Original Contribution Kitasato Med J 2020; 50: 44-51

Novel MprF amino acid mutations contributing to daptomycin non-susceptibility in MRSA

Hayato Kawada,[1]* Tsubasa Hori,[1]* Masaki Nakamura,[1,2] Yuko Kimura,[2]
Kurumi Sato,[2] Mayu Yoshida,[2] Hidero Kitasato[1,2]

[1] Department of Environmental Microbiology, Kitasato University Graduate School of Medical Sciences
[2] Department of Microbiology, Kitasato University School of Allied Health Sciences
*Equal contribution

Objective: Daptomycin (DAP) is one of the anti-methicillin-resistant *Staphylococcus aureus* (MRSA) drugs and sterilizes MRSA by damaging bacterial cell membranes. It was reported that MprF is a bacterial membrane protein that maintains the homeostasis of the bacterial cell membrane. MprF mutation was reported to be one of the causative genes of DAP-nonsusceptibility (NS), but it remains controversial. We analyzed the clinical isolated DAP-NS MRSA to identify the causative gene.
Methods: We analyzed MprF in 3 MRSA clinical isolates from Kitasato University Hospital and evaluated the effect of MprF mutations that were detected from those clinical isolates using the laboratory strain, *S. aureus* N315.
Results: We detected two new mutations: MprF (N450_I451 insI) and MprF (T345P), which, to our knowledge, are first reported here. We introduced those mutations into *S. aureus* N315 and evaluated the drug susceptibility profile. The result was that the minimum inhibitory concentration (MIC) of DAP was increased by the introduction of these mutations into *S. aureus* N315.
Conclusion: We found that these two novel and unique mutations contribute to DAP-NS and suspect that they will help clarify the contribution of MprF to DAP-NS.

Key words: daptomycin, MRSA, antimicrobial resistant, infection control

Introduction

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] MRSA has acquired resistance not only to β-lactam but also to various other antibiotics, including aminoglycosides, macrolides, chloramphenicol, tetracyclines, and fluoroquinolones.[2] Moreover, recently, hospital-associated MRSA and livestock-associated MRSA have been reported, and countermeasures are required globally not only from the clinic for human health but also from a "One Health" approach.[3-6]

Daptomycin (DAP) is a cyclic lipopeptide with potent bactericidal activity against gram-positive bacteria and is used to treat MRSA infections. It forms the micelle positively charged in a calcium ion and binds to the negatively charged bacterial membrane. Finally, bacteria are sterilized by induced membrane depolarization.[7-10] 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.[11] DAP has become a key antibiotic for treating severe acute infections caused by gram-positive bacteria, such as sepsis, because 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] Recently, DAP nonsusceptibility (DAP-NS) has been detected and reported as clinical failures. DAP-NS is caused by a mutation of a membrane protein known as the multiple

Received 27 November 2019, accepted 10 December 2019
Correspondence to: Hidero Kitasato, Department of Microbiology, Kitasato University School of Allied Health Sciences
1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan
E-mail: hkita@kitasato-u.ac.jp

peptide resistance factor (MprF), which synthesizes lysyl-phosphatidylglycerol (LPG) and facilitates its translocation to the outer membrane leaflet from the inner leaflet to maintain a positive membrane charge.[7] However, the contribution of the MprF mutation to DAP-NS and the mechanism of DAP-NS is controversial because several mechanisms have been reported. The mechanism of DAP-NS is generally considered when the electrical repulsion of DAP by increasing the positive charge of the cell membrane as a result of LPG translocation was promoted by the MprF mutation.[15] On the other hand, it has also been reported that the MprF indirectly contributes to increasing cell wall thickness and cell membrane fluidity.[16] Furthermore, D-alanine (*dlt*) operon mutations may contribute to DAP-NS,[17] with various factors being involved in a complex resistance mechanism among different MRSA strains.

In order to identify the causative gene of DAP-NS, we analyzed three clinical MRSA strains isolated from a patient with septic arthritis of the hip. We found that these isolates harbored two previously unreported MprF mutations. In addition, we evaluated those mutations that were introduced into the laboratory strain, *S. aureus* N315, and analyzed the drug susceptibility of those genetically mutated strains to prove the contribution of those mutations to DAP-NS.

Materials and Methods

Ethics approval and consent to participate

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

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 (Figure 1). These MRSA isolates were identified on the basis of colony morphology and by using the WalkAway System (Beckman Coulter, Brea, CA, USA).

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. The *S. aureus* N315 was provided by Yuki Katayama, PhD, Juntendo University, Tokyo,[18] and the *S. aureus* ATCC 29213 was purchased from the American Type Culture Collection (ATCC, Washington, DC, USA).

Measurement of minimum inhibitory concentrations (MICs)

MICs were measured by the microdilution method using dry plates (Eiken, Tokyo) and by the disc susceptibility testing method using the Etest (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, Wayne, PA, USA).

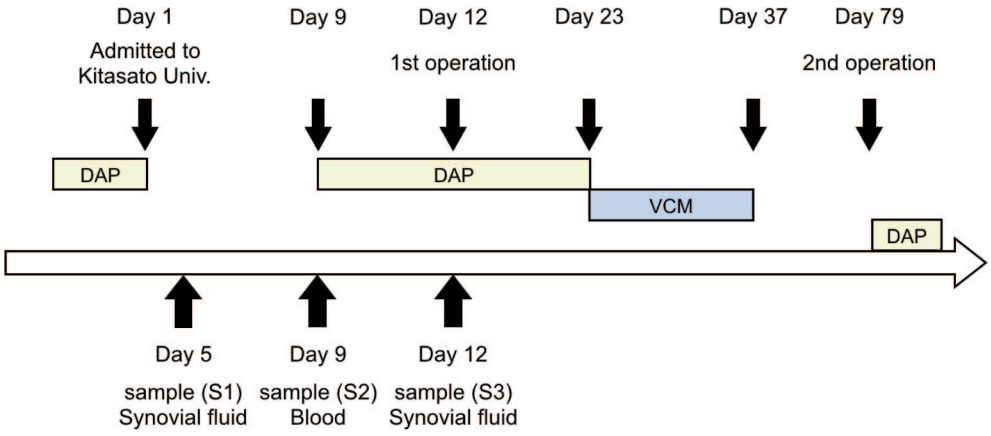

Figure 1. Clinical course of the patient with septic arthritis

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.

Bacterial population analysis

Each isolate was cultured overnight at 37℃ in tryptic soy broth (TSB) and harvested by centrifugation at 8,000 rpm for 5 minutes. After washing 3 times in saline, the pellet was resuspended in saline at optical density (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 (Mast Diagnostics, Merseyside, UK) containing DAP (0－5 μg/ml) and incubated for 48 hours at 37℃, after which colonies were counted.

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

Genotypes of the MRSA isolates from the patient with septic arthritis were analyzed using a Cica Geneus Staph POT Kit (Kanto Chemical, Kanagawa) according to the manufacturer's directions. Certain *S. aureus*-specific genes (including *femA* and *mecA*) were amplified by multiplex PCR and detected by 4% agarose gel electrophoresis, allowing strain-level identification based on the POT number.[19]

Sequencing

Bacterial colonies were subjected to PCR as follows. Single colonies grown on tryptic soy agar (Eiken, Tokyo) 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]) were added then incubated for 10 minutes at 37℃. 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) 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), while the remaining 20 μl of the PCR product was purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA). Then 10 μl of primer mixture (Table 1) was added and sequencing was done according to the guidelines of Eurofins Genomics (Tokyo). The sequence obtained was compared with that of *S. aureus* N315 (GeneBank: BA000018.3) using ApE DNA editing software.

Culture of competent S. aureus N315

S. aureus N315 cells were cultured overnight at 28℃ in 2 ml of B2 broth, followed by dilution to 200 ml and further incubation at 28℃ until an OD at 590 nm (OD_{590}) of 0.4－0.6 was reached. Then bacterial growth was

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

stopped by rapid cooling, and the culture fluid was centrifuged for 5 minutes at 12,000 g and 4℃, after which the pellet was washed in chilled sterile water and subsequently washed in 10% glycerol. The pellet was resuspended in 2 ml of 10% glycerol, and the suspension was dispensed into PCR tubes and immediately frozen by immersing the tubes in an acetone-dry ice freezing mixture.

Introduction of mutant or defective MprF into S. aureus *N315*

The pIMAY plasmid was provided by Timothy J. Foster, PhD, Trinity College, Dublin, Ireland, and was used to introduce MprF mutations into *S. aureus* N315 according to the method published.[20] The *mprF* of *S. aureus* N315 was cloned into a multi-cloning site of the pIMAY vector, which was designated as pIMAY-*mprF*-*wt*. Two recombinant vectors, pIMAY-*mprF*-N450_ I451 insI and pIMAY-*mprF*-T345P, were generated using the PrimeSTAR Mutagenesis Basal kit (TaKaRa) according to the manufacture's manual (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, 100Ω). Subsequently, strains were selected by culture for 48 hours at 28℃ in TSB containing 10 μg/ml chloramphenicol. In addition, point mutations of MprF were introduced into *S. aureus* N315 by homologous recombination using a modification of the reported protocol.[20] Finally, the drop of the vector and introducing the *mprF* mutation was confirmed by PCR and sequencing, respectively.

Results

MICs for the MRSA isolates

We measured the MICs of various antimicrobial agents for the 3 chronological MRSA isolates (S1－S3) by the microdilution method using dry plates (Table 2) and by the disc susceptibility testing method using the Etest (Figure 2A). S1 and S3 were increased in the MIC of DAP and acquired DAP-NS, although these strains were still susceptible to other antibiotics that are used for anti-MRSA antibiotics (vancomycin [VCM], teicoplanin [TEIC], and linezolid [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. Furthermore, the color of the S2 colonies was slightly different from that of the S1 and S3 colonies.

In the Etest of S1, an independent colony was recognized inside the DAP inhibition area, raising the possibility of heteroresistance. Therefore, population analysis of S1, S2, and S3 was carried out, but the results did not suggest heteroresistance of S1 (Figure 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 (Figure 3).

MprF sequence

To identify the causative gene of DAP-NS in those strains, we used DNA editing software to compare the findings between the three MRSA isolates and the reference strain, *S. aureus* N315 (Figure 4). In S1, we identified the 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 to C was noted at nucleotide 1033, corresponding to a change from threonine to proline at

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	NS	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	NS	S	S	S

MIC, minimum inhibitory concentration; DAP, daptomycin; VCM, vancomycin; TEIC, teicoplanin; LZD, linezolid; S, susceptible; NS, nonsusceptible

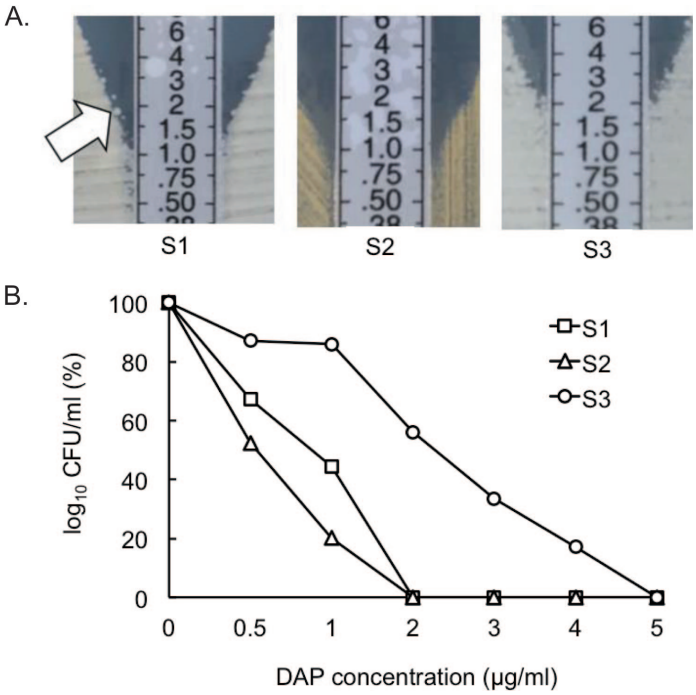

Figure 2. Measurement of MICs using the Etest and population analysis

A. MICs were measured by the Etest, 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%).

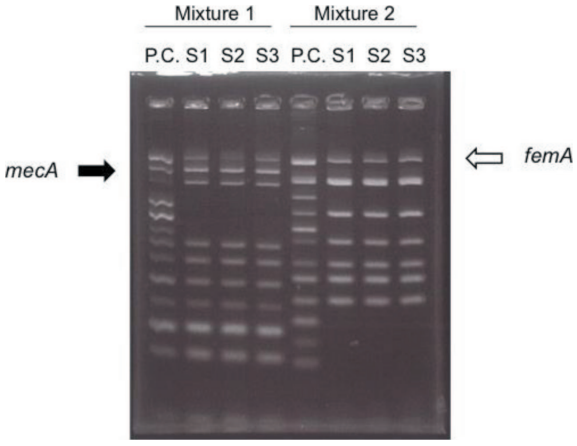

Figure 3. Genotyping by the POT method

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 *femA* (white arrow) and PBP-2a in *mecA* (black arrow).

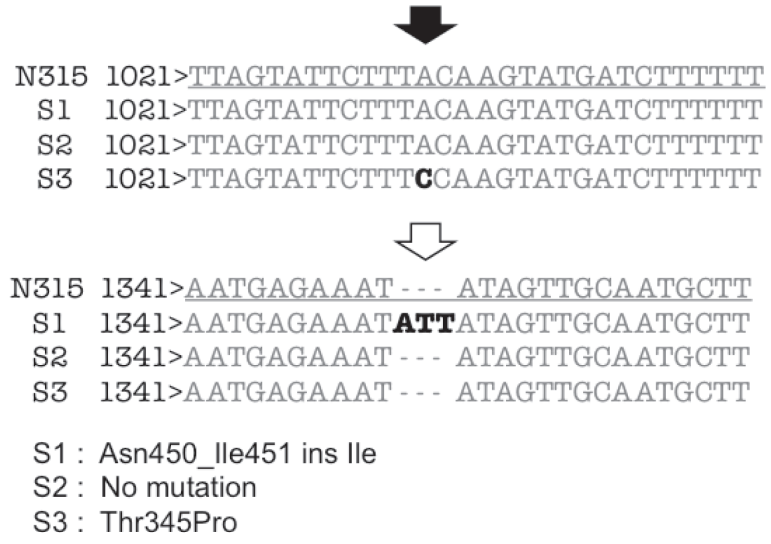

Figure 4. Full-length sequence analysis of *mprF*

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.

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

Strains	Antibiotics	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

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 (Data not shown).

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

In order to prove that those novel mutations were the cause of the elevation of the MIC in clinical isolates, we generated *S. aureus* N315 MprF (N450_I451 insI) and *S. aureus* N315 MprF (T345P) by introducing those mutations into *S. aureus* N315. We then measured the MICs of the antibiotics for these transformed strains by the microdilution method using dry plates, and compared them with *S. aureus* N315 MprF-WT, which was also generated by the same method (Table 3). We found that both the mutations of *S. aureus* N315 were slightly increased compared to the MprF-WT in MIC for DAP. Additionally, the MIC of VCM was increased for the two mutant strains, although it was not increased for the clinical isolates.

Discussion

The causative gene of DAP-NS was not completely identified, because it is reported that various mutations have been detected in clinically isolated MRSA, even in the whole genome sequence. We have evaluated the genetically introduced strains to clarify the influence of MprF mutations to DAP-NS. 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. We also observed that the MIC of DAP was elevated by introducing the MprF mutation in *S. aureus* N315 (Table 3). 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.[21] In particular, many mutations of amino acid 345 have been reported, e.g., T345I, T345A, and T345K; however, to our knowledge, the present study is the first report about the substitution of proline for threonine.[22] Proline is unique among the amino acids because it is a secondary amino acid.[23] In addition, we first detected the insertion in MprF which influences DAP-NS. Taken together, those novel mutations were different compared to the other mutations previously reported. Structural and functional analyses, e.g., three-dimensional analysis, assessment of LPG translocation, and phenotypic studies, may be required to clarify the structural influence of the MprF mutation on DAP susceptibility.

The MIC for DAP in genetically mutated *S. aureus* N315 was lower than that of the clinical isolates (S1 and S3), even if MprF (T345P) and MprF (N450_I451 ins I) were introduced. That may possibly be the cause of the characteristic 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 that 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*, e.g., Mu50, as the recipient.

Only S2 showed susceptibility to DAP 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,[24] 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 survived during DAP administration with a higher fitness cost because 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.[25] 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. If a patient's symptoms do not improve after administration of DAP within 2 weeks, discontinuation of DAP and switching to other antibiotics may be warranted to avoid clinical failure due to DAP-NS.

The mechanism of DAP-NS remains controversial. It is generally consider that DAP is electrically repelled by increasing LPG associated with accelerated MprF function. On the other hand, Mishra et al.[26] reported that DAP-NS of *S. aureus* apparently involved multifactorial strain-specific adaptive mechanisms and suggested that various mechanisms may be associated with multiple phenotypes, such as an increase of teichoic acid in the cell wall or increased membrane fluidity. In their study, MRSA isolated from the patients was used. It is necessary to perform examinations without clinical factors to completely elucidate the mechanism of DAP-NS because cross-resistance with DAP and antimicrobial peptide was reported.[27] Following such examinations, we will attempt to reveal the mechanism by analyzing *S. aureus* N315 in which MprF has been genetically mutated.

In conclusion, we found that the novel MprF mutations, T345P and N450_I451 insI, from clinically isolated MRSAs in this study, and those mutations, deeply contributed to DAP-NS. Moreover, the study of those novel and unique mutations may be expected to elucidate the contribution of MprF to DAP-NS.

Acknowledgments

We thank Fumiaki Kojima, PhD, Department of Pharmacology, Kitasato University School of Allied Health Sciences, and Makoto Kubo, PhD, Department of Clinical Immunology, Kitasato University Graduate School of Medical Sciences, for helpful discussion; and Hideaki Hanaki, PhD, and Hidehito Matsui, PhD, Infection Control Research Center, Kitasato Institute for Life Sciences, Kitasato University, for technical advise. We also thank Yamada Translation Bureau, Tokyo, for proofreading the manuscript.

Funding: This work was supported by research funds from the Kitasato University School of Allied Health Sciences and Graduate School of Medical Sciences.

Conflicts of Interest: None

References

1. Grema HA, Geidam YA, Gadzama GB, et al. Methicillin-resistant Staphylococcus aureus (MRSA): a review. *Adv Anim Vet Sci* 2015; 3: 79-98.
2. Lee JH. Methicillin (Oxacillin)-resistant Staphylococcus aureus strains isolated from major food animals and their potential transmission to humans. *Appl Environ Microbiol* 2003; 69: 6489-94.
3. Umaru GA, Kabiru J, Adamu NB, et al. A review of emerging methicillin-resistant Staphylococcus aureus (MRSA): a growing threat to veterinarians. *Nigerian Vet J* 2011; 32: 174-86.
4. Tietz A, Frei R, Widmer AF. Transatlantic spread of the USA300 clone of MRSA. *N Engl J Med* 2005; 353: 532-3.
5. Donnio PY, Preney L, Gautier-Lerestif AL, et al. Changes in staphylococcal cassette chromosome type and antibiotic resistance profile in methicillin-resistant Staphylococcus aureus isolates from a French hospital over an 11 year period. *J Antimicrob Chemother* 2004; 53: 808-13.
6. Monecke S, Coombs G, Shore AC, et al. A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant Staphylococcus aureus. *PLoS One* 2011; 6: e17936.
7. Muraih JK, Pearson A, Silverman J, et al. Oligomerization of daptomycin on membranes. *Biochim Biophys Acta* 2011; 1808: 1154-60.
8. Ho SW, Jung D, Calhoun JR, et al. Effect of divalent cations on the structure of the antibiotic daptomycin. *Eur Biophys J* 2008; 37: 421-33.
9. Strauss SK, Hancock RE. Mode of action of the new antibiotic for Gram-positive pathogens daptomycin: comparison with cationic antimicrobial peptides and lipopeptides. *Biochim Biophys Acta* 2006; 1758: 1215-23.
10. Scott WR, Beak SB, Jung D, et al. NMR structural studies of the antibiotic lipopeptide daptomycin in DHPC micelles. *Biochim Biophys Acta* 2007; 1768: 3116-26.
11. Tran TT, Munita JM, Arias CA. Mechanisms of drug resistance: daptomycin resistance. *Ann N Y Acad Sci* 2015; 1354: 32-53.
12. Bayer AS, Schneider T, Sahl HG. Mechanisms of daptomycin resistance in Staphylococcus aureus: role of the cell membrane and cell wall. *Ann N Y Acad Sci* 2013; 1277: 139-58.
13. Roveta S, Marchese A, Schito GC. Activity of daptomycin on biofilms produced on a plastic support by Staphylococcus spp. *Int J Antimicrob Agents* 2008; 31: 312-8.

14. Malizos K, Sarma J, Seaton RA, et al. Daptomycin for the treatment of osteomyelitis and orthopedic device infections: real-world clinical experience from a European registry. *Eur J Clin Microbiol Infect Dis* 2016; 35: 111-8.
15. Ernst CM, Staubitz P, Mishra NN, et al. The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion. *PLoS Pathog* 2009; 5: e1000660.
16. Mishra NN, Yang SJ, Sawa A, et al. Analysis of cell membrane characteristics of in vitro-selected daptomycin-resistant strains of methicillin-resistant Staphylococcus aureus. *Antimicrob Agents Chemother* 2009; 53: 2312-8.
17. Kanesaka I, Fujisaki S, Aiba Y, et al. Characterization of compensatory mutations associated with restoration of daptomycin-susceptibility in daptomycin non-susceptible methicillin-resistant Staphylococcus aureus and the role mprF mutations. *J Infect Chemother* 2019; 25: 1-5.
18. Kuroda M, Ohta T, Uchiyama I, et al. Whole genome sequencing of methicillin-resistant Staphylococcus aureus. *Lancet* 2001; 357: 1225-40.
19. Suziki M, Matsumoto M, Minagawa H, et al. Identification of the clonal complexes of Staphylococcus aureus strains by determination of the conservation patterns of small genomic islets. *J Appl Microbiol* 2009; 107: 1367-74.
20. Schneewind O, Missiakas D. Genetic manipulation of Staphylococcus aureus. *Curr Protoc Microbiology* 2014; 32: Unit 9C.3.
21. Yang SJ, Mishra NN, Kang KM, et al. Impact of multiple single-nucleotide polymorphisms within mprF on daptomycin resistance in Staphylococcus aureus. *Microb Drug Resist* 2018; 24; 1075-81.
22. Bayer AS, Mishra NN, Chen L, et al. Frequency and distribution of single-nucleotide polymorphisms within mprF in methicillin- resistant Staphylococcus aureus clinical isolates and their role in cross-resistance to daptomycin and host defense antimicrobial peptides. *Antimicrob Agents Chemother* 2015; 59; 4930-7.
23. Kini RM, Evans HJ. A novel approach to the design of potent bioactive peptides by incorporation of proline brackets: antiplatelet effects of Arg-Gly-Asp peptides. *FEBS Lett* 1995; 375; 15-7.
24. Roch M, Gagetti P, Davis J, et al. Daptomycin resistance in clinical MRSA strains is associated with a high biological fitness cost. *Front Microbiol* 2017; 8; 2303.
25. Li S, Yin Y, Chen H, et al. Fitness cost of daptomycin-resistant Staphylococcus aureus obtained from in vitro daptomycin selection pressure. *Front Microbiol* 2017; 8; 2199.
26. Mishra NN, Bayer AS, Yang SJ, et al. Phenotypic and genotypic characterization of daptomycin-resistant methicillin-resistant *Staphylococcus aureus* strains: relative roles of *mprF* and *dlt* operon. *PLoS One* 2014; 9: e107426.
27. Mishra NN, McKinnell J, Bayer AS, et al. In vitro cross-resistance to daptomycin and host defense cationic antimicrobial peptides in clinical methicillin-resistant Staphylococcus aureus isolates. *Antimicrob Agents Chemother* 2011; 55: 4012-8.