Video Article

Methodology for the Study of Horizontal Gene Transfer in *Staphylococcus aureus*

Fabio Cafini*[1,2], Nguyen Thi Le Thuy*[3], Federico Román[4], José Prieto[5], Sarah Dubrac[6,7], Tarek Msadek[6,7], Kazuya Morikawa[1]

[1]Division of Biomedical Science, Faculty of Medicine, University of Tsukuba

[2]Department of Basic Biomedical Science, Universidad Europea de Madrid

[3]Human Biology Program, School of Integrative and Global Majors, University of Tsukuba

[4]Laboratory of Nosocomial Infections, Department of Bacteriology, Centro Nacional de Microbiología, Instituto de Salud Carlos III

[5]Division of Microbiology, Department of Medicine, School of Medicine, Universidad Complutense

[6]Biology of Gram-Positive Pathogens, Department of Microbiology, Institut Pasteur, Paris, France

[7]ERL3526, CNRS, Paris, France

*These authors contributed equally

Correspondence to: Fabio Cafini at fabio.cafini@universidadeuropea.es

URL: https://www.jove.com/video/55087
DOI: doi:10.3791/55087

Keywords: Immunology, Issue 121, *Staphylococcus aureus*, horizontal gene transfer, conjugation, transduction, transformation, antibiotic resistance.

Date Published: 3/10/2017

Citation: Cafini, F., Thi Le Thuy, N., Román, F., Prieto, J., Dubrac, S., Msadek, T., Morikawa, K. Methodology for the Study of Horizontal Gene Transfer in *Staphylococcus aureus*. *J. Vis. Exp.* (121), e55087, doi:10.3791/55087 (2017).

Abstract

One important feature of the major opportunistic human pathogen *Staphylococcus aureus* is its extraordinary ability to rapidly acquire resistance to antibiotics. Genomic studies reveal that *S. aureus* carries many virulence and resistance genes located in mobile genetic elements, suggesting that horizontal gene transfer (HGT) plays a critical role in *S. aureus* evolution. However, a full and detailed description of the methodology used to study HGT in *S. aureus* is still lacking, especially regarding natural transformation, which has been recently reported in this bacterium. This work describes three protocols that are useful for the *in vitro* investigation of HGT in *S. aureus*: conjugation, phage transduction, and natural transformation. To this aim, the *cfr* gene (chloramphenicol/florfenicol resistance), which confers the Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A (PhLOPSA)-resistance phenotype, was used. Understanding the mechanisms through which *S. aureus* transfers genetic materials to other strains is essential to comprehending the rapid acquisition of resistance and helps to clarify the modes of dissemination reported in surveillance programs or to further predict the spreading mode in the future.

Video Link

The video component of this article can be found at https://www.jove.com/video/55087/

Introduction

Staphylococcus aureus is a commensal Gram-positive bacterium that naturally inhabits the skin and nasal cavity of human beings and animals. This bacterial species is the leading cause of nosocomial infections in hospitals and healthcare settings. Moreover, its ability to develop resistance to different antimicrobial compounds has made the management of the infections caused by this bacterium into a global concern.

Two main pathways involved in the spreading of resistance phenotypes are known: the clonal dissemination of resistant genotypes and the dissemination of genetic determinants among the bacterial pool. In the case of *S. aureus*, different antibiotic resistance genes (as well as virulence determinants) have been found to be associated with mobile genetic elements (MGEs)[1]. The presence of these elements in the genome of *S. aureus* indicates that the acquisition and transfer of genetic material within the bacterial population could play an important role for *S. aureus* adaptation and evolution.

Genetic material can be exchanged through three well-known mechanisms of HGT in Gram-positive bacteria: transformation, conjugation, and phage transduction. Transformation involves the uptake of free DNA. To acquire foreign DNA, bacterial cells need to develop a special physiological phase: the competence stage. When this stage is reached, competent cells are capable of transporting DNA into the cytoplasm, acquiring new genetic determinants. In the case of *S. aureus*, the existence of natural transformation has been recently demonstrated[2]. In line with this, our group has shed light on the relevance of the expression of the SigH factor (a cryptic secondary transcription sigma factor) in the competence stage of development and on how its constitutive expression renders *S. aureus* capable of reaching the competence stage, which allows for the acquisition of resistant phenotypes by natural transformation[2].

Conjugation is a process involving the transmission of DNA from one living cell (donor) to another (recipient). Both cells must be in direct contact, allowing the DNA to be exchanged while being protected by special structures, such as tubes or pores. The transfer of DNA by this method requires the conjugative machinery. In *S. aureus*, the prototype conjugative plasmid is PGO1, which harbors the conjugative operon TraA[3].

Phage transduction involves the transfer of DNA from cell to cell through bacteriophage infection and implies the packing of bacterial DNA, instead of viral DNA, into the phage capsid. Most of *S. aureus* isolates are lysogenized by bacteriophages[1]. Upon stress conditions, prophages can be excised from the bacterial genome and shift to the lytic cycle.

These are the three well known mechanisms for DNA transmission in *S. aureus*. There are some additional transfer mechanisms, such as "pseudo-transformation"[2] and phage-like systems in the transfer of pathogenicity islands[4]. Recently, one group reported that "nanotubes" are involved in the transfer of cellular materials (including plasmid DNA) between neighboring cells[5,6], but a follow-up study has not appeared from other groups so far.

This work provides the necessary methodology to study HGT in *S. aureus* by addressing the three main transfer pathways: conjugation, transduction, and natural transformation. The results obtained with these methodologies were used to study the transmission of the *cfr* gene (chloramphenicol/florfenicol resistance) among *S. aureus* strains[7]. These three techniques are versatile tools for the investigation of MGE transmission in *S. aureus*.

Protocol

NOTE: The strains and materials used in this work are listed in **Table 1** and the **Table of Materials**, respectively. In the transmission experiments, N315 and COL *cfr*-positive derivatives were used as donors of the *cfr* gene (N315-45 and COL-45). These strains were previously obtained by conjugation, using as the donor a clinical *cfr*-positive *Staphyloccocus epidermidis* strain (ST2), following the standard conjugation protocol (see below). This strain harbored the *cfr* gene on a pSCFS7-like plasmid[7].

1. Conjugation Using the Filter-mating Method

NOTE: An *S. aureus* N315 strain carrying the *cfr* gene (N315-45)[7] (Cm^R) was used as the donor. COL[8] or Mu50[9] strains (Tet^R, Cm^S) were used as the recipients. Double-resistant colonies (Tet^R, Cm^R) able to grow in the presence of 32 mg/L of chloramphenicol plus 8 mg/L of tetracycline were considered as putative transconjugants and were analyzed to determine the presence of *cfr* by colony PCR and to determine the recipient susceptibility profile.

1. **Perform conjugation following a previously described protocol[10] with a few modifications:**
    1. Prepare 5 mL of overnight culture of the donor and recipient in Tryptic soy broth (TSB) medium (with and without 32 mg/L chloramphenicol, respectively), with shaking at 37 °C.
    2. Adjust the optical density (OD_{600}) of the overnight cultures to 1 (OD_{600} = 1.0) by using fresh TSB medium. Mix 0.5 mL of the donor culture (N315-45) with 0.5 mL of the recipient culture (COL or Mu50). Add 1 ml of PBS to the mixture.
    3. Transfer the mixture of bacteria onto a 0.45 µm filter membrane using a vacuum pump system.
    4. Put the filter membranes on a sheep blood agar plate. Incubate at 37 °C for 1 day.
    NOTE: In this step, enriched medium is necessary to allow the growth of double-resistant cells.
    5. Take out the filter membrane from the plate and suspend in 10 ml of phosphate-buffered saline (PBS). Vortex well to collect all the bacteria attached on the membrane.
    6. Make a serial 10-fold dilution of the bacterial suspension by using fresh TSB. Plate 100 µl of the 0-10^{-4} diluted samples on TSB agar (TSA) plates supplemented with 32 mg/L chloramphenicol and 8 mg/L tetracycline for the selection of transconjugants. Plate 100 µl of the diluted suspension (10^{-5}-10^{-6}) onto TSA plates with 8 mg/L tetracycline alone to count the total number of recipients. Incubate the plates at 37 °C for 18-24 hr.
    7. Analyze double-resistant colonies (putative transconjugants) for the presence of the *cfr* gene by colony PCR[7] and their susceptibility profiles (antibiogram).
    8. Determine the antibiogram by measuring the minimum inhibitory concentrations (MICs) of appropriate antibiotics according to the standard microdilution method or disk diffusion method[11]. The susceptibility profile of the transconjugants must be identical to the recipient, except for chloramphenicol (and other compounds affected by the *cfr* gene). This determination is essential to rule out tetracycline resistance developed by the donor strain.

2. Phage Transduction

NOTE: The bacteriophage MR83a belonging to the *Siphoviridae* family (laboratory stock) was used in the transduction experiments. N315-45 was used as the *cfr* donor for phage infection. N315, COL, or Mu50 strains were used as the recipients. The acquisition of *cfr* was determined by the ability of the recipient strain to grow in the presence of 32 mg/L chloramphenicol. Colonies growing under this condition were analyzed to determine the presence of *cfr* (by colony PCR) and to determine the susceptibility profile to rule out potential contamination.

1. **Phage amplification on the donor**
    1. Prepare an overnight culture of N315-45 in 5 ml of nutrient broth supplemented with 3.6 mM Ca^{2+} ($NBCaCl_2$) at 37 °C with shaking (180 rpm).
    NOTE: Calcium is required for phage infection. See the **Materials Table**. Nutrient broth from other companies might have issues, such as calcium precipitation.
    2. Prepare subcultures by diluting the overnight culture 1:1,000 in a final volume of 10 mL of $NBCaCl_2$ in 50 ml glass flasks or glass vials. Grow the bacteria for 1 hr at 37 °C with shaking (180 rpm).
    3. Prepare a series of diluted phage MR83a in $NBCaCl_2$ medium (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}). Add 20 µl of the diluted phage into the bacterial culture. Also prepare a control culture without phage infection, which serves as the positive control for bacterial cell growth and can be used to determine the phage titer the next day.
    4. Grow the cultures at 37 °C with gentle shaking (100 rpm) overnight.

NOTE: The cells will grow to some extent when the infecting phage is not in excess; then, they will enter into lysis phase due to the phage amplification through the lytic cycle. The culture without phage will show full growth, while cultures with phage will show distinct rates of transparency.
5. Select one or two cleared culture vials with the highest dilution of the added phage.
6. Transfer the cultures into 15 ml centrifuge tubes. Add 250 µl of chloroform and mix thoroughly by inverting the tubes.
7. Centrifuge the tubes at 5,000 *x g* for 20 min at 4 °C.
8. Transfer the supernatants to fresh tubes and store them at 4 °C until use.
NOTE: The phage is stable for at least a few months, but storing the phage preparation for too long reduces the titer and transduction efficiency.

2. **Measuring the phage titer (phage plaque assay)**
1. Prepare nutrient broth agar (1.5% agar) medium; keep it warm in a water bath at 55 °C. Add autoclaved 0.5 M $CaCl_2$ solution to the medium to a final concentration of 3.6 mM. Pour it into 90 mm Petri dishes ($NBCaCl_2$ plates).
2. Prepare an overnight culture of N315 in 5 ml of $NBCaCl_2$ at 37 °C with shaking; this can be substituted with the control culture described above (step 2.1.3).
3. Add 10 µl of the overnight culture into 200 µl of $NBCaCl_2$ and evenly spread it onto the $NBCaCl_2$ plate. Stop the spreading when the surface of the plate is covered with liquid. Let the plate dry.
4. Make a serial 1:10 dilution (from 10^{-5}-10^{-10}) of previously prepared phage (step 2.1.8) using $NBCaCl_2$ medium.
5. Spot 3 µl of each phage dilution onto the plate covered with bacteria.
6. Incubate the plate overnight at 30 °C.
7. Count the plaque numbers and calculate the phage titer using the following equation: Plaque forming unit (pfu)/ml = Number of plaques x Dilution factor/spotted volume (3 x 10^{-3} ml)

3. **Phage transduction**
NOTE: The phage pool prepared in the previous step (2.1.8) is used to test the transduction of the *cfr* gene into the *S. aureus* strains. In this experiment, strains N315, COL, or Mu50 are used as the recipients.
1. Prepare the overnight culture of N315, COL, or Mu50 in 5 ml of $NBCaCl_2$ at 37 °C with shaking (180 rpm).
2. Dilute the phage in $NBCaCl_2$ to ~10^9 pfu/mL.
3. In a 50 ml glass vial, mix 500 µl of overnight culture, 500 µl of fresh $NBCaCl_2$, and 1 ml of phage (~10^9 pfu/ml); the expected multiplicity of infection (MOI) must not exceed 1. Incubate the mixture at 37 °C with gentle shaking (100 rpm) for 30 min.
NOTE: Heat treatment of recipient cells just prior to the addition of phage (52 °C for 2 min to inactivate the endogeneous restriction enzymes) may increase the efficiency[12].
4. Add 50 µl of 20% Na_3-Citrate. Continue gentle shaking for 30 min at 37 °C.
NOTE: Na_3-Citrate acts as a moderate chelator of calcium.
5. Prepare melted brain-heart infusion (BHI) agar (1.5% agar) medium and keep it in a water bath at 55 °C.
6. Transfer the bacterium-phage mixture to a 100 ml flask, add 50 ml of warm BHI agar supplemented with 32 mg/L chloramphenicol, and mix well. Pour the mixture into the 90 mm Petri dishes.
NOTE: This method enables the detection of a small number of growing colonies (putative transductants).
7. Incubate the plate at 37 °C for 24-48 hr.
8. Further test the generated colonies (transductants) for resistance by transferring the colonies onto new BHI agar plates supplemented with 32 mg/L chloramphenicol. Confirm the presence of the *cfr* gene by colony PCR[7].

3. Natural Transformation

NOTE: The natural transformation assay in *S. aureus* was carried out following the method described in our previous study[2]. The N315 derivative, N2-2.1[2,7], was used as the recipient. In this strain, the *sigH* locus was duplicated so as to constitutively express SigH[2]. For detailed procedures on how to isolate SigH-expressing competence variants, please see a previous description[2]. If the resistance marker to be transferred is not chloramphenicol, pRIT-sigH (Cm^R) can be used to express SigH, as described previously[2]. Purified plasmid or whole DNA extract from *cfr*-acquired *S. aureus* COL-45[7] is used as the donor DNA for transformation.

1. **Preparation of donor DNA**
1. Cultivate COL-45 overnight with shaking at 37 °C in a 300 ml flask containing 50 mL of TSB supplemented with 32 mg/L chloramphenicol. Culture *E. coli* HST04 carrying pHY300 plasmid$^{(TetR)}$ to extract the plasmid for the control experiment.
NOTE: HST04 (*dam*$^-$/*dcm*$^-$) lacks the DNA methylase genes[13]. Other conventional strains with DNA methylases can also be used to prepare the DNA donor, because the DNA methylation state does not affect the transformation efficiency. Alternatively, pT181 plasmid purified from the *S. aureus* COL strain can also be used as a positive control for the transformation assay[2].
2. Collect the cells by centrifugation (8,000 *x g* for 10 min at 4 °C).
3. Extract the plasmids using a plasmid DNA extraction kit or a conventional DNA purification method to purify the whole DNA.
4. Quantify the purified DNA by spectrometer and keep it at 4 °C until use.
NOTE: Use a fresh DNA preparation for the transformation assay, usually less than one week old. Old DNA preparations reduce the efficiency of transformation.

2. **Transformation assay**
1. Culture the recipient cell (N2-2.1) overnight in 5 ml of TSB at 37 °C with shaking.
2. Transfer 0.5 ml of overnight culture into a 1.5 ml tube. Precipitate the cells by centrifugation (10,000 *x g* for 1 min at 4 °C).
3. Suspend the cells with 10 mL of CS2 medium in a 50 ml tube.
NOTE: CS2 medium is a complete synthetic medium that induces competence for natural transformation in *S. aureus*[2] (see the medium composition in the Material Table). Other standard laboratory media, such as TSB or BHI, are not suitable for transformation[2,13].

4. Grow the bacteria at 37 °C with shaking (180 rpm) until the late exponential phase (about 8 hr).
5. Harvest the cells by centrifugation (5,000 *x g* for 5 min at 4 °C).
6. Resuspend the cells in 10 ml of fresh CS2 medium.
7. Add 10 µg of purified plasmid or genome DNA (step 3.1.4) to the cell suspension. Shake at 37 °C and 180 rpm for 2.5 hr.
   NOTE: Shorter incubation times with DNA (<2.5 hr) result in lower transformation frequencies.
8. Collect cells by centrifugation (5,000 *x g* for 5 min at 4 °C).
9. Resuspend the cells in 10 mL of BHI medium. Mix the cell suspension with 90 ml of melted BHI agar (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. Swiftly cool and let the agar solidify.
10. Incubate the plates at 37 °C for 2 days.
11. Replicate generated colonies (transformants) by transferring the colonies (using toothpicks) to new BHI agar plates containing the appropriate antibiotics to confirm their resistance characteristics. Confirm the acquired resistance gene (*cfr* or *tetM*) by colony PCR[7].

Representative Results

The results represented here have been previously published (adapted from reference[7] with the publisher's permission). We studied the potential transmission pathways of the *cfr* gene, which causes low-level linezolid resistance and the expression of the PhLOPSA-resistance phenotype[14,15] in *S. aureus* strains, by investigating three mechanisms of HGT.

Figure 1 shows the results obtained in conjugative assays. The conjugation protocol is useful for inter-species transmission (A) as well as intra-species transmission (B), giving similar results using the same conjugative vector in different conjugative donors. The efficiency of conjugation is calculated as the Nº of transconjugants (colony-forming units, or CFU/ml)/Nº of recipient cells (CFU/ml). The obtained frequencies ranged from 1 x 10^{-6} to 1 x 10^{-5}, with similar values using *S. epidermidis* or *S. aureus* as the donor.

The results are summarized in **Table 2.** *cfr*-acquired *S. aureus* were able to further transfer the *cfr* gene to other *S. aureus* strains by conjugation as well as by phage transduction. However, the results suggest the absence of natural transformation for *cfr* transmission, although it was detected for a different resistance marker (*tetM* gene on the pHY300 plasmid).

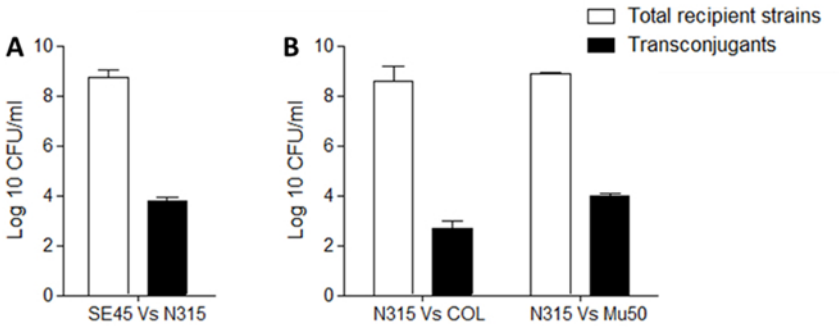

Figure 1: Representation of recipient and transconjugant CFUs obtained in conjugative assays. The clear bars represent the total recipient strains isolated after 18-24 hr of culture in selective media for recipient strains. The filled bars represent the total double-resistant strains obtained after 18-24 hr of culture in selective media for transconjugant strains. (**A**) Inter-species conjugative assay. *Staphylococcus epidermidis* (SE45) was used as the *cfr* donor, and the *Staphylococcus aureus* N315 strain was used as the recipient. The N315-45 transconjugant strain harbored the *cfr* gene inserted in a pSCFS7-like plasmid. This strain was used as the source of *cfr* in the MRSA-to-MRSA transmission assays. (**B**) MRSA-to-MRSA conjugative experiments. The previously obtained N315-45 was used as the donor, and the COL or Mu50 strains were used as the recipients. The average values of two independent experiments are shown with the standard deviation (SD).
Please click here to view a larger version of this figure.

Strain name	Description	Reference source
Bacterial strains		
SE45	Clinical isolatated *S. epidermidis*	7
N315	pre-MRSA, KmR, ErmR	9
COL	MRSA, carrying tetracycline resistance gene on pT181 plasmid	8
Mu50	MRSA, VISA	9
N315-45	derivative of N315, carrying *cfr* gene on a pSCFS7-like plasmid obtained from *S. epidermidis* by conjugation	7
COL-45	derivative of COL, carrying *cfr* gene on a pSCFS7-like plasmid obtained from *S. epidermidis* by conjugation	7
RN4220-45	derivative of RN4220, carrying *cfr* gene on a pSCFS7-like plasmid obtained from *S. epidermidis* by conjugation	7
N2-2.1	SigH active cell derived from N315, allowing cell natural competence for transformation	7
E. coli HST04 *dam*-/*dcm*- pHY300	*E. coli* HST04 *dam*-/*dcm*- (Takara) carrying tetracycline resistance pHY300 plasmid	11
Bacteriophages		
MR83a	Siphoviridae family	Laboratory stock
MR83-45	phage MR83a packing a pSCFS7-like plasmid carrying *cfr* gene after infection into N315-45	This study

Table 1: List of strains used in this work.

HTG	Donor	Recipient	Frequency
Conjugation	N315-45	COL	1.00×10^{-6}
	N315-45	MU50	1.29×10^{-5}
Transduction	N315-45	COL	1.00×10^{-11}
	N315-45	MU50	3.68×10^{-10}
	N315-45	N315	6.88×10^{-10}
Transformation	Plasmids (COL-45)	N2-2.1	ULD
	Whole DNA (COL-45)	N2-2.1	ULD
	pHY300 (control)	N2-2.1	6.52×10^{-10}

Table 2: HTG frequencies of *cfr* gene transmission obtained in MRSA-to-MRSA experiments. Conjugative transmission was evaluated using the N315 *cfr*-positive derivative (N315-45) as the donor. The frequency of transmission in these experiments is expressed as the Nº of transconjugants/recipient cells. Transduction was evaluated using the transducing phage MR83a, amplified from the N315-45 strain. The transduction frequency was calculated as the Nº of transductants/pfu. Transformation assays were performed using purified DNA (plasmid or whole cellular DNA) as donors. The transformation frequency was calculated as the number of transformants/recipient cells. ULD: under limit detection.

Discussion

This work describes the three major methods to study the HGT of genetic determinants in *S. aureus*. Although transduction and conjugation have been studied for decades, the existence of natural transformation was only recently recognized[2]. Thus, *S. aureus* is equipped with all of the three major modes of HGT, and testing all of them is required to clarify the possible dissemination pathways of genetic determinants. The aim of this work is to compile complete protocols and to provide practical information on the methodologies used in a previously published work[7]. Although conjugation and transduction protocols are available, this is the first paper in which a detailed transformation protocol is described.

Conjugation using the filter-mating method is a simple technique and can be applied to the study of conjugative transfer in different bacterial species[7,10]. By using the standardized inocula, recipient counts after 18-24 hr reach a value of ~10^9 CFU/mL. Transconjugant counts are variable, and values show strong strain-to-strain dependence, but typically, a transconjugant range from 10^2 to 10^5 was obtained when the

conjugation results were positive. Using the protocol provided here, the limit of detection achieved was <10 transconjugants/mL. This limit can be optimized by concentrating the filter suspension.

The natural transformation protocol described here was established in *S. aureus* N315-derivate strains. The use of CS2 medium is critical for transformation, since the transformation is undetectable in other standard laboratory media, such as TSB and BHI[13].

The use of long-term stored plasmids *(e.g.,* pT181 and pHY300) as the donor resulted in about a 10- to 50-fold reduced frequency (data not shown), suggesting that DNA quality could affect the transformation frequency. It is known that nicked plasmids are not suitable for natural transformation in *B. subtilis*[16].

DNA obtained from both *S. aureus* and *E. coli* can be used as the donor in natural transformation assays, suggesting that a restriction barrier does not inhibit the natural transformation. We also observed the same transformation frequency between the DNA prepared from *E. coli* HST04 (*dam*$^-$/*dcm*$^-$) lacking the DNA methylase genes and from JM109, supporting the idea that methylation status does not affect the transformation frequency.

It should be noted that the transformation frequency detected by using this protocol was low (~10^{-9}-10^{-10}), and the transformable strains are limited to N315 derivates[2]. It is likely that the transformation efficiency in *S. aureus* could be strain-specific, as has also been reported in other transformable bacteria[15,16,17]. Further studies are in progress to improve the transformation efficiency; this will be described elsewhere.

Phage transduction seems to be the most prevalent HGT mechanism in *S. aureus* because most *S. aureus* isolates are lysogenized by bacteriophages[18]. The infection ability of the transducing phage depends on host susceptibility and needs to be checked by plaque assay. Another limitation of phage transduction is the size of the DNA. Small DNA can be efficiently transferred, but DNA fragments larger than 45 kb cannot be packed in the staphylococcal phage head[19]. However, a novel giant staphylococcal phage has recently been isolated from the environment, and itcould conceivably be able to transfer larger DNA fragments[20].

The plaque assay method described in this work is easier than the conventional protocol, where top-agar is used. However, the present method requires that the bacterial cells are spread evenly in order to detect tiny plaques generated on the surface. To achieve the completely even spreading, we recommend spreading enough volume of the bacterial suspension on the agar surface, stopping when the liquid evenly covers the agar surface. Then, dry the plates in air-flow in a clean bench. If it is necessary to avoid the lysogenization of the transducing phage, a low multiplicity of infection is recommended (the MOI should not exceed 1). Lysogenized cells can be distinguished by their diminished susceptibility to phage in the plaque assay.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was partly supported by Takeda Science Foundation, Pfizer Academic Contribution and JSPS Postdoctoral Fellowship for Foreign Researchers (FC).

References

1. Lindsay, J. A. Genomic variation and evolution of *Staphylococcus aureus. IJMM.* **300**, 98-103 (2010).
2. Morikawa, K. *et al.* Expression of a cryptic secondary sigma factor gene unveils natural competence for DNA transformation in *Staphylococcus aureus. PLoS Pathog.* **8**, e1003003 (2012).
3. Caryl, J. A., & O'Neill, A. J. Complete nucleotide sequence of pGO1, the prototype conjugative plasmid from the Staphylococci. *Plasmid.* **62**, 35-38 (2009).
4. Novick, R. P., Christie, G. E., & Penades, J. R. The phage-related chromosomal islands of Gram-positive bacteria. *Nat. Rev. Microbiol.* **8**, 541-551 (2010).
5. Dubey, G. P., & Ben-Yehuda, S. Intercellular nanotubes mediate bacterial communication. *Cell.* **144**, 590-600 (2011).
6. Dubey, G. P. *et al.* Architecture and Characteristics of Bacterial Nanotubes. *Dev cell.* **36**, 453-461 (2016).
7. Cafini, F. *et al.* Horizontal gene transmission of the *cfr* gene to MRSA and *Enterococcus*: role of *Staphylococcus epidermidis* as a reservoir and alternative pathway for the spread of linezolid resistance. *J. Antimicrob. Chemother.* **71**, 587-592 (2016).
8. Dyke, K. G., Jevons, M. P., & Parker, M. T. Penicillinase production and intrinsic resistance to penicillins in *Staphylococcus aures. Lancet.* **1**, 835-838 (1966).
9. Kuroda, M. *et al.* Whole genome sequencing of meticillin-resistant *Staphylococcus aureus. Lancet.* **357**, 1225-1240 (2001).
10. Marraffini, L. A., & Sontheimer, E. J. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science.* **322**, 1843-1845 (2008).
11. Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Growth Aerobically - Seventh Edition: Approved Standard M7-A7.* CLSI, Wayne, PA, USA, (2006).
12. Edwards, R. A., Helm, R. A., & Maloy, S. R. Increasing DNA transfer efficiency by temporary inactivation of host restriction. *BioTechniques.* **26**, 892-894, 896, 898 passim (1999).
13. Thi, L. T., Romero, V. M., & Morikawa, K. Cell wall-affecting antibiotics modulate natural transformation in SigH-expressing *Staphylococcus aureus. J. Antibiot.* (2015).
14. Long, K. S., Poehlsgaard, J., Kehrenberg, C., Schwarz, S., & Vester, B. The *Cfr* rRNA methyltransferase confers resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A antibiotics. *Antimicrob. Agents Chemother.* **50**, 2500-2505 (2006).

15. Ando, T. *et al.* Restriction-modification system differences in *Helicobacter pylori* are a barrier to interstrain plasmid transfer. *Mol microbiol.* **37**, 1052-1065 (2000).
16. Evans, B. A., & Rozen, D. E. Significant variation in transformation frequency in *Streptococcus pneumoniae*. *ISME J.* **7**, 791-799 (2013).
17. Wilson, D. L. *et al.* Variation of the natural transformation frequency of *Campylobacter jejuni* in liquid shake culture. *Microbiology.* **149**, 3603-3615 (2003).
18. McCarthy, A. J., Witney, A. A., & Lindsay, J. A. *Staphylococcus aureus* temperate bacteriophage: carriage and horizontal gene transfer is lineage associated. *Front Cell Infect Microbiol* **2**., 6 (2012).
19. Lindsay, J. A. *Staphylococcus aureus* genomics and the impact of horizontal gene transfer. *IJMM.* **304**, 103-109 (2014).
20. Uchiyama, J. *et al.* Intragenus generalized transduction in *Staphylococcus spp.* by a novel giant phage. *ISME J.* **8**, 1949-1952 (2014).

