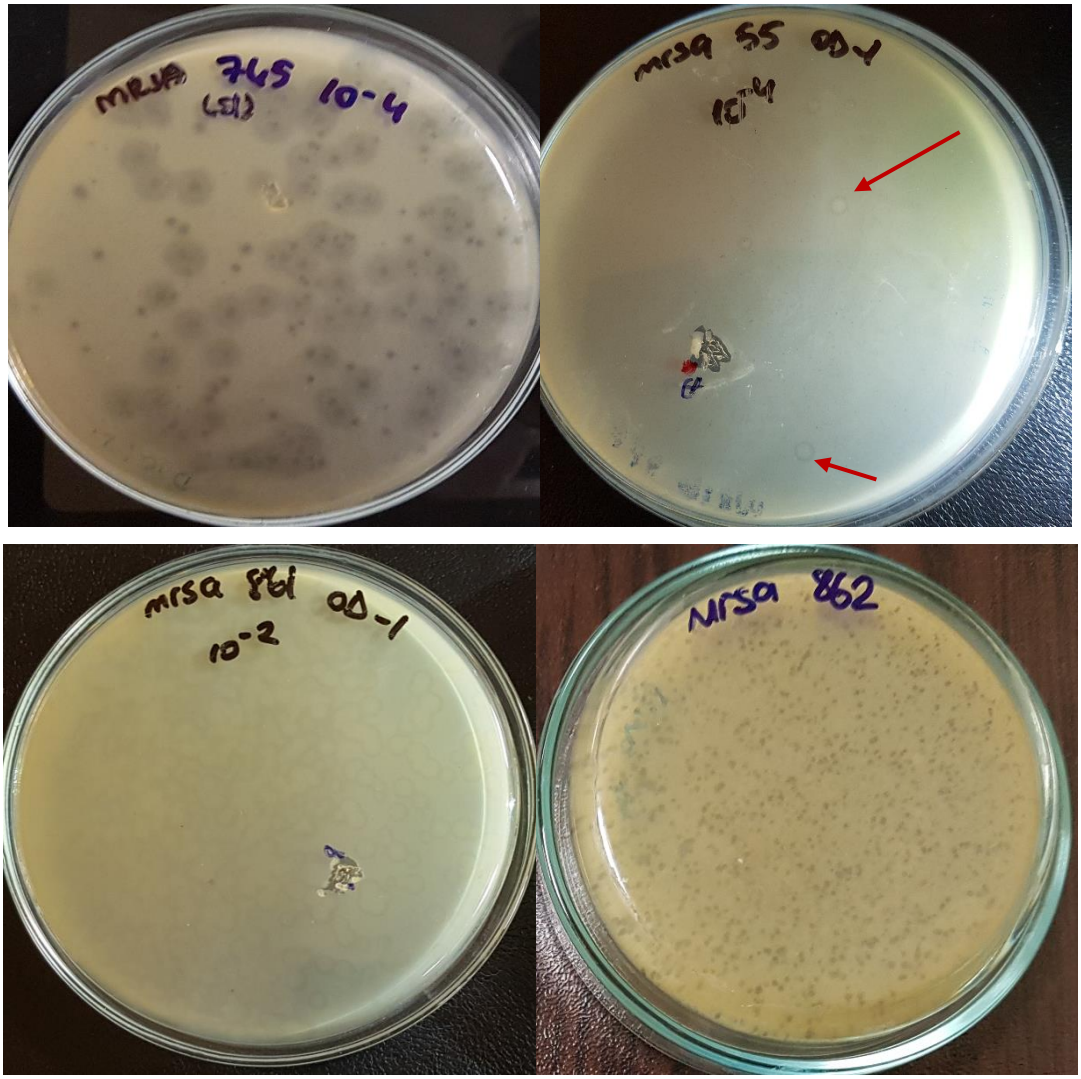

Figure 3.1 Plaques of phages (MikSA745, MikSA55, MikSA861 and MikSA862). Arrows indicate single plaques.

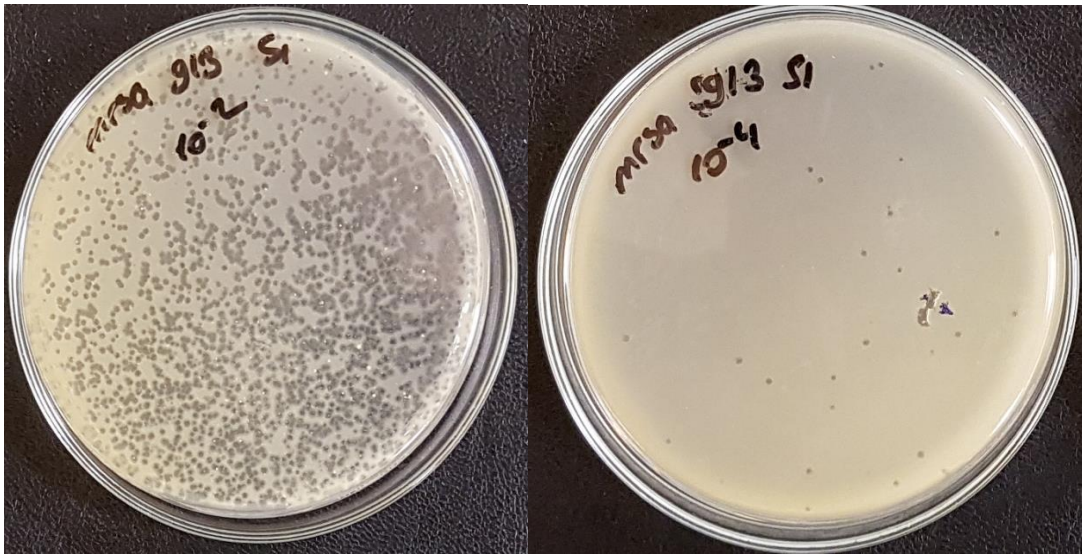

Figure 3.2 Plaques of phage MikSA913 **(**high titer (10^{-2}) to low titer (10^{-4})).

3.2. Optimal MOI selection

MOI of was tested with values of 0,001 to 100 range and the results demonstrated when the MOI of 0.001 the phage titer was highest, reaching 9.7 x 10^9 pfu /ml **(Fig. 3.3)**. Therefore, the optimal MOI, phage to bacteria concentration (pfu/cfu), is 0,001.

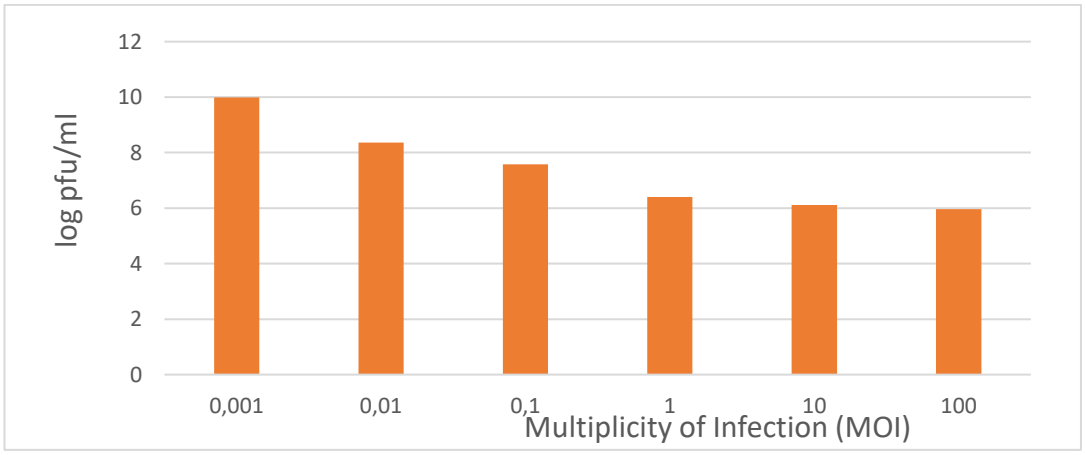

Figure 3.3 Optimal multiplicity of infection (MOI) of phage MikSA913. Comparison of titer for 6 hours at MOI ratios of phage to bacteria (pfu/cfu) in LB medium.

3.3. Effect of Calcium on Adsorption Kinetics

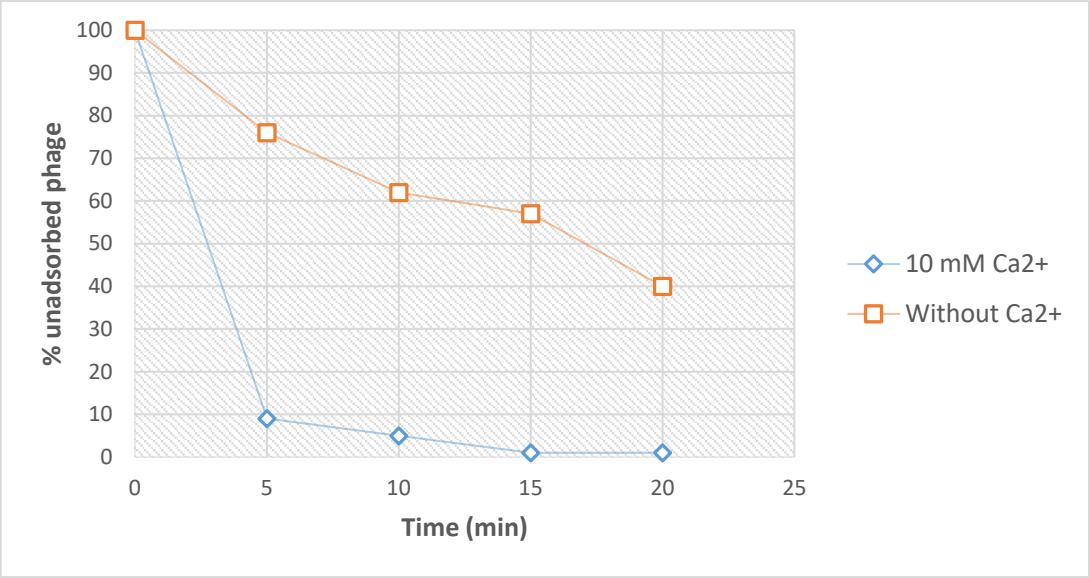

Figure 3.4 Adsorption kinetics of MikSA913.

As shown in **Fig. 3.4** and **Table 3.1**, adsorption occurred rapidly, indeed, more than 90% of the phage adsorbed within 5 min, and the adsorption reached 99% within 15 min.

Table 3.1 Percentages of free phages with or without calcium.

Time (min)	**% unadsorbed phage w/o Ca^{2+}**	**% unadsorbed phage 10 mM Ca^{2+}**
0	100	100
5	76	9
10	62	5
15	57	1
20	40	1

Samples were removed at 5 min intervals and the number of free infectious phage particles was calculated by phage titration. The absorption rate constant was calculated as following formula where k is the adsorption rate constant (ml/min), B is the concentration of bacterial cells, and t is the time interval in which the titer falls from P_0 to P (final).

$$k = \frac{2.3}{Bt} log \frac{P_o}{P}$$

Adsorption rate of phage MikSA913 with 10 mM Ca^{2+} as calculated from the data within the interval 5 min to 15 min:

k= 2.3 / ((3x 10^8) x 10 min) x log (9/1) = 7,32 x 10^{-10} ml/min

Adsorption rate of phage MikSA913 without Ca^{2+} as calculated from the data within the interval 5 min to 15 min:

k=2.3/ ((3x 10^8) x10 min) x log (76/57) = 9,57 x 10^{-11} ml/min

3.4. MikSA913 Host Range

Of the tested isolates, the phage MikSA913 was lytic against 35 out of 50 strains (70%) of MRSA and 4 MSSA strains tested. The phage also lysed the standard strain *S. aureus* ATCC 25923. However, the phage could not lyse the other species of Gram-negative or Gram-positive bacteria (**Table 3.2**).

Table 3.2 Phage MikSA913 host range analysis by spot testing.

Bacteria	**Strain Number**	**Spot Testing**
MRSA	55	-
	56	+++
	711	-
	719	++++
	744	++++
	745	-
	748	+++
	753	++++
	757	-
	783	++++
	794	++
	802	+++
	820	-
	821	+++
	831	++++
	836	-
	838	+++
	845	+++
	846	++
	849	-
	850	++++
	861	-
	867	++++
	871	-
	875	++
	888	-
	905	++++
	907	++
	913	++++
	918	++++
	919	-
	922	+++
	948	++++
	953	-
	958	++++
	982	++
	988	+++
	989	++++
	997	+++
	1000	++

Table 3.2 cont.

MRSA	1001	+
	1003	-
	1007	++++
	1018	++
	1019	+++
	1029	-
	1031	+++
	1034	-
	1036	-
	1045	++++
MSSA	730	+
	747	+++
	752	-
	773	++
	796	++++
S.aureus ATCC 25923		++++
Other Gram Positive Bacteria		
B. subtilis		-
E. faecalis		-
S. lugdunensis	937	-
	1008	-
Other Gram Negative Bacteria		
K.pneumoniae	147	-
	220	-
E.coli ATCC 25922		-
P. aeroginosa ATCC 27853		-

3.5. Burst Size and Latent Period

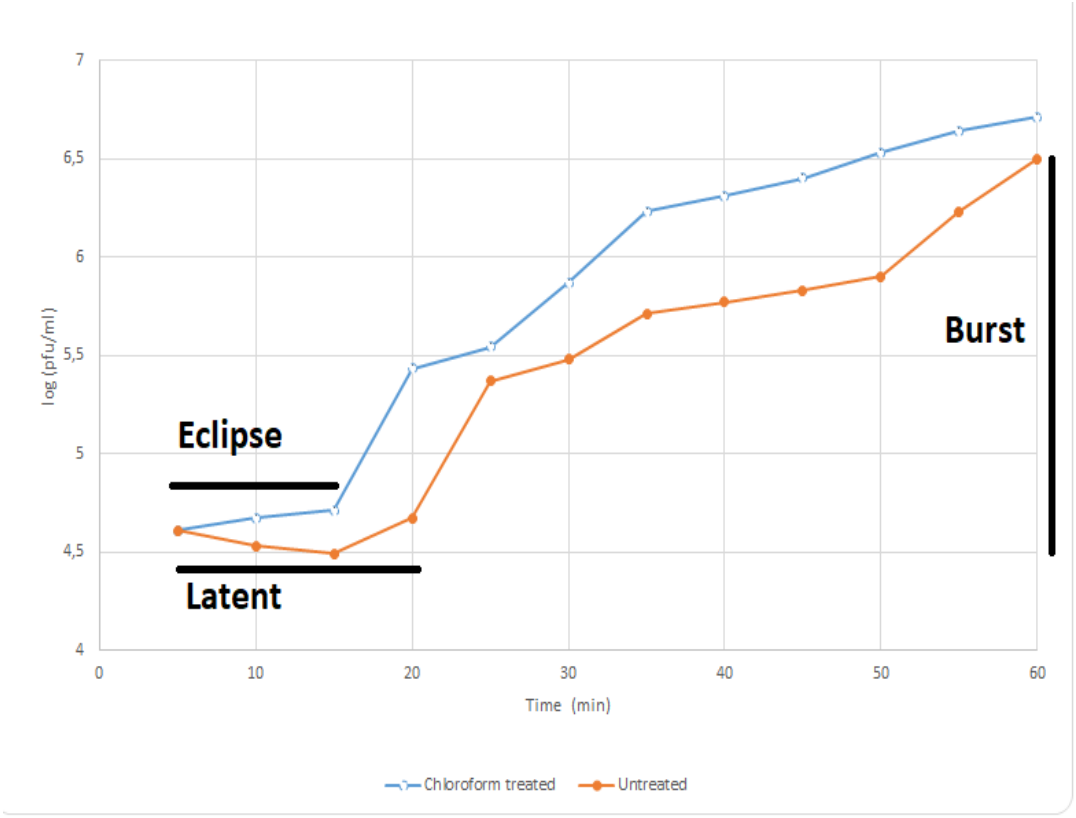

Figure 3.5 One-step growth curve of phage MikSA913.

One-step growth studies were performed to identify the different phases of a phage infection process. After infection, phage growth cycle parameters, latent, eclipse and burst size were determined **(Fig. 3.5)**. The eclipse and latent periods of MikSA913 was measured 15 min and 20 min, respectively and the burst size is 112 PFU/infected cell.

Burst size = $(10^{6.53} – 10^{4,5})$ / (3×10^{4}) = **112 pfu/infected cell**

3.6. Thermal and pH stability

The stability of the phage MikSA913 was evaluated over a wide range of pH and temperature. As shown in **Fig. 3.6** the activity of phage MikSA913 was stable between 30 and 37 °C, however, when kept at 50 °C for 2 hours the phage survival decreased by 30%. The phage was stable at 60 and 70 °C in the first 40 minutes although the activity decreased. However, complete inactivation occurred at 80 °C in about 30 minutes. Lytic activity was stable between of pH 5 and 11 and activity was completely diminished at pH 4 or below and beyond pH 11 (**Fig. 3.7**)

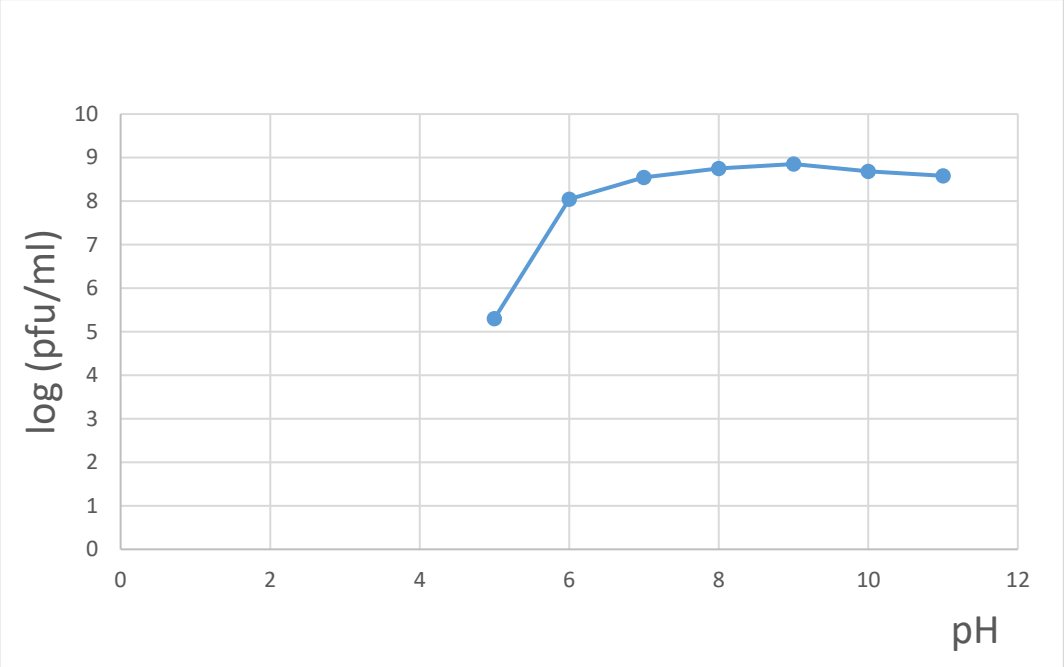

Figure 3.6 pH stability assay of phage MikSA913.

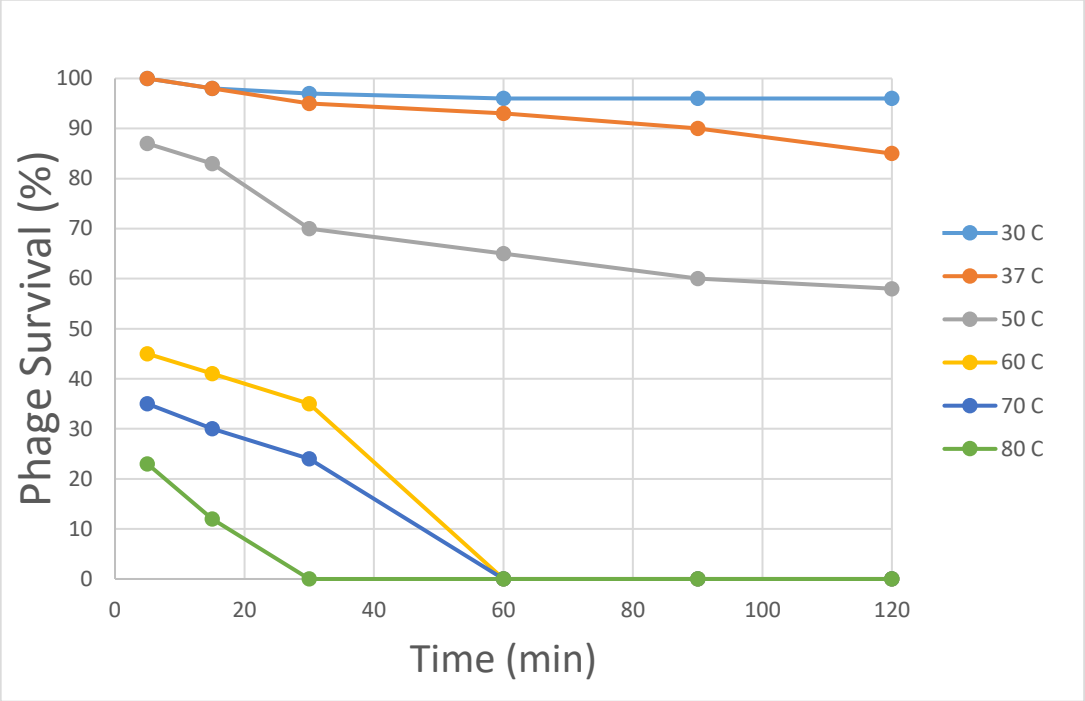

Figure 3.7 Stability of phage MikSA913 at different temperatures.

3.7. MikSA913 Genome Analysis

3.7.1. Genome Overview

The phage MikSA913 genome size is a 134193 bp, of linear, double-stranded DNA with a G+C content of 30.4 %. The bioinformatics analysis for functional annotation of the genes gives 206 ORFs with GeneMarkS database and 103 (50%) of them have given a predicted function while the half have hypothetical protein with no homology to any protein in databases. 191 of the ORFs' translation sites starts with an AUG start codon but only 9 ORF have UUG codon at start site.

According to BLAST analysis and ICTV classification, vB_SauM_MikSA913 belong to order; *Caudovirales*, family; *Myoviridae*, subfamily; *Spounavirinae* and genus *Kayvirus*. ICTV suggestion for *Kayvirus* characterization is terminally redundant genome with an average of 142 kb encoding 215 proteins and 3 to 4 tRNAs. The low

percentage of G+C (30.3%) is also a characteristic of Kayvirus genus. No GATC site was found in this genus so far (Adriaenssens et al., 2018). All of these characteristics are in coherent with the phage MikSA913 genome confirming that it belongs to the *Kayvirus* genus.

Overall, genes of the MikSA913 organized into functional modules of structural, DNA/RNA manipulation, lysis and some other additional functions **(Table 3.3).** The large terminase subunit (*orf81* and *orf83*) of MikSA913 contains a group I intron protein called a VRS endonuclease (*orf82*).

The ends of *Spounavirinae* phages are long terminal repeat genes, which are encoding small proteins functioning in takeover of the host cell metabolism and redirect to phage propagation (Stewart, Yip, Myles, & Laughlin, 2009). Long terminal repeats are first parts of the injected genome of the Twort-like phages. This region size is different in each Staphylococcal *Spounavirinae* family. Long ***t***erminal ***r***epeat ***e***ncoded proteins are written as ***tre*** and in MikSA913 genome 15 *tre* genes were observed (*tre, treH ,tre,treJ, treK, treN, treP, tre, treS, treT, treA, treC, treD, treE and treF*) in a region of 9930 bp. The core genome region is suggested to be between the boundary between TreA (*orf202*) and BofL (*orf22*).

Using the ARNOLD web server, the existence and location of the rho-independent transcription terminators were predicted and the total number of predicted transcription terminators was found to be 70 for phage MikSA913 genome.

Phage MikSA913 genome was searched with the virulence factor database (VFDB) and antibiotic resistance genes database (ARDB) and there was no hit to any known virulence or resistance gene.

Table 3.3 Functional modules of MikSA913 genome.

ORF	Putative Function	Amino Acid Length (MikSA913)	Average Amino Acid Length other Kayviruses)
DNA Manipulation			
125	DNA helicase A	582	582
126	Rep protein	537	537
127	DNA helicase B	480	480
128	Recombination exonuclease A	341	345
130	Recombination exonuclease B	639	639
132	DNA primase	355	355
135	Resolvase	202	202
143	DNA polymerase A	1072	1008-1072
147	Repair recombinase	418	418
149	RNA polymerase sigma factor	220	220
174	DNA sliding clump inhibitor	58	58
Structural			
93	Major capsid protein	463	463
96	Capsid protein	292	158-293
100	Major tail sheath protein	587	587
101	Tail tube protein	142	142
109	Tail morphogenetic protein (TmpB)	178	178
110	Tail tape measure	1352	1341-1377
111	Tail murein hydrolase (TAME)	808	808

112	Peptidoglycan Hydrolase (TmpE)	295	296
116	Baseplate wedge subunit (BmpA)	234	234
117	Baseplate morphogenetic protein (BmpB)	348	348
118	Tail morphogenetic protein (TmpF)	1021	1019
119	Baseplate morphogenetic protein (BmpC)	173	173
120	Adsorption-associated tail protein (TmpG)	1152	1152
124	Tail fiber protein	458	458
RNA Manipulation			
131	Anti-sigma factor	198	198
136	Ribonucleotide reductase, stimulatory protein	143	143
137	Ribonucleotide reductase, large subunit	704	704
138	Ribonucleotide reductase, minor subunit	349	349
58	DNA/RNA ligase	297	298
57	Nucleoside 2-deoxyribosyltransferase	198	208
62	Ribonuclease	141	141
Lysis			
70	Endolysin (N-acetylmuramoyl-L-alanine amidase)	495	495
71	Holin	167	167
Additional Functions			
82	Putative intron-encoded nuclease	256	324
113	Glycerophosphoryl diester phosphodiesterase	848	849

140	Thioredoxin	106	106
142	DNA binding protein	101	101
150	Ig like protein	210	210
82	Group I intron endonuclease	256	245
56	HNH homing endonuclease	261	162-194
22	BofL	82	78-82
31	Serine/Threonine protein phosphatase	233	235
46	AAA family ATPase	372	372
60	PhoH related protein	246	246
65	Trancriptional regulator	76	70-76
67	Transglycosylase	230	210-232

Four tRNA genes were found in the phage MikSA913 genome coding for tRNA-Met, tRNA-Trp, tRNA-Phe and tRNA-Asp. The G+C content of the tRNA genes ranges from 38.9% to 51.3% **(Table 3.4)**. The three of these tRNAs are located next to each other between orf72 and orf73 region.

Table 3.4 tRNAs encoded by phage MikSA913 genome.

tRNA type	**Number of Bases**	**Genome region**	**G+C %**
tRNA-Met (cat)	72	[12982,13053]	45.8
tRNA-Trp (cca)	72	[35358,35429]	38.9
tRNA-Phe (gaa)	73	[35436,35508]	41.1
tRNA-Asp (gtc)	76	[35514,35589]	51.3

3.7.2. Lytic Proteins

The lysis module contains genes involved in bacterial lysis, endolysin (*orf70*) having and holin (*orf71*) having 495 and 167 amino acids, respectively (**Table A1**). The endolysin contains two catalytic domains: CHAP (PF05257), N-acetylmuramoyl-L-alanine amidase domain (PF01510) and one cell wall-binding domain: SH3-like domain (PF08460) **(Fig. 3.8)**. Endolysins that have the highest homology with the endolysin of MikSA913 were compared in **Table 3.5**.

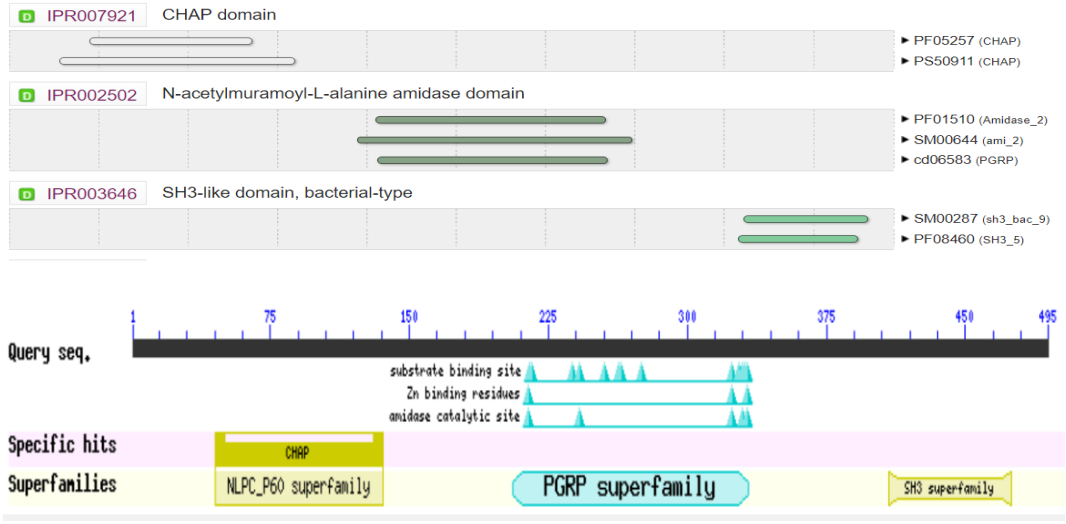

Figure 3.8 Phage MikSA913 endolysin domains. InterProScan (top) and Conserved Domain Database (bottom).

```
MAKTQAEINK RLDAYAKGTV DSPYRVKKAT SYDPSFGVME AGAIDADGYY HAQCQDLITD 60
YVLWLTDNKV RTWGNAKDQI KQSYGTGFKI HENKPSTVPK KGWIAVFTSG SYQQWGHIGI 120
VYDGGNTSTF TILEQNWNGY ANKKPTKRVD NYYGLTHFIE IPVKAGTTVK KETAKKSASK 180
TPAPKKKATL KVSKNHINYT MDKRGKKPEG MVIHNDAGRS SGQQYENSLA NAGYARYANG 240
IAHYYGSEGY VWEAIDAKNQ IAWHTGDGTG ANSGNFRFAG IEVCQSMSAS DAQFLKNEQA 300
VFQFTAEKFK EWGLTPNRKT VRLHMEFVPT ACPHRSMVLH TGFNPVTQGR PSQAIMNKLK 360
DYFIKQIKNY MDKGTSSSTV VKDGKTSSAS TPATRPVTGS WKKNQYGTWY KPENATFVNG 420
NQPIVTRIGS PFLNAPVGGN LPAGATIVYD EVCIQAGHIW IGYNAYNGNR VYCPVRTCQG 480
VPPNQIPGVA WGVFK
```

Figure 3.9 Amino acid sequence of endolysin MikSA913 . The domains of are illustrated as: CHAP (45-136) in yellow, N-acetylmuramoyl-L-alanine amidase (206-333), and SH3-like domain (409-475) in green.

Table 3.5 Comparison of endolysin of phage MikSA913 with other similar lysins

Phage	Amino Acid Length	Amino acid residue position												
		26	83	113	165	171	231	266	300	372	469	484	485	486
			CHAP				N-acetylmuramoyl-L-alanine amidase				SH3			
MikSA913	495	V	S	Q	A	E	N	G	A	D	N	N	Q	I
K	495	V	S	E	A	E	N	G	A	D	N	N	Q	I
JD007	495	V	S	E	A	E	N	G	A	D	N	N	Q	I
P4W	495	V	S	E	A	K	N	G	A	D	N	N	Q	I
qdsa002	495	I	S	Q	A	E	N	G	A	D	N	N	H	I
phiSA12	495	V	N	E	A	E	N	G	A	D	N	N	Q	I
vB_Sau_CG	495	V	N	Q	A	E	T	G	A	D	N	N	Q	I
phiSA039	495	V	N	Q	A	E	N	G	A	N	N	N	Q	I
GH15	495	I	S	Q	A	E	N	G	A	D	D	N	H	I
MCE-2014	**496**	V	S	E	A	E	N	G	A	D	N	S	H	V
S25-4	495	V	N	H	E	E	N	G	T	D	N	N	Q	I
Sb-1	**494**	V	S	E	A	E	N	-	A	D	N	N	Q	I

Amino acid changes are indicated in red.

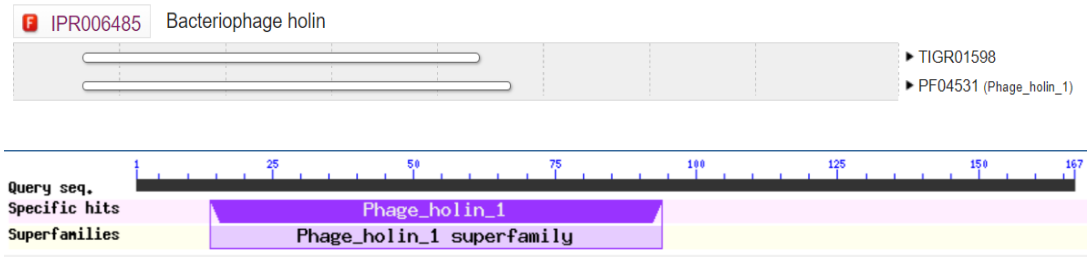

Figure 3.10 Phage MikSA913 holin domains. Results from InterProScan (top) and Conserved Domain Database (bottom).

Besides endolysin and holin,tail lysins are deduced from BLASTp and domain search. While ORF110 codes for tail lysin having phage tail lysozyme domain, ORF111 encodes for tail murein hyrdolase containing CHAP domain (**Fig. 3.11**). These tail lysin proteins belong to kinds of virion-associated peptidoglycan hydrolase proteins.

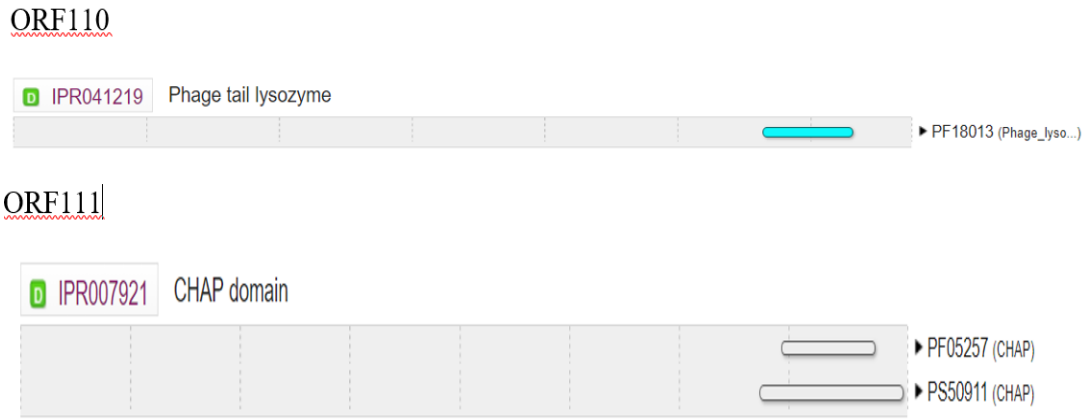

Figure 3.11 VAPHGs of MikSA913. ORF110 codes for tail hydrolase (808 aa) and ORF111 encodes for tail lysin (1352 aa) lytic proteins.

3.7.3. Comparative Genomics

The phage MikSA914 is more closely similar to phages qdsa002, GH15, vB_Sau_CG, and phiSA039 with more than 90% query cover and identity as shown in **Table 3.6**.

Table 3.6 Comparison of phage MikSA913 with its mostly closed phages.

Phage	**Max Score**	**Query Cover**	**Identity**	**Genome Size**	**G+C content %**	**CDS**	**tRNA**
qdsa002	79567	94%	95%	142499		229	3
GH15	79427	93%	95%	139,806	30.23	214	4
vB_Sau_CG	74602	91%	93%	142934	30.51	224	5
phiSA039	74592	91%	93%	141038		228	
Phage K	37218	91%	93%	148317	30.39	233	4
MikSA913				**134193**	**30.40**	**206**	**4**

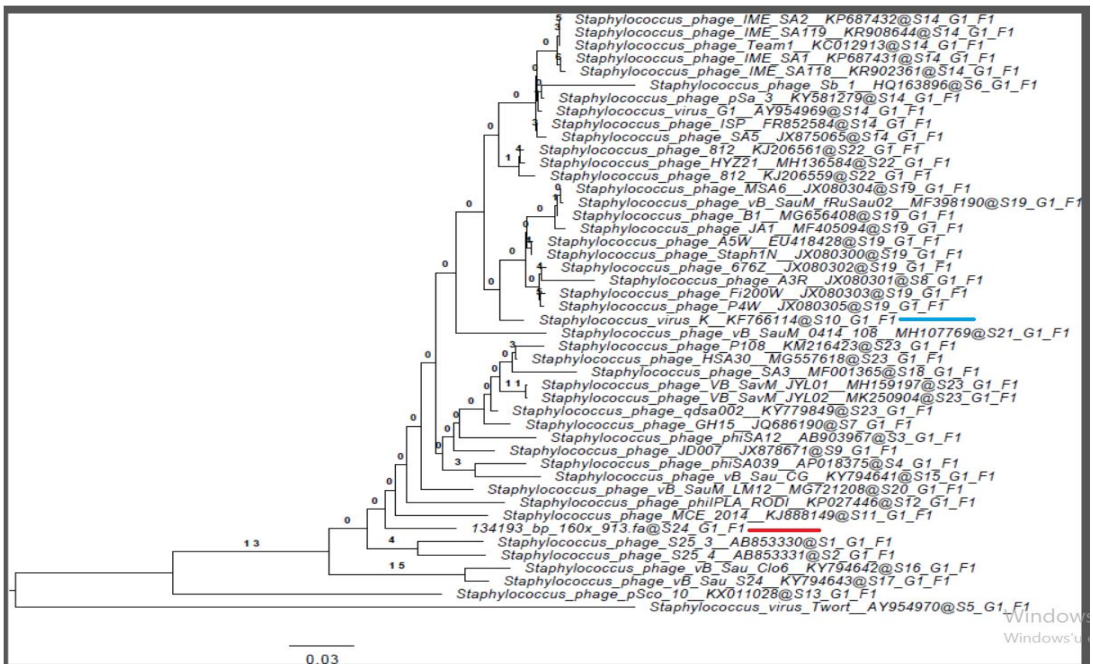

Figure 3.12 Phylogenetic comparison of *Staphylococcus aureus Myoviridae* Phages. MikSA913 (Red line), phage K representative type (blue line) (created by VICTOR Virus Classification and Tree Building Online Resource).

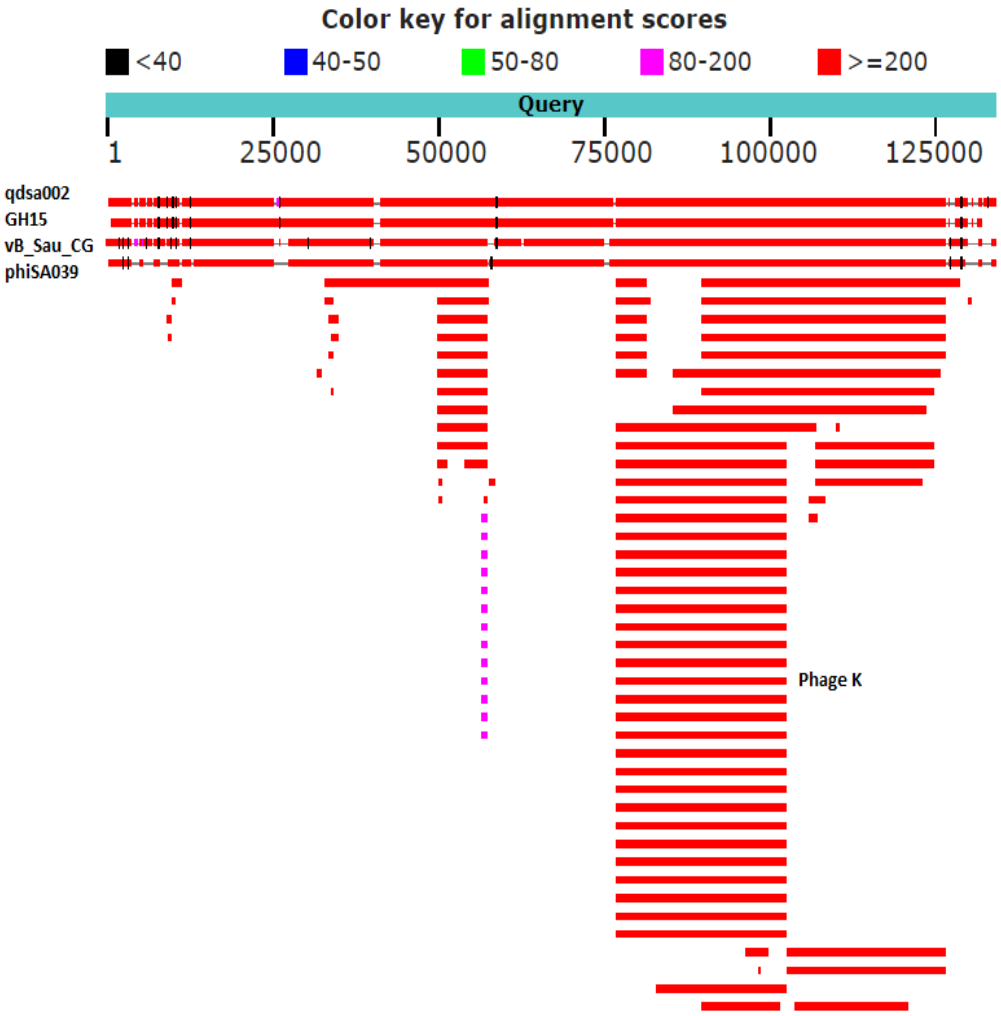

Figure 3.13 Nucleotide BLAST of phage MikSA913**.** The highest homology is with the myophages qdsa002, GH15, vB_Sau_CG and phiSA039 and phage K is less similar compared to those phages.

CHAPTER 4

DISCUSSION

S. aureus is one of the most common pathogen causing serious infections and lytic bacteriophages can provide a solution to this problem. There is a lack of information about genomes of bacteriophages in our region. Thus, in this study, we aimed to find phages active against MRSA strains circulating in Turkey. We searched sewage waters, isolated a few bacteriophages, and chose only one to characterize with high lytic efficacy and broad host range. The isolated phage named as vB_SauM-MikSA913 was classified into *Myoviridae* family, and *Kayvirus* genus. Based on genome size and organization vB_SauM-MikSA913 belongs to the class III staphylococcal phages classified by Kwan (Kwan, Liu, DuBow, Gros, & Pelletier, 2005). Myoviridae class III phages generally contain introns in the genes of lysin, DNA polymerase, ribonucleotide reductase, large terminase and DNA helicase (Kwan et al., 2005)**.** MikSA913 large terminase subunit has a group I intron protein coding for VRS endonuclease and there seems no intron in other genes.

The eclipse and latent periods of MikSA913 was measured 15 min and 20 min, respectively and the burst size is 112 pfu/infected cell in one-step growth experiments. Calcium ion was reported to increase adsorption rate constant of the phage. MikSA913 is stable at a wide range of pH and temperature.

Phage MikSA913 has a broad host range (lysing 75% of MRSA strains) like other Kayviruses known for their broad host range. This phenomenon is mainly accounted to the presence of multiple receptor binding proteins in the viral capsid that allow them to utilize at least two adsorption apparatuses and recognize different structures (Takeuchi et al., 2016).

Currently, *Kayvirus* genus contains 13 members, including phage K, GH15, JD007, MCE-2014, P108, S25-3, S25-4, G1, Team1, philPLA-RODI, phiSA12, 812 and Sb-1 on NCBI. A typical feature for *Kayvirus* genus is the presence of long terminal repeats, several thousand base pair-long direct repeats at the ends of the genome (King et al. 2011). The ends of the phage genome are flanked by LTRs. The region between TreA and Bofl encoding genes is considered LTR and this is 9930 bp in phage MikSA913. The nucleotide sequence and length of LTR regions differ among the representatives of the genus and may influence the host range. (Łobocka et al., 2012). According to nucleotide BLAST analysis, the most variable regions are the LTRs between MikSA913 and its closed phages.

S. aureus Myoviridae phages contain a large deal of promoters recognized by *S. aureus* σ^{70}. In addition, anti-σ factors and an alternative σ factors are located in this phage class. MikSA913 ORF131 codes for the anti-sigma factor while ORF 149 encodes sigma factor.

MikSA913 genome has no 'GATC' sites, which are the targets of *S. aureus* restriction enzymes thus allowing the phage to avoid bacterial defensive immune system. The safety assessment based on genome sequence showed that phage MikSA913 is strictly lytic with no integrase that is responsible for recombination event, which is characteristics of temperate phages. In addition, there is no virulence factor, toxin or antibiotic resistance coding genes.

The *in silico* analysis of the phage derived lytic enzymes was also performed with BLAST analysis and domain search. As seen in most *Myoviridae* staphylococcal phages, MikSA913 contain a lysis cassette containing two proteins: endolysin and holin. The endolysin modular structure have three domains: N-terminal CHAP domain, and internal N-acetylmuramoyl-L-alanine amidase domain and a C-terminal SH3-like domain. In addition, the other lytic proteins, virion associated peptidoglycan hyrdolases are shown in the MikSA913 genome. ORF110 and ORF111 have lytic domains of lysozyme and CHAP domain, respectively.

MikSA913 endolysin BLAST analysis results showed that it is highly homologous to other isolated phage endolysins with only one or a few amino acid changes. Jun et al. showed that despite the high degree of similarity SAL-1 and the phage endolysin LysK, SAL-1 has higher cell wall hydrolyzing activity than LysK. The authors suggested that this enhanced enzymatic change is due to the glutamic acid to glutamine at the residue at 114. However, I believe that this reported residue is 113 and erroneously reported as 114 since LysK has glutamic acid at residue 113 and glutamine at residue 114. Based on BLASTp analysis, the MikSA913 endolysin differs from LysK and endolysin derived from JD007 only at one residue: glutamine instead of glutamic acid at the 113th residue. According to the this previous study, we can claim that endolysin of MikSA913 have higher staphylolytic activity than LysK and endolysin of JD007 phage (Jun et al., 2011). In structural studies of the LysK, Cys54-His117-Glu134 residues are shown to be proteolytic site of the CHAP domain (Keary et al., 2016). These residues are also conserved in CHAP domain of MikSA913 endolysin spanning the residues 45-136.

The gene sequences and organization of *Myoviridae* phages isolated in different geographical regions show high similarity and this suggests that the evolutionary forces lead to maintain core functional activities on *S. aureus* isolates.

CHAPTER 5

CONCLUSION

In this thesis, we isolated a new bacteriophage active against local MRSA strains and carried out biophysical and genomic characterization. To sum up all of the characteristics: strictly lytic cycle, and lack of virulence or resistance genes, highly lytic activity, wide host range on MRSA strains and specificity to *S. aureus* strains make the phage MikSA913 suitable for clinical applications to eradicate MRSA. Further work on the activities of the phage and its purified recombinant endolysin should be carried out to validate their efficacy *in vivo*.

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APPENDICES

A. **Table A1** ORF annotation of phage MikSA913 genome by BLASTp and tRNAs

Phage MikSA ORF	**Strand**	**Amino Acid Length**	**Gene Length**	**LeftEnd RightEnd**	**BLASTp Best Hit**
1	+	81	246	32 -277	**terminal repeat-encoded protein [Staphylococcus phage phiSA12]**
2	+	114	345	288 -632	**TreH [Staphylococcus phage vB_SauM_LM12]**
3	-	112	339	833 -1171	**terminal repeat-encoded protein [Staphylococcus phage phiSA12]**
4	+	102	309	1480 - 1788	**TreJ [Staphylococcus phage vB_Sau_CG]**
5	+	94	285	1994 -2278	**TreK [Staphylococcus phage A5W]**
7	+	63	159	3066 -3224	**TreN [Staphylococcus phage JD007]**
8	+	52	324	3392 -3715	**TreP [Staphylococcus phage B1]**
16	+	161	486	6417- 6902	**terminal repeat-encoded protein [Staphylococcus phage phiSA12]**
18	+	54	174	7158 - 7331	**TreS [Staphylococcus phage vB_Sau_CG]**
19	+	89	270	7331 -7600	**TreT [Staphylococcus phage phiIPLA-RODI]**
22	-	82	249	8586 - 8834	**BofL [Staphylococcus phage vB_Sau_S24]**
24		81	246	9094 - 9339	**putative DUF1024 domain protein [Staphylococcus virus K]**
25	-	63	192	9339 - 9530	**membrane protein [Staphylococcus phage vB_SauM_0414_108]**
26	-	161	486	9527 -10012	**membrane protein [Staphylococcus phage phiSA12]**
28	-	164	495	10461-10955	**GTP cyclohydrolase II [Staphylococcus phage phiSA_BS1]**
31	-	233	702	11843 - 12544	**serine/threonine protein phosphatase [Staphylococcus phage MCE-2014]**
32	-	182	549	13333 - 13881	**putative non-cytoplasmic protein [Staphylococcus phage 812h1]**
39	-	57	174	15928 - 16101	**transglycosylase [Staphylococcus caprae]**
42	-	175	528	17216 - 17743	**peptidase membrane protein [Staphylococcus phage VB_SavM_JYL01]**
43	-	54	165	17746 - 17910	**putative membrane protein [Staphylococcus phage vB_SauM_LM12]**

44	-	92	279	17913 - 18191	**putative membrane protein [Staphylococcus phage qdsa002**]
45	-	281	846	18191 - 19036	**AAA family ATPase [Staphylococcus phage VB_SavM_JYL01]**
46	-	372	1119	19049 - 20167	**AAA family ATPase [Staphylococcus phage P108]**
49	-	100	303	21185 - 21487	**NTP pyrophosphohydrolase [Staphylococcus phage Twillingate]**
52	-	683	2052	21881 - 23932	**diguanylate cyclase/phosphodiesterase [Staphylococcus phage vB_SauM_0414_108]**
54	-	57	174	24291 - 24464	**LysM domain-containing protein [Staphylococcus virus SA11]**
55	-	192	579	24471 - 25049	**MbpB [Staphylococcus phage Staph1N]**
56	-	261	786	25042 - 25827	**putative HNH endonuclease [Staphylococcus phage GH15**]
57	-	198	597	25802 - 26398	**nucleoside 2-deoxyribosyltransferase [Staphylococcus phage phiIPLA-RODI]**
58	-	297	894	26391 - 27284	**RNA ligase [Staphylococcus phage phiIPLA-RODI]** **putative DNA ligase [Staphylococcus phage P108]**
60	-	246	741	27581 - 28321	**PhoH-related protein [Staphylococcus phage phiIPLA-RODI]**
62	-	141	426	29003 - 29428	**putative ribonuclease [Staphylococcus phage GH15]** **ribonuclease H [Staphylococcus phage vB_SauM_Romulus]**
65	-	76	231	30263 - 30493	**transcriptional regulator [Staphylococcus phage JD007]**
67	-	230	693	30832 - 31524	**putative transglycosylase IsaA [Staphylococcus phage GH15]** **immunodominant staphylococcal antigen A precursor [Staphylococcus phage MCE-2014]**
68	-	264	795	31722 - 32516	**putative membrane protein [Staphylococcus phage GH15]**
70	-	495	1488	32938 - 34425	**autolysin (n-acetylmuramoyl-l-alanine amidase) [Staphylococcus phage JD007]**
71	-	167	504	34425 - 34928	**holin [Staphylococcus phage phiIPLA-RODI]**
76	-	108	327	38002 - 38328	**putative membrane protein [Staphylococcus phage GH15]**
78	+	88	267	38889 - 39155	**membrane protein [Staphylococcus phage phiIPLA-RODI]**

81	+	116	351	39833 - 40183	**terminase large subunit [Staphylococcus phage MCE-2014]**
82	+	256	771	40423 - 41193	**VSR homing endonuclease [Staphylococcus phage phiIPLA-RODI]** **group I intron protein [Staphylococcus phage vB_SauM_Romulus]**
83	+	486	1461	41260 - 42720	**Ter [Staphylococcus phage MSA6]** **putative terminase large subunit [Staphylococcus phage GH15]**
84	+	273	822	42713 - 43534	**putative structural protein [Staphylococcus phage S25-4]**
87	+	392	1179	44263 - 45441	**membrane protein [Staphylococcus phage phiIPLA-RODI]**
88	+	116	351	45517 - 45867	**putative membrane protein [Staphylococcus phage GH15]**
89	+	123	372	45886 - 46257	**putative portal protein [Staphylococcus phage GH15]**
90	+	563	1692	46261 - 47952	**portal protein [Staphylococcus phage vB_Sau_CG]**
91	+	257	774	48148 - 48921	**prohead protease [Staphylococcus phage pSco-10]**
93	+	463	1392	50003 - 51394	**major capsid protein [Staphylococcus phage JD007]**
96	+	292	879	52717 - 53595	**capsid protein [Staphylococcus phage JD007]**
100	+	587	1764	55315 - 57078	**major tail sheath protein [Staphylococcus phage phiIPLA-RODI**]
101	+	142	429	57151 - 57579	**putative tail tube protein [Staphylococcus phage phiSA12]**
105	+	64	195	58470 - 58664	**putative membrane protein [Staphylococcus phage phiSA12]**
108	+	152	459	59343 - 59801	**tail tape measure chaperone [Staphylococcus phage Terranova]**
109	+	178	537	59845 - 60381	**tail morphogenetic protein [Staphylococcus phage P108]**
110	+	1352	4059	60434 - 64492	**tail tape measure [Staphylococcus phage vB_SauM_LM12]** **putative tail lysin [Staphylococcus phage GH15]**
111	+	808	2427	64570 - 66996	**N-acetylmuramoyl-L-alanine amidase [Staphylococcus phage SA3]** **tail murein hydrolase [Staphylococcus phage vB_SauM_LM12]**

112	+	295	888	67010 - 67897	**protease [Staphylococcus phage phiIPLA-RODI] putative tail protein [Staphylococcus phage SA5]**
113	+	848	2547	67897 - 70443	**glycerophosphoryl diester phosphodiesterase [Staphylococcus phage MCE-2014]**
114	+	263	792	70550 - 71341	**structural protein [Staphylococcus phage pSco-10]**
116	+	234	705	71865 - 72569	**putative baseplate protein [Staphylococcus phage GH15]**
117	+	348	1047	72584 - 73630	**putative baseplate J protein [Staphylococcus phage JA1]**
118	+	1021	3066	73651 - 76716	**tail morphogenetic protein [Staphylococcus phage vB_SauM_0414_108] TmpF [Staphylococcus phage A5W]**
119	+	173	522	76827 - 77348	**baseplate morphogenetic protein [Staphylococcus phage vB_Sau_CG]**
120	+	1152	3459	77369 - 80827	**adsorption-associated tail protein [Staphylococcus phage phiIPLA-RODI] TmpG [Staphylococcus phage P4W]**
122	+	640	1923	81035 - 82957	**carbohydrate binding domain-containing protein [Staphylococcus phage vB_SauM_0414_108] receptor binding protein [Staphylococcus phage 812]**
124	+	458	1377	83352 - 84728	**capsid and scaffold protein [Staphylococcus phage MCE-2014] putative receptor binding protein [Staphylococcus virus K]**
125	+	582	1749	84820 - 86568	**DNA helicase [Staphylococcus phage MCE-2014]**
126	+	537	1614	86580 - 88193	**putative Rep protein [Staphylococcus phage P108]**
127	+	480	1443	88186 - 89628	**DNA helicase [Staphylococcus phage MCE-2014]**
128	+	341	1026	89707 - 90732	**recombination exonuclease [Staphylococcus phage MCE-2014] recombination exonuclease A [Staphylococcus phage vB_Sau_CG]**
130	+	639	1920	91109 - 93028	**ATPase [Staphylococcus phage phiIPLA-RODI] putative recombination related exonuclease [Staphylococcus phage JA1]**
131	+	198	597	93028 - 93624	**anti-sigma factor [Staphylococcus phage vB_Sau_Clo6]**
132	+	355	1068	93639 - 94706	**DNA primase [Staphylococcus phage phiIPLA-RODI]**
135	+	202	609	95549 96157	**resolvase [Staphylococcus phage vB_SauM_LM12]**

136	+	143	432	96135 96566	**ribonucleotide reductase stimulatory protein [Staphylococcus phage Team1]** **ribonucleotide reductase flavodoxin [Staphylococcus phage phiIPLA-RODI]**
137	+	704	2115	96581 98695	**putative ribonucleotide reductase large subunit [Staphylococcus phage GH15]**
138	+	349	1050	98709 99758	**ribonucleotide reductase small subunit [Staphylococcus phage phiIPLA-RODI]**
140	+	106	321	100089 100409	**oxidoreductase [Staphylococcus phage MCE-2014]** **thioredoxin-like protein [Staphylococcus phage qdsa002]**
142	+	101	306	101223 101528	**integration host factor [Staphylococcus phage MCE-2014]**
143	+	1072	3219	101604 104822	**DNA polymerase [Staphylococcus phage phiIPLA-RODI]** **DNA polymerase A [Staphylococcus phage vB_Sau_CG]**
147	+	418	1257	107051 108307	**DNA repair protein [Staphylococcus phage phiIPLA-RODI]** **DNA repair recombinase [Staphylococcus phage vB_SauM_LM12]**
149	+	220	663	108651 109313	**RNA polymerase sigma factor [Staphylococcus phage vB_Sau_Clo6]**
150	+	210	633	109440 110072	**putative Ig-like protein [Staphylococcus phage phiIPLA-RODI]**
151	+	170	513	110096 110608	**major tail protein [Staphylococcus phage JD007]** **putative bacterial adhesin/Ig-like protein [Staphylococcus virus K]**
152	+	75	228	110623 110850	**putative major tail protein [Staphylococcus phage GH15]** **putative tail morphogenetic protein [Staphylococcus phage phiSA12]**
155	+	416	1251	111957 113207	**putative DNA repair exonuclease [Staphylococcus phage P108]**
156	+	122	369	113221 113589	**putative membrane protein [Staphylococcus phage phiSA12]**
164	+	147	444	118178 118621	**transposase domain-containing protein [Staphylococcus phage Terranova]**
166	+	132	399	119405 119803	**putative membrane protein [Staphylococcus phage GH15]**
170	+	82	249	120959 121207	**putative membrane protein [Staphylococcus phage vB_SauM_LM12]**
172	+	214	645	121514 122158	**ribulose carboxylase/oxygenase [Staphylococcus phage phiIPLA-RODI]** **ribulose 1,5-biphosphate carboxylase/oxygenase small subunit [Staphylococcus phage vB_SauM_LM12]**

174	+	58	177	122434 122610	**DNA sliding clump inhibitor [Staphylococcus phage vB_Sau_Clo6]**
176	+	60	183	122947 123129	**MbpK [Staphylococcus phage A5W]** **membrane protein [Staphylococcus phage pSco-10]**
179	+	95	288	123861 124148	**putative membrane protein [Staphylococcus phage GH15]**
184	+	136	411	125909 126319	**putative membrane protein [Staphylococcus phage vB_SauM_LM12]**
186	+	75	228	126630 126857	**putative membrane protein [Staphylococcus phage vB_SauM_LM12]**
191	+	134	405	128309 128713	**putative membrane protein [Staphylococcus phage vB_SauM_LM12]**
202	+	100	303	132547 132849	**TreA [Staphylococcus phage phiIPLA-RODI]** **TreA [Staphylococcus phage vB_Sau_CG]**
203	+	96	291	132957 133247	**TreC [Staphylococcus phage vB_SauM_LM12]**
204	+	95	288	133247 133534	**TreD [Staphylococcus phage vB_SauM_LM12]**
205	+	97	294	133534 133827	**TreE [Staphylococcus phage vB_Sau_CG]**
206	+	82	249	133831 134079	**TreF [Staphylococcus phage Fi200W]**

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name	: Çotak, Medine
Nationality	: Turkish (TC)
Date and Place of Birth	: 3 January 1986, Afyon
Phone	: +90 5550098603
E-mail	: e157287@metu.edu.tr

EDUCATION

Degree	Institution	Year of Graduation
MS	Bonn University Molecular Biotechnology	2014
BS	METU Molecular Biology and Genetics	2010
High School	Kütahya Science High School, Kütahya	2006

WORK EXPERIENCE

Year	Place	Enrollment
2015-Present	Mikroliz Biotech	Founder R&D

FOREIGN LANGUAGES

Advanced English, Fluent German

PUBLICATIONS

HOBBIES

Gardening, Crime Science Investigation