Study on Horizontal Transfer of Drug Resistance in *Staphylococcus aureus*

(黄色ブドウ球菌における薬剤耐性の水平伝達の研究)

2017

筑波大学グローバル教育院

School of the Integrative and Global Majors in University of Tsukuba

PhD Program in Human Biology

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筑波大学

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Abstract

Staphylococcus aureus is recognized as the leading cause of nosocomial infections in the hospitals and healthcare settings. This bacterium can cause a broad spectrum of infection such as skin, soft tissue, respiratory infections and sepsis. Its ability to rapidly develop resistance to antimicrobial compounds by horizontal gene transfer (HGT) has been the global concern. There are three major mechanisms responsible for HGT in bacteria: conjugation, transduction and transformation. Conjugation is responsible for the transfer of DNA from one living cell to another through the direct contact allowing the DNA to be exchanged while being protected by special structure, such as tubes or pores. Conjugation is not widespread in *S. aureus*. Transduction is the process in which DNA is transferred by bacterial virus (bacteriophage), and is considered as the major route to acquire new DNA in *S. aureus*. However, phage transduction has the limitation in the size of DNA to be transferred (< 45 kb). Transformation is the direct uptake of free DNA by the cells that developed the "competence" stage. Natural transformation in *S. aureus* was recently described by our group.

In this thesis, I describe the researches to elucidate the resistance acquisition through the HGT in *S. aureus*. The thesis consists of four chapters. In Chapter 1, general introduction of *S. aureus* with the attention to the major issue of drug resistance was described. Later part in this chapter focused on the HGT and the recent discovery of natural transformation in *S. aureus*.

In Chapter 2, the impact of HGT pathways in the spread of resistance among *S. aureus*, and between *S. aureus* and other staphylococci species was evaluated. In this work, we investigated the dissemination of *cfr* (chloramphenicol/florfenicol resistance) gene, which is responsible for the resistance to linezolid, the emerging problem of resistant *S. aureus*.

In Chapter 3, the investigations to unravel the factor(s) affecting on natural transformation in *S. aureus* are summarized. Here, I found that antibiotics have distinct effects on transformation; some drugs that target on the bacterial cell wall could increase transformation frequencies. Thus, the results raise a caution regarding medical prescription in the treatment of *S. aureus* considering the induction of HGT.

Finally, summary of the studies and future directions of the investigations on natural competence and transformation in *S. aureus* are discussed in the last chapter (Chapter 4).

List of Abbreviations

AIDS: Acquired immune deficiency syndrome

Agr: Accessory gene regulator

BHI: Brain heart infusion

CC: Clonal complex

CDC: US Center for disease control and prevention

CDDEP: The center for disease dynamics, economics & policy

CF: Cefoxitin

Cfr: Chloramphenicol/florfenicol resistance

CFU: Colony forming unit

CoNS: Coagulase negative staphylococci

CS2: Complex synthetic medium 2

FOM: Fosfomycin

GFP: Green fluorescent protein

HGT: Horizontal gene transfer

LB: Lysogeny broth

LRSA: Linezolid-resistance *Staphylococcus aureus*

MGE: Mobile genetic element

MIC: Minimal inhibitory concentration

MLST: Multilocus sequence typing

MRSA: Methicillin-resistant *Staphylococcus aureus*

MSSA: Methicillin-susceptible *Staphylococcus aureus*

NB: Nutrient broth

OD: Optical density

OX: Oxacillin

PBP: Penicillin binding protein

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PFU: Plaque forming unit

PhLOPSA: Phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A antibiotics

SCC*mec*: Staphylococcal cassette chromosome *mec*

ST: Sequence type

TCS: Two-component system

TSA: Tryptic soy agar

TSB: Tryptic soy broth

VRSA: Vancomycin-resistant *Staphylococcus aureus*

VRE: Vancomycin-resistant Enterococci

WHO: World health organization

Chapter 1

General Introduction – Horizontal Gene Transfer in Bacteria.

First evidence of natural transformation in *Staphylococcus aureus*

1.1 *Staphylococcus aureus* – The leading cause of nosocomial infection

Staphylococcus aureus belongs to Firmicutes group of Gram-positive bacteria. Its natural habitats are the skin and nasal cavity of human and warm-blooded animals. This bacterium is known as a commensal as well as opportunistic pathogen. *S. aureus* can cause a broad spectrum of infectious diseases, ranging from food-poisoning and superficial skin abscess to more life-threatening diseases such as bacteremia, osteomyelitis, endocarditis, and toxic shock syndrome. The number of deaths caused by *S. aureus* bacteremia alone is greater than total deaths caused by AIDS, tuberculosis and hepatitis infection [1]. In the most recent worldwide estimation published by the World Health Organization (WHO) in 2014, *S. aureus* is among the top three greatest antimicrobial resistance concerns associated with both hospital-and community-acquired infections [2]

1.2 Drug resistance in *S. aureus*

Treatment of *S. aureus* infection has been difficult due to multidrug-resistance strains. Methicillin-resistant *Staphylococcus aureus* (MRSA) is the commonly isolated antibiotic-resistant pathogen in the hospitals and community [3]. MRSA rates exceed 20 percent in most of the regions and above 80 percent in some regions (WHO Report, 2014). The increase and spread of MRSA and other resistant *S. aureus* strains such as vancomycin resistant *S. aureus* (VRSA) and linezolid resistant *S. aureus* (LRSA) with high burden in mortality and morbidity are major concerns of staphylococcal infection.

S. aureus has an extraordinary ability to rapidly develop resistance (Figure 1-1). It has acquired resistance to almost all antibiotics that have been developed, ranging from penicillin which was introduced in the early 1940s to the more recent drugs such as linezolid, daptomycin, and ceftaroline (the fifth-generation of cephalosporin) [4], [5], [6], [7].

1.3. Mechanisms of drug resistance in *S. aureus*

Mechanisms of resistance to major antibiotics used for *S. aureus* treatments are summarized in Table 1-1. Resistance mechanisms in *S. aureus* include enzymatic inactivation of antibiotics (β-lactamase [8], aminoglycoside-modifying enzymes [9]), alteration of the target to reduce the affinity to the drugs (penicillin-binding protein PBP2a [10], D-Ala-D-Lac [11]), trapping the antibiotics (vancomycin intermediate resistance [12]), efflux pumps (tetracycline [13], fluoroquinolone [14], and so on [15], [16]. These mechanisms are associated with either intrinsic way by modifying the bacterial genes or extrinsic way through the acquisition of genes responsible for the resistance. The later route is predominant and plays the major role in the resistance evolution in *S. aureus* (Table 1-1). Indeed, genome sequence studies revealed that *S. aureus* carries many resistance and virulence genes located in mobile genetic elements (MGEs) including plasmids, transposons, insertion sequences, bacteriophages, pathogenicity islands and staphylococcal cassette chromosomes (SCC) [17]. The presence of these elements in the genome of *S. aureus* indicates that the acquisition and transfer of genetic materials play an important role for the adaptation and evolution of *S. aureus*.

1.4. Horizontal gene transfer in bacteria and *S. aureus*

Transfer of genetic materials between organisms is known as horizontal gene transfer (HGT) or lateral gene transfer, which is distinct from the transfer between parent-offspring relationship, the vertical gene transfer. There are three major HGT mechanisms in bacteria: conjugation, transduction and transformation [18, 19] (Figure 1-2).

Conjugation is a process involving the transmission of DNA from one living cell to another. Both cells must be in direct contact, allowing the DNA to be protected by special structures such as tubes or pores [20]. Conjugation occurs in *S. aureus*, but it requires a

series of *tra* genes or conjugative plasmids, which are not widespread among *S. aureus* strains [21], [22], [23].

Phage transduction is the transfer of DNA through the bacteriophage (phage). Transduction is though to be more predominant, since most of *S. aureus* isolates are lysogenized by bacteriophages [24]. Phage transduction in *S. aureus*, however, is limited to transfer of small DNA fragments (less than 45 kb) due to the size of phage capsid. Also, *S. aureus* phages are particularly host specific [25].

Transformation is a process to actively acquire free DNA in the environment by the bacterial cell. To uptake extracellular DNA, bacterial cell needs to develop a special physiological phase: the competence stage. When the stage is reached, competent cells are capable of transporting DNA into the cytoplasm, acquiring new genetic traits. It had long been the question whether *S. aureus* was able to develop competence for transformation. A part of my previous study contributed to demonstrate the natural transformation in *S. aureus* for the first time [26].

Studies of transduction and conjugation have long history and useful review articles are available [24], [27], [21]. On the other hand, natural transformation was recently found, and the regulation of the competence development is distinct from other species. What was known for the natural genetic transformation in *S. aureus* is summarized in the following section.

1.5. Natural competence and transformation in *S. aureus*

Only small number of transformable species has been reported (approximately 0.02% among bacterial species) [28]. Unlike conjugation and transduction, transformation is entirely directed by the recipient bacterial cell. The cell develops the competence stage in which a series of membrane-associated and cytoplasmic proteins so called competence machinery are activated, allowing the uptake of foreign DNA into the cell. Studies in the

most well known transformable bacteria such as *Streptococcus pneumoniae, Bacillus subtilis* (Gram-positive bacteria) or *Helicobacter pylori, Hemophilus influenza, Neisseria* spp. (Gram-negative bacteria) have shown that these species share the conserved competence machinery for DNA uptake and processing [28]. However, the cues and regulation of competence for transformation are different among species [28]. The information obtained from previous studies also suggests the activation of competence could provide the benefit to the needs of each bacterium [29].

Pseudo-competence in *S. aureus* was reported a few decades ago [30], but it requires phage tail [31], and was not the true competence [26]. However, *S. aureus* possesses the competence gene orthologues that share among transformable Gram-positive bacteria such as *B. subtilis* and *S. pneumoniae* [26]. The expressions of competence genes in *S. aureus* are induced by SigH [32]. SigH is one of the alternative sigma factors in *S. aureus*. It associates with the core RNA polymerase, and renders the resultant holoenzyme the ability to recognize the promoter sequence and initiate the transcription of competence genes. Overexpression of SigH induced the expression of *comG* operon (*comGA, comGB, comGC, comGD, com GE, comGF*) and *comE* operon *(comEA, comEB, comEC)* encoding for DNA binding, transport and uptake machinery [32], [26], and other competence genes (*dprA, coiA*) required for DNA recombination [33].

The expression of SigH is in the minor population and undetectable during growth under standard laboratory growth conditions [26]. At least two distinct mechanisms regulating the expression of SigH was clarified. One is the spontaneous duplication of *sigH* locus that occurs at a low frequency ($\leq 10^{-5}$) generating the chimeric *sigH*. The other involves the post-transcriptional regulation through an inverted repeat sequence upstream of *sigH* coding sequence, which suppresses the expression of SigH [26]. The limited SigH

expression could explain the difficulties to detect transformation through competence in *S. aureus*.

The artificial overexpression of SigH does not make *S. aureus* transformable in normal growth conditions. The growth in a complete synthetic medium (CS2 medium) facilitates the transformation with the frequencies up to $10^{-8} \sim 10^{-9}$ when purified plasmids were used as donor DNAs and about $10^{-7} \sim 10^{-9}$ in the co-culture with the living donor cells [26]. Importantly, we could detect the transformation of the entire 53 kb type II Staphylococcal Cassette Chromosome *mec* (SCC*mec*) conferring the resistance to methicillin (Figure 1-3).

In this thesis, the impact of transformation together with other HGT pathways in the spread of the emerging linezolid resistance in *S. aureus* was examined (Chapter 2). Furthermore, the factors affecting on transformation, here the effects of antibiotics, were examined to get insights into the regulation of competence and transformation in *S. aureus*. Summary of the studies and future directions on natural competence and transformation in *S. aureus* are discussed in the last chapter (Chapter 4).

1. 6. Tables and Figures

Table 1-1. Mechanisms of resistance to major antibiotics in *S. aureus*

Antibiotic class	Target	Resistance gene	Gene product	Mechanism of resistance	MGE	Reference
Fluoroquinolones (ciprofloxacin, norfloxacin, levofloxacin, moxifloxacin)	DNA replication	*gyrA, gyrB* *grlA, grlB* *norA*	GyrA, GyrB GrlA, GrlB NorA, efflux pump	Mutations on drug target – decreased binding Active efflux		[34] [35] [14]
Lipopeptides (daptomycin)	Cell membrane	*mprF* *yycG* *rpoB, rpoC*	Phosphatidylglycerol lysyltransferase Two-component sensor kinase WalK. RNA polymerase	Gene mutation. Change in cell membrane charge – decreased drug binding		[36]
Oxazolidinones (linezolid)	Translation	*rrn* *cfr*	Ribosomal RNA Methyltransferase	Mutation of ribosome. Reduce the susceptibility Methylation of ribosome - decreased binding	Plasmid	[4]; [37] [38], [39]
β-lactam (penicillins, methicillins, cephalosporins, carbapenems)	Peptidoglycan synthesis	*blaZ* *mecA*	β-lactamase Penicillin-binding protein (PBP2a)	Enzymatic hydrolysis of β-lactam Reduced affinity for PBP	Plasmid Chromosome: SCC*mec*	[8] [10]
Chloramphenicol	Translation	*cat*	Chloramphenicol acetyltransferase	Acetylation of drug – inactivation	Plasmid	[40]
Tetracycline	Translation	*tetK, tetL* *tetM, tetO*	Tetracycline efflux pump Tetracycline resistance protein	Active efflux Ribosome protection – competitive binding	Plasmid Plasmid	[13]
Macrolides and Lincosamides (erythromycin, clindamycin)	Translation	*ermA,* *ermB,* *ermC*	Ribosomal methylase	Methylation of ribosome – decreased binding	Plasmid	[41]
Trimethoprism/Sulfamethoxazole	Enzyme modification	*dhfr* *dhps*	Dihydrofolate reductase Dihydropteroate synthase	Mutations on drug target – decreased binding	Chromosome, Plasmid Chromosome	[42] [43]
Streptogramins (quinupristin/dalfopristin)	Translation	*ermA,* *ermB,* *ermC,* *vat, vatB*	Ribosomal methylase Acetyltransferase	Methylation of ribosome – decreased binding Acetylation of drug – inactivation	Plasmid Plasmid	[44] [45]
Aminoglycosides (amikacin, gentamicin, tobramicycin, kanamycin, streptomycin)	Translation	*aac,* *ant,* *aph*	Acetyltransferase Adenylyltransferase Phosphotransferase	Aminoglycoside modifying enzymes – inactivation	Chromosome, Plasmid	[9]
Glycopeptides	Peptidoglycan synthesis	*vanA*	D-Ala-D-Lac	Modified drug target - reduced affinity for vancomycin	Chromosome, Plasmid	[11]
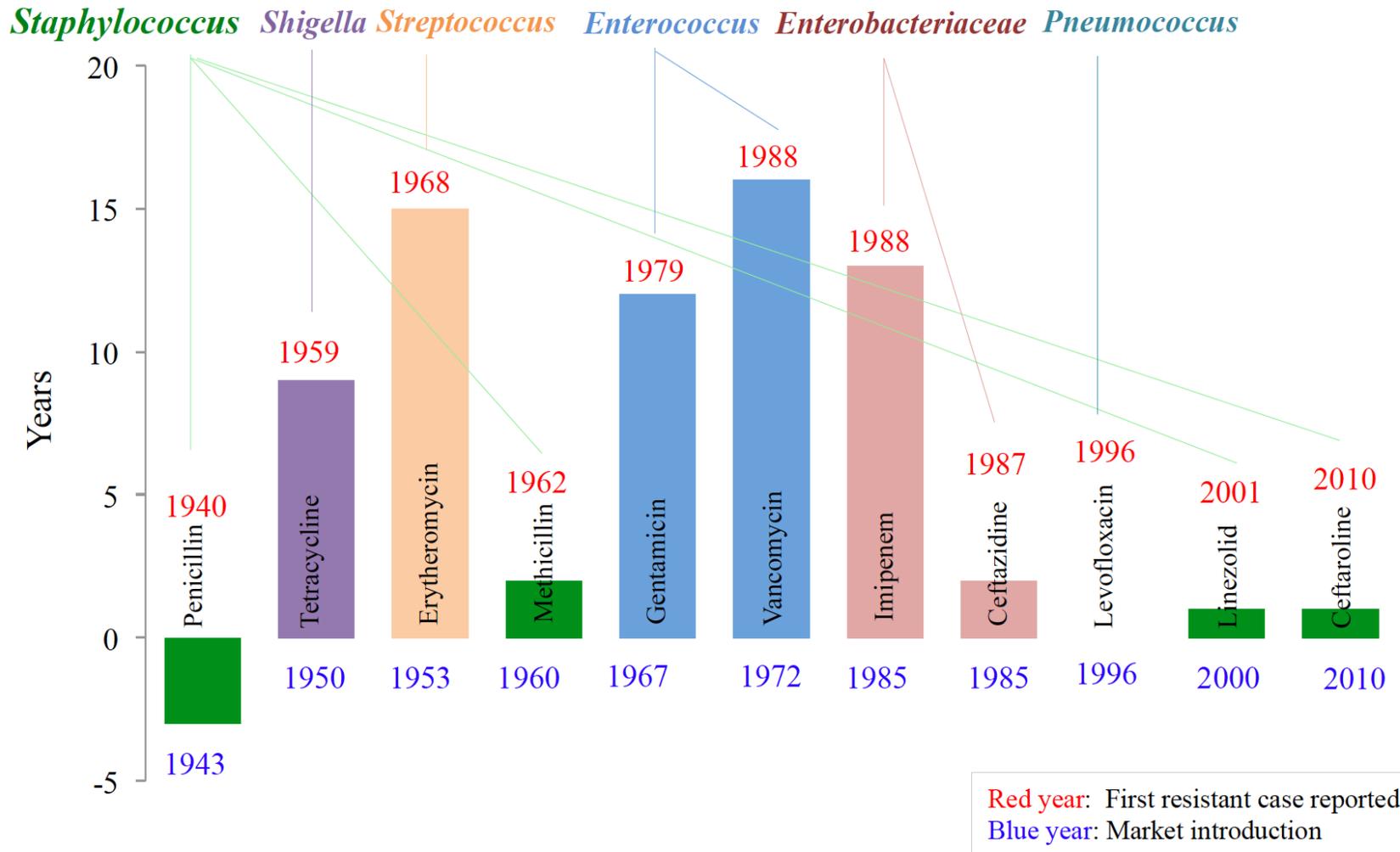
Figure 1-1. First reported cases of bacterial resistance against key antibiotics.

Data source: Antibiotic Resistance Threats in the United States, 2013, US Center for Disease Control and Prevention (CDC).

Transformation

- Active uptake of free DNA by cells developing the competence state.
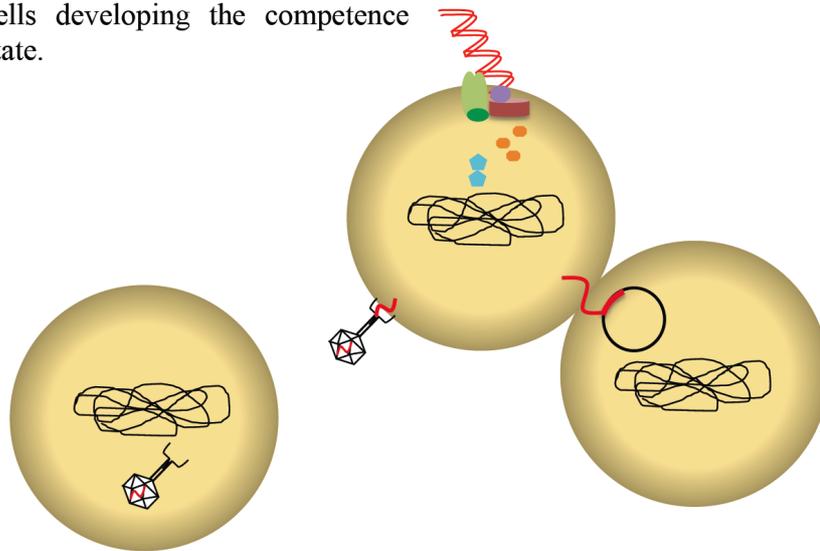
Transduction

- Through bacteriophage.
- Predominant in *S. aureus;* is limited to transfer small DNA ($\leq$ 45 kb).

Conjugation

- Requires a series of conjugative genes.
- Not widespread among *S. aureus*.

Figure 1-2. Three major horizontal gene transfer mechanisms in *S. aureus*.
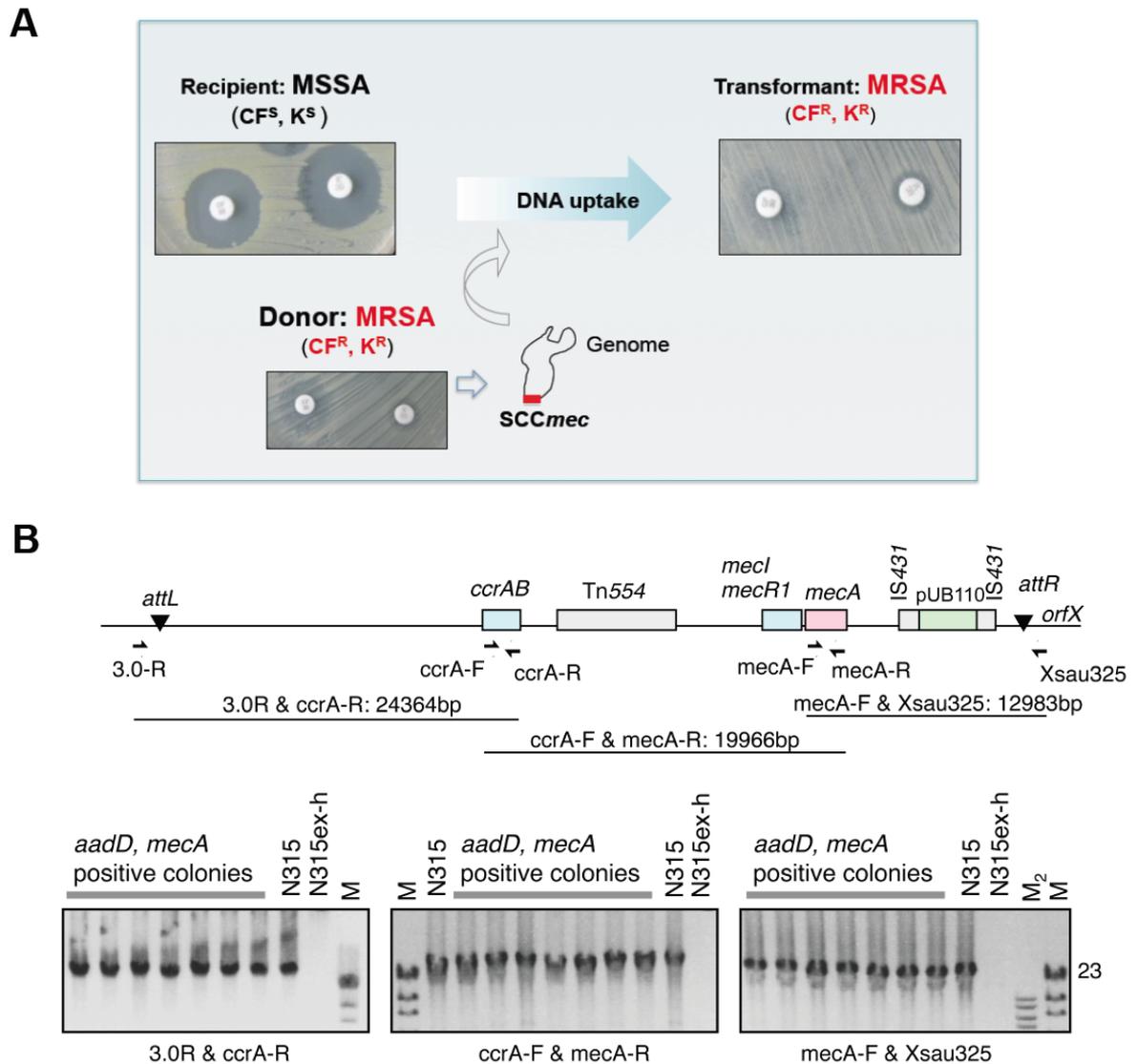
Figure 1-3. Natural transformation of type II SCC*mec*. (A) Acquisition of drug resistance through the competence in *S. aureus*. Methicillin sensitive *S. aureus* (MSSA) overexpressing SigH were incubated with purified genome harboring SCC*mec*II from methicillin resistant *S. aureus* (MRSA). The transformant became resistant to β-lactam Cefoxitin (CF, left side) and Kanamycin (K, right side). (B) Long PCR analyses confirming transfer by transformation of the entire SCC*mec*II element in seven transformants where *aadD* and *mecA* were both present. Primer locations are shown on the SCC*mec*II map. All of the transformants gave signals with the expected size. Chromosomal DNA from strains N315 and N315ex-h was used for positive and negative controls, respectively. M: λHindIII, M2: 1 kb ladder [26].

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Chapter 2

Horizontal transmission of *cfr* gene to MRSA

2.1. Summary

Linezolid resistance mediated by the *cfr* (chloramphenicol/florfenicol resistance) gene represents a global concern due to its dissemination among multidrug resistant nosocomial pathogens such as MRSA. This work aimed to clarify the potential of global transmission of *cfr* on the pSCFS7-like plasmids from two *Staphylococcus epidermidis* strains isolated in Spanish hospitals to various clinically isolated *S. aureus* strains in Japan in which *cfr* has not been detected. An efficient transfer of *cfr* was observed in this interspecies transmission. Furthermore, the transmission of *cfr* among *S. aureus* pool after acquiring the gene from *S. epidermidis* was investigated by addressing the three major pathways of HGT: conjugation, transduction and transformation. Even though conjugation is thought to be the main mechanism of *cfr* dissemination, transduction was found to be an alternative pathway for the transmission of *cfr* gene between *S. aureus* strains.

2.2. Introduction

Linezolid is an oxazolidinone antimicrobial drug that is most effectively used in the treatment of resistant Gram-positive bacteria, including MRSA, VRSA, vancomycin resistant enterococci (VRE), and streptococci [46]. However, linezolid-resistant *S. aureus* (LRSA) was reported just one year after the drug was approved for clinical use [4]. Three major linezolid resistance mechanisms have been described. The first mechanism involves the mutations in the domain V region of the 23S rRNA, thus preventing the binding of the antibiotic. Second mechanism is related to the mutations or deletions in the ribosomal protein L3 and L4, reducing the binding to drug target. These mutations and deletions are often associated with the prolonged and/or intermittent use of linezolid [47]. The third mechanism is due to the acquisition of a plasmid-borne ribosomal methyltransferase gene, the *cfr* (chloramphenicol/florfenicol resistance). The methyltransferase encoded by *cfr* gene methylates the adenosine at position A2503 in 23S rRNA within the overlapping binding sites for phenicols, lincosamides, oxozolidinones, pleuromutilins and streptogramin A, resulting in the resistance to all of these antibiotics, known as PhLOPSA resistance phenotype [48], [49]. Even though the presence of *cfr* gene only slightly increase the minimal inhibitory concentration (MIC) to linezolid in the absence of ribosomal mutations, its ability to be transmitted between strains and species has raised a global concern [50].

The *cfr* gene was initially described in a 16.5-kb transmissible plasmid (pSCFS1) from a livestock-associated *Staphylococcus sciuri* strain [51]. The *cfr* gene located in different types of plasmids was later detected in other staphylococcal species [52]. The first report of *cfr* gene isolated from *S. aureus* in a human patient sample in Columbia was described in 2005 [39]. Following this report, the *cfr*-associated linezolid resistances have been documented in clinical staphylococcal isolates and hospital outbreaks in different countries [53], [54], [55], [56], [57], [58], [59], [60]. The coagulase negative staphylococci (CoNS), in particular *S. epidermidis*, have been speculated as the reservoir of *cfr* gene for

the transmission to *S. aureus* and other pathogenic bacteria [61]. The *cfr*-positive CoNS are increasingly isolated in hospitals due to high consumption of linezolid. This entails the subsequent increase in the *cfr*-positive *S. aureus* strains, which are currently maintained at low frequencies [50]. In Japan, the resistance to linezolid in CoNS and *S. aureus* strains remains hitherto low. No *cfr* detection in clinical isolates has been reported [62]. In this situation, it is important to determine the risk of *cfr* dissemination among clinical isolates. Regarding to the mechanisms of dissemination, conjugation has been the only route demonstrated for *cfr* transmission.

This work aimed to evaluate the ability of the transmission of *cfr* gene from two *S. epidermidis* strains isolated from Spanish hospitals to a series of clinically isolated *S. aureus* strains in Japan, and examine the HGT mechanisms involved in the further dissemination among *S. aureus* strains. The results showed a potential of global transmission of *cfr* from *S. epidermidis* to *S. aureus;* and demonstrated that in addition to conjugation, phage transduction could be responsible for the dissemination of *cfr* among *S. aureus* pool while natural transformation was unable to be detected in this transmission.

2.3. Materials and Methods

Bacterial strains

Bacterial strains used in this study are listed in Table 2-1. *S. epidermidis* strains were isolated in Spanish hospitals and stored in the reference collection of the Spanish National Center for Microbiology. The SE45 strain was isolated in Madrid and the SE50 was isolated in Andalucia region. Both strains belonged to the ST2 group (defined by multilocus sequence typing - MLST) and harbored the *cfr* gene on ~ 40 kb plasmids with a pSCSF7-like *cfr* insertion region, determined by mapping PCR (Figure 2-1).

S. aureus clinical MRSA strains (n=16) were isolated from the Kanto area in Japan [63]. Most of the clinical isolates belonged to clonal complex 5 and all of them harbored the type II SCC*mec* (Table 2-1) defined by clonal complex designation and SCC*mec* typing, respectively, as described in the below.

Clonal complex designation

Clonal complex (CC) designation of *S. aureus* clinical isolates was performed following the method described by Cockfield *et al.*, 2007 [64]. Three PCR assays were carried out to determine the CC type of each isolate. Each PCR containing one forward and two different reverse primers (Table 2-2). Chromosomal DNAs were extracted from the strains following the conventional whole DNA isolation method [65] and used as DNA templates for the PCRs.

SCCmec typing

A multiplex PCR to assign major SCC*mec* types for *S. aureus* was carried out as previously described method [66]. A QIAGEN multiplex PCR kit was used. Whole DNA extracts were used as DNA template for the PCR. Prototype strains used for SCC*mec* typing were COL (SCC*mec* I), N315 (SCC*mec* II), 85/2082 (SCC*mec* III), MW2 (SCC*mec* IV), and WIS (SCC*mec* V). The PCR products (5 µL/each) were visualized using UV light

(FasIII UV Transilluminator, Toyobo) after electrophoresis in a 3% (w/v) agarose gel.

Molecular analysis of cfr-carrying vectors

Primers used in the molecular analysis of pSCFS7-like vectors are listed in Table 2-2. Amplifications were carried out in SE45, SE50 and their N315 *cfr*-positive derivatives (N315-45 and N315-50, see below). Amplification of *traA* and *nes* genes was performed using primers designed based on the pGO1 sequence (accession number FM207042) [67], which is considered as the prototype conjugative staphylococcal plasmid. Primers for backbone amplification of the *cfr* vectors were designed based on the available sequence of pSCFS7 vector p12-02300 (accession number KM521837) [59].

Conjugation

Conjugative transfer of *cfr*-carrying plasmids was performed using filter-mating method described previously [68].

Interspecies conjugation: S. epidermidis to MRSA

SE45 and SE50 were used as donor strains. MRSA strains; N315, COL and their phage-cured derivatives (N315ex w/oφ and COL w/oφ) were used as recipients. The recipient strains used were chloramphenicol susceptible and erythromycin resistant, except COL and COL w/oφ strains, which was chloramphenicol susceptible and tetracycline resistant. The susceptibility profile of recipient strains allowed the use of chloramphenicol (32 mg/L: to select *cfr* positives) plus erythromycin or tetracycline (32 or 8 mg/L respectively: to eliminate donor *S. epidermidis*) in the selection of transconjugants. Conjugation was performed following a previously described protocol [68] with a few modifications. Overnight cultures of the donor and recipient were prepared in Tryptic soy broth (TSB) medium with and without 32 mg/L chloramphenicol, respectively, with shaking at 37 °C. The cultures were adjusted to the optical density 1 ($OD_{600} = 1$) using fresh TSB medium. 0.5 mL of the donor (SE45 or SE50) was mixed with 0.5 mL of the

recipient and 1 mL of PBS. The mixture was transferred onto a 0.45-µm filter membrane using a vacuum pump system. The membrane was put on a sheep blood agar plate and incubated at 37 °C for 1 day. The membrane was collected and resuspended in 10 mL of phosphate-buffered saline (PBS) by vortex. One hundred µL of the 10^{-4} diluted samples were plated on TSB agar (TSA) plates supplemented with 32 mg/L chloramphenicol plus erythromycin or tetracycline (32 or 8 mg/L, respectively: to eliminate donor *S.epidermidis*) in the selection of transconjugants. Another one hundred µL of the diluted suspension (10^{-5}-10^{-6}) were plated onto TSA plates with erythromycin (32 mg/L) or tetracycline (8 mg/L) alone to count the total number of recipients. The plates were incubated at 37 °C for 18-24 h. Double-resistant colonies obtained in the interspecies (*S. epidermidis* to MRSA) transmission experiments were confirmed by plating on mannitol salt agar (*S. aureus* is positive while *S. epidermidis* is negative in mannitol utilization), and detection of the *cfr* gene by PCR (Table 2-2).

N315-45 and N315-50 derivatives were obtained by filter mating using as donors SE45 and SE50 strain, respectively. These *cfr*-positive strains were subsequently used in the characterization of pSCFS7-like vectors.

Intraspecies conjugation: MRSA-to-MRSA

For intraspecies conjugation between *S. aureus* strains, N315-45, COL-45, N315ex w/oφ-45, COLw/oφ-45 *cfr*-positive derivatives (obtained by filter-mating using as donor SE45), the T-N315-45 *cfr*-positive transductant strain (obtained by phage transduction) and their original *cfr*-negative strains were used as donor and recipient respectively. Putative transconjugants were confirmed by checking the antibiotic susceptibility profile and detection of the *cfr* gene by colony PCR.

Phage transduction

The bacteriophage MR83a (laboratory stock) is a staphylococcal transducing phage,

belonging to Siphoviridae family, suitable for transduction from/to N315 [69]. First, phage MR83a was used to infect the N315-45 transconjugant strain. The resultant phage pool (MR83a-45) was harvested and used to test the transmission of *cfr* gene by generalized transduction. One mL of the phage pool (2×10^{12} plaque forming unit pfu/mL) was mixed with 0.5 mL of recipient strains (N315, COL or Mu50) overnight-cultured in nutrient broth (NB, Oxoid) supplemented with 3.6 mM $CaCl_2$ ($NBCaCl_2$). The mixture was incubated at 37^oC for 30 min. After the addition of 0.1 mL of 20% sodium citrate, the mixture was further incubated for 30 min. Transductants were selected in brain heart infusion (BHI)-agar medium supplemented with 32 mg/L chloramphenicol. Putative transductants were confirmed by assessment of recipient susceptibility profile and detection of the *cfr* gene by colony PCR.

Natural transformation

A natural competent strain derived from N315 (N2-2.1) was used as the recipient in this study. N2-2.1 carries a *sigH* locus duplication that constitutively expresses the *sigH* fusion gene, rendering this strain naturally competent [26]. Donor DNA in transformation experiments was obtained from the COL-45 transconjugant strain. Plasmid purified by QIAfilter plasmid Midi kit (QIAGEN) or whole DNA prepared by conventional purification method [65] was used as the source of *cfr* in these experiments. Transformation of pT181 and pHY300PLK plasmids, purified from COL and *E. coli* HST04 *dam⁻/dcm⁻* (Takara) respectively, was performed in parallel as the positive control. Transformation assay was carried out using a previously described method [26]. Briefly, 0.5 mL of overnight culture of N2-2.1 in TSB supplemented with chloramphenicol (12.5 g/L) was precipitated by centrifugation (10,000 x g for 1 min at 4 °C). Cells were resuspended into 10 mL of CS2 medium. The bacteria were grown at 37 °C with shaking (180 rpm) until the late exponential phase (about 8 h). Cells were harvested and resuspended in 10 mL of fresh

CS2 medium. Ten 10 μg of purified plasmid or genomic DNA was added to the cell suspension and continuously grown for 2.5 hours. After centrifugation, cells were resuspended in 10 mL of BHI medium and mixed with 90 mL of melted BHI agar (kept warm at 55 °C) supplement with 32 mg/L chloramphenicol (or 5 mg/L tetracycline in the control experiment). Pour the mixture into the 90-mm petri dishes and incubate the plates at 37 °C for 2 days. Generated colonies (transformants) were confirmed the acquired resistance gene (*cfr* or *tetM*) by colony PCR.

2.4. Results

2.4.1. Conjugative transmission of cfr from S. epidermidis to MRSA

In the first transmission experiment, the interspecies transmission of *cfr* from *S. epidermidis* strains (SE45 and SE50) to MRSA strains was investigated. The data are summarized in Figure 2-2. The SE45 strain was able to transfer *cfr* gene to all tested *S. aureus* strains with a mean frequency of 8.57×10^{-5} transconjugants per recipient cell. In contrast, transfer of *cfr* from SE50 could only be detected in half of the recipient strains and the frequency of transmission was lower (mean 5.21×10^{-9}). The results indicate that frequency of *cfr* transmission depends on background of the donors.

Next, the existence of the conserved conjugative element (*tra*) and the associated nickase gene (*nes*) in the *S. epidermidis* donors (SE45, SE50) and the *S. aureus* transconjugants (N315-45, N315-50, respectively) was analyzed by PCRs. Negative amplification was observed in case of SE45, whereas positive amplification results for both genes were obtained in the SE50 strain (Figure 2-3). Negative amplifications were obtained in both N315-45 and N315-50, indicating the absence of these genes in SE45 and SE50 pSCFS-7 like vectors. To determine the structural similarity of the SE45 and SE50 pSCFS-7 like plasmids to the recently reported *cfr*-carrying plasmid, the p12-02300 [59], a set of primers located outside the *cfr* insertion context was designed based on the available p12-02300 sequence. Amplifications were tested in SE45, SE50, N315-45 and N315-50 strains. Amplifications gave expected size products in the four strains (Figure 2-4) suggesting the backbone similarities between SE45 and SE50 plasmids and p12-02300.

2.4.2. S. aureus can transfer cfr by conjugation

The *cfr* transmission between *S. aureus* strains was further investigated. The *cfr*-positive *S. aureus* strains, N315-45 and COL-45 obtained from the interspecies conjugative transmission described above were used as *cfr* donors in the conjugation to COL and N315

respectively. As shown in Figure 2-5A, N315-45 and COL-45 were able to further transfer the *cfr* to COL and N315 by filter-mating method indicating that these strains retained the transmission ability after they had acquired it from *S. epidermidis*.

To exclude the possible involvement of other HGT pathways in this transmission, a set of intraspecies conjugative experiments were performed in the absence of native phages and competence machinery. Phage cured strains N315ex woφ-45 and COL woφ-45, were used as donors, while their original *cfr*-negative strains were used as recipient. We observed a similar transconjugant generation in these transmission (Figure 2-5B) showing that residence phages might not involve in the transmission of *cfr*-carrying vectors by conjugation. In the absence of competence components, the *comG* operon deletion mutant derived from N315ex woφ (N315ex woφ Δ*comG*) was used as recipient; COL woφ-45 strain was used as donor in this conjugative experiment. As shown in Figure 2-5B, no significant difference in the transconjugant generation was obtained in this case. Thus, the result suggests the absence of transformation event in the filter-mating conjugative experiments.

2.4.3. Phage-mediated transmission of cfr among S. aureus

In *S. aureus*, phage transduction is thought to play a major role in HGT, since most of *S. aureus* isolates carry prophage in their genome. The DNA size which can be packaged by staphylococcal phage transduction is up to ~ 45 kb. Thus, the pSCFS-7 like vectors (~ 40 kb) was expected could be transferred by phage transduction. The potential transmission of *cfr* gene by transduction was tested by using a staphylococcal transducing phage MR83a (see Materials and methods). First, phage MR83a was amplified by infecting the N315-45 to generate the *cfr*-packaged phage MR83a (MR83a-45). Phage MR83a-45 was then used to infect *S.aureus* strains N315, COL and Mu50. The transduction of *cfr* gene was observed

in all strains tested as shown in Table 2-3. The results indicate that phage transduction could contribute to the spread of *cfr* among *S. aureus* pool.

To further evaluate the contribution of transduction in the spreading of *cfr* gene, the *cfr*-acquired N315 transductant strain obtained from transduction experiment (T-N315-45) was investigated. This strain was analyzed to determine the integrity of pSCFS7-like plasmid after transduction, as well as its conjugative ability. Analyses of the genetic environment of *cfr* insertion (Figure 2-1) and the plasmid backbone (Figure 2-6) by PCRs showed an indistinguishable amplification pattern between SE45, N315-45 and T-N315-45. The results showed the transmission of complete pSCFS7-like plasmid by phage transduction. Checking for its further transmission ability showed a comparable transconjugant generation to that previously obtained in the case of transmission from N315-45 to COL in the conjugative experiments (Figure 2-5A). Thus, these data suggest the retention of conjugative capability of pSCFS7-like plasmid after phage transduction.

2.4.4. Natural transformation of cfr

The involvement of natural transformation in the transmission of *cfr* among *S. aureus* was evaluated. A native SigH-active strain constitutively expressing SigH (N2-2.1) was used as recipient in the transmission. Purified plasmid or whole DNA extracts from COL-45 strain was used as *cfr* donor (see Materials and methods). As shown in Table 2-3, no *cfr* transformant was observed in our experimental condition, while transformation frequencies of pT181 and pHY300PLK plasmid was at $10^{-9} \sim 10^{-10}$. The results suggest a low relevance of transformation in the dissemination of *cfr* among *S. aureus* strains.

2.5. Conclusions and Discussion

In this study, the *cfr* transmission to *S. aureus* from the CoNS species (*S. epidermidis*) was confirmed. Moreover, different HGT pathways involved in the dissemination of *cfr* among *S. aureus* pool were investigated. The results showed that the *cfr*-carrying pSCFS7-like plasmids in SE45 could be efficiently transferred from *S. epidermidis* to various clinically isolated *S. aureus*. The efficiency of the transmission depended on the characteristics of the donor (Figure 2-2). Of concern, after acquisition, the pSCFS7-like plasmid could further spread to other *S. aureus* strains by different HGT routes (Table 2-3). Even though conjugative transmission has been thought to be the only way for *cfr* spreading, here the contribution of phage transduction was demonstrated. To our knowledge, this is the first report of a conjugation-independent HGT mechanism of the *cfr* gene in *S. aureus*. Importantly, the pSCFS7-like plasmid was entirely transduced by phage transduction and retained the conjugative ability of the plasmid for further transmission (Figure 2-1, Figure 2-6 and Figure 2-5). No transformation of the *cfr* gene was observed in the employed condition, suggesting a low relevance of natural transformation in the spread of pSCSF7-like plasmid. The reason for the failure to detect the pSCSF7-like plasmid transformation might be attributed to its large size ($\sim$ 40 kb) compared to other plasmids (pT181, 4.4 kb and pHY300, 4.9 kb) that have been transferred by transformation as described in this study and previous study [26]; transformation could be detected at a lower frequency when 0.1 µg of pT181 was used, less than 10^{-9} while 10 µg of the plasmid generated the frequency at $\sim 10^{-8}$ (data not shown), but not for 10 µg of pSCSF7-like plasmid. There also remains a possibility that plasmid quality of the purified pSCSF7 was low due to its large size, and it is known that intact DNA topology is important for natural transformation in *B. subtilis* [70]. It must also be noted that the transfer of large MGE (a 53 kbp SCC*mec* type II) is possible by transformation [26]. Plasmid transformation and chromosomal transformation utilizes distinct systems to be stably incorporated as the

genetic information into the recipient, where several factors differently participate in *S. pneumonia*: DprA-RecA facilitate homologous recombination, SsbA facilitates the plasmid transformation, SsbB stabilizes single strand DNA in both chromosomal and plasmid transformation, while the C-terminal region of SsbB stimulate the recombination [71]. Another point that needs to be discussed is that the transformation efficiency in the employed protocol was low, which might have made it difficult to properly evaluate the transfer of large MGEs. There remains a possibility that *S. aureus* becomes highly active in transformation in certain unknown conditions. Further investigation is necessary to find better conditions for transformation as discussed in Chapter 3.

The *cfr*-carrying pSCFS7-like vector could be transferred to clinical *S. aureus* in Japan, a country in which this gene has not been yet detected. Therefore, surveillance for the *cfr* gene in both *S. aureus* and 'reservoir' species, i.e. CoNS, is important. Particularly, *S. epidermidis* would be of great concern, because it is the major species carrying the *cfr* gene.

In this work, the presence of the conjugative elements (*traA* and *nes* gene) in SE45, SE50, and in the *cfr*-acquired N315-45, N315-50 transconjugant was analyzed. The detection of these conjugative components in SE50 strain but not on its N315-50 derivative suggested that conjugative genes were not associated with the pSCSF7-like vector, but might be related to chromosomal or additional native conjugative plasmid in this strain. In addition, the absence of these conjugative genes in the SE45 strain suggests an alternative route of transfer of the pSCSF-7 like plasmid in the filter-mating transmission.

2.6. Tables and Figures

Table 2-1. Bacterial strains and bacteriophage used in this work

Strain	Species	*cfr*	CC assignment	SCC*mec* type	Reference or Source
Bacteria					
SE45[a]	*S. epidermidis*	+	2		This study
SE50[a]	*S. epidermidis*	+	2		This study
r406[a]	*S. aureus* (MRSA)	-	5	II	[63]
r3[a]	*S. aureus* (MRSA)	-	5	II	[63]
r39[a]	*S. aureus* (MRSA)	-	5	II	[63]
r59[a]	*S. aureus* (MRSA)	-	5	II	[63]
r32[a]	*S. aureus* (MRSA)	-	5	II	[63]
r132[a]	*S. aureus* (MRSA)	-	5	II	[63]
r68[a]	*S. aureus* (MRSA)	-	5	II	[63]
r91[a]	*S. aureus* (MRSA)	-	5	II	[63]
r62[a]	*S. aureus* (MRSA)	-	5	II	[63]
r703[a]	*S. aureus* (MRSA)	-	5	II	[63]
r418[a]	*S. aureus* (MRSA)	-	5	II	[63]
r434[a]	*S. aureus* (MRSA)	-	5	II	[63]
r83[a]	*S. aureus* (MRSA)	-	5	II	[63]
r408[a]	*S. aureus* (MRSA)	-	NT	II	[63]
r2040[a]	*S. aureus* (MRSA)	-	5	II	[63]
r70[a]	*S. aureus* (MRSA)	-	5	II	[63]
N315[a,b]	*S. aureus* (MRSA)	-	5	II	[17]
N315-45[b,d]	*S. aureus* (MRSA)	+	5	II	Transconjugant N315 strain harboring *cfr* from SE45 (This study)
T-N315-45[b]	*S. aureus* (MRSA)	+	5	II	Transductant strain harboring *cfr* from N315-45 through phage MR83a (This study)
N315-50	*S. aureus* (MRSA)	+	5	II	Transconjugant N315 strain harboring *cfr* from SE50 (This study)
N315ex w/oφ-45[b]	*S. aureus*	+	5	-	Transconjugant N315ex w/oφ strain harboring *cfr* from SE45 (This study)
N315ex w/oφ *ΔcomG*[b]	*S. aureus*	-	5	-	[72]
N2-2	*S. aureus*	-	5	-	*SigH* active derived from N315, natural competent strain [26]
N2-2.1[c]	*S. aureus*	-	5	-	N2-2 derivative, reporter plasmid cured (This study)
COL[a,b,d]	*S. aureus* (MRSA)	-	8	I	[73]
COL-45[b,c,d]	*S. aureus* (MRSA)	+	8	I	Transconjugant COL strain harboring *cfr* from SE45 (This study)
COLw/oφ[a,b]	*S. aureus* (MRSA)	-	8	I	Phage cured COL strain [26]
COLw/oφ-45[b]	*S. aureus* (MRSA)	+	8	I	Transconjugant COL w/oφ strain harboring *cfr* from SE45 (This study)

T-COL-45	*S. aureus* (MRSA)	+	8	I	Transductant COL strain harboring *cfr* from N315-45 through phage MR83a (This study)
Mu50d	*S. aureus* (MRSA, VISA)	-	5	II	[17]
T-Mu50-45	*S. aureus* (MRSA, VISA)	+	5	II	Transductant Mu50 strain harboring *cfr* from N315-45 through phage MR83a (This study)
Bacteriophages					
MR83ad	*Siphoviridae* family				[69]
MR83a-45d	*Siphoviridae* family				Phage obtained after the infection of N315-45 strain (This study)

a: strains used in *cfr*-conjugation experiments (*S. epidermidis*-to-MRSA)

b: strains used in *cfr*-conjugation experiments (MRSA-to-MRSA)

c: strains used in *cfr*-transformation experiments

d: strains used in *cfr*-transduction experiments

NT: not typeable

Table 2-2. Oligonucleotides used in this study

Product or region	Primer	Sequence (5' → 3')	Annealing temperature	Amplicon size (bp)	Reference
cfr	cfr-fw	TGAAGTATAAAGCAGGTTGGGAGTCA	58ºC	746	[52]
	cfr-rv	ACCATATAATTGACCACAAGCAGC			
pSCFS7 mapping	tnpB-fw	GGAACAGTGATAAGCCGGATG	56ºC	4000	[52]
	tnpB-rv	ACATGGTCCTGCAATTACTGGTA			
	1F	TTGCGAAATGGGAAGAGGTAT	56ºC	743	This study
	1R	TCGCATTGGTTATTTGTTTTATTC			
	cfrR-F2	CTGCTTGTGGTCAATTATATGGT	56ºC	681	This study
	bin3R	AGGGTCTACTCTAATCAAACATAA			
traA	traAF	AATGGCCGAAGATAGAGAGGA	56ºC	557	This study
	traAR	TTGCTATTTGTCCCGAGGCG			
nes	nesF	ATCATTGAATCTAGCCCAGCA	56ºC	380	This study
	nesR	GGCACAGAAAACACGCTTGA			
b1 (28064-32584)*	b1F	TCGGAACTCCAATCGCAAGAA	56ºC	4521	This study
	b1R	GCCCCCTGATCGTCCTTTAT			
b2 (4490-8343)*	b2F	AGTGACGCGCAAGAACAGTA	56ºC	3854	This study
	b2R	TTTCCAGGCCTCGTCAAGTC			
b3 (36848-612)*	b3F	CGCAATTGGAGATCAGCACG	56ºC	2648	This study
	b3R	GCAAGAATTGCACCAGCGAT			
CC typing	AF	AGGGTTTGAAGGCGAATGGG	55ºC		[64]
	AR30	CAACAGAATAATTTTTTAGTTC		203 (with AF)	

	AR22	TCAGAGCTCAACAATGATGC		990 (with AF)	
	AR45	GGAGCATTATCTGGTGTTTTCC		722 (with AF)	
	AR1	GGGTTGCTCCTTGCATCATA		1037 (with AF)	
	BF	CCCAAAGGTGGAAGTGAAAA			
	BR8	CCAGTTGCACCATAGTAAGGGTA		680 (with BF)	
	BR5	TCGTCCGACTTTTGAAGATTG		1071 (with BF)	
SCC typing	Type I-F	GCTTTAAAGAGTGTCGTTACAGG		613	
	Type I-R	GTTCTCTCATAGTATGACGTCC			
	Type II-F	GATTACTTCAGAACCAGGTCAT		287	
	Type II-R	TAAACTGTGTCACACGATCCAT			
	Type III-F	CATTTGTGAAACACAGTACG		243	
	Type III-R	GTTATTGAGACTCCTAAAGC			
	Type IVa-F	GCCTTATTCGAAGAAACCG		776	
	Type IVa-R	CTACTCTTCTGAAAAGCGTCG			
	Type IVb-F	AGTACATTTTATCTTTGCGTA	57ºC	1000	[66]
	Type IVb-R	AGTCATCTTCAATATGGAGAAAGTA			
	Type IVc-F	TCTATTCAATCGTTCTCGTATT		677	
	Type IVc-R	TCGTTGTCATTTAATTCTGAACT			
	Type IVd-F	AATTCACCCGTACCTGAGAA		1242	
	Type IVd-R	AGAATGTGGTTATAAGATAGCTA			
	Type IVh-F	TTCCTCGTTTTTTCTGAACG		663	
	Type IVh-R	CAAACACTGATATTGTGTCG			
	Type V-F	GAACATTGTTACTTAAATGAGCG		325	

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Type V-R	TGAAAGTTGTACCCTTGACACC	
mecA-F	TCCAGATTACAACTTCACCAGG	162
mecA-R	CCACTTCATATCTTGTAACG	

* Numbers correspond to the available p12-02300 plasmid sequence (accession number KM521837).

Table 2-3. HGT mechanisms involved in *cfr* transmission in *S. aureus* strains

HTG	Donor	Recipient	Frequency
Conjugation	N315-45	COL	1.00×10^{-6}
	N315-45	Mu50	1.29×10^{-5}
Transduction	N315-45	N315	6.88×10^{-10}
	N315-45	COL	1.00×10^{-11}
	N315-45	Mu50	3.68×10^{-10}
Transformation	plasmids (COL-45)	N2-2.1	ULD[*]
	whole DNA (COL-45)	N2-2.1	ULD

[*] ULD: under the limit of detection

The frequency is expressed as transconjugants/recipient cell in conjugation experiments, whereas it is expressed as transductants/pfu in transduction experiments. The values correspond to average data obtained from two independent experiments.
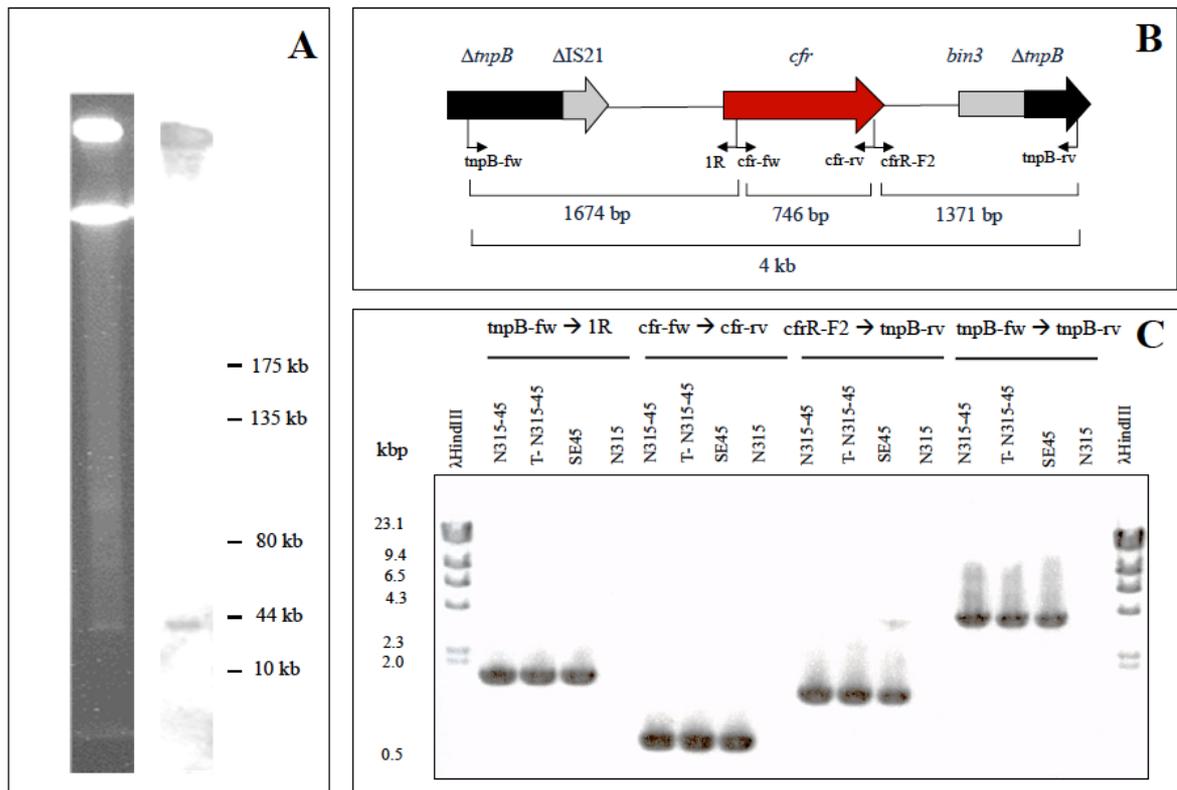
Figure 2-1. Molecular characterization of representative pSCFS7-like plasmid of *S. epidermidis* strains. A: The S1 nuclease PFGE assay and hybridization with *cfr* probe performed on SE45 strain showed *cfr* gene located on a ca. 40 kb plasmid. B: Scheme of mapping PCR used for pSCFS7 genetic environment determination. C: Amplification of *cfr* genetic environment of N315-45 (*cfr*-positive transconjugant strain obtained in filter mating experiments using as donor SE45), T-N315-45 (*cfr*-positive transductant strain obtained *cfr* by transduction from N315-45 through MR83a phage), SE45 (clinical *S. epidermids cfr*-positive) and N315 (*cfr*-negative strain).
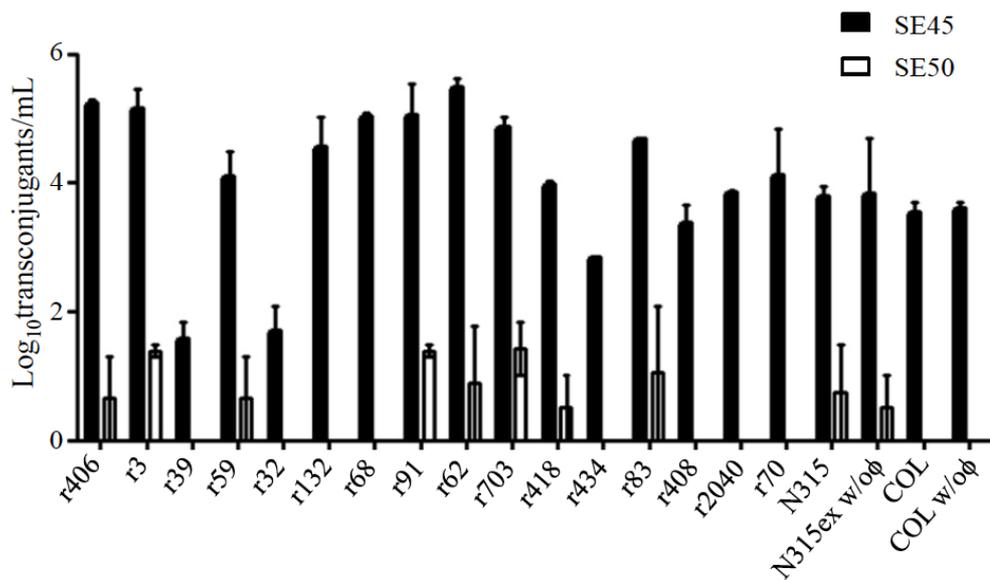
Figure 2-2. Interspecies dissemination of *cfr* from *S. epidermidis* to *S. aureus* obtained in filter-mating experiments. Two clinical *cfr*-positve *S. epidermidis* strains (SE45 and SE50) were used as donors. Clinical MRSA (n = 16), N315, COL and their phage-cured derivatives N315ex woϕ and COL woϕ were used as the recipients in these experiments. Representation of transconjugant generation is expressed as Log_{10}transconjugants/mL. Average data obtained from two independent experiments are shown with SD. Black bars represent values of SE45 transmission; while bars represent values of SE50 transmission.
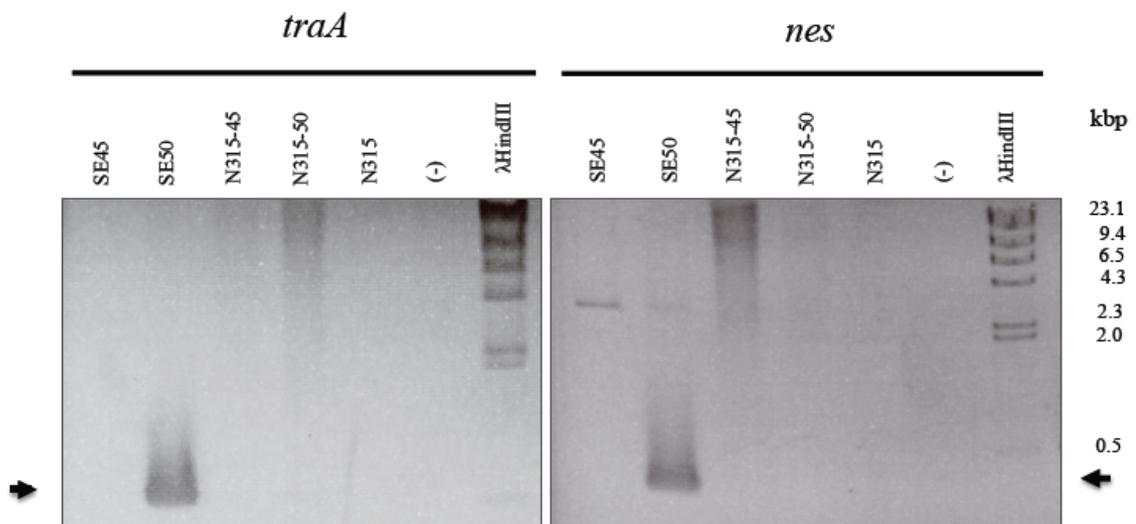
Figure 2-3. Amplification of the conserved conjugative elements *traA* and nickase (*nes*) in the two *S. epidermidis* strains (SE45 and SE50) and their transconjugants (N415-45 and N315-50) obtained in filter-mating experiments.
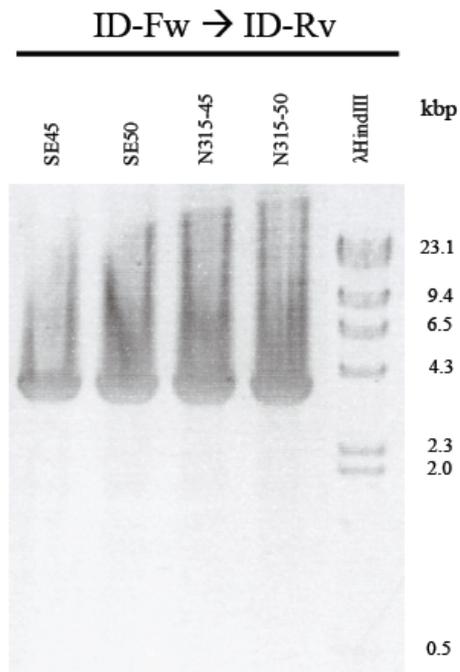
Figure 2-4. Amplification of the pSCFS7-like plasmid backbone in the two *S. epidermidis* strains (SE45 and SE50) and their transconjugants (N415-45 and N315-50) obtained in filter-mating experiments.
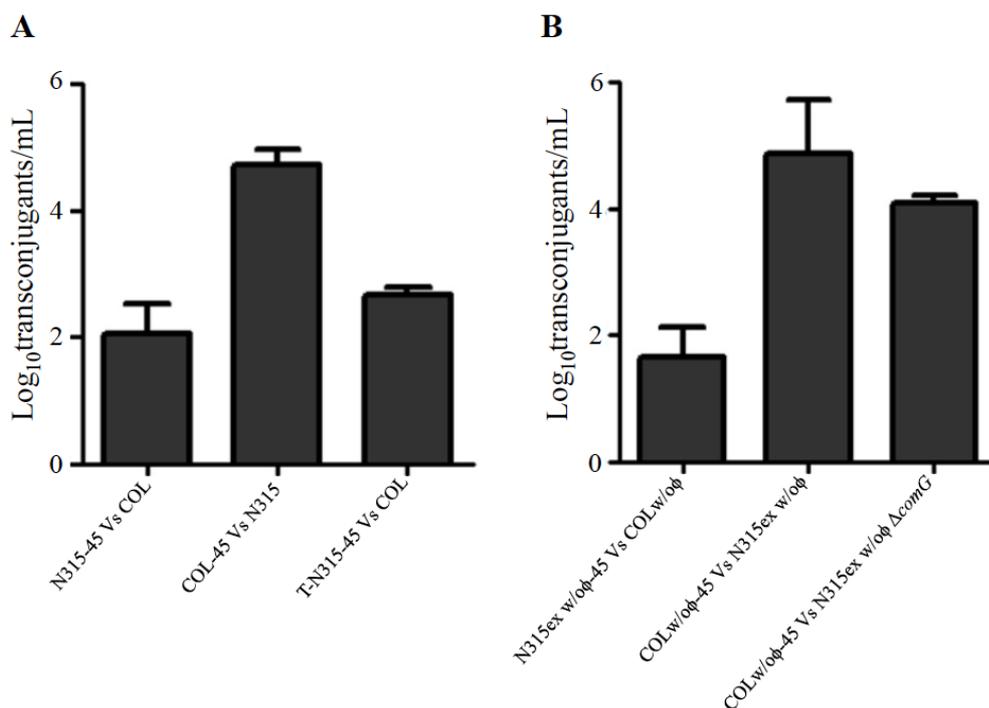
Figure 2-5. Intraspecies dissemination of *cfr* from MRSA to MRSA in the presence (A) and absence (B) of bacteriophage and competence genes obtained in filter-mating experiments. All donor strains harbor the pSCSF-7 like plasmid from the SE45. (A) Transmission results obtained from the unmodified N315 and COL *cfr*-positive strains. N315-45 and COL-45 acquired by conjugation from SE45; T-N315-45 acquired *cfr* by transduction from N315-45 through the MR83a phage. (B). Transmission results obtained using N315 and COL phage-cured strains. N315ex woφ-45 and COL woφ-45 acquired *cfr* by conjugation from SE45. N315ex woφ ΔcomG is a DNA uptake defective mutant derived from N315ex woφ. The values corresponding to average data of two independent experiments are shown with SD.
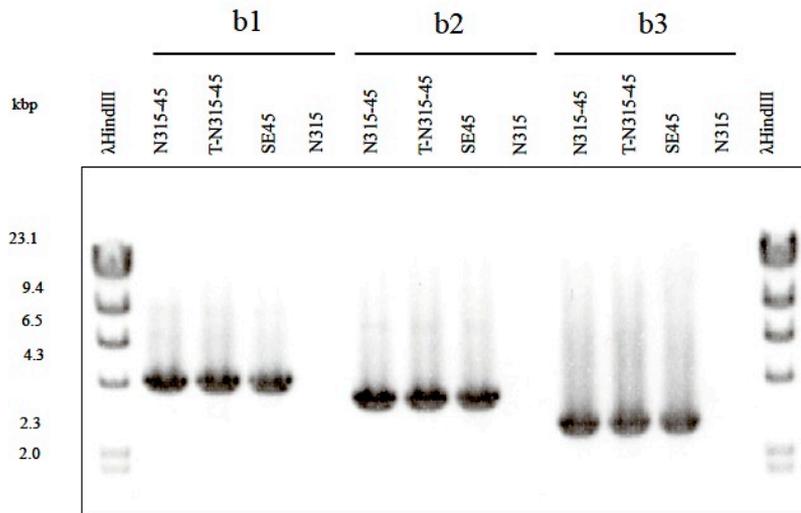
Figure 2-6. Backbone similarity amplifications of N315-45 (*cfr*-positive transconjugant strain obtained in filter mating experiments using as donor SE45), T-N315-45 (*cfr*-positive transductant strain obtained *cfr* by transduction from N315-45 through MR83a phage), SE45 (clinical *S. epidermidis cfr*-positive) and N315 strain.

Chapter 3

Effects of antibiotics on transformation in SigH-expressing
S. aureus

3.1. Summary

Subpopulations of *Staphylococcus aureus* can develop competence for natural transformation in a manner dependent on SigH, which is an alternative sigma factor responsible for competence machinery expression. In addition to SigH expression, unknown environmental factors are thought to be required for natural transformation, because the transformation frequency in SigH expressing cells is variable depending on the culture conditions. In this chapter, I aimed to clarify the effects of antibiotics on transformation using *S. aureus* N315 carrying a *sigH*-expression vector. Cell wall-affecting antibiotics such as bacitracin, fosfomycin and vancomycin, but not β-lactams, increased the transformation frequency. Mitomycin C suppressed transformation. These results substantiate the involvement of additional factors, importantly cell wall affecting reagents, in the control of transformation, and also suggest that some antibiotics may induce staphylococcal horizontal gene transfer in the clinical settings.

3.2. Introduction

In *S. aureus*, our group has revealed that competence is regulated by a transcriptional sigma factor – SigH, whose expression is tightly controlled to be expressed in only a minor population [26].

In the competence-inducing CS2 medium, transformation frequency in the wild type strain is almost undetectable ($<10^{-11}$) and was found to be low ($\sim 10^{-9}$) when SigH was overexpressed [26]. Considering that *S. aureus* has a prominent ability to quickly acquire resistance, there could be the favorable conditions to develop competence for transformation.

Drug resistance is initially manifested in the setting where antibiotics constitute as selective pressure [74]. However, the effects of antibiotics on *S. aureus* transformation have not been explored yet. In this study, I investigated the effect of antibiotics used in the treatment of staphylococcal infection on the efficiency of transformation in SigH-expressing cells. I found that antibiotics have distinct effects on transformation: antibiotics targeting on the bacterial cell wall modulate natural transformation, mitomycin C suppresses transformation.

3.3. Materials and methods

Bacterial strains

Bacterial strains and plasmids used in this study are listed in Table 3-1.

Antibiotic susceptibility test

Minimal inhibitory concentration (MIC) values of the antibiotics in TSB medium were determined by the microdilution method. A 96-well tissue culture test plate (TPP®, Zellkultur und Labortechnologie) inoculated with 5×10^4 colony forming unit (CFU)/well (100 µL) was incubated at $37^{\circ}C$ for 18h. The antibiotics used in this study and their MIC values in TSB medium are described in Table 3-3.

Natural transformation assay

The strain N315ex w/oφ h was used as the recipient. In this strain, the prophage was eliminated to exclude the possibility of "pseudo-competence" DNA transfer with the help of phage components, which is distinct from real competence [26]. SigH is expressed by a plasmid, pRIT-sigH [32]. The pRIT-sigH plasmid was also introduced into the competence deficient strains N315ex w/oφ ΔcomG and N315ex w/oφ ΔcomE by phage transduction to generate the SigH-overexpressing strains N315ex w/oφ ΔcomG h and N315ex w/oφ ΔcomE h (Table 3-1).

Transformation assay was carried out as previously described with some modifications [26]. Briefly, glycerol stocks of *S. aureus* were inoculated in 5 mL of TSBcm and grown overnight at 37˚C with shaking 180 rpm. The overnight culture was diluted 20-fold with fresh TSBcm (in 10 mL) and grown to mid-log phase (about 3 hours). Cells were then harvested by centrifugation (5000 rpm, 5 min) and suspended in fresh TSB with or without the antibiotics to be tested. After 5-hour incubation, cells were washed and replaced with fresh medium. Ten µg of unmethylated pHY300 (Tet[R]) purified from *Escherichia coli* HST04 (*dam⁻/dcm⁻*) was added. Following 2.5 hour incubation at 37°C

with shaking, transformants were selected in BHI–agar medium supplemented with 5 mg/L tetracycline. A part of transformants was tested for the presence of tet^R gene by colony PCR (Table 3-2). In line with our previous study [26], no spontaneous Tet^R mutant was detected throughout this study. The transformation frequency was calculated as the ratio of total number of transformants to the total viable cells after the antibiotics treatment and incubation with DNA. Total recipient cells were counted by plating a series dilution of the culture onto TSB-agar (TSA) plates.

Mechanical and enzymatic disruption of cell walls

Fastprep® device (MP Biomedicals) was used to physically disrupt the bacterial cell walls. A tryptic soy broth supplemented with 12.5 mg/L chloramphenicol (TSBcm) overnight culture was diluted 10-fold with fresh TSBcm (total 5 mL), and successively cultured for 8h. Cells were harvested, resuspendend in 0.4 mL TSB, and transferred to 2 mL lysing matrix tube containing 0.1 mm silica beads (MP Biomedicals). The tube was shaken in Fastprep® device at 4 m/s for 10 s, 20 s and 30 s, once or twice, at 4˚C. After beating, cells were collected by centrifugation (10000 rpm, 10 min) and cell-bead mixture was resuspended in 1 mL TSB. The cell suspension was recovered in a new tube, and DNA was added to test the transformation frequency.

For lysostaphin treatment, cells grown in 5 mL TSBcm were harvested at the mid-log phase. Cells were then resuspended in 10 mL TSB medium containing lysostaphin at different concentrations and incubated for 5h at 37^oC with shaking. After treatment, cells were washed and mixed with DNA for transformation.

Statistical analysis

Statistical analysis was carried out for the log values using Student's t-test.

3.4. Results

3.4.1 Antibiotics have distinct effects on transformation in SigH expressing cell

The effects of antibiotics on transformation were tested by at least 3 independent experiments (Figure 3-1). Bacitracin reproducibly increased the transformation frequency at low concentrations, but showed suppressive effect at higher concentrations. D-cycloserin showed no significant effect. Transformants were rarely detected in 1 mg/L cefazolin treatment (frequency 0.8×10^{-11}, n = 1; none detected, n = 2). Oxacillin abolished transformation: no transformants were detected when cells were treated at ½ MIC of oxacillin. Mitomycin C suppressed transformation in a concentration-dependent manner. Ciprofloxacin, and norfloxacin that target on DNA gyrase had no significant effect. Streptomycin interfering the bacterial protein synthesis showed no effect on transformation.

Vancomycin and fosfomycin increased the transformation frequencies (Figure 3-2A, 2B), and the effects were statistically significant (P = 0.016, n = 9 for vancomycin; P = 0.012, n = 10 for fosfomycin) (Figure 3-2C). SigH-expresing cells lacking the competence genes (N315ex w/oφ ΔcomE h, N315ex w/oφ ΔcomG h) generated no transformant in the presence of these antibiotics (n = 2), confirming that this is due to the transformation by natural genetic competence, rather than other horizontal gene transfer mechanisms. The transformation frequency in the presence of fosfomycin was highly variable depending on the experiment (Figure 3-2C). This variation might be due to the killing effect of fosfomycin (Figure 3-2B).

3.4.2 Physical damages on the cell wall do not facilitate transformation in SigH-expressing cells

Increased transformation frequencies observed in several cell wall-targeting antibiotics suggested that cell wall integrity could be important for transformation. The Gram staining images of antibiotics treated cells indicated that treatment with some

antibiotics increases negatively stained subpopulation (red cells in Figure 3-3). However, the frequencies of the red subpopulation did not correlate with transformation frequencies, suggesting that the damages in cell wall alone cannot explain the increase in transformation frequencies. To test this implication, I examined the effect of cell wall damage by silica beads, and lysostaphin.

First, the effect of physical disruption on the cell wall of SigH-overexpressing cell grown in TSB was tested using silica beads. CFUs were measured at different time points of bead beating to find the appropriate condition. Beating for 10 sec x 2, 20 sec x 2 and 30 sec x 2 resulted in 30%, 45% and 60% reduction in CFU. None of the treatments improved the transformation efficiency (Figure 3-4A).

I also tested the effect of lysostaphin, an enzyme that cleaves *S. aureus* cell walls [75]. As shown in Figure 3-4B, I did not observe any increase in the transformation frequency of TSB grown cells by lysostaphin treatment up to the concentration that reduced the CFU by half (0.1 mg/L). Higher concentrations of lysostaphin resulted in undetectable transformants due to cell death (Figure 3-4B).

Taken together, these results indicate that external physical damages to the cell wall do not simply facilitate the transformation and suggested that certain complex cellular activity would be involved in the control of transformation.

3.5. Conclusions and Discussion

Complex mechanisms participate in the control of natural transformation in a species-specific manner, which has been well studied in the model bacteria such as *Streptococcus* and *Bacillus*. In *S. aureus*, we recently showed that SigH plays a key role in competence development, but many aspects of the regulation in competence development and the following transformation are not yet known. As part of the effort to gain insight into the regulation after SigH expression, we first showed the effects of antibiotics on transformation.

I found that antibiotics targeting cell wall synthesis modulate transformation. The cell wall integrity or metabolism is known to be important in streptococcal transformation: the involvement of autolytic activity has been described in some species [76], [77], and the cell wall integrity can be an important signal that modulates transformation [78]. However, positive effects of bacitracin, vancomycin and fosfomycin are not simply caused by the damages in the cell wall as external physical damages to the cell wall by silica beads or lysostaphin did not facilitate the transformation. This suggests that effects of cell wall-affecting antibiotics on transformation could be involved in certain cellular response.

One important finding was that bacitracin facilitated transformation at a low concentration (1/20 of MIC). Utaida *et al.* previously reported the change in transcriptome in response to cell wall-affecting antibiotics, and found that 46 genes were up-regulated specifically by bacitracin. The most highly up-regulated gene was the ORF 1764 (SACOL2727) encoding a putative integral recombinase [79], but the corresponding gene is missing in the N315 genome. Three genes in the lysine biosynthesis pathway were also up-regulated by bacitracin (*dapB*, *dapB*, and *lysA*); Lysine is a component of the cell wall, as well as the substrate to modify a phospholipid, phophatidylglycerol, affecting the net charge on the cell surface [80]. It is also known that bacitracin is sensed by the two

component system BraSR (or TCS17) together with the ABC transporter, BraDE [81]. Whether such bacitracin-related factors would be involved in the transformation regulation is an interesting question to be addressed in the future study.

Treatment with β-lactam antibiotics could induce multiple responses. Antignac *et al.* showed that growing *S. aureus* in the presence of sub-inhibitory concentrations of β-lactam antibiotics provoked strong repression of the expression and activity of major autolytic enzymes [82]. On the other hand, β-lactams activate the SOS response [83], [84], [85]. Each response might interfere with transformation. Indeed, the involvement of autolytic activity in the induction of transformation has been described in some species [76], [77] whereas SOS response and transformation seem to be phenomena exclusive from each other [86]. SOS inducers such as fluoroquinolone or mitomycin C induce competence in species lacking the SOS system [87], [88], [89], but suppress competence in species harboring the SOS system [90]. Although the SOS response in *S. aureus* is more limited and its accessories are simple, it does exist [91], [92]. Our results showed that treatment with mitomycin C suppressed transformation in *S. aureus* (Figure 3-1E). However, the lack of effect of fluoroquinolone (ciprofloxacin and norfloxacin) (Figure 3-1F, 1G) might indicate the regulation is not so simple.

Low concentrations of bacitracin are often combined with other antibiotics in triple-antibiotic ointments used in the treatment of soft tissue infections [93]. Vancomycin remains an effective resource for MRSA treatment, though vancomycin resistant *S. aureus* has been reported [94]. Fosfomycin previously selected resistant staphylococci (fosfomycin resistant *S. aureus* increased in Japanese hospitals in 1980's), but it is still among the choices for treatment, often in combination with other antibiotics [95]. The present study potentially raises a caution regarding medical prescription in the treatment of *S. aureus* considering the induction of HGT.

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Mitomycin C showed an inhibitory effect on transformation frequencies in a dose dependent manner. Although the mechanism of how mitomycin C suppressed the transformation is unknown, the present finding indicates that some antibiotics currently used in hospitals could be applied for the control of natural transformation of *S. aureus*. Mitomycin C is mainly used as anti-cancer drug, but it has an inhibitory effect on bacteria. Notably, Kwan *et al.* proposed its effect on the "persister" *S. aureus* that is responsible for chronic infections, where prolonged contact with other resistant bacterial species might increase the chance of HGT under the selective pressures of prescribed antibiotics [96].

3.6. Tables and Figures

**Table 3-1. Bacterial strains and plasmids used in this study**

Strain	Description	Reference
N315	pre-MRSA	[97]
N315ex	SCC*mec* cured derivative of N315	[98]
N315ex w/oφ	N315ex cured of the N315 prophage	[26]
N315ex w/oφ h	N315ex w/oφ carrying pRIT-sigH (Cm^R)	[26]
N315ex w/oφ ΔcomG	*ΔcomG* mutant of N315ex w/oφ	[26]
N315ex w/oφ ΔcomG h	N315ex w/oφ ΔcomG pRIT-sigH (Cm^R)	This study
N315ex w/oφ ΔcomE	*ΔcomE* mutant of N315ex w/oφ	[26]
N315ex w/oφ ΔcomE h	N315ex w/oφ ΔcomE pRIT-sigH (Cm^R)	This study
E.coli HST04 *dam⁻/dcm⁻* pHY300	*E.coli* HST04 *dam⁻/dcm⁻* (Takara) carrying tetracycline resistance pHY300 plasmid	[26]
Plasmids		
pHY300PLK	Shuttle vector, ori-pAM✔1, Amp^R (*E. coli*), Tet^R (*S. aureus*)	Takara, Japan
pRIT-sigH	SigH-expressing plasmid (Cm^R)	[32]

Table 3-2. Oligonucleotides used in this study

Primer name	Sequence (5' $\rightarrow$ 3')
IF-tet-I	AGCCTGATAAAATACATCCTATTCACAAT
IF-tet-II	AATTCCGGAACTCTCTCCCAAAGTTGATC

Table 3-3. Characteristics of antibiotics used in this study

| Drug	Target	Effect	MIC (mg/L)
Fosfomycin	UDP-N-acetylglucosamine-3-enolpyruvyltransferase (MurA)	Inhibits peptidoglycan synthesis	4
Vancomycin	D-alanyl-D-alanine moieties of the NAM/ (N-acetylmuramic acid NAG-peptides	Inhibits peptidoglycan synthesis	4
Bacitracin	Undecaprenyl pyrophosphate (UPP), the lipid carrier	Inhibits peptidoglycan synthesis	300
D-Cycloserin	D-Ala:D-Ala ligase A and alanine racemase	Inhibits peptidoglycan synthesis	128
Cefazolin	Penicillin binding proteins (PBPs)	Inhibits peptidoglycan synthesis	2
Oxacillin	Penicillin binding proteins (PBPs)	Inhibits peptidoglycan synthesis	1
Mitomycin C	DNA	Replication-fork stalling	1
Ciprofloxacin	DNA gyrase	Replication-fork stalling	2
Norfloxacin	DNA gyrase	Replication-fork stalling	4
Streptomycin	Ribosome 30S subunit	Inhibits protein synthesis	16
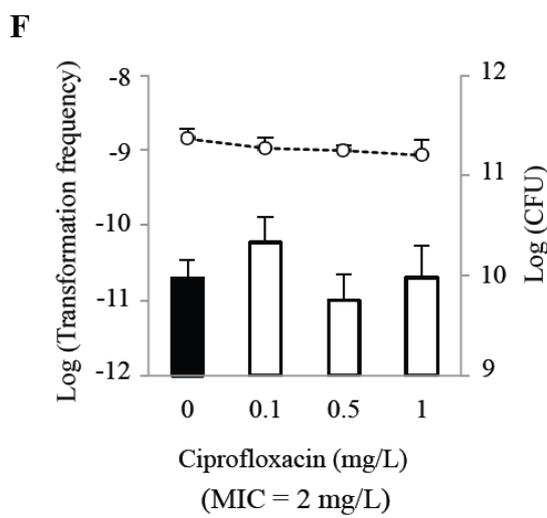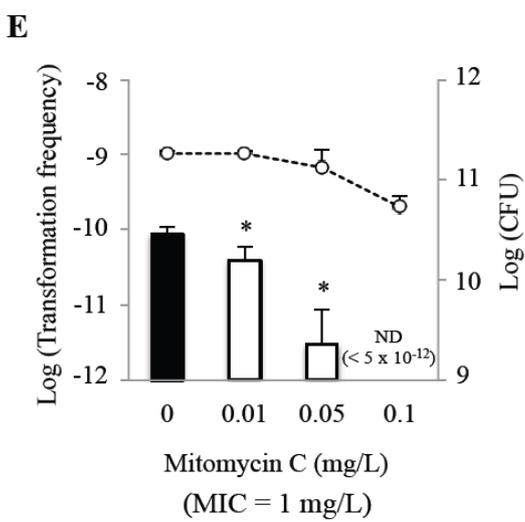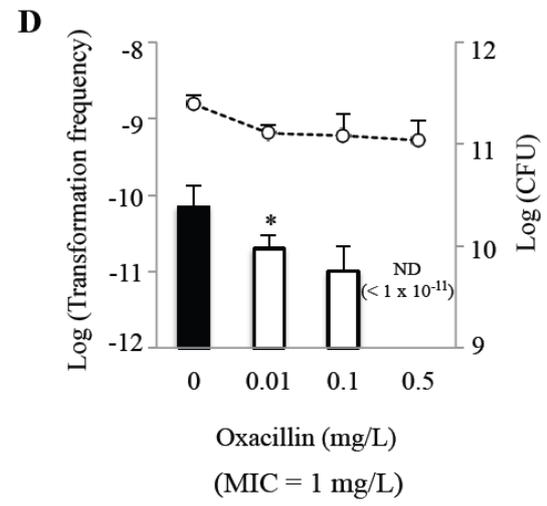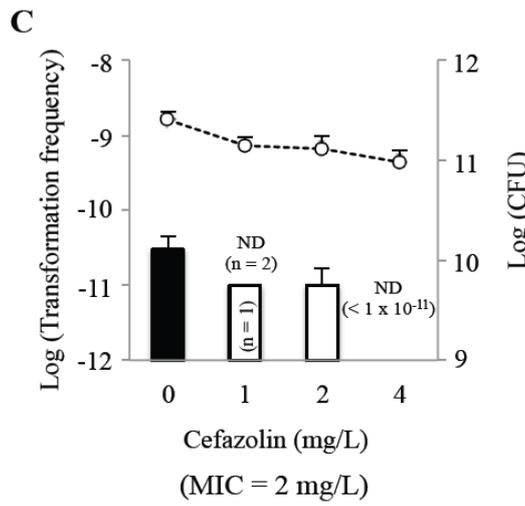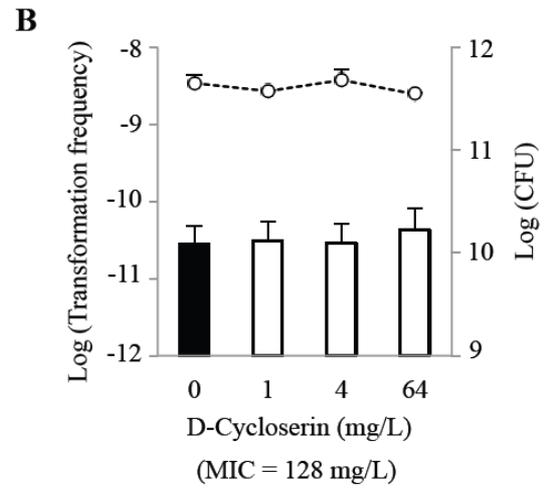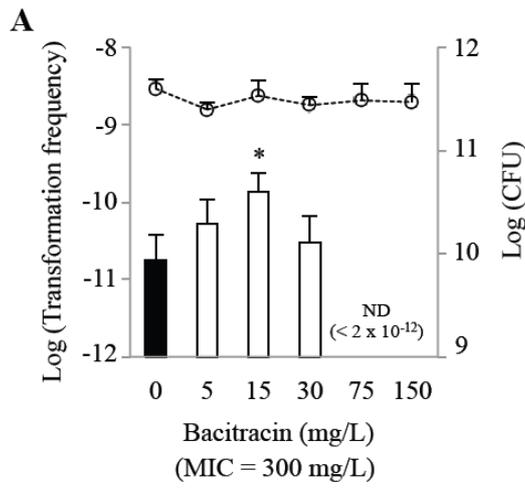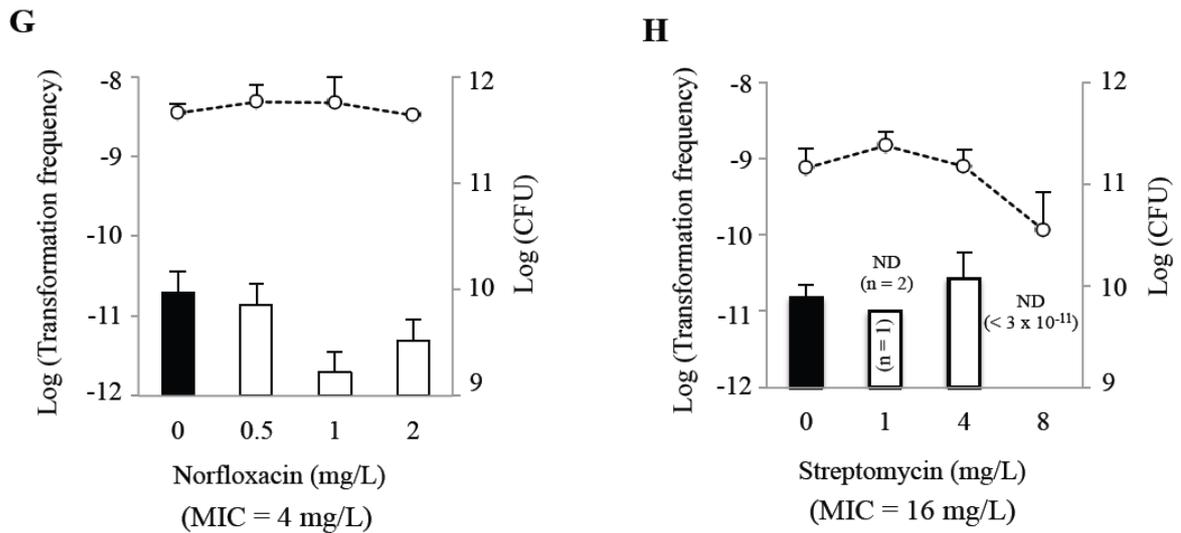
Figure 3-1. Effects of antibiotics on transformation. N315ex w/oφ h was exposed to different concentrations of antibiotics followed by the transformation with 10 μg of pHY300 plasmid. Bars: Log_{10} (transformation frequency); dotted lines: Log_{10} (CFU). Average values of at least three independent experiments are shown with SD. * $P < 0.05$ by Student's T-test for log values of frequencies. MIC values of antibiotics were determined in TSB medium. ND: none detected.
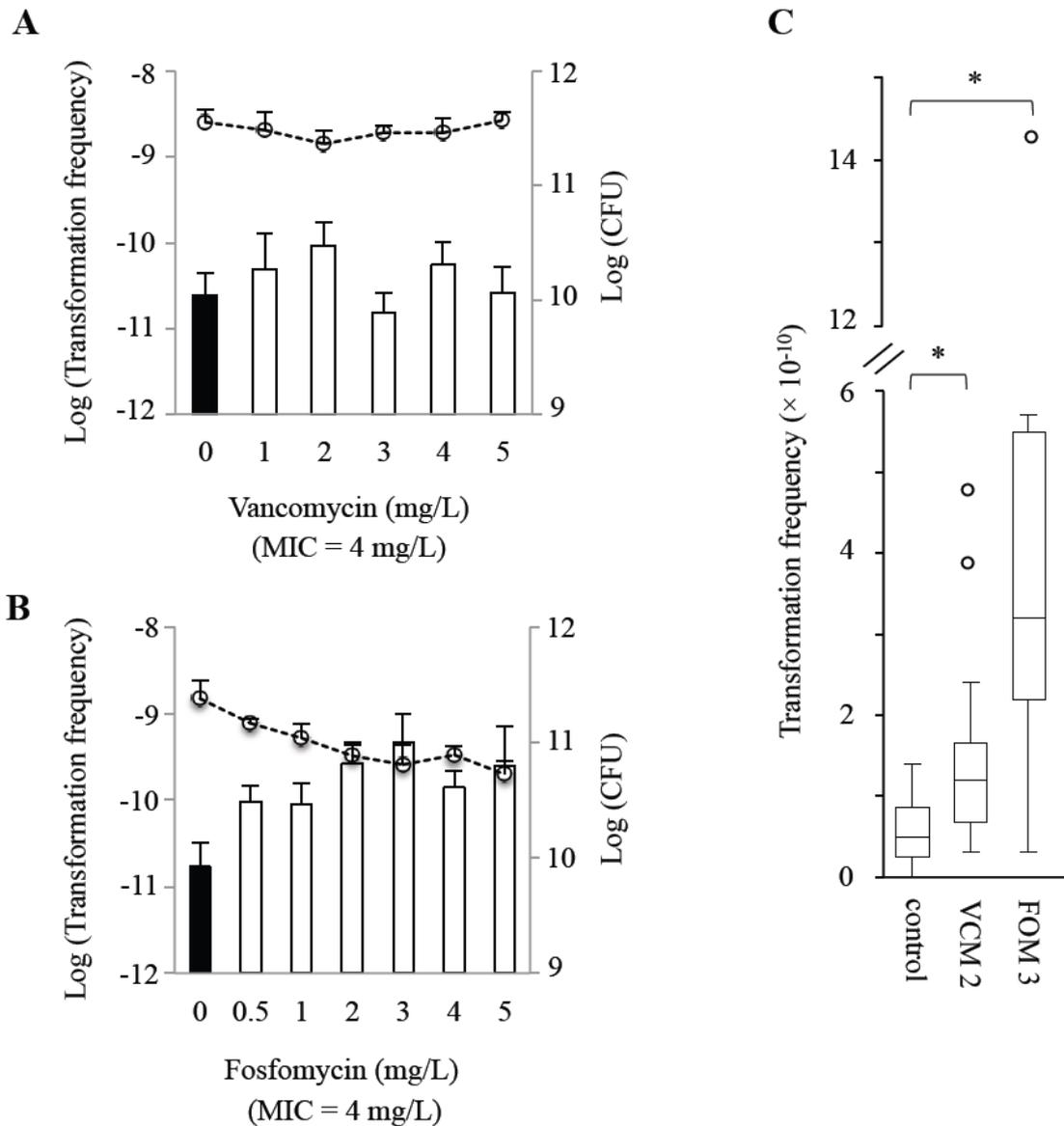
Figure 3-2. Effects of vancomycin (A) and fosfomycin (B) on transformation. (C) Repeated tests of the effects of vancomycin 2 mg/L (VCM2) and fosfomycin 3 mg/L (FOM3) on transformation frequencies are shown by box-plot. Boxes span the upper and lower quartile, lines inside the boxes indicate median, whiskers present the maximum and minimum values within the 1.5 quartile range, empty circles represent data points that are outside of this range. Control (no antibiotics), n = 10; VCM2, n = 9; FOM3, n = 10; * P < 0.05.
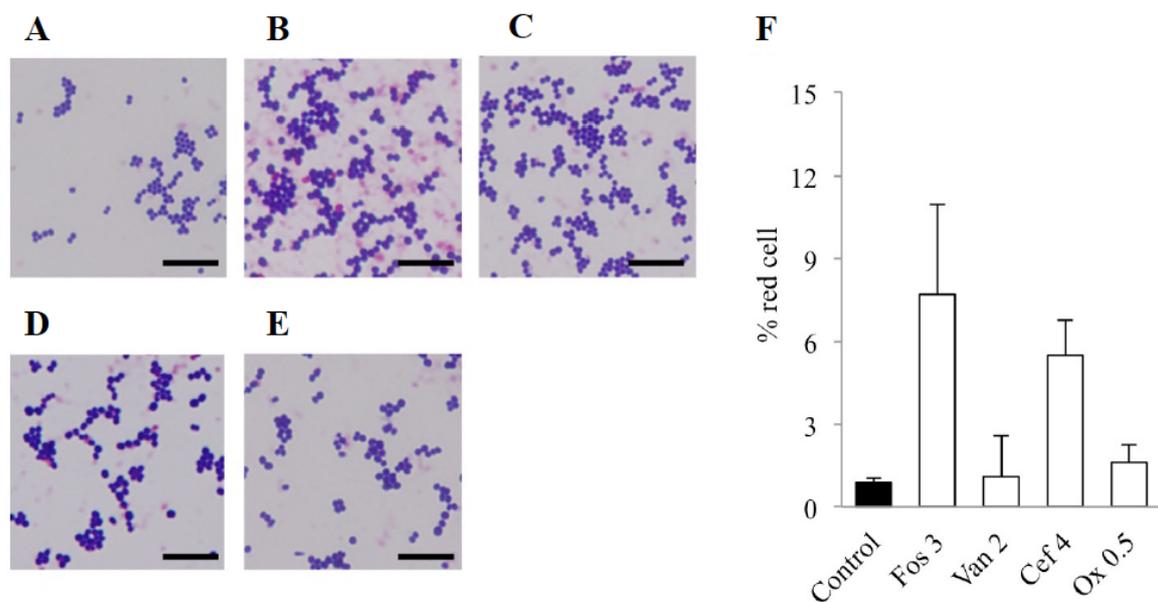
Figure 3-3. Gram staining images of *S. aureus* (N315ex w/oφ h) grown in TSB. (A) without antibiotics, (B) Fosfomycin 3 mg/L, (C) Vancomycin 2 mg/L, (D) Cefazolin 4 mg/L, (E) Oxacillin 0.5 mg/L. Scale bars = 10 μm. (F) Average percentages of negatively stained cells (red cells) from two independent experiments. Spherical cells alone were counted, and red cell debris was excluded from the calculation.
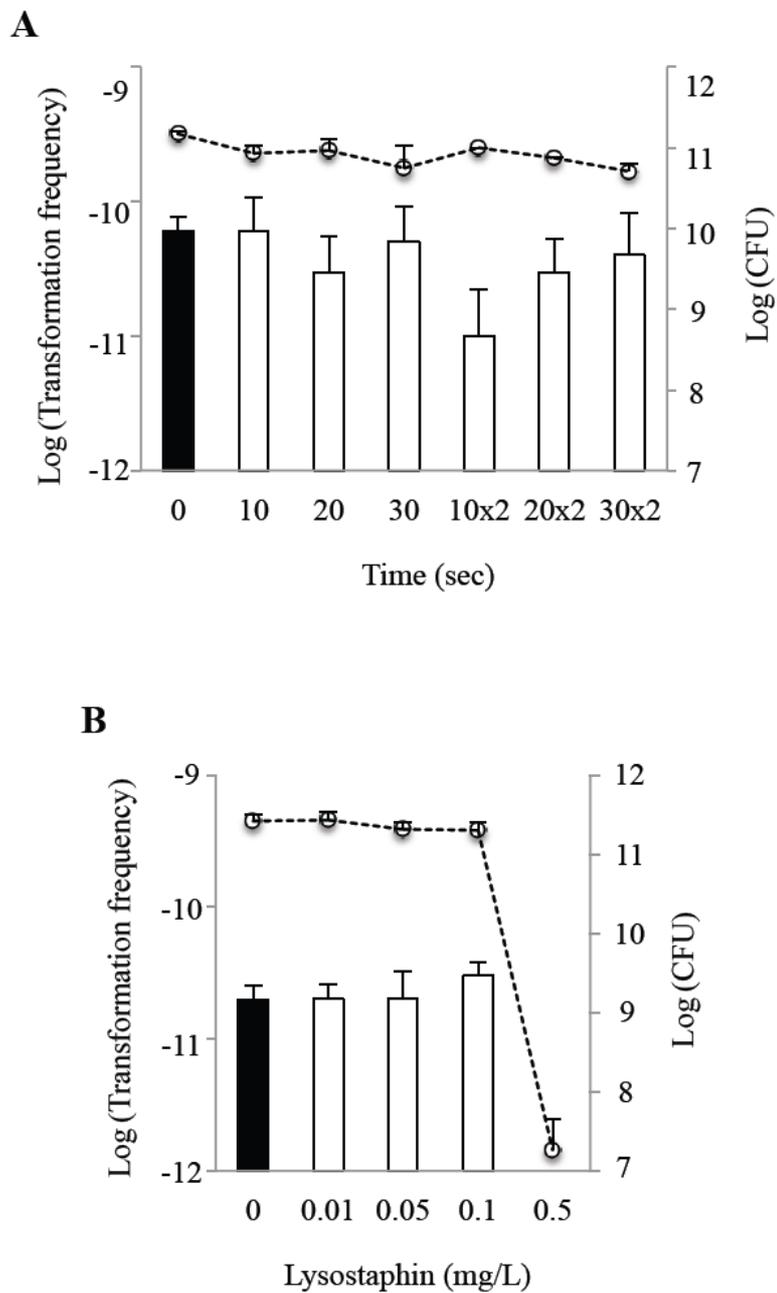
Figure 3-4. Transformation frequencies in bead beating or lysostaphin treated cells.
(A) Cells were treated by Fastprep system for the indicated time period prior to the transformation assay. (B) Cells were treated by lysostaphin. Mean and SD values are shown ($n = 3$).

Chapter 4

Summary and Perspective

The prominent evolutionary ability of *S. aureus* was evident from its history of the antibiotics resistance, which was further demonstrated by the genome sequencing study reported in 2001, where most of the virulence factors and antibiotics resistance genes were found in the MGEs [17]. For this reason, HGT ability of *S. aureus* has been a great concern, and each of the antibiotics resistance genes in distinct context of the MGEs were tested on their modes of transmission, as documented in a series of review articles and textbooks [99], [27], [25]. However, our knowledge had been limited to conjugation, transduction, and pseudo-transformation, because natural transformation was not known before 2012 [26]. In this context, it is now critical to re-evaluate the evolutionary ability of *S. aureus* from the aspect of natural transformation. Aiming to start this effort, I examined the transmission mechanisms of the *cfr* gene. By testing three major HGT pathways including natural transformation, the relevance of two pathways was clarified. In future studies, similar efforts need to focus onto other resistance determinants to fully understand the overall characteristics of the staphylococcal evolutionary ability.

As described before, the transformation efficiency in our first report was very low, and there remains the argument that the role of natural transformation would be quite limited in the evolution of *S. aureus* [25]. The bottleneck step(s) is still unknown: it can be DNA binding, internalization, double strand synthesis, or DNA recombination. It might also be the competence component(s) that are not regulated by SigH [32], [33] such as ComC (a signal peptidase that is indispensible for the maturation of the major pili ComGC), Com FA and ComFC (an ATP-binding protein and unknown function protein, respectively that are involved in the DNA transport across the membrane), and RecA (a protein required for homologous recombination). Tracking of competence developing steps can be addressed by observing the maturation of the competence machinery using immunofluorescence microscopy for the tagged components. With in this line, I have

prepared a series of strains carrying tagged competence genes, which will be useful for the future studies.

It is also important to explore the unknown situation where *S. aureus* becomes highly transformable. This effort would be reasonable, because competence-inducing conditions in Gram-positive bacteria are diverse among species [28], and we might not know the *bona fide* stimuli to induce the transformation. In order to get insight into this question, one strategy we took was to test a series of conditions using SigH expressing cells. Previous efforts focused on distinct growth media, and found the specific complete synthetic medium (CS2 medium) was the most appropriate medium for transformation. Notably, in the CS2 medium, wild-type *S. aureus* N315 strain increases the percentage of the SigH expressing cells [26]. This indicates that certain conditions where the expression of the cryptic SigH factor is released might be preferable conditions for the process of natural transformation. Therefore, revealing the signaling mechanisms for SigH expression is also important to know the hidden conditions for the efficient transformation. Here, the involvement of a series of signal sensing molecules needs to be tested including the SigB general stress responsive system (SigB-RsbW partner switching mechanism that is regulated by upstream stress sensing complex [100]), the transcription factors that directly sense stimuli (e.g. PerR, a transcription repressor that can sense oxidative stress [101]), and the two-component systems (TCSs). TCSs play important roles to sense and regulate the expression of genes in response to environmental stimuli. TCS are composed of a sensor kinase (membrane sensor protein carrying cytosolic kinase domain) and a response regulator (transcription factor regulated by phosphorylation). In the regulation of natural competence, the involvements of TCS have been reported in some species [102], [103], [104], [105], [106]. *S. aureus* possesses 17 TCSs [17]. So far, we have successfully obtained deletion mutants for each of the TCSs from the N315ex woφ as parental strain.

SigH activities in the TCS mutants can be measured by using a GFP reporter construct with SigH-dependent *comG* promoter [26]. Understanding the involvement of the specific TCS(s) in the control of competence would reveal the environmental cues affecting competence and transformation.

A part of the efforts to find out the conditions in which transformation can be elevated, I found that antibiotics have the effects on transformation: cell wall-affecting antibiotics modulate transformation; mitomycin C suppresses transformation. Antibiotics were previously tested in *S. pneumoniae* showing that mitomycin C and other DNA replication inhibitors (ciprofloxacin and norfloxacin) induced the expression of the late competence gene (*ssbB*) while cell wall-targeting antibiotics (vancomycin and ampicillin) were not involved. Thus effects of antibiotics on transformation are distinct among species, probably reflecting the distinct cellular processes as discussed in the Chapter 3. Antibiotics inducing *S aureus* transduction have also been reported [107]. Thus, it seems to be general that antibiotics have effect on HGT in bacteria.

Another interesting aspect would be to investigate the molecular basis of the interplay between natural transformation and other HGT pathways. Mitomycin C is often used to induce the lytic cycle from the lysogenic prophage, suggesting that it would induce the phage transduction, while suppressing the natural transformation. Previous studies showed that SigH plays a role in stabilizing the lysogenic state [108]. How HGT systems interact and control each other is also interesting question that must be addressed in future studies.

The long-term goal in the studies of antibiotics resistance and their dissemination is to conquer the problem of antimicrobial resistance. To prevent and stop the dissemination of resistance, we need to take it into account not only the dissemination among human society, but also the livestock and the environment ("One health" concept) (CDC, 2016).

Indeed, in the case of *S. aureus*, vancomycin resistance is thought to be transferred via vancomycin-resistant enterococci (VRE) [109]. VRE was developed through the excess use of antibiotics in poultry farms around South-East Asia [110]. Linezolid resistance would be similar situation. CNS species found in livestock are considered as the genetic pool of the antibiotics resistance. In such a context, epidemiological efforts of comprehensive surveillance of resistance, politics to reduce the use of unnecessary antibiotics, and education of a broad range of specialists were proposed as the critical action plan to cope with this global issue. I have studied the HGT mechanism from the aspect of molecular genetics, but now we need to consider how we could contribute to this global effort. In principle, we think conjugation and transduction can be blocked by inactivating the donor side, e.g. proper food cooking. However, free DNA alone can be the donor in the case of natural transformation, and to find how we could stop the DNA transformation would have significance. In this sense, the finding of the inhibitory effect of Mitomycin C was encouraging.

Acknowledgements

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LIST OF PUBLICATIONS

1. Fabio Cafini[*], **Le Thuy Nguyen Thi**[*], Federico Román, José Prieto, Sarah Dubrac, Tarek Msadek, Kazuya Morikawa. Methodology for the study of horizontal gene transfer in *Staphylococcus aureus*. J. Vis. Exp. (), e55087, doi: 10.3791/55087 (2016) – Peer reviewed.

2. **Le Thuy Nguyen Thi**, Veronica Medrano Romero, Kazuya Morikawa. Cell wall-affecting antibiotics modulate natural transformation in SigH-expressing *Staphylococcus aureus*. Journal of Antibiotics. 2016; 69(6):464-6. doi: 10.1038/ja.2015.132 – Peer reviewed.

3. Fabio Cafini, **Le Thuy Nguyen Thi**, Masato Higashide, Federico Román, José Prieto, Kazuya Morikawa. Horizontal Gene Transmission of *cfr* gene to MRSA and *Enterococcus*: role of *S. epidermidis* as reservoir and alternative pathway for the spread of linezolid resistance. Journal of Antimicrobial Chemotherapy. 2016; 71(3):587-92. doi: 10.1093/jac/dkv391 – Peer reviewed.

4. Aya J Takemura, Yuri Ushijima, **Le Thuy Nguyen Thi** and Kazuya Morikawa. Classical *rsbU*- strains of *S. aureus* exhibit better growth in synthetic nasal medium. "Microbes in the Spotlight: Recent Progress in the Understanding of Beneficial Harmful Microorganisms". ISBN-10: 1-62734-612-0 (2016). Book chapter.

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6. Kazuya Morikawa, Aya J. Takemura, Yumiko Inose, Melody Tsai, **Le Thuy Nguyen Thi**, Toshiko Ohta, Tarek Msadek. Expression of a Cryptic Secondary Sigma Factor Gene Unveils Natural Competence for DNA Transformation in *Staphylococcus aureus*. PLoS Pathog. 2012; 8(11):e1003003. doi: 10.1371/journal.ppat.1003003 – Peer reviewed.