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**Host-recognition mechanism of phage ϕ MR003 and its
application to controlling of *Staphylococcus aureus***

Doctoral Thesis by PENG Chanthol

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List of abbreviation

aux: auxiliary gene

CA-MRSA: Community-associated methicillin-resistant *Staphylococcus aureus*

CAMHB: Cation adjusted mueller hinton broth

CFU: Colonies forming unit

CLSI: Clinical laboratory standard institute

EOP: Efficiency of plating

fem: factor essential for methicillin resistance gene

GlcNAc: *N*-acetylglucosamine

GroP: Glycerolphosphate

HA-MRSA: Healthcare-associated methicillin-resistant *Staphylococcus aureus*

ICTV: International committee on taxonomy of viruses

LA-MRSA: Livestock-associated methicillin-resistant *Staphylococcus aureus*

ManNAc: *N*-acetylmannosamine

MIC: Minimum inhibitory concentration

MRSA: Methicillin-resistant *Staphylococcus aureus*

OD₆₆₀: Optical density at 660 nm

ORF: Open reading frame

PBPs: Penicillin binding proteins

POT: Phage open reading frame

PVL: Panton-valentine leucocidin

RboP: Ribitol phosphate

RBPs: Receptor binding proteins

SCC*mec*: Staphylococcal cassette chromosome *mec*

TEM: Transmission electron microscopy

VALs: Virion-associated lysin

VAPGHs: Virion-associated peptidoglycan hydrolases

WTA: Wall teichoic acid

1 CHAPTER 1 General Introduction

1.1 An overview of *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive bacterium, appears in a spherical shape and is commonly in clusters similar to a bunch of grapes when observed under the light microscope. The name ‘Staphylococcus’ was derived from Greek, meaning a bunch of grapes (staphyle) and berry (kokkos). While the term ‘aureus’ which is derived from Latin, refers to the color of gold. The diameter of the cells ranges from 0.5 to 1.0 μm . *S. aureus* forms fairly large yellow or white colonies on nutrient rich agar media. The yellow color of the colonies is known by carotenoids produced by the cell (Gnanamani et al. 2017). *S. aureus* causes various infectious diseases such as skin infections, bacteremia, pneumonia, food poisoning and so on. The ability of *S. aureus* to become resistant to multiple classes of antibiotics is a challenge in its infectious disease treatment (Gnanamani et al. 2017). The World Health Organization has declared the accelerated development of antibiotic-resistant bacteria as one of the top ten threats to public health (WHO 2019).

1.2 Methicillin-resistant *Staphylococcus aureus*

S. aureus strains that are resistant to methicillin or oxacillin are called methicillin-resistant *S. aureus* (MRSA). MRSA is a major human pathogen and multi-drug resistant bacterium widely found in healthcare and community settings worldwide (WHO 2014).

Healthcare-associated (HA) MRSA often infects patients in intensive care units or patients who have stayed for a long period in hospitals. HA-MRSA can cause various infections including bloodstream infections, respiratory tract infections, urinary tract infections, and surgical-wounds and device-associated infections (Doulgeraki et al. 2017; Papadopoulos et al. 2018). In Japan, MRSA has been identified at high level (40–70%) in healthcare settings which is critical to infection treatment (Shigemura et al. 2005; Kunishima et al. 2010). HA-MRSA strains such as New York/Japan clone have been dominantly isolated in Japan. However, the number of community-associated (CA) MRSA clones is increasing and the ratio of these clones is more than 50% (Harada et al. 2018).

CA-MRSA causes skin and soft tissue infection in healthy people and those who have had no recent healthcare exposure. CA-MRSA has been reported to be more pathogenic than HA-MRSA due to the production of a cytotoxin known as Pantone-Valentine leukocidin (PVL) (Doulgeraki et al. 2017; Papadopoulos et al. 2018). Approximately 97% of MRSA isolates from

skin and soft tissue infection were identified as USA300 (CA-MRSA), and about 98% harbored staphylococcal cassette chromosome *mec* (SCC*mec*) type IV, with isolates from emergency clinics testing positive for PVL toxin (Moran et al. 2006). MRSA infections that are prevalent in Europe, Africa, and other countries in the Asia-Pacific region are of great concern as well (Lee et al. 2018).

Besides from HA-MRSA and CA-MRSA, the emergence of livestock-associated (LA) MRSA has provoked a great concern in food-producing animals and associated foodstuff. Food and animal are vectors of transmission of the MRSA strains (Leone et al. 2010; Doulgeraki et al. 2017). Many reports have identified the presence of MRSA in different retailed meat products include raw meat and dairy products (milk and cheese) (Kamal et al. 2013; Doulgeraki et al. 2017). In some cases, the MRSA isolates are identified as HA-MRSA or CA-MRSA, indicating that food handlers are likely to be the source of the bacteria. In other surveys, LA-MRSA strains are the primary isolates, indicating an animal source of contamination. LA-MRSA has been isolated from both human and animal infections, as well as from bovine mastitis cases. When SCC*mec* characterization is carried out with MRSA isolates of food origin, SCC*mec* III and IV are identified in isolates recovered from cows' milk and chicken. Foodborne illness causing severe symptoms due to MRSA has been documented, illustrating the potential impact of this pathogen on human health (Doulgeraki et al. 2017).

1.3 Mode of action and resistant mechanism to β -lactam antibiotic in *S. aureus*

The antibiotic-resistant development is a threat to public health. The treatment of staphylococcal infection has been with β -lactam antibiotics. However, as the emergence of MRSA, alternative antibiotics have been used such as vancomycin, daptomycin, and linezolid (Haaber et al. 2017). In order to adapt to the antibiotic attack, *S. aureus* commonly use two different major genetic strategies such as a mutation in a gene which often linked with the antibiotic target and the acquisition of antibiotic-resistant gene through horizontal gene transfer (Foster 2017).

The major targets of antibiotics in staphylococci include the cell envelop, the ribosome and nucleic acids (Foster 2017). The *S. aureus* can develop resistant through horizontal transfer of resistance genes encoded by plasmids, transposons and the SCC*mec* gene (mobile genetic elements) or by mutations in chromosomal genes. The horizontally acquired resistance mechanism can occur via one of the following: enzymatic drug modification and inactivation, enzymatic modification of the drug binding site, drug efflux, bypass mechanisms involving the acquisition of a novel drug-resistant target, and displacement of the drug to protect the target. On

the other hand, the acquisition of resistance by mutation can result from alteration of the drug target that prevents the inhibitor from binding, multidrug resistance efflux pumps and multiple stepwise mutations that alter the structure and composition of the cell wall to reduce drug access to its target (Foster 2017).

1.3.1 Mode of action of β -lactam antibiotics

The β -lactam antibiotics inhibit bacterial growth by interfering with the bacterial cell wall synthesis (Kong et al. 2010). The cell wall is a rigid outer layer that maintains cell integrity and prevents cell lysis from high osmotic pressure. The cell wall is composed of a complex, cross-linked polymer of polysaccharides and peptides known as peptidoglycan (Entenza et al. 2005). The polysaccharides composed of amino sugar *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). A five-amino-acid peptide (L-ala-D-glu-L-lys-D-ala-D-ala) is linked to the NAM sugar (Entenza et al. 2005) (Fig. 1-1a).

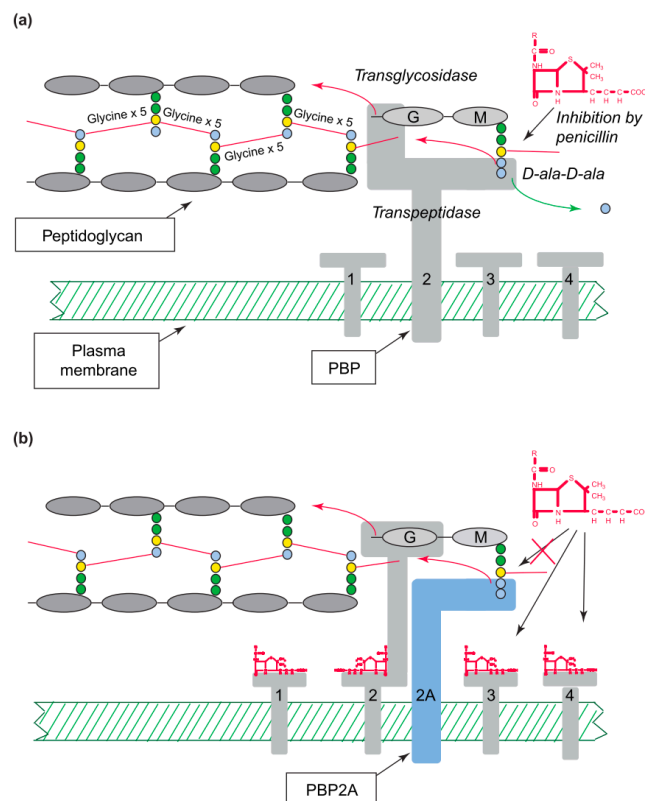


Figure 1-1 Peptidoglycan assembly in *S. aureus* and in MRSA. (a) Cell wall precursors consist of the disaccharide pentapeptides NAG and NAM-L-ala-D-glu-L-lys-D-ala-D-ala. (b) MRSA carry an additional PBP called PBP2A that has a low binding affinity to most β -lactam antibiotics. Colored circles represent the amino acids of the stem peptides. Green circles: L-alanine and D-isoglutamine, respectively; yellow circles: L-lysine; and blue circles: D-alanine; G: NAG; M: NAM (Entenza et al. 2005)

The enzyme called penicillin-binding proteins (PBPs) removes the terminal alanine in the process of forming a cross-link with a nearby peptide. The cross-links help the rigidity of cell wall. Several of these PBPs are bifunctional and retain both a transglycosidase and a transpeptidase activity.

The transglycosidase step links NAG to NAM in the nascent wall, and a transpeptidase step links the penultimate D-ala to a glycine acceptor in the nascent wall. In *S. aureus*, the lysine in position 3 of the stem peptide is almost always decorated with a pentaglycine side-chain (red bars, Fig 1-1). Penicillin is in the same class to oxacillin (β -lactam), inhibits the transpeptidase domain of PBPs. *S. aureus* carries only one bifunctional PBP (PBP2) and three monofunctional transpeptidases (PBP1, 3 and 4) (Fig 1-1a).

β -lactam antibiotics covalently bind to the active site of PBPs. This binding inhibits the transpeptidation reaction and peptidoglycan synthesis, and the cell die. The exact mechanism of cell death is not completely understood, but autolysins are involved in addition to the disruption of cross-linking of the cell wall. β -lactam antibiotics kill actively growing or synthesizing cell wall cell (Entenza et al. 2005; Pinho 2008).

1.3.2 *blaZ* and *mecA* genes as the determinants of β -lactam resistance

The most common resistant mechanism of *S. aureus* to β -lactam antibiotics is mediated by β -lactamase which hydrolyzes β -lactam-susceptible compounds. β -lactamase is encoded in the *blaZ* gene which is usually carried on a plasmid. Moreover, MRSA produces a newly acquired PBP2A encoded by the *mecA* gene (is carried in mobile genetic element, SCC*mec*), which is a wall-building transpeptidase that resists blockage by β -lactam. Under the presence of β -lactam antibiotics, the normal PBPs are blocked but not PBP2A. PBP2A has only a transpeptidase domain, and must 'hijack' the transglycosidase domain of normal PBP2 to be active (Entenza et al. 2005) (Fig 1-1b). PBP2A is absolutely required for high-level β -lactam-resistance in MRSA. Blocking its activity in isolation, as in PBP2A- negative mutants restores susceptibility to β -lactam (Pinho 2008).

Although *mecA* is the principal oxacillin resistance determinant in MRSA, several additional native genes of *S. aureus* also are essential for the full expression of oxacillin resistance in MRSA. Most of these genes called *aux* (auxiliary) or *fem* (factor essential for methicillin resistance). The inactivation of *fem* genes in the presence of *mecA* in MRSA results in strains with a heterogeneous profile of oxacillin resistance. For example, the majority of cells have a low resistant, while a subpopulation appears as highly resistant. The identified *fem* genes are mostly

housekeeping genes that encode putative sensory or regulatory activities, transcription factor, proteins that have a direct or indirect role in peptidoglycan metabolism or structure. The identified *fem* genes and their function in peptidoglycan metabolism include *pbp2*, *fntA*, *vraSR* and so on (Pinho 2008).

1.4 Bacteriophage

The widespread use of antibiotics has generated selective pressures that have driven the emergence of resistant strains, and have consequently limited treatment options for MRSA infections (Enright et al. 2002). Therefore, the use of bacteriophages (phages) has been suggested as an alternative therapy (Lin et al. 2017; Moelling et al. 2018; Azam and Tanji 2019a).

The phage therapy give several advantages over antibiotic: phage can lyse not only the susceptible but also the antibiotic-resistant bacteria; phage has high host specificity so that it does not affect normal microflora; it has antibiofilm activity; if the resistance strain arises, phages mutate alongside the bacteria to effectively infect the resistant strain (Gordillo Altamirano and Barr 2019).

Phages are viruses which can infect and replicate within a bacterial host. The term "phage" refers to "bacterium eater" that was discovered over a century ago. Since their discoveries, phages were used to treat bacterial infections in the human patient. However, after the introduction of antibiotics, penicillin since 1940, the phage studies were collapsed. Nevertheless, phage therapy was not completely abandoned in Eastern Europe such as Georgia, Russia, and Poland, phage therapy steadily investigated (Gordillo Altamirano and Barr 2019).

Phage therapy is the administration of lytic phage directly to a patient in order to lyse the bacterial pathogen that causes a clinical infection (Gordillo Altamirano and Barr 2019). The phage infection pathway involves several steps: phage adsorption on the host cell surface, DNA injection into the host, DNA replication, phage particle assembly, and host lysis (lytic cycle, Fig 1-2). In comparison, the lysogenic cycle is displayed particularly by temperate phages and results in the integration of the prophage (viral genetic material) with the bacterial genome. With this process, viral genetic is ensured to replicate through the division of host without causing any fatal consequence to the host. However, because of the viral genetic material incorporated into the host, the infected host is commonly encountered phenotypic changes such as inducing the pathogenicity of the host (Sharma et al. 2017).

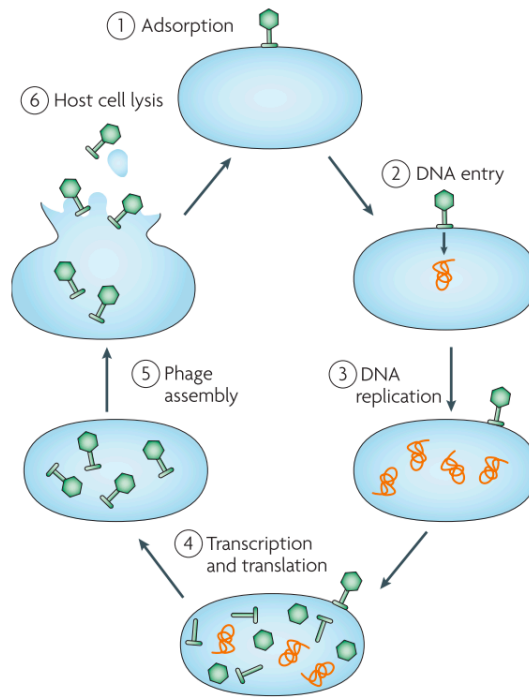


Figure 1-2 Lytic cycle of phage (Sharma et al. 2017)

1.5 Staphylococcal phage

All known *S. aureus* phages belong to order *Caudovirales* (tailed phages) which are composed of an icosahedral capsid filled with double-stranded DNA and a thin filamentous tail (Xia and Wolz 2014). Based on the tail morphology, they can be further clustered into five major families and the three most characterized include *Siphoviridae* (have a long non-contractile tail); *Myoviridae* (have a long, contractile, double-sheathed tail) and *Podoviridae* (have a very short tail) (Fig 1-3). And recently, two more families: *Ackermannviridae* and *Herelleviridae* (ICTV 2019) were added into this order.

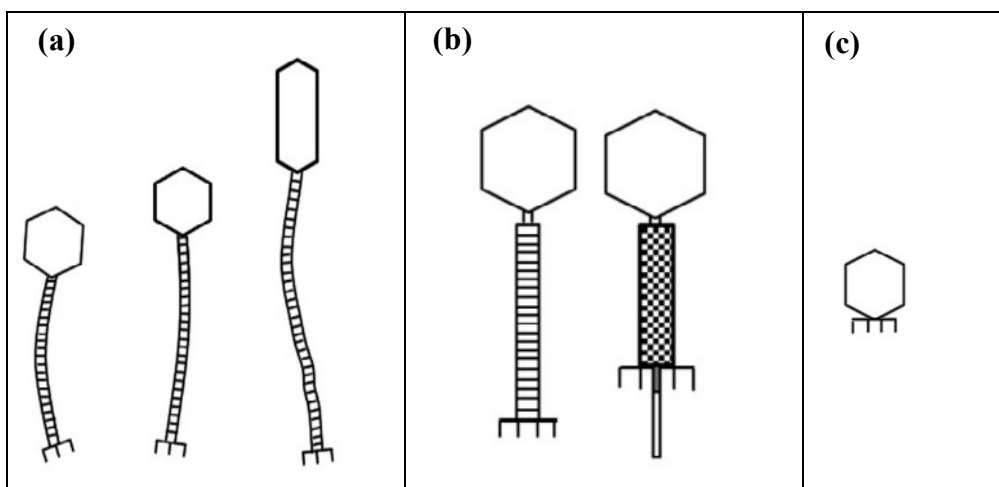


Figure 1-3 The representative phages morphology. Siphoviridae (a), Myoviridae (b), and Podoviridae family (c) (Xia and Wolz 2014)

According to the International Committee on Taxonomy of Viruses (ICTV) (ICTV 2019), a new classification has been updated. The most well-studied *Kayvirus* genus (representative phage K) was updated from *Spounavirinae* subfamily of *Myoviridae* family to *Twortvirinae* subfamily of *Herelleviridae* family. Similarly, *Twortvirus* (representative phage Twort) and *Silviavirus* (representative phage Remus) were also updated into *Herelleviridae* family.

In most *S. aureus* strains, wall teichoic acid (WTA), the most abundant molecule found in the outermost layer of bacterial cell envelopes, is composed of repeated units of ribitol phosphate (RboP) modified by D-alanine and *N*-acetylglucosamine (GlcNAc). The GlcNAc residues are transferred to WTA by the β -GlcNAc transferase TarS and α -GlcNAc transferase TarM (Xia et al. 2010) (Fig. 1-4) (Swoboda et al. 2010; Azam and Tanji 2019b). The WTA attaches to the peptidoglycan by the linker which is synthesized by the initiative of the glycosyltransferase TarO (transferring GlcNAc).

Moreover, WTA serves as the main receptor for most of the *S. aureus* phages (Azam and Tanji 2019a). Members of genus *Kayvirus* such as ϕ SA012 and phage K require backbone of WTA. While ϕ SA039 which is in the same genus requires backbone and β -GlcNAc as its host receptor and recognition site on the *S. aureus* cell wall.

Phage adsorption is mediated by phage receptor binding proteins (RBPs) and receptors on the bacterial cell surface. This primary step is critical for determining the success of phage infection (Silva et al. 2016). Fig 1-5 represents the typical structure of phage and phage DNA insertion into a host cell through the cell wall.

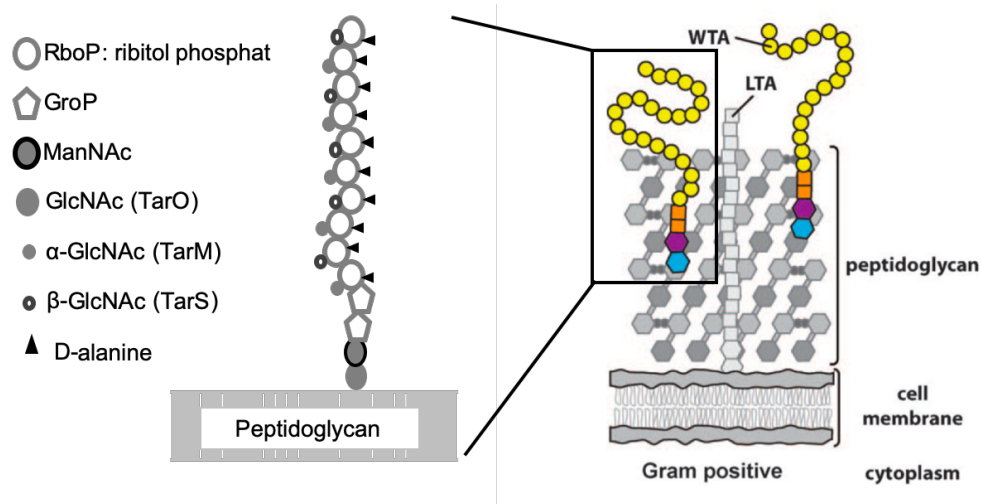


Figure 1-4 WTA structure of *S. aureus* (Swoboda et al. 2010; Azam and Tanji 2019b). Glycerolphosphate (GroP); *N*-acetylmannosamine (ManNAc)

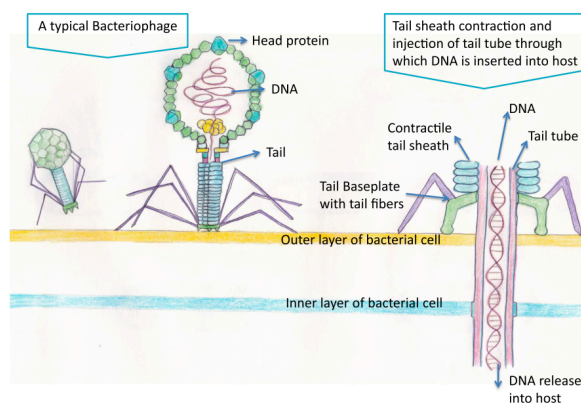


Figure 1-5 Typical structure of a phage emphasizing tail region and contractile ability to insert its DNA into a host (Sharma et al. 2017)

1.6 Enzymes and mechanisms employed by phages to breach the bacterial cell barriers

The initial step of phage infection is the recognition of phage RBPs to the specific host receptor on the host cell surface. This is followed by the phage genome penetration to the site of replication within host cells, the genome assembly to form new virus particles and finally, virion progeny escape from the host cell. Therefore, during an infection cycle, viruses need to overcome the host cell envelope at least twice, first to get inside cells and then to escape from them after virus multiplication (Fernandes and São-José 2018).

Most of the Gram-positive bacteria have lipoteichoic acids anchored to the cytoplasmic membrane via a glycolipid anchor, with a phosphate-rich polymeric moiety extending through the cell wall (Percy and Gründling 2014; Fernandes and São-José 2018). The major structural component of the cell wall is peptidoglycan, also known as murein, which forms a polymeric network surrounding the cytoplasmic membrane (Brown et al. 2013; Fernandes and São-José 2018). In Gram-positive bacteria, the peptidoglycan is also covalently modified with carbohydrate polymers, frequently with anionic WTA (Brown et al. 2013; Fernandes and São-José 2018) (Fig. 1-6).

Some phages synthesize enzymes that attack the bacterial peptidoglycan during the infection process. Those enzymes are known as virion-associated peptidoglycan hydrolases (VAPGHs) or virion-associated lysin (VALs), are phage-encoded lytic enzymes that specifically degrade peptidoglycan. The peptidoglycan-degrading enzymes can be classified into three major groups according to the bond they cleave in the murein network: glycosidases, amidases, and peptidases. Glycosidases cleave one of the two glycosidic bonds in the glycan chain and can be subdivided into *N*-acetyl- β -D-glucosaminidases (glucosaminidases domain), *N*-acetyl- β -D-muramidases

(muramidases or lysozymes) and lytic transglycosylases (Fig. 1-7). The peptidoglycan amidases hydrolyze the amide bond between the first amino acid residue of the peptide stem (generally L-Ala) and NAM, while peptidases cleave within or between the peptide strands. Peptidases are subdivided into carboxypeptidases, which remove C-terminal amino acid residues, and endopeptidases that cleave internal bonds of the peptide (Fig. 1-7).

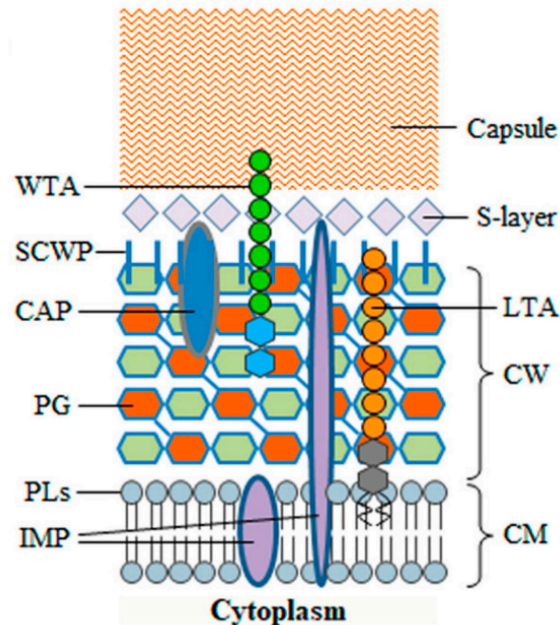


Figure 1-6 Schematic representation of the structure of the bacterial cell envelope in Gram-positive bacteria. SCWP: secondary cell wall polymers; CAP, covalently attached protein; PG: peptidoglycan; PLs: Phospholipids; IMP: inner membrane proteins; CM: cytoplasmic membrane; CW: cell wall; LTA, lipoteichoic acids; The S-layer and capsule are extracellular structures (Fernandes and São-José 2018)

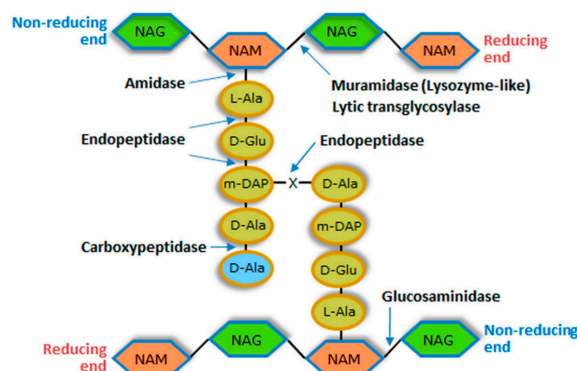


Figure 1-7 Basic structure of the bacterial cell wall peptidoglycan and the cleaving bond of the enzymatic activities of the peptidoglycan degrading enzymes (Pires et al. 2016)

VALs most often correspond to individual components or to domains of tail proteins, like the tape measure protein, central fibers, tail tip knobs, and tail tip puncturing devices, but they can also be capsid inner proteins that are ejected upon virus opening (Pires et al. 2016). An example of virion structure and VAL action is found in *E. coli* phage T4 and its contractile tail. Irreversible binding to host receptors induces tail sheath contraction that cause the inner tail tube with a puncturing device at its tip to penetrate the bacterial cell envelope. One of the proteins composing the piercing apparatus is gp5 that has muralytic activity (Hu et al. 2015).

Moreover, VALs promote a local digestion of the peptidoglycan in order to facilitate penetration or extension of the tail tube to cross the cell wall. Some reported phages which their virus particle are harbored with a VAL (e.g., *E. coli* phage T7, mycobacteriophage TM4 and *S. aureus* phage phi11). The presence of the lytic enzyme in their virion was shown to be important for infection in the laboratory conditions (Moak and Molineux 2000; Piuri and Hatfull 2006; Rodríguez-Rubio et al. 2013; Fernandes and São-José 2018). Importantly, VALs facilitate phages to infect cells which have a thickening and/or increased cross-linking peptidoglycan (e.g., under stationary growth or low temperature) (Moak and Molineux 2000; Piuri and Hatfull 2006; Rodríguez-Rubio et al. 2013; Fernandes and São-José 2018).

1.7 Application of bacteriophage

Due to a crisis of emergence of the antibiotic-resistant pathogen, phage has become attractive as an alternative therapeutic agent to the antibiotic which can be used in various field of application. In biotechnology aspect, phage has been used as a substitute for the antibiotic to control antibiotic-resistant bacteria, as biocontrol agents in agriculture and food safety as well as in aquaculture. Phage also important in medicine and clinical application such as use as a therapeutic agent to treat staphylococcal infection both in vitro and in vivo (Sharma et al. 2017; Azam and Tanji 2019a). In order to obtain a high bactericidal effect, the use of phage cocktails consisting of several phages that bind to different host receptor has been proposed (Synnott et al. 2009). The use of phage cocktail may reduce the inactivation of phage by the immune system and the emergence of phage-resistant bacteria (Azam and Tanji 2019a). Similarly, combined of phage with antibiotic also suggests a synergy effect in controlling bacterial pathogens (Torres-Barcel and Hochberg 2016; Kumaran et al. 2018).

1.8 Aim of this study

Appropriate phage selection is crucial to the success of phage therapy. A wide host range is essential for a phage therapy candidate and a crucial component of phage therapy research. Due

to the limitation of a broad host range phage and the need for phage therapy candidate against clinical MRSA, this study aims to:

1. Isolate phage which has a broad host range against MRSA of human origin
2. Analyze host-recognition mechanism of the newly isolated phage
3. Study the synergistic effects of the isolated phage and antibiotic on controlling of MRSA of human origin.

1.9 Structure of the thesis

Fig. 1-8 shows the structure of the thesis composing of 6 chapters.

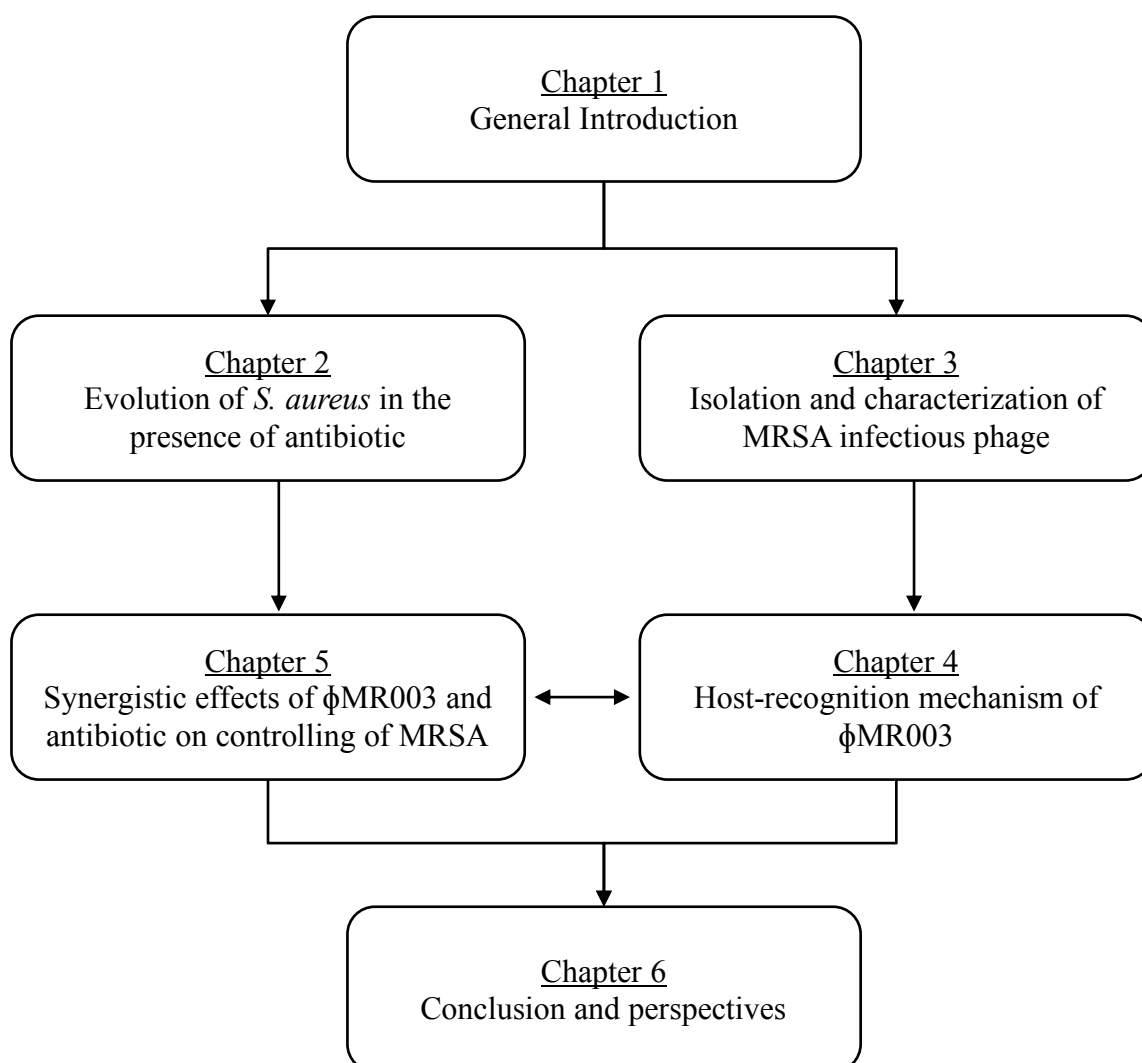


Figure 1-8 The structure of the thesis

CHAPTER 2 Evolution of *S. aureus* in the presence of the antibiotic

2.1 Introduction

Antibiotic-resistant bacteria can evolve through the multiple mutation accumulations under stress environment, such as an antibiotic. Antibiotic-resistant evolution through spontaneous mutation can confer higher resistant to the antibiotic from just a single mutation. However, for most of the antibiotics, multiple mutations are required to develop high levels of resistance (Toprak et al. 2011).

S. aureus can develop resistance through horizontal gene transfer by acquiring a resistant gene from the resistant strain. The resistant mechanism includes β -lactamase production, antibiotic target alteration, efflux pumps, and so on. *S. aureus* also develops resistant through spontaneous mutation (Tang et al. 2014). The detection of the resistant mechanisms and their genetic evolution under antibiotic stress is an important support to antibiotic susceptibility surveillance in *S. aureus*. This chapter aims to trace *S. aureus* evolution under stepwise batch culture with the antibiotic.

2.2 Materials and methods

2.2.1 Bacterial strains, antibiotic and mediums

A drug-sensitive wild type (WT) strain *S. aureus* ATCC6835 was adapted to increased concentration of oxacillin (Wako, Japan). Cation Adjusted Mueller Hinton broth (CAMHB) supplemented with 2% of NaCl was used in the stepwise batch culturing of the *S. aureus* ATCC6835 with oxacillin and Minimum Inhibitory Concentration (MIC) test.

2.2.2 Batch culturing of *S. aureus* ATCC 6538 with increased dose of oxacillin

An overnight culture of *S. aureus* ATCC 6538 was inoculated 1% into L shaped tube in a series of increased concentration of oxacillin (Figure 2-1). The cultivation was conducted in TVS062CA compact rocking incubator (ADVANTEC, Tokyo, Japan) at 35°C with shaking 40 rpm in CAMHB supplemented with 2% of NaCl. Optical density (OD₆₆₀) value of cultivation was automatically recorded in every 15 min at 660 nm wavelength. One culture without oxacillin was used as a control. The cultivation period was conducted until the culture reached stationary phase (needs several days). The culture where bacterial growth was present at the highest concentration was used as serial transfer inoculum into fresh medium for the next concentration challenge. Each

serial transfer with increased concentration challenges was called round. Totally, 17 rounds were conducted.

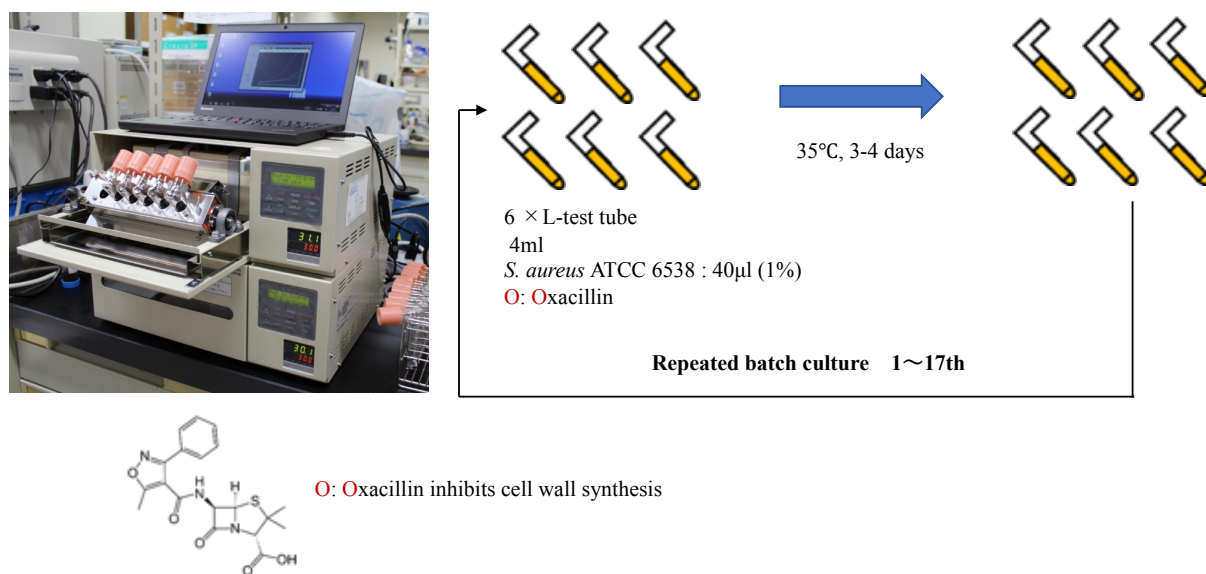


Figure 2-1 Batch culture of *S. aureus* ATCC 6538 with the increased dose of oxacillin

2.2.3 MIC and Cross-resistant test

The MIC and Cross-resistant test were conducted according to Clinical Laboratory Standard Institute (CLSI) protocol with some modification (CLSI 2012). An overnight culture was adjusted to concentration of 10^6 Colonies Forming Unit (CFU) per ml using CAMHB medium supplemented with 2% of NaCl for testing with oxacillin or without supplement of NaCl for testing with vancomycin (Wako, Japan), kanamycin (Wako, Japan), ciprofloxacin (Wako, Japan), and erythromycin (Wako, Japan). All of the antibiotics used belong to different classification. The vancomycin belongs to glycopeptide which targets the precursor of cell wall. The ciprofloxacin belongs to the fluoroquinolones which targets the DNA gyrase. The kanamycin belongs to aminoglycoside which targets the 30S rRNA and the erythromycin that belongs to macrolides targets the 50S rRNA (Bal and Gould 2005). The adjusted overnight cultures were transferred into 96 microwell plates with the pre-two-fold dilution of antibiotic concentration. The final concentration of the culture was in between $2-8 \times 10^4$ CFU/ml in each well. The cultures were incubated for 24h at 35°C. The MIC was determined by the minimum concentration of antibiotic which prevents the visible growth of the bacteria after the incubation.

2.2.4 Whole-genome analysis and bioinformatics

WT strain and its mutant strains which were recovered from the repeated batch culture, were

extracted their genome by using GenElute Bacterial Genomic DNA kits (Sigma, Germany). Next, the genome was submitted for analysis by illumination Miseq at Hokkaido System Science (Japan). The sequence reads of WT were mapped to the reference strain *S. aureus* ATCC 6538 available in the database with the accession number CP020020 by Burrows-Wheeler Aligner (BWA) (Li 2013). Next, the mapped genome sequence of WT was used as a reference for mapping with the mutant strains by BWA and Single Nucleotide Polymorphisms were detected by SAMtools/BCFtools (Li et al. 2009) and Pilon (Walker et al. 2014). Open Reading Frames (ORFs) were predicted and annotated by using the RAST server (<http://rast.nmpdr.org/>).

2.3 Results

2.3.1 Batch culture of *S. aureus* ATCC 6538 with the oxacillin

(a)

Serial transfers	Oxacillin concentration ($\mu\text{g/ml}$)																		
	0.031	0.062	0.125	0.25	0.5	1	2	4	8	16	32	50	100	200	400	800	1600	3200	
1	O	O	O	O	O														
2						Δ	O	Δ	Δ	Δ									
3							O	O											
4								O*	O	O	Δ	Δ							
5									O	O	x	Δ	Δ						
6										O	O	Δ	Δ	x					
7											O	O	Δ	x	x				
8												O	O	Δ	x	x			
9													O	O	Δ	x	x	x	
10														Δ	O*	x	x		
11															O	x	x		
12															O	Δ	x		
13															O	Δ	x		
14															O	x	x		
15															O	x	x		

(b)

Round	Oxacillin concentration ($\mu\text{g/ml}$)																				
	0.031	0.062	0.125	0.25	0.5	1	2	4	8	16	32	50	100	200	400	800	1000	1200	1400	1600	
16																O	O	O			
17																		O	O	Δ *	

Figure 2-2 Summary of the stepwise batch culture of *S. aureus* ATCC 6538 with oxacillin. The concentration of oxacillin was increased from one batch to another with a total of 17 batch cultures. (a) round 1-15; (b) round 16-17; O: $\text{OD}_{660_{660}} > 1$ at stationary phase after incubation; Δ : $\text{OD}_{660} < 1$ at stationary phase after incubation, x: no growth observed. Shade indicates the seed used for the next batch culturing; * indicates the culture where the mutant strains were screen.

Fig. 2-2 shows the total batch culture of *S. aureus* ATCC 6538 with stepwise increasing oxacillin concentration. In round 1, all of the oxacillin concentration allowed the growth of the bacteria even in the concentration higher than oxacillin-MIC (Fig. 2-3, round 1). The MIC of WT is 0.062 $\mu\text{g}/\text{ml}$ of oxacillin. With the increased oxacillin concentration up to 8 $\mu\text{g}/\text{ml}$, the cell was not able to grow well in round 2 (Fig. 2-3).

With the concentration of 2 $\mu\text{g}/\text{ml}$ or lower, the cell could grow but the growth rate was lower than the culture without oxacillin in round 2 (Fig. 2-3). In the 3rd batch, the cell that grew at 2 $\mu\text{g}/\text{ml}$, was used as inoculum with 2 times increased concentration of oxacillin. The cell was able to grow at the concentration that was previously grew and the 2 times increased concentration of oxacillin (4 $\mu\text{g}/\text{ml}$) (Fig. 2-2).

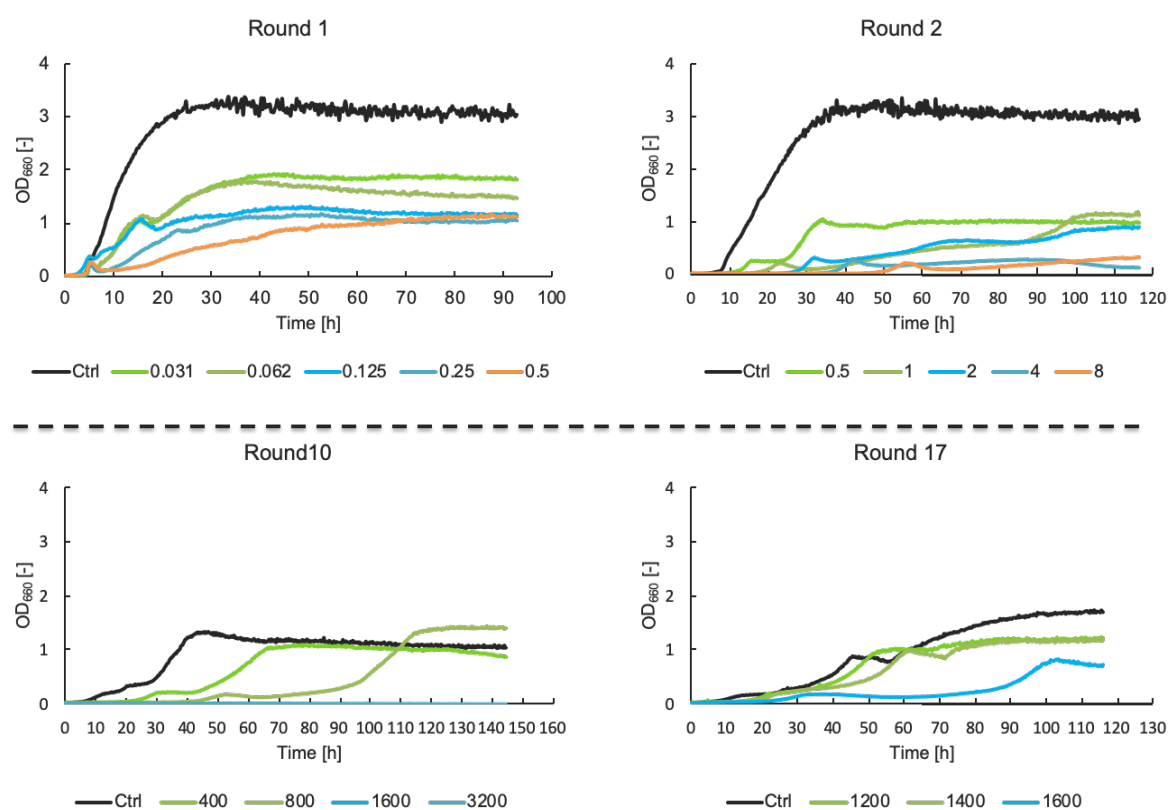


Figure 2-3 Growth curve in batch culture of *S. aureus* ATCC 6530 with the oxacillin. 4 batch cultures were selected as representative. Ctrl: culture without oxacillin. Value of the legend represents the concentration of oxacillin used.

Next, the culture at the highest concentration (4 $\mu\text{g}/\text{ml}$) in which the cell grew, was used as the seed for the following round. With this criterion, the highest concentration which the cell could grow was at 1600 $\mu\text{g}/\text{ml}$ at the 17th round. From round 4 until round 9, the bacteria could grow at 2 times increase of the oxacillin concentration from which they grew. However, until reaching

800 µg/ml (round 10), the cell was not able to grow at concentration higher than 800 µg/ml (Fig. 2-2).

Next, several batch cultures were repeated with the same oxacillin concentration to the round 10 in the presence of oxacillin dose up to 3200 µg/ml. But the bacteria could not grow at this concentration. The oxacillin concentration reduced to 1200 and 1600 µg/ml in the round 16 and 17, respectively. The decreased concentration of oxacillin allowed bacteria to grow.

By repeating 17 times batch cultures, the bacteria cell was able to grow at the highest concentration of 1600 µg/ml. However, the growth rate was slower than the culture with lower concentration (Fig. 2-3) (Round 17).

2.3.2 MIC and cross-resistant of mutant strains

The oxacillin-MIC of *S. aureus* ATCC6538 was 0.062 µg/ml which indicates that the strain is a drug-sensitive. According to CLSI (CLSI 2012), MIC breakpoint of *S. aureus* is determined as resistant when they grow with the a concentration of oxacillin equal to or higher than 4 µg/ml or susceptible with concentration lower or equal to 2 µg/ml. The MIC breakpoint based on CLSI of the antibiotics used are listed in Table 2-1.

The round 4, 10 and 17 which the culture was exposed to 4, 800 and 1600 µg/ml of oxacillin, respectively, were selected to isolate the mutant strains by picking the single colony. The cross-resistant of mutant isolates was conducted. Table 2-1 shows the cross-resistant of the selected mutant isolates to oxacillin and other classes of antibiotics. CLSI-MIC of each tested antibiotic is different. The mutant strains were resistant to oxacillin but not cross-resistant to other antibiotics tested. MIC of C4 and C800 increased up to 100 and 1600 µg/ml of oxacillin, respectively. MIC of C1600 toward oxacillin was not able to determine because of the slow growth of the mutant strain during the long storage period. At the high concentration of MIC test, the mutant strain isolated from 1600 µg/ml was slow growth so that it was difficult for MIC judgment. But considering that this strain was recovered from the culture of 1600 µg/ml of oxacillin, its MIC was considered higher than this concentration.

Table 2-1 MIC of WT ATCC6538 and its mutants to different antibiotics

Antibiotic	CLSI-MIC	MIC			
		WT	C4	C800	C1600
Oxacillin	4	0.062	100	1600	n/a
Vancomycin	16	0.25	0.25	0.25	0.25
Ciprofloxacin	4	2	1	1	1
Kanamycin	64	4	4	1	4
Erythromycin	8	0.125	0.125	0.062	0.062

n/a not available

2.3.3 Whole-genome analysis of mutant strains

Based on whole-genome analysis of mutant strains compared to WT, spontaneous mutations were found conferring resistant to oxacillin (Table 2-2). The mutations which conferred silent mutation was not discussed. The mutations which were outside of ORF were excluded except mutations that were related to a promoter.

Table 2-2 presents the mutations in mutant strains C4, C800 and C1600 compared to WT strain. The fictional protein encoding in mutated genes were search in BLAST search. The strain C4 harbored less mutations than the strain C800 and C1600. Some mutations existed in all of the three strains such as gene *rpoD* encoding RNA polymerase sigma factor; gene *yvgF* encoding cell wall-active antibiotic response protein; gene *tetR/acrR* encoding DNA-binding transcriptional regulator. Mutation in gene *pbp4* encoding penicillin binding protein was found only in strain C4. Several mutations by insertion in gene *tarO* encoding glycosyltransferase responsible for initiation of WTA synthesis, were observed in strain C800 and C1600. Mutation in *rmhC* encoding Ribonuclease HIII that is responsible for DNA replication and repair, was observed in both strain C800 and C1600. While mutation in gene *valS* encoding valine tRNA ligase was found only in strain C1600.

Table 2-3 shows mutation by the deletion in the strain C4, strain C800 and strain C1600. The strain C4 contained less deletion compared to strain C800 and strain C1600. However, strain C800 had more deletion mutation than strain C4 and strain C1600. While in strain C4, the partial deletion was observed just in *lytR* gene. There was no deletion in *fntA* gene was observed in C4. Moreover, strain C800 and C1600 acquired a whole deletion of *lytR* gene and a partial of *fntA* gene. C800 harbored additional deletion in *mutS2* gene.

Table 2-2 Spontaneous mutation in mutant strains

Position	Amino acid		Mutant strains			Gene	Accession number
	WT	ALT	C4	C800	C1600		
663449	TTC (F)	TCC (S)	○	x	x	<i>pbp4</i>	AYU98954.1
1584053	TGT (C)	TAT (Y)	○	○	○	<i>rpoD</i>	WP_064264749.1
1931576	GCA (A)	GAA (E)	○	○	○	<i>yvqF</i>	VFS07056.1
2569725	G	GG	○	○	○	<i>tetR/acrR</i>	WP_070021141.1
772974	.	AACTAC (N) (Y)	x	○	○	<i>tarO</i>	WP-112380577
1098187	.	T	x	○	○	<i>rnhC</i>	WP_079199726.1
1674767	TGG (Y)	TCG (S)	x	x	○	<i>valS</i>	WP_042727698.1

○: present, x: absent, ALT: alternation, underline: insertion

Table 2-3 Deletion in mutant strains

Position in WT (deletion length in bp)	Deletion			Gene	Accession number
	C4	C800	C1600		
1009831-1012173 (2343 bp)	Δ	○	○	<i>lytR</i> ; <i>fntA</i>	WP_031871891.1
1103641-1104552 (912 bp)	x	○	x	<i>mutS2</i>	VDZ34952.1

○: present, x: absent, Δ: partial deletion in *lytR* gene

2.4 Discussions

This chapter describes the possible resistant mechanism of mutant *S. aureus* recovered from the stepwise batch culture of *S. aureus* ATCC 6538 with oxacillin. This study revealed that antibiotic-sensitive *S. aureus* developed into antibiotic-resistant strain through spontaneous mutation during the stepwise batch culturing which is similar to previously reported (Toprak et al. 2011; Schenk and de Visser 2013; Lenhard et al. 2015). The MIC of the mutant strains were higher than the standard MIC breakpoint of being resistant (Table 2-1). Moreover, the mutant strains did not confer resistant to other classes of antibiotic (Table 2-1). The horizontal gene transfer plays a crucial role in distributing antibiotic resistant gene among *S. aureus*. However,

the reported resistant mechanism to all known classes of antibiotics has been linked to genes found within the *S. aureus* chromosome or due to spontaneous mutation resulted from selection pressure (Espedido and Gosbell 2012).

Based on whole genome analysis in mutant strains, various mutations, insertion and deletion were observed (Table 2-2, Table 2-3). In strain C4, mutation in PBP4 which is one of an important enzyme involves in peptidoglycan synthesis was observed (Vollmer et al. 2005). Since oxacillin targets PBPs, in order to escape from the oxacillin, *S. aureus* often mutates in this gene (Foster 2017). The mutation in gene *pbp4* was not found in strain C800 and strain C1600, which implies that mutation is random among strains. Nevertheless, the three mutant strains acquired the same mutation in gene *yvqF* encoding cell wall active antibiotic response protein in *S. aureus*, especially in MRSA. This protein induced MRSA resistant expression to the methicillin, vancomycin, and daptomycin (Boyle-Vavra et al. 2013). Moreover, a protein encoded in YvqF facilitates resistance by playing a necessary regulatory role in the *VraSR* (regulatory system) that is a cell wall stimulant mediator. The *VraS* and *VraR* autoactivate the expression of the *vra* operon and about 46 other unlinked genes in the *vra* regulon, several of which encode known or putative cell wall biosynthesis enzymes that can presumably repair cell wall damage (Boyle-Vavra et al. 2013). Thus, the protein encoded in *yvqF* help to repair the damaged cell wall caused by the antibiotic (Boyle-Vavra et al. 2013). Deletion in gene *fntA* gene was observed in strain C800 and strain C1600. This protein encoded in *fntA* plays an important role in cell wall synthesis and methicillin-resistant in MRSA (Rahman et al. 2016). Deletion of gene *fntA* gives abnormality of cell growth (Rahman et al. 2016). Although the deletion of this gene did not affect antibiotic resistance in the mutant strains (strain C800 and C1600), the slow growth of strain C800 and C1600 were observed.

3 CHAPTER 3 Isolation and characterization of MRSA infectious phage

3.1 Introduction

The emergence of life-threatening MRSA has led to increased interest in the use of bacteriophages as an alternative therapy to antibiotics (WHO 2014). Phage therapy gives several advantages over the antibiotic treatment such as phage infectivity is host specific so that phage cannot kill the other microflora. The possibility of phage to kill its host is high and it is suitable to use on antibiotic allergies patient. Moreover, if phage-resistant strains emerge, the phage mutates to counter adapt with the host (Gordillo Altamirano and Barr 2019).

Phages with wide host ranges that target clinical MRSA strains are limited (Gu et al. 2019). Appropriate phage selection is crucial to the success of phage therapy. A wide host range is essential for a phage therapy candidate and a crucial component of phage therapy research.

In this chapter, I describe the isolation of phage ϕ MR003, which has a broad host range against clinical MRSA. The genome and general features of ϕ MR003 were characterized.

3.2 Materials and methods

3.2.1 Bacterial strains, bacteriophage isolation, and culture conditions

S. aureus strain RN4220 was provided by Prof. Motoyuki Sugai (Hiroshima University, Graduate School of Biomedical & Health Science, Hiroshima, Japan), with the permission of Prof. Richard P. Novick (Skirball Institute of Biomolecular Medicine, NY) and used as a host for phage enrichment. RN4220 is a competent strain used in the laboratory whose parent strain is the clinical isolate, *S. aureus* strain NCTC8325. *S. aureus* strain SA003, previously isolated from bovine mastitic milk (Synnott et al. 2009), was also used. Details of the strains used are listed in Table 3-1.

To determine host range, 104 strains of MRSA clinical isolates were randomly selected from MRSA strains, of which the MIC was more than 4 μ g/ml of oxacillin following to the criteria of the Clinical and Laboratory Standards Institute (CLSI 2012), of Kyorin University Hospital (Tokyo, Japan) in 2015 and 2016. The MRSA strains were classified into HA-MRSA or CA-MRSA according to the SCC*mec* type based on the phage open reading frame (ORF) typing by POT kit (Kanto Chemical Co., Inc., Tokyo) (Suzuki et al. 2009b; Maeda et al. 2012). This kit detects phage-derived ORFs which allows us to understand the genotypes of strains widespread

in specific regions during an outbreak. POT has three scores that indicate different features of MRSA, such as *SCCmec*, prophage, and genomic island (Suzuki et al. 2009b; Suzuki et al. 2009a). Among these, the POT1 score is used to differentiate *SCCmec* types.

Table 3-1 Bacterial strains and phages

Strain name	Description	Reference
<i>S. aureus</i> RN4220	Transformable strain, restriction-deficient (<i>hsdR⁻</i>), <i>rsbU⁻</i> , <i>agr⁻</i>	DSM 26309, DSMZ culture collection, Braunschweig, Germany
<i>S. aureus</i> SA003	<i>S. aureus</i> isolated from bovine mastitic milk	(Synnott et al. 2009)
ϕSA012 and ϕSA039	Isolated from sewage influent in Tokyo possess a broad host range phage against bovine mastitis <i>S. aureus</i>	

Phage was isolated from the influent of a municipal wastewater treatment plant in Tokyo, Japan. The isolation method was previously described (Synnott et al. 2009). Strain RN4220 was used as the primary phage propagation host for newly isolated phage. SA003 was used as the propagation host for previously isolated phages ϕSA012 and ϕSA039 (Synnott et al. 2009) (Table 3-1). Phage purification was conducted by repeated plating and picking of single plaques, followed by plate lysate and the polyethylene glycol (PEG) #6000-NaCl precipitation method (Synnott et al. 2009). Phages ϕSA012 and ϕSA039 were used for comparison to the newly isolated MRSA infectious phage ϕMR003. All bacterial strains and phages were grown in Luria-Bertani (LB) (10 g of NaCl, 10 g of Hipolypepton and 5 g of Yeast extract per liter) medium overnight at 37°C, unless otherwise stated.

3.2.2 Phage host range (spot test)

One hundred and four MRSA isolates were used to test the infectivity of ϕMR003, whose host range was subsequently compared to those of ϕSA012 and ϕSA039. RN4220 was a primary host and used as the control strain for ϕMR003. SA003 was a primary host and used as the control strain for ϕSA012 and ϕSA039. To determine the infectivity of the phages on the bacterial strains, 5 µl of phage lysate at titer 10⁶, 10⁵, and 10⁴ plaque forming units (PFU) was dropped onto an agar plate with *S. aureus* mixed with 0.5% (w/v) top agar and incubated overnight. Infectivity was determined by the turbidity of plaques where the phage lysate was dropped.

3.2.3 Phage morphology

Phage morphology was observed by transmission electron microscopy (TEM). Phage-containing lysate was purified by PEG #6000- NaCl precipitation and CsCl centrifugation using previously described methods with modifications (Synnott et al. 2009). Briefly, 5 μ l of a concentrated phage suspension with a minimum of 10^9 PFU/ml in SM buffer (100 mM NaCl, 8 mM MgSO₄, 50 mM Tris-HCl, and 0.01% (w/v) gelatin, pH 7.5) was spotted on a hydrophilic plastic-carbon-coated copper grid (Nissin EM Corporation, Tokyo, Japan). Phages were allowed for 1 min adsorption. Excess sample was removed by carefully touching the side of the grid with filter paper. Next, 10 μ l of distilled water was spotted on the grid and removed after a short time. Phages were stained by adding 5 μ l of 2% (v/v) uranyl acetate or EM Stainer (Nissin EM Corporation, Tokyo, Japan). After 1 min, the excess stain was removed, and the grid was air-dried for 30 min. The grids were observed with a transmission electron microscope (JEM-1400 Plus, JEOL Ltd, Tokyo, Japan) operating at 80 kV.

3.2.4 Single-step growth

Single-step growth was conducted using a previously described method with some modifications (Osada et al. 2017). Phage was added to the bacterial culture to achieve a multiplicity of infection (MOI) of 0.01 in a total volume of 2 ml, and 10 minutes were allowed for phage adsorption. After adsorption, the culture was washed with LB broth five times on ice to remove free phages. Washed cells with adsorbed phage were incubated again in 2 ml of LB at 37 °C with shaking at 120 rpm. One hundred microliters of culture were removed periodically at 0, 5, 10, 15, 20, 25, 30, 35 and 40 min and used to perform plaque assays against RN4220. The first sampling time after the wash step was defined as time zero. By observing time courses of the number of adsorbed phages plus released phages from lysed host cells, a single-step growth curve was generated. The ten min allowed for phage adsorption was added to the latent period calculation from the single-step growth curve generated and burst size was determined. The phage titer was normalized by the initial phage titer.

3.2.5 DNA extraction, sequencing, and bioinformatics

DNA was extracted from phages as previously reported (Takeuchi et al. 2016). Briefly, the phage genome was extracted from purified phage using a phage DNA isolation kit (Norgen Biotex Corp., Thorold, ON, Canada). Genomes were submitted to BGI (Hongkong) for whole genome sequencing by Illumina HiSeq platform with genome coverage (sequencing depth) of 100-fold

with 100 bp paired-end. Sequencing results were assembled and aligned using Velvet ver. 1.2.10 (Zerbino and Birney 2008). Pairwise sequence alignments (nucleotide) were performed with EMBOSS Stretcher (Myers and Miller 1988; Rice et al. 2000).

3.2.6 Accession number(s)

The genomic sequence of ϕ MR003 was submitted to the DNA Data Bank of Japan (DDBJ) database under the accession number AP019522. The genomes of ϕ SA012, ϕ SA039, and SA003 were available in the GenBank database under accession numbers AB903967, AP018375, and AP018376, respectively.

3.3 Results

3.3.1 Characteristics of selected MRSA isolates

Based on the POT test, all selected clinical MRSA strains from Kyorin University Hospital were classified into SCC*mec* types I, II, and IV based on POT1 score. SCC*mec* types I and II are categorized as HA-MRSA while type IV is categorized as CA-MRSA. POT testing revealed that SCC*mec* type II strains are the New York/Japan strain and six SCC*mec* type IV strains are USA300. Furthermore, other unknown strains harbored SCC*mec* I or IV, showing distinct POT scores revealing different phenotypes.

3.3.2 Isolation of lytic phages and host range

Municipal wastewater was used to screen phages, which resulted in the isolation of various lytic phages against RN4220. As we were attempting to isolate phages with a wide host range, we tested several of the phages we recovered against representative MRSA strains using spot test assay. As a result, we found that one of the phages we had isolated, ϕ MR003, showed high infectivity against all representative MRSA strains, indicating that we had found a promising phage candidate for MRSA treatment. For comparison, previously isolated phages, ϕ SA012 and ϕ SA039 (showed high infectivity against *S. aureus* isolated from bovine mastitic milk) (Synnott et al. 2009), were also tested against MRSA. We used ϕ SA012 and ϕ SA039 to infect MRSA collection because both phages belong to *Kayvirus*, a genus which presents in most commercial phage preparation which has been widely used (Azam and Tanji 2019a).

Phage host range was determined by the spot test assay. Phage solution with different titers of 10^6 , 10^5 , 10^4 PFU were spotted on a bacterial lawn. The infection was determined by the

formation of clear plaques. Based on spot test results, RN4220 showed high sensitivity to ϕ MR003 (clear plaque) but lower sensitivities toward ϕ SA012 and ϕ SA039 (turbid plaque). In contrast, SA003 showed high sensitivity to ϕ SA012 and ϕ SA039 (clear plaque) but is less sensitive to ϕ MR003 (turbid plaque).

Table 3-2 Sensitivity of HA-MRSA and CA-MRSA isolates to ϕ MR003, ϕ SA012, and ϕ SA039 by spot test

Prevalence setting	SCC <i>mec</i>	ϕ MR003	ϕ SA012	ϕ SA039	Number of strains
HA-MRSA	II	+	+	+	34
	I	+	+	-	1
	II	+	+	-	7
	n/a	+	-	+	0
	n/a	-	+	+	0
	I	+	-	-	1
	II	+	-	-	6
	n/a	-	+	-	0
	n/a	-	-	+	0
	n/a	-	-	-	0
	Sub-total		49	42	34
CA-MRSA	IV	+	+	+	23
	IV	+	+	-	9
	n/a	+	-	+	0
	IV	-	+	+	2
	IV	+	-	-	20
	n/a	-	+	-	0
	n/a	-	-	+	0
	IV	-	-	-	1
Sub-total		52	34	25	55
Total		101	76	59	104
RN4220	n/a	+	+/-	+/-	1
SA003	n/a	+/-	+	+	1

(+) sensitive (clear plaque); (+/-): medium (turbid plaque); (-): insensitive (no plaque); (n/a): not available

Table 3-2 shows MRSA strains sensitive to ϕ MR003, ϕ SA012, and ϕ SA039 by spot test. ϕ MR003 showed a broader host range than ϕ SA012 and ϕ SA039. ϕ MR003 infected all HA-MRSA (49 isolates, SCCmec type I and II) and 52 out of 55 of CA-MRSA isolates. Moreover, ϕ MR003 highly infected all USA300 strains, which are life-threatening CA-MRSA strains that emerged in the US. In contrast, ϕ SA012 and ϕ SA039 infected 42 and 34 of HA-MRSA, and 34 and 25 of CA-MRSA, respectively. One other strain of MRSA (MR58) was not sensitive to any of the three phages. Overall, ϕ MR003 showed 97% (101/104) infectivity, while ϕ SA012 and ϕ SA039 showed 73% (76/104) and 57% (59/104) infectivity to MRSA isolates, respectively.

3.3.3 Phage morphology

Based on morphological features as observed by TEM, ϕ MR003 is assigned to the *Herelleviridae* family due to the presence of long contractile tail (Fig. 3-1). This phage has an average head diameter of 93 nm, and tail with a length of 210 nm and a width of 22 nm (Fig. 1b), which is close to Remus (Vandersteegen et al. 2013). Specifically, these features correspond to those of other reported *Herelleviridae* *S. aureus* phages (Klumpp et al. 2010).

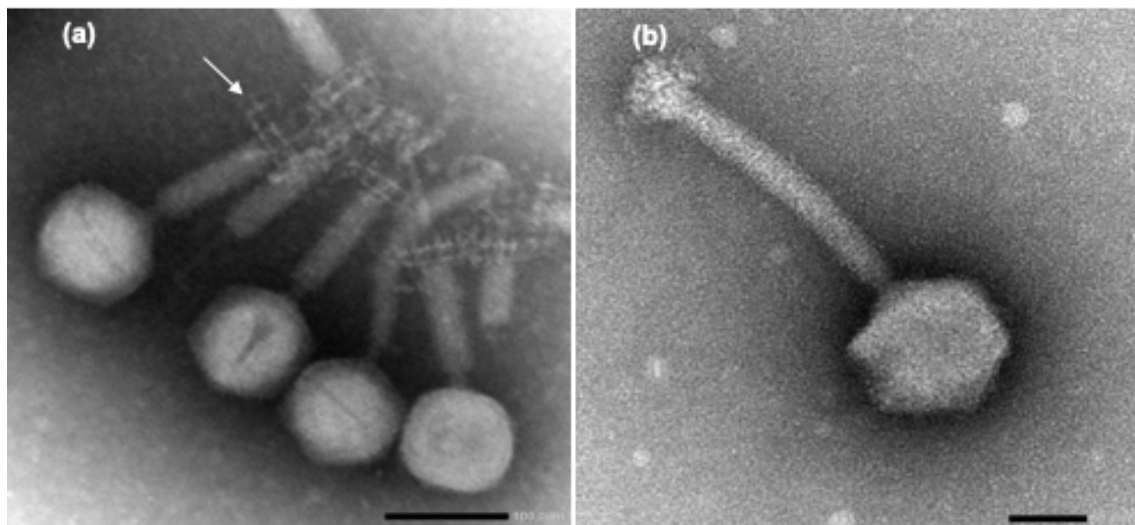


Figure 3-1 Morphology of ϕ MR003. (a) Multiple views of ϕ MR003, the bar indicates 100 nm, the arrow indicates contract tail; (b) single view of ϕ MR003, the bar indicates 50 nm

3.3.4 Single-step growth

Single-step growth was conducted to quantify the phage propagation between ϕ MR003 and RN4220. The latent period of ϕ MR003 was 33 min. The burst size of ϕ MR003 was 35 PFU per cell (Fig. 3-2). This result was comparable to ϕ SA012 against SA003, which yielded an average burst size of 41 PFU per cell (Osada et al. 2017). A *Siphoviridae* family SA97 showed similar

features with a latent period of 20 min and a burst size of 32 PFU per infected RN4220 cell (Chang et al. 2015).

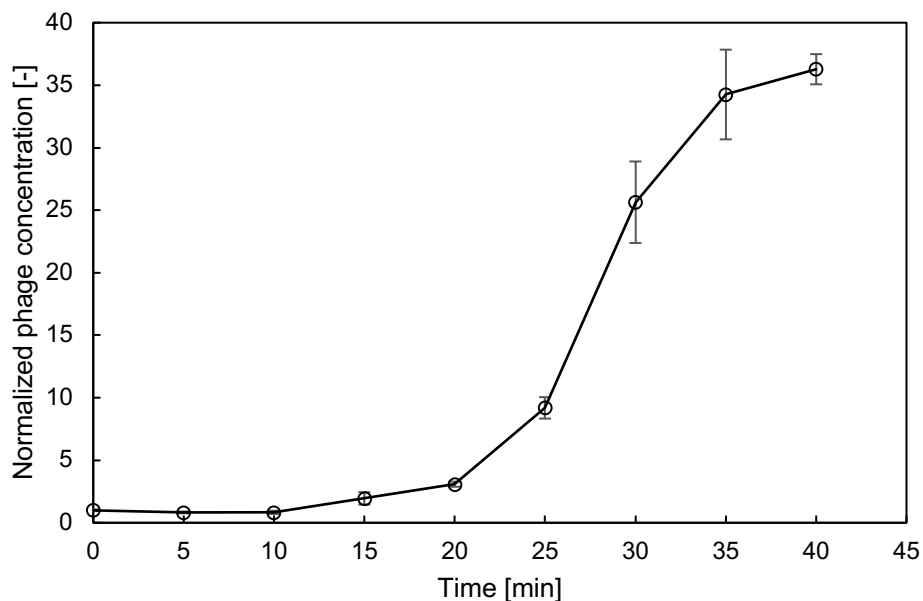


Figure 3-2 Single-step growth of ϕ MR003 with RN4220. Time zero represents the time of first sampling after five washes followed by 10-min adsorption, (n = 3, means \pm SD)

3.3.5 Genome analysis of ϕ MR003

Based on whole genome analysis, ϕ MR003 contains a double-stranded DNA comprising 132,152 bp with 185 putative predicted ORFs and 30% GC content. No tRNA gene was detected in its genome. Antibiotic-resistant genes and virulence genes were not detected, suggesting that this phage is safe for phage therapy.

A comparison of the genome of ϕ MR003 with those of studied *S. aureus* phages, including Remus (JX846612), SA11 (JX194239), ϕ SA012 (AB903967), ϕ SA039 (AP018375), and phage K (KF766114) is presented in Fig. 3-3. The genome organization of ϕ MR003 is similar to that of Remus (Vandersteegen et al. 2013). ϕ MR003 showed 80.2% and 87.1% nucleotide identity to phages Remus and SA11, respectively, in the genus *Silviavirus* (Vandersteegen et al. 2013; Cui et al. 2017). Therefore, ϕ MR003 represents a new species within this genus based on the criteria of less than 95% genome similarity to the phage genome available in the database (Adriaenssens and Rodney Brister 2017). In addition, we compared some genome features of Remus to those of ϕ MR003, especially genes interrupted by self-splicing elements that were reported as distinct from other phages. In the genome of ϕ MR003, the terminase large-subunit gene was fragmented by *orf73* and *orf74* which encoded hypothetical proteins. In Remus, the terminase gene is

fragmented into *orf2* and *orf5* which encode group I introns. Therefore, the introns inserted into the terminase gene of Remus are absent in ϕ MR003. Similar to Remus, *orf82* and *orf85*, which encode portal proteins of ϕ MR003, were fragmented by *orf83* and *orf84*, which encoded transposase. However, the degree of amino acid identity between the transposase of Remus and that of ϕ MR003 was under 59% (Vandersteegen et al. 2013). In addition, the helicase gene of ϕ MR003 was split into two ORFs (ORF120 and ORF121), a quality similar to Remus (ORF49 and ORF50) (Vandersteegen et al. 2013). In Remus, ribonucleotide reductase large-subunit genes (ORF66, ORF68, ORF70) are interrupted by two group I introns (Vandersteegen et al. 2013). On the other hand, in ϕ MR003, these genes were interrupted by one group I intron. ORF148 and ORF150, which encoded the DNA repair protein in ϕ MR003, was fragmented by an intron as observed in Remus (ORF80 and ORF82).

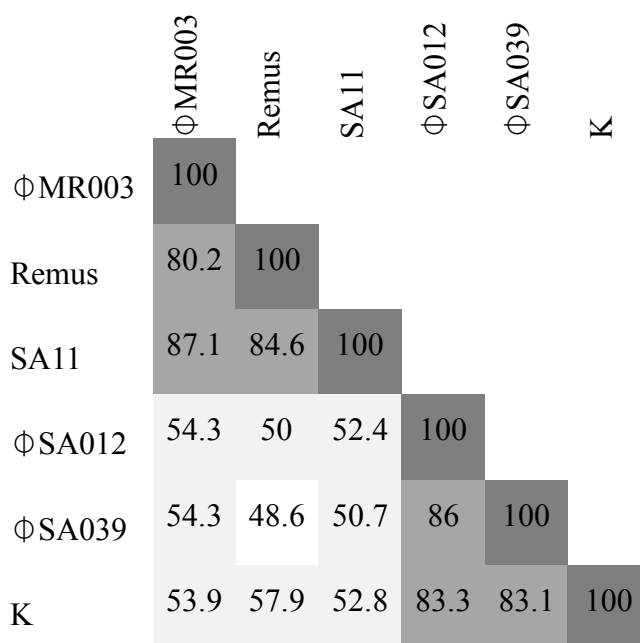


Figure 3-3 Genome comparison between *Silviavirus* (ϕ MR003, Remus, and SA11) and *Kayvirus* (ϕ SA012, ϕ SA039, and phage K)

The genome of ϕ MR003 showed about 54% nucleotide identity to those of phages belonging to the genus *Kayvirus*, namely ϕ SA012, ϕ SA039, and phage K. The Remus genome is very similar to that of SA11, as well as that of ϕ MR003 (Fig. 3-3). The genome of Remus and SA11 show less than 60% nucleotide identity to ϕ SA012, ϕ SA039, and phage K. Moreover, ϕ SA012, ϕ SA039, and phage K are closely related to nucleotide identities exceeding 80%.

3.4 Discussions

This study described the isolation of ϕ MR003 with a broad host range against HA-MRSA and CA-MRSA. Member of *Kayvirus*, including ϕ SA012, ϕ SA039, K, ISP, and ϕ 812, were reported as potential phage candidates for the control of staphylococcal infections (Cui et al. 2017). This study revealed that ϕ MR003 belonged to *Silviavirus*, and showed a broader host range against MRSA than ϕ SA012, and ϕ SA039. ϕ MR003 infected 97% of MRSA strains that originated from humans (i.e. 101 out of 104 MRSA strains of human origin). In contrast, ϕ SA012 and ϕ SA039 infected 73% and 57% of human origin MRSA, respectively. Phage K, ISP, and ϕ 812 were reported to infect 61%, 86%, and 83% of *S. aureus* isolates (including MRSA), respectively. Remus (*Silviavirus*) also infected human and animal origin of 70% of the tested *S. aureus* strains (Vandersteegen et al. 2013). The same representative panel of MRSA strains would be essential to assess their different lytic efficiencies. Nevertheless, ϕ MR003 has a wide host range against MRSA of human origin in Japan, therefore making it a potential phage candidate for controlling MRSA infections.

4 CHAPTER 4 Host-recognition mechanism of phage ϕ MR003

4.1 Introduction

Phages depend on their host for new viral particles production and release inside and outside, respectively. Phage tails are considered as molecular machines that specifically recognize bacterial host cells, penetrate the cell envelope and deliver the phage genome into the cytoplasm (Nobrega et al. 2018). The adsorption of phage onto host cell is the primary step and crucial for phage replication. This process mediated by phage receptor binding proteins (RBPs) and receptors on the bacterial cell surface. The phage recognizes a sensitive host and next positions itself for DNA ejection into the host (Silva et al. 2016). Therefore, this step is critical for not only determining the success of phage infection but also represents the initial point of phage-host contact and the phage's host range specificity (Silva et al. 2016). Despite this usefulness event, the phage-host interaction is poorly characterized.

The outmost layer of gram-positive cell envelopes, such as *S. aureus* consist of WTA that plays as phage receptor for most of the *S. aureus* phages (Xia and Wolz 2014). There are two major types of WTA which are composed of either repetitive 1,3-glycerol-phosphate (GroP) or 1,5-ribitol-phosphate (RboP) and are modified with sugar residues and alanyl groups (Weidenmaier and Peschel 2008). Most of the *S. aureus* strains express RboP substitute with *N*-acetylglucosamine (GlcNac) and D-alanine (Xia et al. 2010; Xia and Wolz 2014).

In the environment, in order to survive, bacteria have to evolve and develop their defense mechanism against constant phage attack. However, unlike antibiotic, phage also has the ability to evolve a counter-adapt mechanism against phage-resistant bacteria. This process is called coevolution, the process that involves the adaptation and counter-adaptation of the two or more species evolve and exert selection pressure on each other. The counter-adaptation of phage against resistant host is an advantage of using phage as a therapeutic agent (Örmälä and Jalasvuori 2013).

Comprehension of the mechanisms involved and the factors affecting phage-host recognition and infection is important for phage-host interaction investigation for phage therapy candidate. This chapter describes host receptors involved in recognition and adsorption of phage ϕ MR003 by adsorption assay and in silico analysis of the whole genome of the phage. Since the counter-adaptation of phage is important to investigate the success of phage therapy, this chapter also aims to investigate such characteristic of ϕ MR003 with its host. I conducted a coevolution experiment of the phage with each host independently by repeated batch culturing for 5 rounds.

Next, the whole-genome of mutant phages and host were analyzed as compared with the WT strains.

4.2 Materials and methods

4.2.1 Bacterial strains and bacteriophages

S. aureus strain RN4220, *S. aureus* strain SA003 (Synnott et al. 2009) and WTA-deficient strains, including SA003 Δ TarS, RN4220 Δ TarM, and RN4220dTO (Table 4-1) (Azam et al. 2018) were used for the adsorption assay. The strains RN4220 Δ TarS and RN4220 Δ TarSTarM (Kurokawa et al. 2013) (Table 4-1) were kindly provided by Prof. Kenji Kurokawa (Nagasaki International University, Nagasaki, Japan).

Phage propagation method was conducted following the method described in section 3.2.1. MRSA strains, MR116 and MR144 were used in the coevolution with ϕ MR003 that was isolated from the wastewater treatment plant. The detail characteristics of this phage are described in chapter 3. ϕ SA012 and ϕ SA039 were also used.

Table 4-1 Bacterial strains and phages

Strain name	Description	Reference
RN4220 Δ TarM	<i>S. aureus</i> RN4220 lacking <i>tarM</i> gene	(Takeuchi et al. 2016)
RN4220 Δ TarS	<i>S. aureus</i> RN4220 lacking <i>tarS</i> gene	(Kurokawa et al.
RN4220 Δ TarSTarM	<i>S. aureus</i> RN4220 lacking <i>tarS</i> gene and <i>tarM</i> gene	2013)
RN4220dTarO	<i>tarO</i> gene in <i>S. aureus</i> RN4220 was disrupted	(Azam et al. 2018)
SA003 Δ TarS	<i>S. aureus</i> SA003 lacking <i>tarS</i> gene	(Azam et al. 2018)
MR116 and MR144	MRSA isolated from patients in Kyorin University Hospital	This study

4.2.2 Efficiency of plating (EOP)

EOP was conducted based on the previously described protocol (Kutter 2009; Hyman and Abedon 2010). 10 μ l of phage solution at titer 10^2 , 10 and 10^{-1} PFU were spotted on the bacterial lawn and incubated for 24 h at 37°C. Next, plaques were counted on each strain and EOP was calculated by dividing PFU counts on each MRSA strains with PFU counts on control host. RN4220 was assigned as control host of ϕ MR003, while SA003 was assigned as control host of ϕ SA012 and ϕ SA039. The EOP was classified as “High” when the ratio was equal or more than 0.5. An EOP was greater than 0.1, but below 0.5 was considered as “Medium” efficiency, and equal or lower than 0.1 was classified as “Low” efficiency (Mirzaei and Nilsson 2015). “No” was assigned when there was no plaque observed.

4.2.3 Adsorption assay

To measure the adsorption of ϕ MR003 onto *S. aureus* strains, we measured free phage present in the supernatant of cell-phage contact (Takeuchi et al. 2016). *S. aureus* cells were prepared by inoculating 10% (v/v) of overnight culture into 4.5 ml of LB and incubate the diluted culture at 37 °C with 120 rpm shaking until the OD₆₆₀ reached 1.0. Phage lysate at 10^7 PFU/ml was added into the bacterial culture. After 25 min of infection at 37 °C and 120 rpm, the bacterial culture was centrifuged at 9730 g for 1 min to collect the free phage and perform a plaque assay using RN4220. Either chloramphenicol or erythromycin was added into the cell culture at a concentration of 50 μ g/ml, with the cell culture equilibrated for 10 min prior to infection in order to inhibit cell growth and phage development during incubation with phage (Baptista et al. 2008). Adsorption efficiency was calculated by dividing the number of adsorbed phages by the initial number of phages. A two-tailed Student’s *t*-test was used to determine statistical significance.

4.2.4 Gene base analysis of WTA in MRSA

Genes *tarO*, which encode a transferase that initiates WTA synthesis, and *tarS* and *tarM*, which are glycosyltransferases of β -GlcNAc and α -GlcNAc on WTA, respectively, were detected. A prophage encoding *tarP*, which has a similar function to *tarS* and modifies the location of β -GlcNAc residue on WTA (Gerlach et al. 2018), was also detected in some MRSA strains. The 23 representative’s MRSA strains were selected for detection of these four genes based on their sensitivity to ϕ MR003, ϕ SA012, and ϕ SA039. RN4220 and SA003, were used as controls. PCR was used to amplify the four genes from genomic DNA. Overnight cultures of MRSA isolates were harvested for DNA extraction using the phenol-chloroform extraction method (Lee 2013; Ung et al. 2018). Primers used to detect the genes are listed in Table 4-2. All PCR programs were performed as following: 95 °C (5 min), 25 cycles of 95 °C (15 s), 53 °C (30 s), 68 °C (70 s for

tarO and *tarM*, 2 min for *tarS*, and 1 min for *tarP*) and 68 °C (5 min). The resulting amplicons were verified by electrophoresis on 1% (w/v) agarose.

Table 4-2 Primers for PCR

Primer	Sequence (5' to 3')	Reference
F_ <i>tarP</i>	ATGAAAAAAGTAAGTGTTATAATGCCAACATTC	(Gerlach et al. 2018)
R_ <i>tarP</i>	CTATAATAGCTTATCTGCAATCATCACAGC	(Gerlach et al. 2018)
F_ <i>tarS</i>	GTTGACGTTTCTGACTTTAGAG	This study
R_ <i>tarS</i>	G GCTTCTATACTTACTTGTCCGC	This study
F_ <i>tarO</i>	AGATTCCAGCGACTATAACAG	This study
R_ <i>tarO</i>	GCTTTAGGCTACACACATAGAC	This study
F_ <i>tarM</i>	AATGGATCGAAGAACGAAAATGT	(Takeuchi et al. 2016)
R_ <i>tarM</i>	CAAATATAAAAAACATTAACATAAGGCGT	(Takeuchi et al. 2016)

F: forward primer; R: reverse primer

4.2.5 In silico genome comparison of ϕ MR003 and ϕ SA012

Phage DNA extraction and sequencing of ϕ MR003 were described in section 3.2.5. ORFs were predicted and annotated by the RAST server (Aziz et al. 2008). The genome of ϕ SA012 was analyzed previously (Takeuchi et al. 2016) with accession number AB903967. Nucleotide and amino acid sequences were scanned for homologs using the Basic Local Alignment Search Tool (BLAST) (Altschup et al. 1990). Genome matcher (Ohtsubo et al. 2008) was used to visualize the comparisons between the two phage's genomes. HHpred (Söding et al. 2005) was used to predict protein function. Pairwise sequence alignments (protein) were performed with EMBOSS Stretcher (Myers and Miller 1988; Rice et al. 2000).

4.2.6 Batch-coculturing of ϕ MR003 and MRSA

The coevolution between MR116 and MR144 and ϕ MR003 was conducted with batch co-culture with the serial transfer. The batch co-culturing was conducted in a compact rocking incubator (TVS062CA, Advantec). Initially, 5 ml of Luria-Bertani (LB) broth in an L-shaped glass test tube was inoculated with 50 μ l of an overnight culture of WT MR116 and incubated

aerobically at 37 °C with shaking at 40 rpm. After 1 h of incubation, approximately 10^7 CFU/ml of bacterial culture was infected with phage at a titer of 10^7 PFU/ml, MOI=1. After confirming that bacterial cells entered stationary phase (approximately 4 days of batch co-culture), 50 μ l of the culture was transferred into 5 ml of fresh LB broth. Using this procedure, batch co-culture was repeated 5 times by serial transfer (Fig. 4-1).

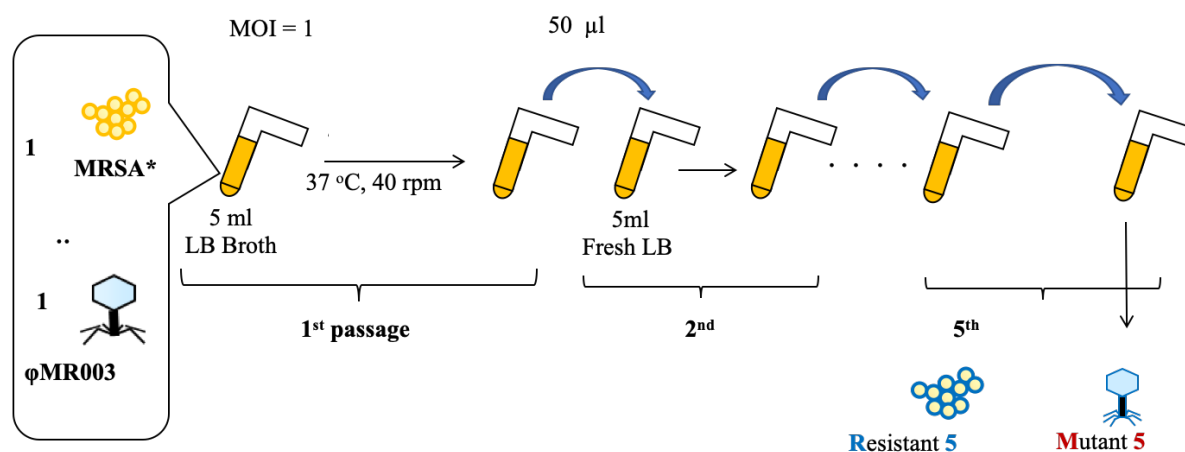


Figure 4-1 Batch co-culturing of MR116 and MR144 with ϕ MR003. 5 passage of co-culturing were conducted. The culture at the final round (5th), was screened for mutant host and mutant phage.

After 5th batch culture, 2 ml of culture was centrifuged at $9730 \times g$ for 5 min at 4 °C. The supernatant was used for phage genome extraction. And pellet was washed several times to remove free phage and then was used to extract the genome. The pellet of culture was used for DNA extraction using GenElute Bacterial Genomic DNA kits (Sigma, Germany). Next, the genome was submitted for their whole genome analysis by illumination Miseq at BGI (Hongkong). The whole-genome of WT host was also extracted from the overnight culture using the same method. The sequence reads were analyzed by MICRA (Caboche et al. 2017). WTA gene cluster of RN4220 (accession number: NC_007795) were searched against annotation mapped sequence generated by MICRA of the MRSA strains. The amino acid sequence alignment was conducted in EMBOSS stretcher (Myers and Miller 1988; Rice et al. 2000). The homolog sequence was searched and compared to a reference sequence by BLAST (Altschup et al. 1990). Phage genome was extracted with the same procedure in section 3.2.5 and sequence reads were assemble by Velvet ver. 1.2.10 (Zerbino and Birney 2008).

4.3 Results

4.3.1 Adsorption assay and EOP of ϕ MR003

The adsorption assay and EOP were conducted to assess the adsorbed ϕ MR003 and their infectivity against *S. aureus* RN4220, its mutants, as well as wild-type *S. aureus* SA003 and its mutant *S. aureus* SA003 Δ TarS (Fig. 4-2a, b). The common structure of WTA of *S. aureus* was presented in Fig. 4-2c. For RN4220, adsorption of ϕ MR003 was (63.2% \pm 10.0%) which was not significant when compared to its mutants, RN Δ TarM with (49.6% \pm 3.65%) adsorbed phage ($p>0.05$); RN Δ TarS with (71.8% \pm 3.6%) adsorbed phage ($p>0.05$); RN Δ TarSTarM with (52.4% \pm 14.6%) adsorbed phage ($p>0.05$) (Fig. 4-2a). The EOP consistently showed high efficiency that implied successful infection of those hosts (Fig. 4-2a). However, adsorption of phage on RN Δ TarO was significantly lower (4.1% \pm 6.6%) compared to RN4220 ($p<0.01$) (Fig. 4-2a). The EOP showed medium efficiency, implying the reduced infectivity compared to RN4220. The adsorption of phage on SA003 was 99.8% \pm 0.1%, which was not significant to SA003 Δ TarS, with adsorption of (90.7% \pm 5.2%) ($p>0.05$). Although the amount of phage adsorbed on SA003 was significantly higher than the amount adsorbed on RN4220 ($p<0.01$), this phage inefficiently infected onto SA003 (Fig. 4-2a). Taken together, the absence of *tarO* affects the absorption of ϕ MR003, but not the deletion of either *tarS* or *tarM*, or of both *tarS* and *tarM*. Nevertheless, I observed medium EOP of ϕ MR003 on RN Δ TarO although there was a significant decrease in the amount of adsorbed phage.

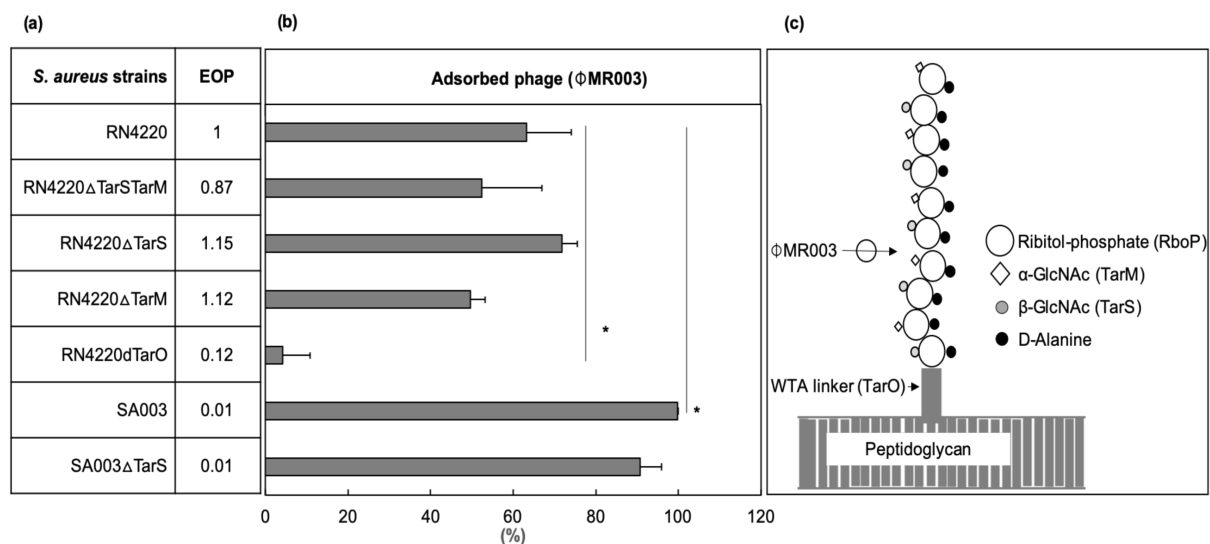


Figure 4-2 (a) EOP, (b) adsorption of ϕ MR003 on *S. aureus* and (c) the common WTA structure of *S. aureus*. Data are presented as means \pm standard deviations (SD, n=3). A two-tailed *t*-test was used to determine statistical significance. The arrow with a circle indicates the infectivity of ϕ MR003

4.3.2 Detection of WTA gene clusters and prophage gene in MRSA

Table 4-3 shows the presence of the four genes in various *S. aureus* strains. All strains harbor *tarO* and *tarS*, with *tarM* being selective amongst strains. On the other hand, *tarP* was detected in two strains out of all sampled strains.

Table 4-3 Presence of *tarO*, *tarS*, *tarM*, and *tarP* of the MRSA strains and EOP

Strain	SCC <i>mec</i>	<i>tarO</i>	<i>tarS</i>	<i>tarM</i>	<i>tarP</i>	EOP		
						ϕMR003	ϕSA012	ϕSA039
RN4220	n/a	○	○	○	x	1.0 (H)	0.1 (L)	< 0.1 (L)
SA003	n/a	○	○	x	x	0.2 (M)	1.0 (H)	1.0 (H)
MR003	II	○	○	x	x	0.7 (H)	0.4 (M)	0.8 (H)
MR005	II	○	○	x	x	1.3 (H)	0.7 (H)	0.7 (H)
MR022	IV	○	○	○	x	0.9 (H)	0.5 (H)	0.6 (H)
MR039	IV	○	○	○	x	1.6 (H)	0.8 (H)	0.8 (H)
MR045	IV	○	○	○	x	1.4 (H)	0.6 (H)	0.7 (H)
MR053	II	○	○	x	x	0.7 (H)	0.4 (M)	0.5 (H)
MR057	II	○	○	x	x	1.8 (H)	0.3 (M)	0.2 (M)
MR102	IV	○	○	○	x	0.6 (H)	0.7 (H)	0.3 (M)
MR116	IV	○	○	○	x	1.2 (H)	0.3 (M)	0.3 (M)
MR035	I	○	○	○	x	0.8 (H)	0.3 (M)	(N)
MR050	I	○	○	○	○	0.8 (H)	0.6 (H)	(N)
MR054	IV	○	○	○	x	1.2 (H)	0.3 (M)	(N)
MR113	II	○	○	x	○	1.3 (H)	0.7 (H)	0.1 (L)
MR121	IV	○	○	○	x	1.4 (H)	0.3 (M)	(N)
MR124	IV	○	○	○	x	0.9 (H)	0.3 (M)	(N)
MR009	IV	○	○	○	x	0.7 (H)	(N)	(N)
MR049	IV	○	○	○	x	0.9 (H)	0.1 (L)	(N)
MR051	II	○	○	x	x	0.5 (H)	0.1 (L)	< 0.1 (L)
MR108	IV	○	○	○	x	0.5 (H)	< 0.1 (L)	(N)
MR144	IV	○	○	○	x	0.9 (H)	0.1 (L)	(N)
MR058	IV	○	○	○	x	(N)	(N)	(N)
MR132	IV	○	○	x	x	(N)	0.5 (H)	0.2 (M)

EOP was determined as a ratio of PFU on MRSA strains versus PFU on control strains. RN4220 was a primary host and used as the control strain for ϕMR003. SA003 was a primary host and used as the control strain for ϕSA012 and ϕSA039. EOP ≥ 0.5 defined as High (H); 0.1

< EOP < 0.5 defined as Medium (M); EOP \leq 0.1 defined as Low (L); No plaque observed (N).
(o) detected; (x) not detected

The presence of *tarO* genes in MRSA strains were not correlate to the infection of the three phages based on EOP. Other genes involved in WTA synthesis between RN4220 and SA003 were compared using their whole genome (genome accession number: NC_007795 and AP018376, respectively) (Nair et al. 2011; Azam et al. 2018). Since WTA is the main receptor for ϕ MR003 adsorption, the key enzyme involved in WTA synthesis of SA003 and RN4220 was hypothesized to be different because of the different EOP of ϕ MR003 to both hosts. The amino acid sequences of key enzymes of WTA synthesis between both hosts were aligned. However, the sequence of *tarO* of RN4220 and SA003 was 100% identical while *tarS* showed 99% identity. *tarM* was absent in SA003, however, the absence of *tarM* did not affect the infection of ϕ MR003 into the host cell.

4.3.3 Putative tail and base plate proteins ϕ MR003

In chapter 3, ϕ SA012 and ϕ MR003 showed different host specificities against MRSA strains. Since RBPs play an important role in host range specificities, I hypothesized that ϕ SA012 and ϕ MR003 possess different RBPs. In this chapter, the putative RBPs of both phages (Fig. 4-3a) were compared.

A previous study described *orf103* and *orf105* of ϕ SA012 as encoding RBPs against *S. aureus* (Takeuchi et al. 2016). The homologous to *orf103* and *orf105* of ϕ SA012 were searched in ϕ MR003 genome by BLAST (Fig. 4-3a). ORF103 of ϕ SA012 contains 640 amino acids, whereas its homolog, ORF117 in ϕ MR003, contains 637 amino acids with about 75% amino acid identity. In addition, 72% of amino acid identity was observed between *orf105* in ϕ SA012 and its homolog *orf119* in ϕ MR003.

Other regions encoding putative tail and baseplate proteins (Fig. 4-3b) between ϕ SA012 and ϕ MR003 were investigated. Most of them showed amino acid similarities under 85%. *orf89* of ϕ SA012 was homologous to a split region (*orf103*, 104, and 105) in ϕ MR003. *orf103* and *orf105* showed a higher similarity of amino acid alignment than that of *orf104* in ϕ MR003 to *orf89* in ϕ SA012 (Fig. 4-3b). *orf89* of ϕ SA012 encoded putative phage lysin which is similar to *orf103* and *orf105* of ϕ MR003. In addition, *orf104* of ϕ MR003 encodes an *N*-acetylglucosaminidase motif based on HHpred (protein motif prediction). Located primarily within the phage tail, this enzyme's function is to hydrolyze peptidoglycan which facilitates phage genome entry into host

cells (Moak and Molineux 2004; Xiang et al. 2008). So, the differences in tail and baseplate protein of ϕ MR003 and ϕ SA012 contribute to their different host specificities.

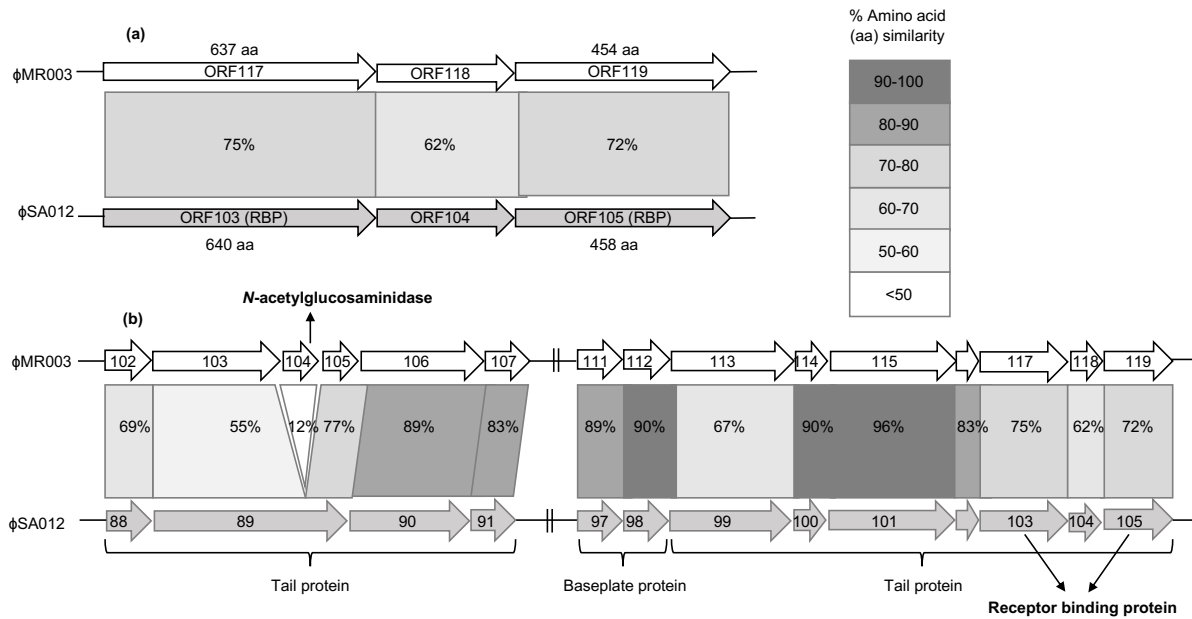


Figure 4-3 (a) Comparison of the RBPs and (b) putative tail and baseplate protein of ϕ MR003 and ϕ SA012

4.3.4 Genomic analysis of mutant phages ϕ MR003

The whole-genome of WT ϕ MR003 was revealed in chapter 3. ϕ MR003 contains a double-stranded DNA comprising 132,152 bp with 185 putative predicted ORFs and 30% GC content. Based on whole genome comparison within phage database and the morphology observation, ϕ MR003 belongs to the genus *Silviavirus* of *Herelleviridae* family.

After five passage of batch culturing of ϕ MR003 with either MR116 or MR144, mutant phage was isolated. The supernatant obtained from the batch culture was used to perform a plaque assay with RN4220. Next, the single plaque was pick and purified followed by DNA extraction and sequencing. The sequence reads of mutant phages were mapped against WT phage so that point mutation was extracted. In mutant phage, ϕ MR003/R5-MR116 (mutant phage isolated from round 5 under coevolution of ϕ MR003 and MR116), mutations were found in 2 ORFs (*orf117* and *orf95*). While the ϕ MR003/R5-MR144 (mutant phage isolated from round 5 under coevolution of ϕ MR003 and MR144), mutations were found in 3 ORFs (*orf117*, *orf114*, and *orf134*) (Table 4-4). The *orf117*, *orf95*, *orf114*, *orf134* are located on the tail of the phage.

Table 4-4 Mutations in mutant ϕ MR003 during coevolution with MRSA strain

ORF (ϕ MR003)	Amino acid alternation	
	ϕ MR003 \rightarrow ϕ MR003/R5-MR116	ϕ MR003 \rightarrow ϕ MR003/R5-MR144
117	GAT (D) \rightarrow AAT (N) GTA (V) \rightarrow GCA (A)	AGA (R) \rightarrow ATA (I) GAA (E) \rightarrow AAA (K) GAT (D) \rightarrow AAT (N)
95	GAT (D) \rightarrow AAT (N)	x
114	x	AAA (K) \rightarrow AAC (N)
134	x	GGT (G) \rightarrow GAT (D)

x: absent

4.3.5 Identification of mutations in WTA gene

Sequence reads of WT and mutant host were analyzed. Genes *tarO*, *tarS*, and *tarM* which are involved in WTA synthesis were compared between WT and mutant host. Gene *tarS* of mutant R5-MR116 acquired 1 mutation. While gene *tarS* in mutant host R5-MR144 acquired 8-point mutations (Table 4-5).

Table 4-5 Point mutation in the mutant host during coevolution

WTA genes	Amino acid alternation between WT and mutant host	
	WT \rightarrow R5-MR116	WT \rightarrow R5-MR144
<i>tarS</i>	GAT (D) \rightarrow AAT (N)	GAT (D) \rightarrow AAT (N)
		ACA (T) \rightarrow GCA (A)
		GAT (D) \rightarrow AAT (N)
		GAA (E) \rightarrow AAA (K)
		ATT (I) \rightarrow GTG (V)
		GAA (E) \rightarrow CAA (Q)
		ATG (M) \rightarrow ATC (I)
		TCG (S) \rightarrow CCA (P)

4.4 Discussions

4.4.1 ϕ MR003 requires WTA for adsorption

The deletion of *tarO* resulted in the deletion of a whole set of WTA from RN4220, leading to a significant decrease of adsorption by ϕ MR003 into the host (Fig. 4-2b). Deletion of glycosyltransferase TarM and TarS resulted in the deletion of α -GlcNAc and β -GlcNAc residues on WTA, respectively, but this modification did not affect the ability of ϕ MR003 to adsorb on to the host (Fig. 4-2b). These results imply that ϕ MR003 requires backbone of WTA as its host receptor which is consistent with most phages of the *Herelleviridae* family, including ϕ SA012 (Azam et al. 2018), phage K, and ϕ 812 (Xia et al. 2011). Nevertheless, ϕ SA039 which is in the same family was recently reported to utilize both backbone and β -GlcNAc residues on WTA as its host receptor (Azam et al. 2018).

WTA is conserved in all of the *S. aureus* strains because it is important for cell division (Winstel et al. 2014). The presence of *tarO* gene was consistently detected, indicating the presence of WTA in all of the selected MRSA (Table 4-3). The presence of WTA on the cell surface of MRSA strains could support ϕ MR003 infection and thus promote the wide host range of this phage. MR132 and MR58 harbor *tarO* (Table 4-3). However, MR132 showed resistance to ϕ MR003 and susceptibility to ϕ SA012 and ϕ SA039, whereas MR58 showed resistance to all three phages. Phage-resistant mechanisms involve the restriction-modification (R-M) system in bacteria (Azam and Tanji 2019b). The Sau3AI restriction enzyme is present in some *S. aureus*, which targets GATC sites in the viral genome. In contrast to phage K, ϕ SA012, and ϕ SA039, the genome of ϕ MR003 has a Sau3AI restriction site (GATC) which may influence the infectivity of the phage against *S. aureus* harboring Sau3AI. The clonal complex of MRSA has a different R-M system which inhibits horizontal gene transfer from one clonal complex to another and overcomes phage attack (Roberts et al. 2013). The R-M system of MR58 may contribute to the insensitivity to ϕ MR003, ϕ SA012, and ϕ SA039. Therefore, whole genome data from MR132 and MR58 would be essential for future studies. Although a high adsorption activity of ϕ MR003 on SA003 was observed, the infectivity of ϕ MR003 against SA003 was low (Fig. 4-2a, b). Based on whole genome analysis in SA003, the R-M system of type I were detected with some of them absent in RN4220. This R-M may influence the infectivity of ϕ MR003 against SA003.

4.4.2 In silico analysis of ϕ MR003 reveals potential viral proteins contributing to wide host range

Although ϕ SA012 infection requires WTA on the host cell surface, this phage shows a narrow host range to MRSA compared to ϕ MR003. The difference in baseplate and tail proteins could be a key factor in the difference in their host specificities. *Orf117* and *orf119* of ϕ MR003 showed identity (75% and 72%) to the homologous region (*orf103* and *orf105*) of ϕ SA012. RBPs (ORF103 and ORF105) of ϕ SA012 recognized different components of WTA for host adsorption.

RBP (ORF103) of ϕ SA012 binds to α -GlcNAc residues of RboP-WTA, whereas, RBP (ORF105) binds to the WTA backbone of *S. aureus* (Takeuchi et al. 2016). The functional protein encoded by *orf119* of ϕ MR003 may also bind to the WTA backbone because the entire region of the protein encoded by this ORF, which includes a PHA01818 domain, is similar to the RBP-encoding *orf105* of ϕ SA012. In contrast, in *orf117* of ϕ MR003, a PHA01818 domain is located in the C-terminus, similar to the RBP-encoding *orf103* of ϕ SA012. The PHA01818 domain is conserved in the hypothetical proteins of staphylococcal lytic phages that belong to the genus *Kayvirus* (Uchiyama et al. 2014b). The function of the PHA01818 domain remains unknown but this domain could be associated with phage adsorption activity (Uchiyama et al. 2014a; Takeuchi et al. 2016). The functional protein encoded by *orf117* of ϕ MR003 possesses a carbohydrate binding domain motif that is similar to phage K (59% amino acid identity). The RBP encoding ORF103 of ϕ SA012 also has a similar carbohydrate binding domain to that of phage K (99% amino acid identity) which recognizes different sites on WTA compared with RBP ORF105 (Takeuchi et al. 2016). Thus, the functional protein encoded by *orf117* could bind to another component on WTA different from that of the functional protein encoded by *orf119*.

According to a previous study, ϕ SA012 is closely related to ϕ SA039, but they have distinct host specificities. ϕ SA039 requires backbone and β -GlcNAc residues of WTA, but ϕ SA012 only requires the backbone of WTA. The baseplate and tail proteins of both phages show a similar level of identity (83–88%) which contributes to the difference in host specificity between them (Takeuchi et al. 2016; Azam et al. 2018). The low amino acid alignment between the RBP-encoding genes of ϕ SA012 (*orf103* and *orf105*) to homologous regions in the ϕ MR003 genome (*orf117* and *orf119*) may contribute to the difference in host specificity.

The *orf104* encodes *N*-acetylglucosaminidase (glucosaminidase domain) presents in the gene encoding phage tail fiber of ϕ MR003 and absent in ϕ SA012, although this ORF had a low identity to C-terminal of ORF89 in ϕ SA012 (12% identity). *N*-acetylglucosaminidase is one of many VALs that hydrolyze glycosidic crosslinks between alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues that make the peptidoglycan lattice of bacterial cell walls. Several types and functions of VALs were reviewed elsewhere (Fernandes and São-José 2018). Each VAL cleaves specific peptidoglycan bonds. During the phage infection cycle, the phage needs to degrade the host cell envelope for entry with the support of the enzymes produced by the phage itself (Fernandes and São-José 2018). Gram-positive bacterial cell walls contain a thick peptidoglycan layer. In *S. aureus*, penicillin-binding protein 4 is a nonessential transpeptidase that is required for high levels of peptidoglycan crosslinking (Loskill et al. 2014). This protein is essential for β -lactam resistance in CA-MRSA. The presence of *N*-acetylglucosaminidase in the

tail of ϕ MR003 may facilitate penetration of the phage genome into *S. aureus* host cell with either normal or high levels of peptidoglycan crosslinking. The C-terminal of gp49 (located in baseplate) of phage ϕ 11 encodes a peptidoglycan degrading enzyme (glucosaminidase domain) that is similar to functional protein encoded in ORF104 of ϕ MR003. The presence of this enzyme in ϕ 11 support host lysis. Deletion of gp49 results in the delay of host lysis (Rodríguez-Rubio et al. 2013).

Moreover, in the disruption of *tarO* in RN4220, although ϕ MR003 showed low adsorption activity, the EOP result showed medium efficacy (slightly infect) (Fig. 4-2). But ϕ SA012 cannot infect RN4220dTarO (Azam et al. 2018). VALs produced by ϕ MR003 encoded in ORF104 may facilitate the infection of ϕ MR003 onto WTA-null RN4220. A depolymerase has also been reported to show the peptidoglycan hydrolysis motif (Pires et al. 2016). Depolymerase is involved in the degradation of biofilms by *S. aureus*, and some strains of MRSA usually appear as biofilms (Paharik and Horswill 2016). Therefore, the difference in the baseplate and tail protein and the presence of *orf104*, which encodes peptidoglycan hydrolase in ϕ MR003, helps promote a wider host range against MRSA compared with ϕ SA012.

4.4.3 Prophage-encoded *tarP* in MRSA confers resistance to ϕ SA039

Although the *tarS* gene, which encodes β -GlcNAc transferase, was detected in all representative MRSA strains, ϕ SA039 did not infect all of them. TarP protein, which was recently reported to be a prophage-encoded protein in MRSA strains, can translocate β -GlcNAc residues in WTA, and inhibit infection by β -GlcNAc targeting phage, e.g., *Podoviridae* phage (Gerlach et al. 2018). In this aspect, the presence of TarP protein in MRSA may confer resistance to ϕ SA039. I detected the *tarP* gene in MR050 and MR113 with no plaque observed and low EOP, respectively (Table 4-3). Although gene *tarP* was not detected in other MRSA strains because of either limited primer specificity or the absence of *tarP*, it may suggest that *tarP* is one of the limiting factors in using β -GlcNAc-targeting phage on MRSA.

The gene of *tarP* was not detected in the ϕ MR003 genome. Thus, the transfer of this gene to MRSA is unlikely to occur, making this phage safer to use for therapeutic purposes. Moreover, since the backbone of WTA is essential for infection by ϕ MR003, I can expect promising results in its use in phage therapy.

4.4.4 ϕ MR003 acquired the spontaneous mutations in RBP during coevolution

In silico analysis of ϕ MR003 and its mutant phages shows that all of the mutations were linked to RBP and genes encoding tail protein. The *orf117* is a putative RBP which is homolog to *orf103* and *orf100* of ϕ SA012 and of ϕ SA039, respectively.

In the previous study, ϕ SA012 and ϕ SA039 harbored 2 RBPs which are responsible for their wide host range against *S. aureus*. ϕ MR003 encodes 2 homologs RBPs (encoded in ORF117 and ORF119) to the RBPs (encoded in ORF103 and ORF105) of ϕ SA012 and to the RBPs (encoded in ORF100 and ORF102) of ϕ SA039. The mechanism of phage-resistant in bacteria involves several factors such as inhibition of phage adsorption by altering the phage receptor. For example, in *S. aureus* SA003 removes a β -GlcNAc residue on the WTA to prevent adsorption of the specific phages ϕ SA039. As a counter-adaptation, phages can adapt to changes in a receptor by acquiring a point mutation in its gene of RBPs (Azam et al. 2018). ϕ SA039 requires backbone of WTA and β -GlcNAc. The removal of the gene encoding β -GlcNAc from WTA inhibit the infectivity of ϕ SA039. Mutation in *orf100* of ϕ SA039 enables this phage to infect SA003 lacking β -GlcNAc (Azam et al. 2018). During the coevolution, mutations in *orf103* of ϕ SA012 inhibit the infectivity of ϕ SA012 on RN4220. While deletion of *tarM* which results in deletion of α -GlcNAc of the WTA, enable infectivity of ϕ SA012 (Takeuchi et al. 2016).

During the coevolution with its host, ϕ MR003 mutated in RBP ORF117 and other genes encoding phage tail proteins. In mutant host, spontaneous mutations were observed in *tarS* gene encoding β -GlcNAc transferase on WTA. So, the alteration of *tarS* is linked to the counter-adaptation of mutant ϕ MR003.

CHAPTER 5 Synergistic effects of ϕ MR003 and antibiotic on control of MRSA

5.1 Introduction

The inability to treat MRSA is a major clinical challenge. This has been caused by the ability of MRSA strains to be tolerance to antibiotic and resistant to multiple antibiotic classes (Kumaran et al. 2018). Phage therapy has been proposed as a promising alternative to antibiotics, but some studies suggested that phage is similar to antibiotic when using alone, it can create a selection pressure for resistant strain emergence either in vitro or in vivo (Osada et al. 2017; Azam et al. 2018; Azam and Tanji 2019b). In this aspect, combined therapy has been proposed to provide better treatment outcome than single therapy (Torres-Barcel and Hochberg 2016).

In this chapter, I describe synergistic effects of ϕ MR003 and antibiotic on controlling of MRSA strain. Different treatment conditions were analyzed including single treatment and combined treatment of the different administration time of ϕ MR003 or antibiotic.

5.2 Materials and methods

5.2.1 Bacterial strains, bacteriophage, and culture conditions

ϕ MR003 infection of representative MRSA strain MR116 was assessed in vitro for 72 h. Briefly, 50 μ l of bacterial overnight culture was inoculated into 5 ml of LB and incubated for 1 h prior to either phage infection or antibiotic addition in a TVS062CA BioPhoto recorder (Advantec, Tokyo, Japan), with the optical density at 660 nm (OD_{660}) measured every 15 min. Under all experimental conditions, the bacterial cell concentration was adjusted to 10^7 CFU/ml, while phage concentration was 10^7 PFU/ml (MOI of 1). Oxacillin (Wako, Japan) which is a common antibiotic used to treat *S. aureus* infection, was used at 5 μ g/ml, either alone or in combination with phage either added simultaneously or in sequence. After incubating the bacterial culture for 1 h, either phage (P) or oxacillin (O) was added. For simultaneous combination treatment, oxacillin and phage were added at the same time at 1 h (M). In sequential combination treatment, oxacillin was added at 1 h followed by phage at 3 h (OP), or else phage added at 1h followed by oxacillin at 3h (PO). Bacterial cultures without phage or antibiotic added were conducted as controls.

Bacterial cell and phage were enumerated at 24 h, 48 h, and 72 h. Bacterial CFU and phage PFU counts were \log_{10} transformed. The interaction of synergy, additive or antagonistic of

combination treatments of phage and antibiotic against *S. aureus* were determined according to the previous study (Kumaran et al. 2018). The interaction was defined as being synergy if the combination treatments resulted in greater bacterial reduction than the sum of the individual treatments. And as being additive if bacterial reductions in combination treatments equal to the sum of the individual effects. While an antagonistic if the bacterial reduction in combination treatments were lower than the sum of individual effects. A two-tailed Student's *t*-test was used to determine statistical significance between bacterial reduction in combination treatments and the sum of the individual phage and antibiotic treatment.

5.3 Results

Growth curves of MR116 in the presence of either ϕ MR003 (P), oxacillin (O), or both ϕ MR003 and oxacillin adding at the same time (M), oxacillin first followed by phage (OP), and phage first followed by oxacillin (PO) are presented in Fig. 5-1. In (O)-treated MR116, although the maximum OD₆₆₀ of MR116 was lower than that of the control culture by almost half, OD₆₆₀ did not decline, which indicated that oxacillin was ineffective against MR116. Unlike (O) treatment, in (P) and (PO) treatment, a decline in OD₆₆₀ due to cell lysis was observed after 3 h. During (M) and (OP) treatment, OD₆₆₀ declined after about 7 h. Under these conditions, prior to bacterial lysis, a high bacterial growth peak was observed. Among all, the peaks observed during (P) and (PO) treatment were the lowest, indicating high bacterial fragmentation. However, at around 18 h of (P) treatment, phage-resistant bacteria appeared (Fig. 5-1). Counteradaptation of host and phage was observed at around 25 h in (OP) and (M) and 35 h in (PO) (Fig. 5-1).

The observation of OD₆₆₀ did not reflect the actual viable cell. So, the viable cell was count. And the synergistic effects were calculated based on the viable cell. (M) and (OP) treatments resulted in synergy interaction (24 h to 72 h) because the bacterial reduction in the combined treatments was greater than the sum of the individual phage and oxacillin ($p < 0.05$) (Fig.5-2). In (PO) treatment, synergy interaction was observed within 24 h to 48 h. But the additive interaction was observed at 72 h because the bacterial reduction was greater yet not significantly different ($p > 0.05$) than the sum of the individual treatment (Kumaran et al. 2018). Viable density in (M) and (PO) increased around 1 to 3 logs, respectively between 24 h to 72 h. However, cell density in (OP) decreased around 2 logs between 24 h to 72 h. Cell density in control, (O) and (P) were decreased around 1 log from 24 h to 48 h and almost stable till 72 h (Fig. 5-2). The phage concentration in single phage and combination treatment were increased in relative to the initial inoculation concentration (Fig. 5-3). However, in (P) and (PO) treatment, phage concentrations were higher than in (M) and (PO) at 24 h. Phage concentration in (P) was decreased around 1 to

2 logs at 48 h and 72 h, respectively. While, in (PO), the titer was decreased around 1 log at 48 h and 72 h. Phage titer in (M), (OP) were relatively stable within the treatment period.

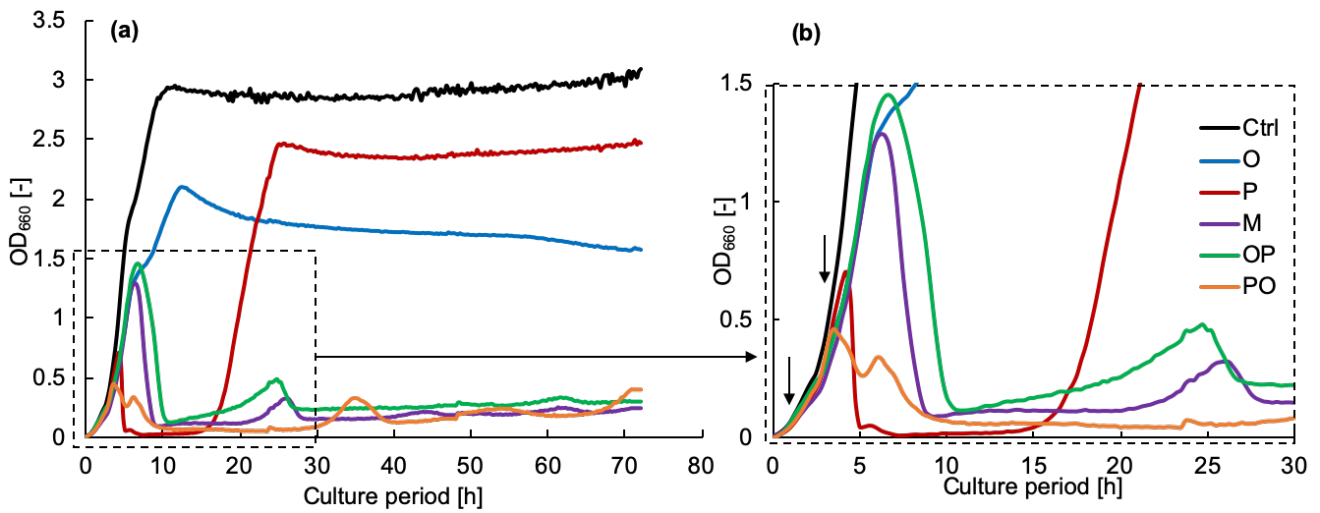


Figure 5-1 Killing curves of MR116 treated with ϕ MR003 and oxacillin. P: single phage treatment; O: 5 μ g/ml oxacillin treatment; M: phage and oxacillin were added simultaneously at 1 h; OP: oxacillin was added after 1 hour followed by phage at 3 h; PO: phage was added after 1 hour followed by oxacillin at 3 h. Arrow indicates the time of ϕ MR003 and/or oxacillin addition.

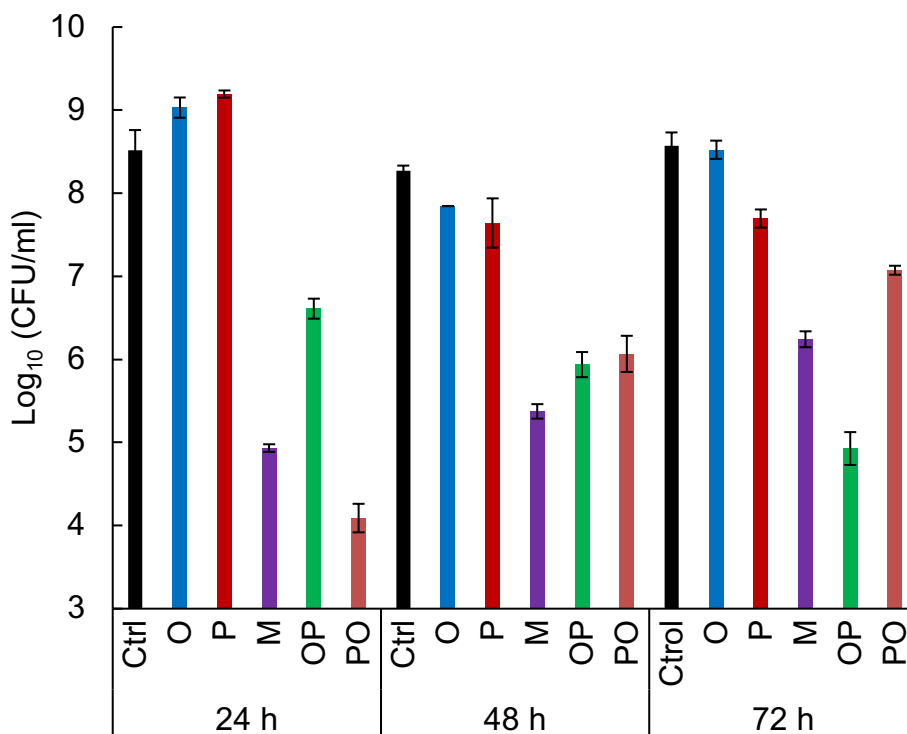


Figure 5-2 Viable count of MR116 in ϕ MR003 and/or oxacillin treatment

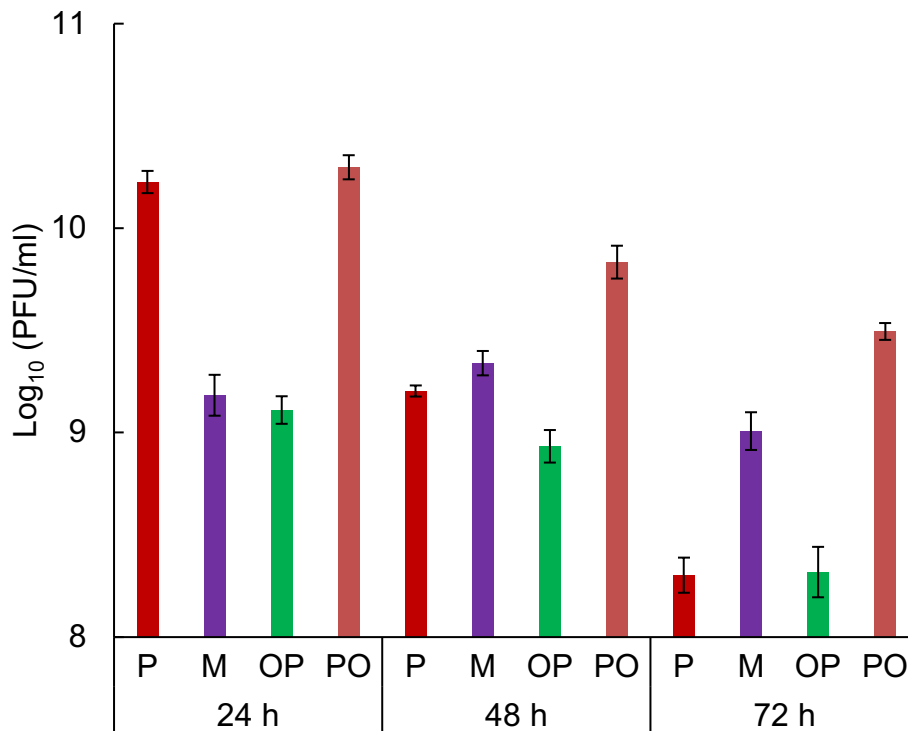


Figure 5-3 Titers of ϕ MR003 in single or combination treatment

5.4 Discussions

In the current study, the combination effects of ϕ MR003 and oxacillin in comparison to single ϕ MR003 or oxacillin treatment in controlling MRSA (MR116) in vitro were assessed. MR116 harbored *SCCmec* IV that conferred resistant to oxacillin. In the present of oxacillin, MR116 paid much cost for growing with OD_{660} max was lower than that of control culture (Fig.5-1). With the dual effect of oxacillin and phage, the synergistic effects were observed in the combination treatment.

In the short treatment at 24 h, (PO) gave better cell reduction because of the rapid lysis of the phage followed by oxacillin treatment (Fig. 5-1, Fig. 5-2). The curve of (M) and (OP) showed relatively similar trend, time of cell lysis and phage host counteradaptation (peak) (Fig. 5-1). However, the addition of oxacillin and phage at the same time (M) gave a greater effect that the addition of phage after oxacillin (OP) for short treatment period (24 h) (Fig. 5-2). Cell continued to grow in relative to a prolonged treatment period in (M). While in (OP), cell decreased (Fig. 5-2). The result implies that synergy interaction was observed in combination treatment yet it depended on the duration of the treatment. Previous studies also observed the synergy interaction of the combination treatments of phage and antibiotics (Chaudhry et al. 2017; Kumaran et al. 2018). I observed that between 10 h to 15 h, all conditions except control and (O), decreased of

OD were observed. The OD decreased implies lower cell concentration. From that point of view, either single phage or combination, the efficacy was observed. However, in the prolonged treatment, it allowed the selection of mutants bacterial so that single phage treatment alone may be not sufficient (Osada et al. 2017).

Nevertheless, our study is limited to in vitro study, therefore further observation in vivo experiment would be essential and may give a different outcome. The success of phage therapy in vivo was reviewed recently (Azam and Tanji 2019a) as well as the combined effects of phage and antibiotics (Tagliaferri et al. 2019).

CHAPTER 6 Conclusion and perspectives

MRSA is one of the biggest current threat to global health. An increasing number of its infections are becoming harder to treat resulting in a high mortality rate. The acquisition of antibiotic-resistant in MRSA is not only through horizontal gene transfer but also by spontaneous mutation. In chapter 2, I found that through stepwise batch culturing of *S. aureus* with oxacillin, the sensitive *S. aureus* strain developed into the resistant strain. Based on in silico analysis between the genome of WT strain and the mutant strains, several point mutations were found such as in PBP4 in strain C4 (*S. aureus* resistant to 4 µg/ml of oxacillin). The PBP4 is one of the key enzymes involves in peptidoglycan crosslink synthesis. Since oxacillin targets PBPs, thus mutation in this gene interfere with the binding of oxacillin, so the strain can survive under oxacillin treatment. I also found a mutation in *ycqF* gene in strain C4, strain C800, and strain C1600. This gene encoding protein plays as a cell wall stimulant active antibiotic response and help in repairing the damaged cell wall caused by the antibiotic action in *S. aureus*.

The therapeutic agent such as phage is a suitable strategy to combat MRSA. Phage which is strictly lytic and possesses a broad host range is an ideal phage therapy candidate. In chapter 3, φMR003 was isolated from influent of a municipal wastewater treatment plant in Tokyo and this phage possesses a broad host range against MRSA of human origin. φMR003 infected 97% of MRSA strains that originated from humans (i.e. 101 out of 104 MRSA strains screened in Kyorin University Hospital). In contrast, *Kayvirus* phages, φSA012 and φSA039 (previously isolated from influent of a wastewater treatment plant and have a broad host range against bovine mastitis *S. aureus*) infected 73% and 57% of human origin MRSA. Based on the whole-genome analysis and comparison to the phage database, φMR003 belongs to the genus *Silviavirus* which has not been studied extensively. So, φMR003 is considered a potential phage therapy candidate for MRSA infections.

φMR003 recognizes and bind to WTA which is the most abundant molecule on the outer layer of *S. aureus* cell wall. In silico comparisons of the genomes of φMR003 and φSA012 revealed that ORF117 and ORF119 of φMR003 are homologs to the RBPs, ORF103 and ORF105 of φSA012, with amino acid similarities of 75% and 72%, respectively. Moreover, the differences in tail and baseplate proteins of both phages were detected which are key contributing factors to the different host specificities of φMR003 and φSA012. In the tail of φMR003 possess a unique gene encoding *N*-acetylglucosaminidase (glucosaminidase domain which hydrolyzes the peptidoglycan) which may facilitate the infection of φMR003 on WTA-null *S. aureus*.

Unlike antibiotic, phage has the ability to counter-adapt toward the mutant host. That is one of the advantages of phage therapy. In chapter 4, I found that during the coevolution between ϕ MR003 and its host, the spontaneous mutation in mutant ϕ MR003 and mutant host were observed. The mutations were observed in *orf117* of ϕ MR003 which encodes the RBP. While in the mutant host, *tarS* gene encoding β -GlcNAc of the WTA. It implies the importance of RBP ORF117 in counteracting with its mutant host during the coevolution.

Fig 6-1 shows the host recognition (adsorptivity) of ϕ MR003, ϕ SA012, and ϕ SA039 on modified RboP type WTA.

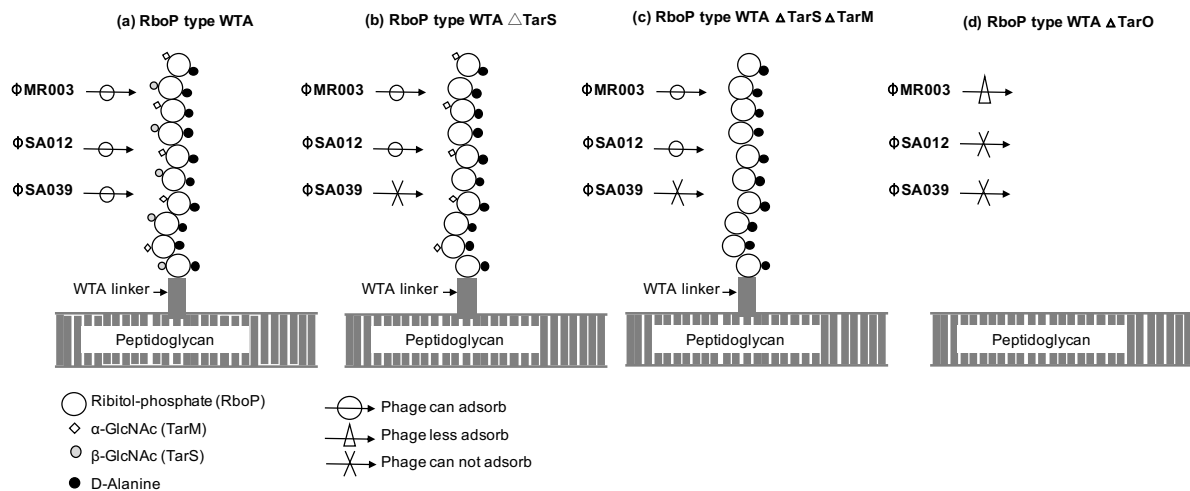


Figure 6-1 The adsorption of ϕ MR003, ϕ SA012, and ϕ SA039 on WTA-deficient *S. aureus*

The drawback of using a single antibiotic or phage is the selection of resistant strains. In order to overcome this, combination treatments suggested being more effective than the single treatment. In chapter 5, ϕ MR003 was combined with oxacillin and found to give a synergistic effect on controlling of MRSA strains in vitro. The synergistic effects were dependent on the period and conditions of the treatment. Therefore, optimization of the combined therapy is crucial before applying it into clinical practice. Moreover, as the future study in vivo of ϕ MR003's therapy on MRSA infection is essential to better understand the potential of ϕ MR003 as a phage therapy candidate.

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2. Ung, P., Peng, C., Yuk, S., Tan, R., Ann, V., Miyanaga, K., Tanji, Y. (2019). Dynamics of bacterial community in Tonle Sap Lake, a large tropical flood-pulse system in Southeast Asia. Science of the Total Environment, 664, 414-423
3. Ung, P., Peng, C., Yuk, S., Ann, V., Mith, H., Tan, R., Miyanaga, K., Tanji, Y. (2018). Fate of *Escherichia coli* in dialysis device exposed into sewage influent and activated sludge. Journal of Water and Health, 16 (3), 380-390