Novel MprF amino acid mutations contributing to daptomycin non-susceptibility

Hayato Kawada
Department of Environmental Microbiology, Kitasato University of Graduate School of Medical Sciences
Tsubasa Hori
Department of Environmental Microbiology, Kitasato University of Graduate School of Medical Sciences
Masaki Nakamura
Department of Environmental Microbiology, Kitasato University Graduate School of Medical Sciences
Yuko Kimura
Department of Microbiology, Kitasato University School of Allied Health Sciences
Kurumi Sato
Department of Microbiology, Kitasato University School of Allied Health Sciences
Mayu Yoshida
Department of Microbiology, Kitasato University School of Allied Health Sciences
Hidehito Matsui
Infection Control Research Center, Kitasato University Institute for Life Sciences, Kitasato University
Hideaki Hanaki
Infection Control Research Center, Kitasato Institute for Life Sciences, Kitasato University
Hidero Kitasato (hkita@kitasato-u.ac.jp)

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Abstract

Background Daptomycin (DAP) is a cyclic lipopeptide antibiotic with potent bactericidal activity against gram-positive bacteria, and use to treat infections due to methicillin-resistant Staphylococcus aureus (MRSA). It was reported that MprF that is bacterial membrane protein and affect to DAP non-susceptible (DAP-NS), and it was generally considered that electrically repulsion by MprF mutation was thought to be contributed to DAP-NS. However, a lot of mechanisms were proposed previously and still controversial. Therefore, in this study, we found new MprF mutations from clinical MRSAs then introduced those MprF mutations into sequenced strain (Staphylococcus aureus N315) in order to evaluate those effects to DAP-NS. Results We analysed the drug susceptibility profile of three MRSA clinical isolates and performed MprF sequence. Two of the three MRSA isolates showed DAP-NS and had different mutations of MprF (N450_I451 insI) and MprF (T345P), which have not been reported before. Then the mutations we identified were introduced into Staphylococcus aureus N315 (S. aureus N315), and we evaluated its susceptibility profile and positive charge of cell envelop to elucidate effect of DAP susceptbility. The MIC of DAP was increased by introduction of these mutations into S. aureus N315. In addition, the positive charge of the cell membrane was significantly increased in MprF(T345P), but not in MprF(N450_I451 insI). Conclusions These findings suggest that the two novel mutations contribute to DAP-NS. Furthermore, we suggested that the mechanism of DAP-NS was different depending on the mutation type and/or position of the amino acid.

Background

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most important gram-positive pathogen causing nosocomial infections and remains a worldwide problem despite the use of various antibiotics and infection control regimens [1]. In addition to hospital-associated MRSA infection, community-associated MRSA infection has recently been spreading among children and healthy young persons without risk factors for nosocomial infection [2-4]. Moreover, livestock-associated MRSA infection has become a global problem affecting all ecosystems [5]. In addition to showing beta-lactam resistance, MRSA has acquired resistance to various other antibiotics, including aminoglycosides, macrolides, chloramphenicol, tetracyclines, and fluoroquinolones [6]. This serious situation has led to worldwide advocacy for better antimicrobial stewardship.

Daptomycin (DAP) is a cyclic lipopeptide with potent bactericidal activity against gram-positive bacteria that is used to treat MRSA infection. It binds to the cell membrane of gram-positive bacteria in a calcium ion-dependent manner and causes membrane depolarization [8-11]. DAP was approved for soft tissue MRSA infections and right heart endocarditis by the US Food and Drug Administration (FDA) in 2003 and 2006, respectively [7]. It has become a key antibiotic for treating severe acute infections caused by gram-positive bacteria, such as sepsis, since the onset of antibacterial activity is rapid without provoking the release of pyrogenic substances such as teichoic acid [12]. DAP is also widely used to treat chronic skin and soft tissue infections, as well as chronic infections associated with biofilm formation like prosthetic joint infections, due to its activity against dormant bacteria [13, 14]. Although resistance to

DAP was uncommon, detection of non-susceptible strains and treatment failure have been reported recently as its use increases. DAP non-susceptibility (DAP-NS) is thought to be caused by mutation of a membrane protein known as multiple peptide resistance factor (MprF), which synthesizes lysyl-phosphatidylglycerol (L-PG) and facilitates its translocation to the outer membrane leaflet from the inner leaflet to maintain a positive membrane charge [7]. However, the contribution of MprF mutation to DAP-NS and the mechanism of DAP-NS have not been fully clarified, although several mechanisms have been suggested. The most common DAP-NS hypothesis is that electrically repulsion of DAP by increasing the positive charge of the cell membrane as a result of L-PG translocation was promoted by MprF mutation [15]. On the other hand, it has also been reported that MprF indirectly contributes to increasing cell wall thickness and cell membrane fluidity [16]. Furthermore, *dlt* operon mutations may contribute to DAP-NS [17], with various factors being involved in a complex resistance mechanism among different MRSA strains.

We investigated the mechanism of DAP-NS by studying three clinical isolates of MRSA from a patient with septic arthritis of the hip. We found that these isolates harbored two previously unreported mutations of MprF. In addition, we consider that the cause of controversial about contribution of MprF and mechanism of DAP-NS was clinical bias. Then, we genetically introduced sequenced MRSA strain (*Staphylococcus aureus* N315) [18], and measured minimum inhibitory concentration (MIC) and positive charge of the cell membrane to evaluate the contribution to DAP-NS.

Results

MICs for the MRSA isolates

We measured the MICs of various antimicrobial agents for the three chronological MRSA isolates (S1-S3) by the microdilution method using dry plates (Table 2) and by the disc susceptibility testing method using the E-test (Fig. 2A). S1 and S3 were found to be DAP-NS, although these strains were still susceptible to other antibiotics that are used to treat MRSA infection (VCM, TEIC, and LZD). On the other hand, S2 (the isolate from a blood sample collected just before starting DAP administration) was susceptible to DAP as well as the other antibiotics. In addition, the color of S2 colonies was slightly different from that of S1 and S3 colonies.

In the E-test of S1, an independent colony was recognized inside the DAP inhibition area, raising the possibility of hetero-resistance. Therefore, population analysis of S1, S2, and S3 was carried out, but the results did not suggest hetero-resistance of S1 (Fig. 2B).

Genotypes of the MRSA isolates

When the genotypes of the three clinical isolates were analysed by the POT method, all three isolates showed the same electrophoretic pattern (POT number: 93-154-125), indicating that these

isolates were derived from the same parent strain of MRSA (Fig. 3).

MprF sequence

To investigate the mechanism of DAP resistance, we analysed the sequence of *mprF* (which has been reported as a cause of DAP-NS), and we used DNA editing software to compare the findings between the three MRSA isolates and the reference strain, *S. aureus* N315 (Fig. 4). In S1, we identified insertion of ATT between nucleotides 1350 and 1351, corresponding to insertion of isoleucine between amino acids 450 and 451 of MprF (white arrow). In S3, substitution of A by C was noted at nucleotide 1033, corresponding to a change from threonine to proline at amino acid 345 of MprF (black arrow). These two mutations of MprF have not been reported previously. On the other hand, no *mprF* mutation was observed in S2, which was a DAP-S isolate. None of the strains showed mutation of *dlt*AB, which has also been reported to influence DAP susceptibility.

Influence of MprF mutation on DAP susceptibility of genetically transformed S. aureus N315

To further investigate the relationship between the novel mutations detected in this study and DAP-NS, we generated *S. aureus* N315 MprF (N450_I451 insI) and *S. aureus* N315 MprF (T345P). Then we measured the MICs of antibiotics for these transformed strains by the microdilution method using dry plates, and compared with S. aureus N315 MprF-WT introduced no mutated MprF. We found that the MICs of antibiotics were higher for both strains of mutant *S. aureus* N315 than for *S. aureus* N315 MprF-WT (Table 3). In addition, the MIC of VCM was higher for the two mutant strains, although it was not increased for the clinical isolates. This finding raised the possibility that MprF mutation could be involved in cross-resistance against glycopeptide antibiotics.

Measurement of positive charge of the cell membrane

Finally, to elucidate the mechanism of DAP-NS, we investigated cytochrome *C* binding assay to evaluate positive charge of cell membrane using three genetically introduced strain (Fig. 5). As a result, the strain introduced MprF(T345P) mutation was significantly increased than N315 MprF-WT. In contrast, no significant changing was observed in MprF(N450_I451 insI), although MIC was increased by this mutation.

Discussion

We detected two novel mutations of MprF in clinical isolates of MRSA displaying DAP-NS. To investigate the contribution of these mutations to DAP-NS, we introduced them into a known strain of MRSA, leading

to an increase in the MIC of DAP in both cases. These findings suggested that the novel mutation could be possible to make a contribution to DAP-NS in MRSA. Furthermore, our data suggest that these strains were acquired DAP-NS by different mechanisms depend on the mutation type and/or position of the amino acid.

Among the three clinical isolates of MRSA that were thought to be derived from the same strain, we found S1 and S3 had acquired DAP-NS, but S2 was still susceptible to DAP. We detected two novel MprF mutations in the DAP-NS clinical isolates (T345P in S1 and N450_I451 insI in S3), while no mutation was observed in the DAP-S isolate. Recently, a number of mutations contributing to DAP-NS have been reported, and the region of MprF from amino acids 276 to 357 is known as a “hot spot” for mutations influencing DAP susceptibility [19]. In particular, many mutations of amino acid 345 have been reported, such as T345I, T345A, and T345K, but this is the first report about substitution of proline for threonine [20]. Proline is unique among the amino acids because it is a secondary amino acid [21]. Structural and functional analyses, such as three-dimensional analysis, assessment of L-PG translocation, and phenotypic studies, may be required to clarify the structural influence of T345P on DAP susceptibility.

Next, we genetically introduced the MprF mutations into a known strain of *S. aureus* to further investigate their contribution to DAP-NS. We demonstrated that introduction of either mutation into *S. aureus* N315 led to an increase of the MIC of DAP (Table 3), suggesting that the two MprF mutations contributed to loss of DAP susceptibility. This study also provided the first evidence that these MprF mutations contribute to DAP-NS without any influence of the clinical background because we used a well-characterized MRSA strain (*S. aureus* N315) that was not a clinical isolate. Furthermore, we analysed the positive charge of the cell membrane in the MprF mutant strains by using the cytochrome *C* binding assay to obtain additional evidence about the mechanism of DAP-NS (Fig. 5). We observed a significant increase of positive charge of the cell membrane with the T345P strain, suggesting that DAP-NS was related to electrical repulsion of DAP in this strain. In contrast, positive charge of the cell membrane was not altered by introduction of N450_I451 insI, suggesting that another mechanism reduced DAP susceptibility in this strain. The role of MprF in DAP-NS has not been fully clarified by previous investigations of clinical isolates [22, 23]. These reports and our findings indicate that the mechanisms may vary among different MRSA strains, with electrical repulsion only being one possibility. Mishra et al. reported that DAP-NS of *S. aureus* apparently involved multifactorial strain-specific adaptive mechanisms, and they suggested that various mechanisms may be associated with certain phenotypes, such as an increase of teichoic acid in the cell wall or increased membrane fluidity [24]. MprF is deeply involved in the two-component system, and it has been reported that the expression of various proteins (especially those contributing to cell membrane synthesis) is increased by MprF mutation [25]. Taken together with our data, it can be suggested that the phenotypic features associated with DAP-NS depend on the type or position of the amino acid mutation, even in the same strain. Analysis of the phenotypic changes related to mutation could help to elucidate the detailed mechanism of DAP-NS. In addition, our MprF mutated strains have been generated without clinical background as compared with previous reports [17, 20, 22, 24]. The possible influence of host antimicrobial peptides on bacterial phenotypes cannot be denied, since cross-resistance to DAP and antimicrobial peptides has been reported because

bacteria use the same strategy to neutralize these peptides and DAP [26, 27]. Hence, we strongly believe that the analysis of genetically introduced strains is important, and that we expect that it is possible to gain important knowledge about the mechanism of DAP-NS by MprF mutation.

We found that the MIC of DAP was lower for the strains with MprF mutations compared to the clinical isolates (S1 and S3), possibly due to the characteristics of the recipient strain. Hiramatsu et al. reported that *S. aureus* N315 might be more sensitive to antibiotics than clinical isolates of MRSA because its nucleotide sequence partly differs from that of other MRSA strains [18] and this difference may influence DAP susceptibility. It is possible that the MIC of DAP would have been increased by using another laboratory strain of *S. aureus*, such as Mu50, as the recipient.

Only S2 showed DAP sensitivity among the three clinical isolates, suggesting that S2 may have lost resistance to DAP during treatment. It was recently reported that DAP-NS MRSA has a higher “fitness cost” than normal MRSA [28], supporting our hypothesis about variation of the DAP susceptibility of clinical MRSA isolates (S1, S2, and S3) due to antibiotic treatment. The “fitness cost” is an indicator of the cost of evolution, and bacteria generally evolve to show greater environmental adaptability with a lower fitness cost. DAP-NS isolates could survive during DAP administration with a higher fitness cost, since their adaptability to an environment containing DAP was superior to that of DAP-S MRSA. Conversely, DAP-S MRSA is better adapted for survival in an environment without DAP, a concept supported by the report that DAP-NS isolates show slower growth than normal MRSA in the absence of DAP [29]. We excluded the possibility that S2 was the original strain, with S1 and S3 gaining DAP-NS during DAP treatment, because the color of its colonies was slightly different. In conclusion, if a patient’s symptoms do not improve after administration of DAP for two weeks, discontinuation of DAP and switching to other antibiotics may be warranted to avoid clinical failure due to DAP-NS.

Conclusions

In conclusion, we found that the novel MprF mutations (T345P and N450_I451 insI) from clinical isolated MRSAs in this study and those mutations were deeply contributed to DAP-NS. In addition, T345P mutation was contributed to increase of positive charge of cell membrane in the mechanism of DAP-NS, using genetically introduced *S. aureus* N315 strain. In contrast, N450_I451 insI was acquired by the other mechanisms, such as cell membrane fluidity, cell wall thickness and so on.

Methods

Bacterial strains

Three clinical isolates of MRSA (S1, S2, and S3) were obtained from a patient with septic arthritis of the hip who was admitted to the Orthopedic Surgery Department of Kitasato University Hospital (Fig. 1). These MRSA isolates were identified on the basis of colony morphology and by using the WalkAway

system (Beckman Coulter, California, USA). This study was approved by the Hospital Ethics Committee of Kitasato University School of Medicine (approval no. B17-013).

S. aureus N315 was used for introduction of mutations and another *S. aureus* strain (ATCC 29213) was used as a standard DAP susceptible (DAP-S) strain for quality control of drug susceptibility tests. *S. aureus* N315 was kindly provided by Dr. Katayama (Juntendo University, Tokyo, Japan), and *S. aureus* ATCC 29213 was purchased from the American Type Culture Collection (ATCC).

Measurement of minimum inhibitory concentrations (MICs)

MICs were measured by the microdilution method using dry plates (Eiken, Tokyo, Japan) and by the disc susceptibility testing method using the E-test (bioMerieux, Marcy l'Etoile, France) according to the protocols of the respective manufacturers. The antibiotic susceptibility profile of each MRSA strain was determined according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI).

Bacterial population analysis

Each isolate was cultured overnight at 37°C in tryptic soy broth (TSB) and was harvested by centrifugation at 8,000 rpm for 5 min. After washing 3 times in saline, the pellet was resuspended in saline at OD = 0.26 ± 0.02 and diluted (10^{-0} to 10^{-6}). Then 100 μl of each bacterial solution was plated onto Mueller Hinton agar (MHA) containing DAP (0 - 5 μg/ml) and incubated for 48 h at 37°C, after which colonies were counted.

Genotype analysis using a PCR-based open reading frame typing (POT) kit

Genotypes of the MRSA isolates from the patient with septic arthritis were analysed by using a CicaGeneus Staph POT kit (Kanto Chemical, Kanagawa, Japan) according to the manufacturer's directions. In brief, certain *S. aureus*-specific genes (including *femA* and *mecA*) were amplified by multiplex PCR and then detected by 4% agarose gel electrophoresis, allowing strain-level identification based on the POT number [30, 31].

Sequencing

Bacterial colonies were subjected to PCR as follows. Single colonies grown on tryptic soy agar (TSA) (Eiken, Tokyo, Japan) were collected and mixed with 10 μl of sterile saline, after which 100 μl of lysis buffer [20 μg/ml of lysostaphin (Sigma, MO, USA) and 1 kU/ml of achromopeptidase (Sigma, MO, USA)] were added then incubated for 10 min at 37°C. Then nuclear protein was denatured by adding 50 μl of 0.5 M KOH, followed by neutralization with 50 μl of 1 M Tris-HCl (pH 6.8). Next, 1 μl of the resulting solution was added to a PCR reaction mixture with a total volume of 25 μl containing TaKaRa Taq HS perfect MIX (TaKaRa, Shiga, Japan) and 10 pmol of primers (Table 1), and the target genes of *S. aureus* (*mprF* and *dlt*AB) were amplified for sequence analysis by using a TaKaRa PCR Thermal Cycler Dice (TaKaRa) according to the manufacturer's instructions. An aliquot (5 μl) of the PCR product was subjected to electrophoresis on 1% TaKaRa agarose gel L03 (TaKaRa, Shiga, Japan), while the remaining 20 μl of the

PCR product was purified using Exo-SAP-IT (Affymetrix, CA, USA). Then 10 µl of primer mixture (Table 1) was added and sequencing was done according to the guidelines of Eurofins Genomics (Tokyo, Japan). The sequence obtained was compared with that of *S. aureus* N315 (GeneBank: BA000018.3) by using ApE DNA editing software.

Culture of competent S. aureus N315

S. aureus N315 cells were cultured overnight at 28°C in 2 ml of B2 broth, followed by dilution to 200 ml and further incubation at 28°C until an optical density at 590 nm (OD_{590}) of 0.4 - 0.6 was reached. Then bacterial growth was stopped by rapid cooling, and the culture fluid was centrifuged for 5 min at 12,000 g and 4°C, after which the pellet was washed in chilled sterile water and subsequently washed in 10 % glycerol. The pellet was re-suspended in 2 ml of 10 % glycerol, and the suspension was dispensed into PCR tubes and immediately frozen with acetone/dry ice.

Introduction of mutant or defective MprF into S. aureus N315

The pIMAY plasmid was kindly provided by Dr. T. Foster (Trinity College, Dublin, Ireland) and was used to introduce MprF mutations into *S. aureus* N315 according to the published method [32]. The *mprF* of *S. aureus* N315 was cloned into a multi-cloning site of the pIMAY vector, which was designated as pIMAY-*mprF-wt*, after which new MprF mutations of interest were introduced into pIMAY-*mprF-wt* by using a PrimeSTAR® Mutagenesis Basal kit (TaKaRa). Two primers (*mprF*-N315_1350_ins-ATT_F, R and *mprF*-N315_1033_A>C_F, R) were designed to generate pIMAY-*mprF*-N450_I451 insI and pIMAY-*mprF*-T345P, respectively (Table 1). The recombinant vectors were amplified in *E. coli* JM109 and then were introduced into competent *S. aureus* N315 cells by electroporation [25 µF, 2.5 µV, 100 Ω]. Subsequently, strains were selected by culture for 48 hours at 28°C in TSB containing 10 µg/ml chloramphenicol (Cm). In addition, point mutations of MprF were introduced into *S. aureus* N315by homologous recombination using a modification of the reported protocol [32]. In brief, integration of vectors containing mutant *mprF* into the bacterial chromosome was achieved by plating cells on TSA+Cm (10 µg/ml) and incubation overnight at 39°C. Integration of the vectors was confirmed by colony PCR using primers that targeted the multi-cloning sites and the integrated *mprF* gene. A colony formed exclusively by cells with the integrated vector was suspended in 2 ml of TSB and incubated overnight at 28°C with shaking. Then the culture fluid was diluted (10^{-1} to 10^{-6}-fold) and plated on TSA containing 1 µg/ml anhydrotetracycline (ATc), followed by incubation for 48 hours at 28°C. Large colonies were streaked onto TSA containing ATc or Cm, and then incubation was done overnight at 37°C. Finally, a colony was selected that grew on TSA+ATc, but not on TSA+Cm, and the *mprF* mutation was confirmed by sequencing.

Cytochrome C binding assay

The cytochrome *C* binding assay was performed by modifying a previously published method [33-35]. Briefly, MRSA strains were cultured in tryptic soy broth until the logarithmic growth phase and then the cultures were centrifuged. The cell pellets were washed three times with 20 mM MOPS buffer (pH 7.0), adjusted to an OD_{590} of 1.00 ± 0.02, and collected by re-centrifugation. Then the pellets were re-

suspended in 250 µl of MOPS buffer, cytochrome *C* (Sigma, Tokyo, Japan) was added at a final concentration of 0.5 mg/ml, and incubation was done at room temperature for 20 min. After centrifugation at maximum speed for 20 min, the supernatant was filtered through a 0.2 µm filter (Sartorius, Göttingen, Germany), and free cytochrome *C* in the supernatant was measured with a GeneQuant 100 (GE HealthCare, Chicago, U.S.A.) at OD_{530}. The amount of cytochrome *C* bound to the bacteria was calculated relative to the control (supernatant incubated without bacteria).

Abbreviations

Cm: chloramphenicol

DAP: daptomycin

DAP-NS: daptomycin non-susceptibility

L-PG: lysyl-phosphatidylglycerol

MprF: multiple peptide resistant factor

MRSA: methicillin-resistant *Staphylococcus aureus*

MIC: minimum inhibitory concentration

S. aureus N315: *Staphylococcus aureus* N315 strain

TSA: tryptic soy broth

TSB: tryptic soy broth

Declarations

[Ethics approval and consent to participate]

This study was approved by the Hospital Ethics Committee of Kitasato University School of Medicine (approval no. B17-013).

Any animal and human data and tissue is not applicable.

[Consent for publication]

Not applicable.

[Availability of data and material]

The sequence of S. aureus N315 strain was referred from GeneBank (data base number: BA000018.3).

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[Author's contribution]

HK and TH carried out molecular genetic studies and drafted the manuscript. MN and HK designed basic of study and contributed helpful discussion. YK, KS and MY helped to analyse of clinical isolated MRSA, such as measuring MIC, sequence analysis and population analysis. HM and HH participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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[Competing interests]

The authors declare that they have no competing interests.

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Tables

Table 1 List of primers.

Primer name	Sequence 5’-3’
mprF-ORF-F	GCACTCATAATCGGCTGTT
mprF-ORF-R	TTGGGCTGATAATAAAAGTT
mprF-Seq1	ACCATATTGTTCTGTTTGAG
mprF-Seq2	TATTGGTGCAGGCGTTAGAG
mprF-Seq3	GGCGCTTTCGATTTAGTTGT
mprF-Seq4	AGCTATTATTTTTGTTCTGC
mprF-Seq5	TTTAACGCAATTTTCAACTT
mprF-N315_1033_A>C_F	ATTCTTTCCAAGTATGATCTTTTTTG
mprF-N315_1033_A>C_R	ATACTTGGAAAGAATACTAAAATTGC
mprF-N315_1350_ins-ATT_F	AGAAATATTATAGTTGCAATGCTTTTA
mprF-N315_1350_ins-ATT_R	AACTATAATATTTCTCATTCTTACTGG
pIMAY-F	TACATGTCAAGAATAAACTGCCAAAG
pIMAY-R	AATACCTGTGACGGAAGATCACTTCG
*dlt*A-ORF-F	CAGTGGCGACACACACAATA
*dlt*A-ORF-R	GACTGGTAATAATGCAATTAAAGCAA
*dlt*B-ORF-F	TGGAACAATTGCCATTGACTT
*dlt*B-ORF-R	TCCAACTGTTTGGAAAGAATCA
*dlt*A-Seq1	TACTGAGTGGATGTTAGAACT
*dlt*A-Seq2	CGATGACGGTATTCGTACAT
*dlt*B-Seq1	GTTACATTCAAAAGTGTGCAG
*dlt*B-Seq2	GATCTTTATTCTACATGTCTC

Table 2 MICs measured in the clinical isolates

Isolates	Antibiotics	DAP	VCM	TEIC	LZD
S1	MIC (μg/ml)	1.5	1.5	1	2
	Susceptibility	N.S.	S	S	S
S2	MIC (μg/ml)	0.75	2	4	1
	Susceptibility	S	S	S	S
S3	MIC (μg/ml)	2	2	4	2
	Susceptibility	N.S.	S	S	S

S：Susceptible, N.S.：Non-susceptible.

Table 3 MICs measured in the transformed strains and control strain.

Strain	Antibiotic	DAP	VCM	TEIC	LZD
MprF-WT	MIC (μg/ml)	0.25	<0.25	0.25	1.5
MprF (N450_I451 insI)	MIC (μg/ml)	0.5	0.75	1	2
MprF (T345P)	MIC (μg/ml)	0.75	0.5	0.5	1

Figures

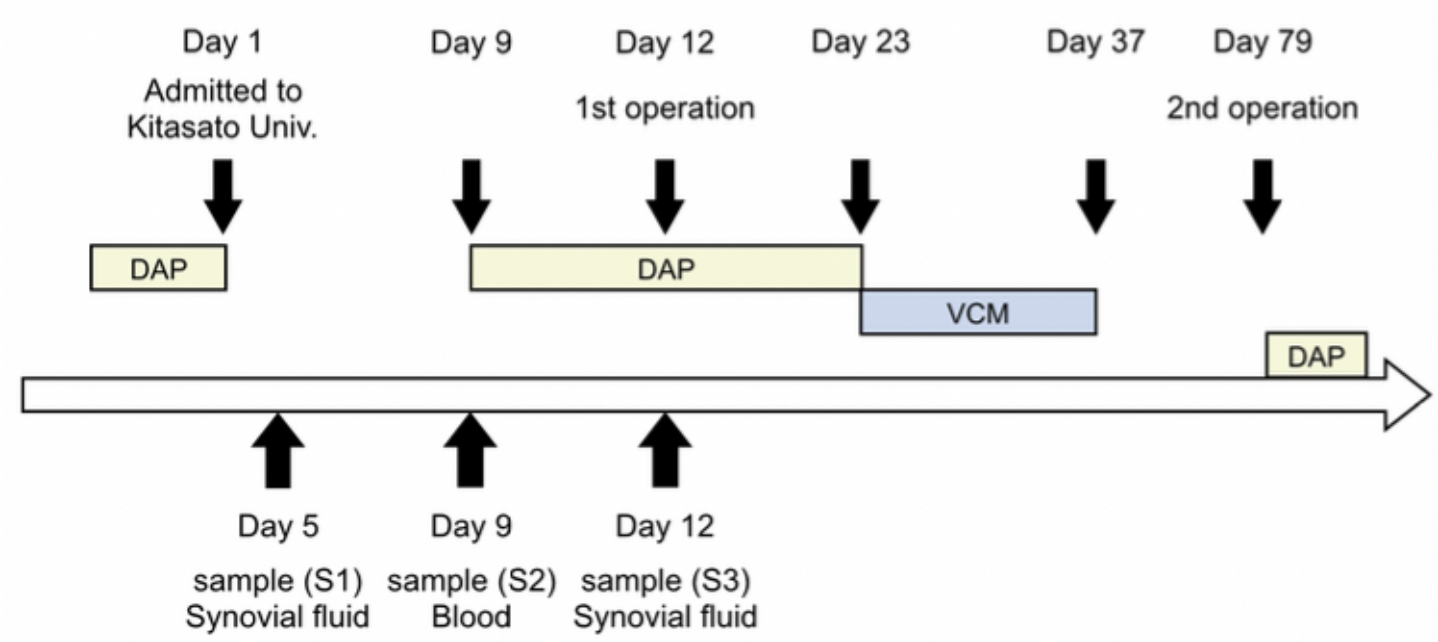

Figure 1

Clinical course of the patient with septic arthritis. Septic arthritis was diagnosed at another hospital, and the patient was transferred to the Department of Orthopedic Surgery at Kitasato University Hospital due to progression of symptoms. The clinical isolates were detected on admission (S1), just before starting

administration of DAP (S2), and at the first operation (S3). These MRSA isolates were identified from colony morphology and the WalkAway assay.

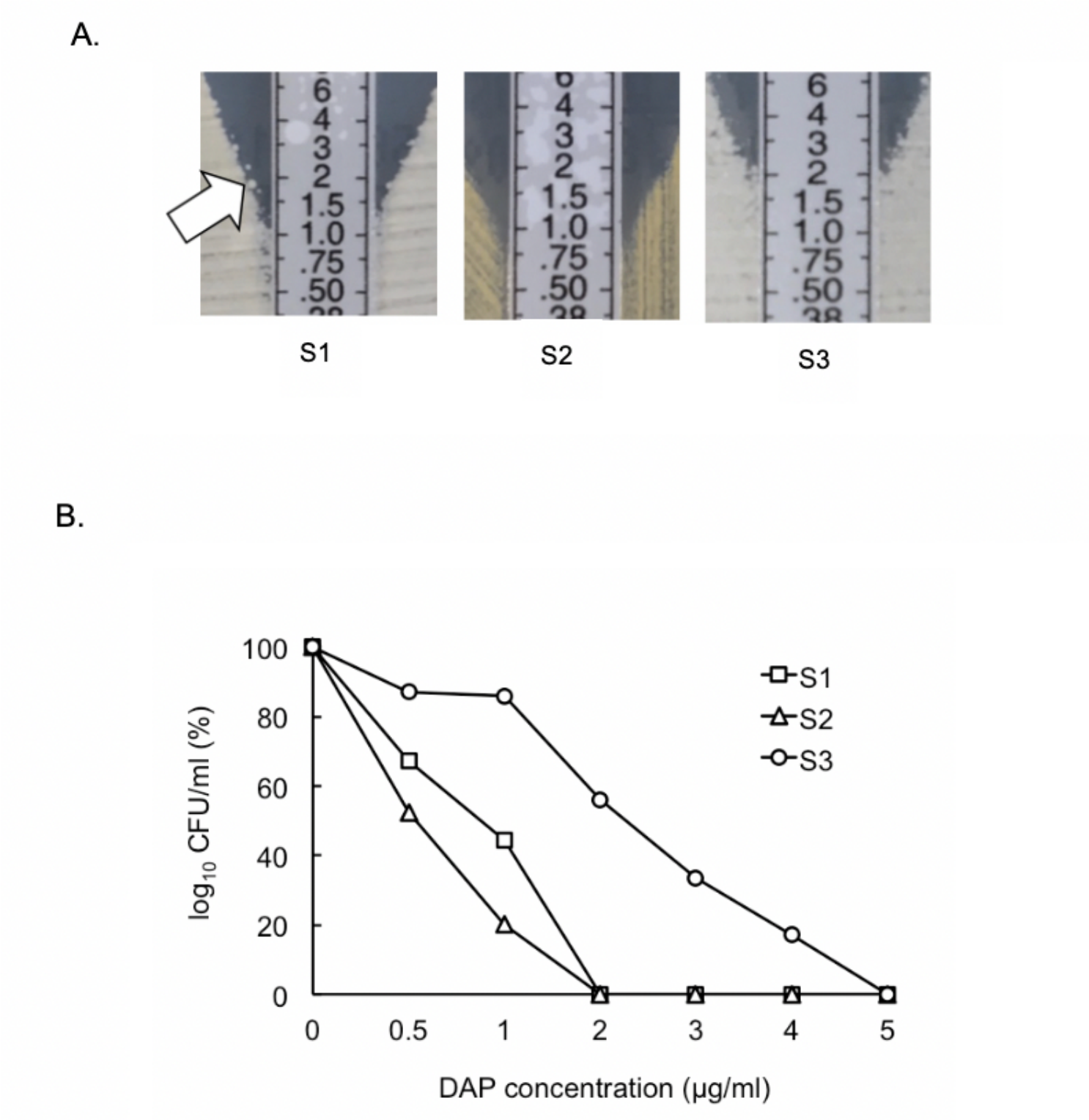

Figure 2

Measurement of MICs using the E-test and population analysis. A. MICs were measured by the E-test, using S. aureus ATCC29213 as the control strain for comparison with the clinical isolates of MRSA. A single colony of S1 was detected in the inhibition zone (white arrow). B. The bacterial populations were

calculated by counting colonies. Then the population at each DAP concentration was expressed as a percentage relative to the number of CFU with 0 μg/ml of DAP (100 %).

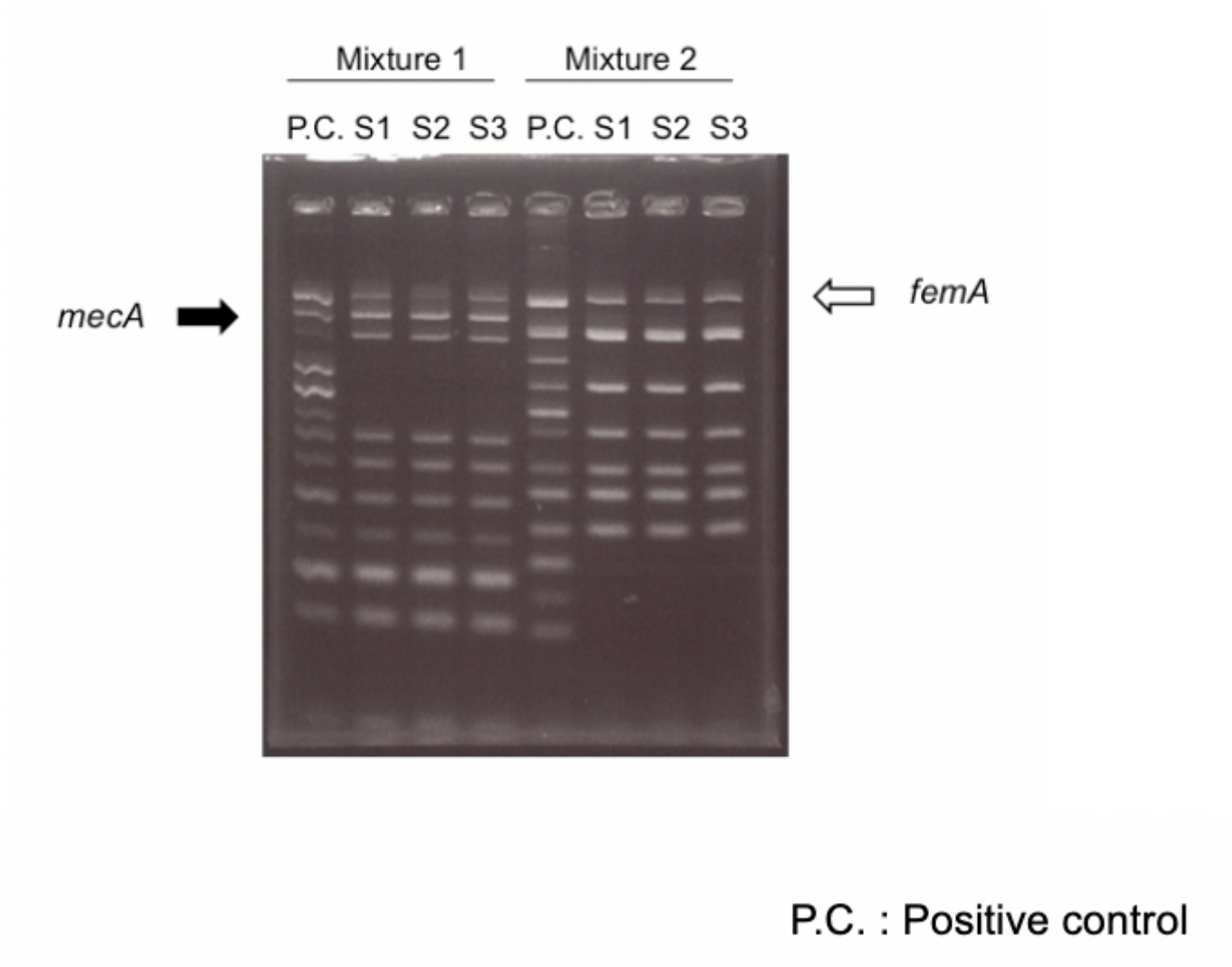

Figure 3

Genotyping by the POT method. DNA samples were extracted from each strain, and PCR was performed using primer mixtures 1 and 2 of a kit designed for S. aureus. Genomes were compared among the strains by electrophoresis on 4% agarose gel. The arrows indicate S. aureus-specific genes in PBP-2a: femA (white arrow) and mecA (black arrow).

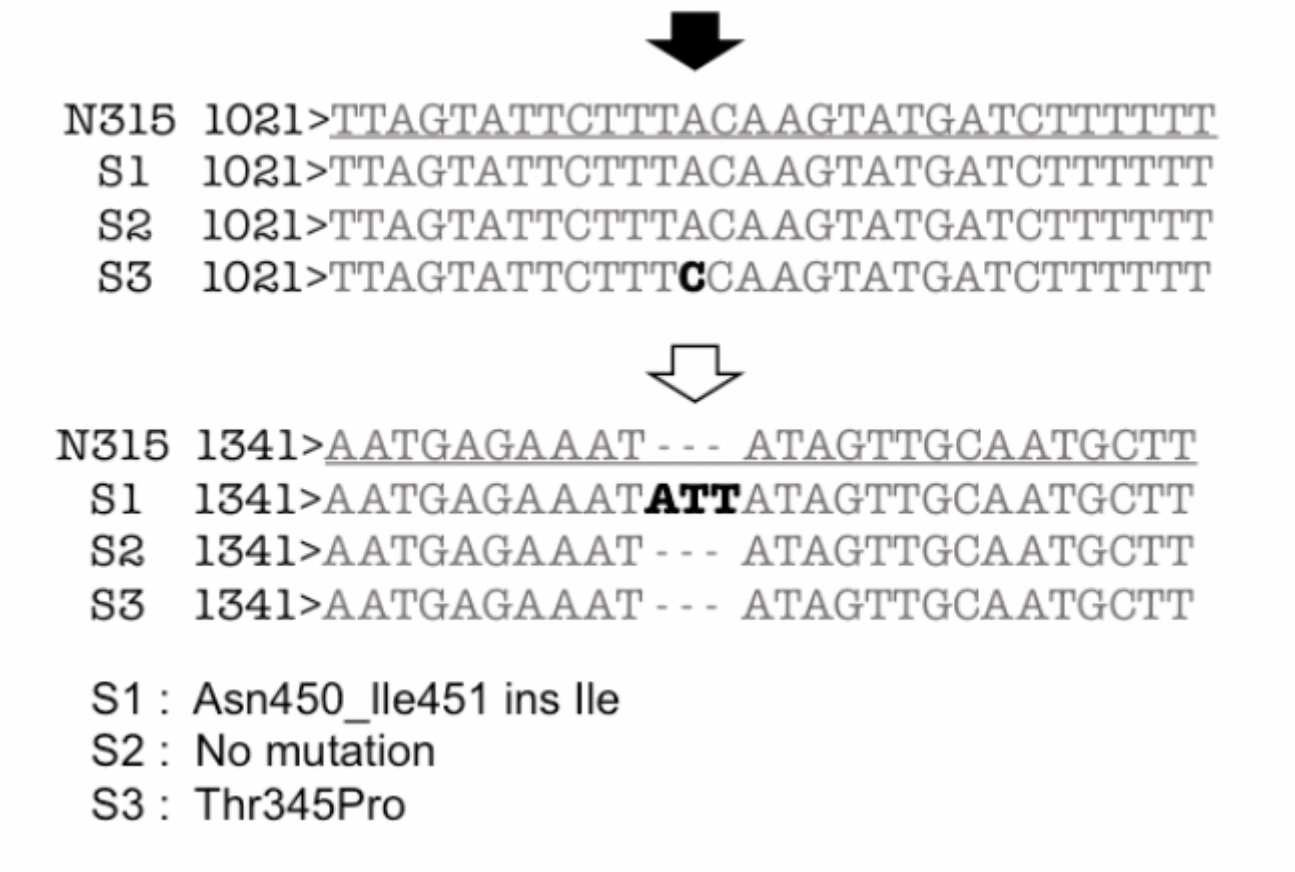

Figure 4

Full-length sequence analysis of mprF. mprF was sequenced and compared with the whole-genome sequence of S. aureus N315 by using ApE DNA editing software. Insertion of ATT between nucleotides 1350 and 1351 (white arrow) was detected in S1, and substitution of adenine by cytosine at nucleotide 1033 (black arrow) was found in S3. No mutation was identified in S2.

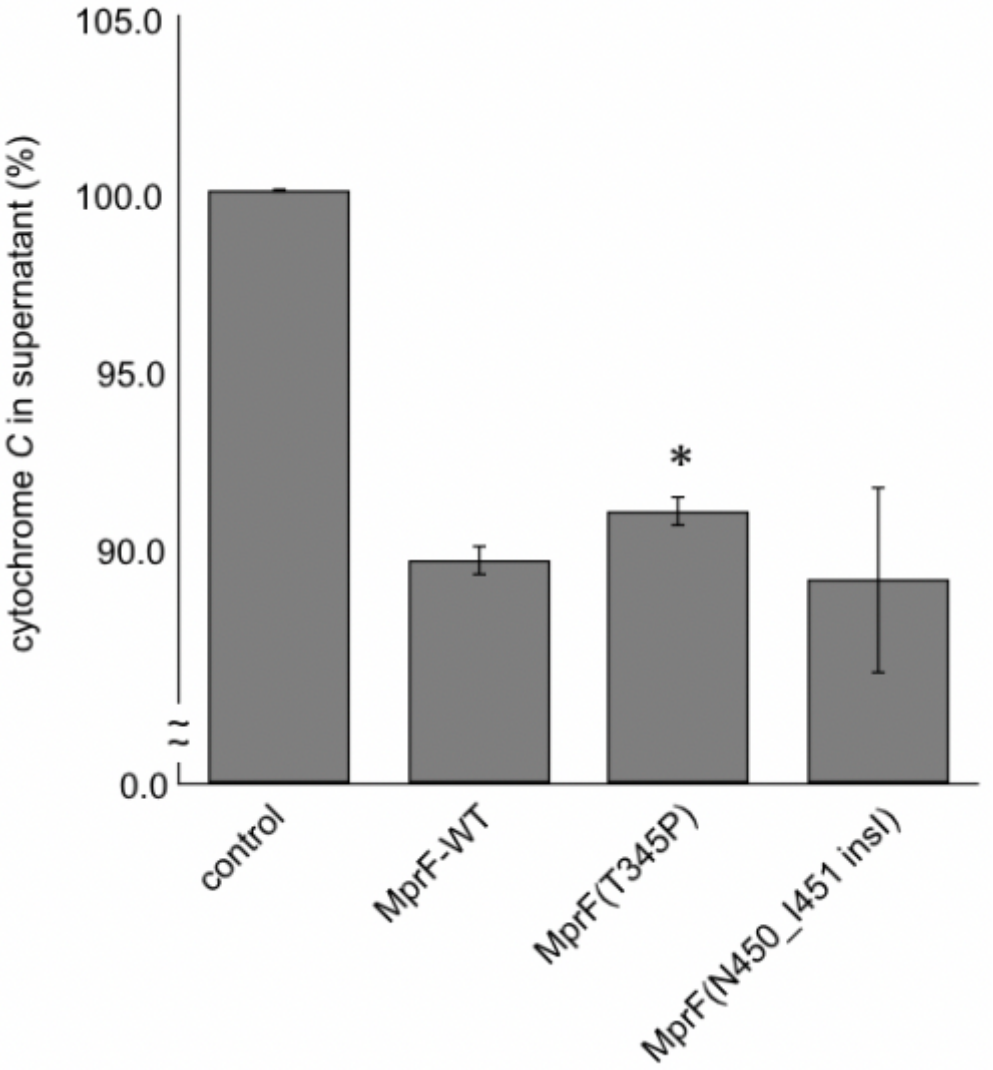

Figure 5

Cytochrome C binding assay. The cytochrome C binding assay was performed to assess the positive charge on the cell membrane of N315 strains with introduced mutations. The cytochrome C level in the supernatant was measured by determining OD530 and was calculated relative to the control (cytochrome C added to 20 mM MOPS buffer at 0.5 mg/ml). Data represent the mean and standard deviation from three independent experiments. Statistical analysis was performed by Student's t-test (n = 3). *p < 0.05 vs. N315(MprF-WT).