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Original Article

Characterization of compensatory mutations associated with restoration of daptomycin-susceptibility in daptomycin non-susceptible methicillin-resistant *Staphylococcus aureus* and the role *mprF* mutations☆

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ABSTRACT

The objective of this study was to investigate the underlying mechanism explaining reversion of clinical DAP non-susceptible (NS) MRSA isolates to DAP-susceptible (S) by analysis of genomic and cell wall characteristics of clinical DAP-NS MRSA and DAP-S MRSA isolates as well as *in vitro* revertant DAP-S MRSA using whole genome sequencing (WGS) and analysis of biological properties.

WGS of the 4 clinical DAP-NS MRSA revealed *mprF* mutations resulting in amino acid substitutions or deletion. These same amino acid substitutions and deletion were also observed in the 4 *in vitro* revertant DAP-S strains. While WGS identified the presence of the same *mprF* mutations in both the DAP-NS and *in vitro* DAP-S revertant strains, new mutations were also detected in other genes and intergenic regions of *in vitro* DAP-S revertant strains. Transmission electron microscopy to assess cell-wall (CW) thickness of 4 sets strains (pre- and post-DAP therapy isolates and *in vitro* DAP-S revertant) showed that 3 of the 4 isolates developed increased thickness of the CW after DAP therapy. After reversion to DAP susceptibility, CW thickness was decreased to the same level as DAP-S MRSA. Our results indicate that *in vitro* conversion of DAP-NS MRSA to DAP-S is independent of *mprF* gene mutations and may be partially explained by a change in CW thickness. However, as some strains showed no change in the CW, further studies are required to elucidate the different mechanisms of resistance to DAP, and factors for conversion of DAP-NS to DAP-S.

1. Introduction

The antibacterial activity of daptomycin (DAP) is based on a mechanism of action that differs from other presently available antibiotics and exhibits strong concentration-dependent, bactericidal activities against gram-positive bacteria. DAP inserts into the bacterial cytoplasmic membrane in a calcium-dependent fashion followed by oligomerization and disruption of the functional integrity of the cytoplasmic membrane, triggering a release of intracellular ions and rapid cell death [1]. Resistance to DAP is rare [2], although there have been reports of DAP resistant methicillin-resistant *Staphylococcus aureus* (MRSA) cases in Japan as well as outside of Japan [3–5]. Of the proposed genes involved in DAP non-susceptible (NS), a mutation in the multiple peptide resistance factor (*mprF*) gene is most often associated with DAP resistance [6,7]. It is felt that this mutation results in the reduction of the negative charge of the cell membrane leading to a positive surface

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charge and making it more difficult for DAP to bind to the cell membrane [8].

A number of studies have reported that reversion of DAP-NS MRSA to DAP-susceptible (S) occurs easily. One study reported on *in vitro* conversion by serial passage in drug-free Mueller-Hinton broth (MHB) for 10 consecutive days [9]. Furthermore, Capone, et al. reported a DAP-NS MRSA that emerged during treatment reverted to DAP-S following termination of DAP therapy [10]. No mutation in the *mprF* gene was detected in this DAP-S revertant, although it has been reported that DAP resistance is an autoregulated phenomenon in which gene expression levels of *mprF* changes in the absence of DAP [10]. In addition, in a study looking into the basis for reversion of DAP-NS MRSA to DAP-S, Iwata, et al. reported up-regulation of the two-component system (TCS) such as *vraSR*, *yycG*, *graS* and cell-wall biosynthesis-related genes in DAP-NS MRSA; however, when DAP therapy was discontinued, expression level of these genes decreased leading to reversion of DAP-NS to DAP-S MRSA. Interestingly, a SNP (L826F) detected in DAP-NS MRSA was also detected in the susceptible strain [11]. At the present time, the role of the *mprF* gene in the mechanism of reversion to DAP-S remains unclear as *mprF* mutation has also been observed in MRSA isolates after reversion to DAP-S [11].

The objective of this study was to investigate the underlying mechanism explaining reversion of clinical DAP-NS MRSA isolates to DAP-S by analysis of genomic and cell wall characteristics of clinical DAP-NS MRSA and DAP-S MRSA isolates as well as *in vitro* revertant DAP-S MRSA using whole genome sequencing (WGS) and examination of biological properties.

2. Materials and methods

2.1. Bacterial isolates and culture conditions

During 2013 to 2014, 27 patients at Toho University Medical Center Sakura Hospital received DAP for treatment of MRSA infections. Of these 27 patients, resistance to DAP developed in 4 patients. The 4 DAP-NS MRSA (A2, B2, C4 and D2) isolates from these patients as well as the 4 DAP-S MRSA (A1, B1, C1 and D1) isolates recovered pre- and post-DAP treatment were stored at −75 °C (Microbank, Iwaki, Ltd, Tokyo, Japan) for further analysis.

DAP susceptibility testing was performed according to CLSI M100-S26 [12] by the broth microdilution method (Dry plate “Eiken”, Eiken Chemical, Ltd, Tokyo, Japan) using MHB adjusted to 50 μg/ml of calcium. DAP concentrations tested were: 0.12, 0.19, 0.25, 0.38, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0 and 8.0 μg/ml.

2.2. Serial passage on non-selective medium for in vitro reversion of DAP-NS MRSA to MRSA with reduced MICs to DAP

Frozen cultures of the 4 DAP-NS isolates (A2, B2, C4 and D2) were thawed, inoculated on Mueller-Hinton Agar (MHA; Becton Dickinson Co., Ltd., Tokyo, Japan) and incubated overnight at 35 °C. Serial passage under non-selective conditions was continued for 20 days [9]. Daily passage using drug-free medium (MHA) was repeated and MICs to DAP and vancomycin (VAN) were determined every 5 days. Confirmation of DAP MIC: ≦1 μg/ml was made on the 20th day. MIC values were determined by the broth microdilution method performed in triplicate. These isolates were used as *in vitro* DAP-S revertants: A2-S, B2-S, C4-S and D2-S (Table 1).

2.3. Whole genome sequencing of S. aureus strains

Genomic DNA was extracted from cells using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) after overnight cultures in tryptic soy broth (TSB; Becton Dickinson Co., Ltd., sparks, MD, USA) were lysed with 50 μg/ml of lysostaphin (Sigma-Aldrich. Co., LLC., St Louis, MO, USA) and 33 μg/ml of RNaseA (Sigma-Aldrich. Co., LLC.) in TE buffer. WGS of DAP-S MRSA clinical strains A1, B1, C1 and D1 was performed as previously described [13]. Briefly, mate-pair sequencing libraries were constructed from genomic DNA of *S. aureus* strains using the Nextera mate-pair sample preparation kit (Illumina, Inc., San Diego, CA, USA) without size selection. Sequencing was performed using the Illumina MiSeq platform (2 × 301 bp) with MiSeq reagent kit version 3 (Illumina, Inc.). The resulting paired-end reads were subjected to quality trimming using the FASTQ toolkit version 2.0.0. The high-quality reads were then assembled with the Velvet de novo assembly version 1.2.10 algorithm.

WGS of DAP-NS *S. aureus* clinical strains (A2, B2, C4 and D2) and *in vitro* DAP-S mutants were determined using the Nextera XT sample preparation kit (Illumina, Inc.) and Illumina MiSeq platform (2 × 301 bp) with MiSeq reagent kit version 3 (Illumina, Inc.). To identify the SNPs, the resulting reads were trimmed and mapped on the genome of each DAP-S parent strain using CLC genomic workbench (Qiagen). The identified mutations were confirmed by using the Sanger-sequencing method [14].

2.4. Transmission electron microscopy

Using transmission electron microscopy (TEM), cell wall thickness of DAP-S, DAP-NS and *in vitro* revertant DAP-S MRSA were examined. Cells for TEM were prepared and examined according to a previously published method [15]. Morphometric evaluation of cell-wall thickness was performed by using photographic images at a final magnification of ×12,000. Cell-wall thicknesses of 10 cells of each strain with nearly equatorially cut surfaces at 16 points were measured. Results were expressed as the mean ± SEM. The two-sided Student's *t*-Test was used to determine statistical significance.

3. Results

3.1. Antibiotic susceptibility results

Table 2 shows DAP and VAN MICs for DAP-S MRSA (A1, B1, C1 and D1) strains recovered pre- and post-DAP treatment, DAP-NS MRSA (A2, B2, C4 and D2) strains from 4 patients and *in vitro* DAP-revertant MRSA strains. DAP MICs for all *in vitro* DAP-S revertants were 0.5 μg/ml.

3.2. Whole-genome analysis of clinical DAP non-susceptible and in vitro DAP-revertant MRSA strains

WGS of the 4 clinical DAP-NS MRSA revealed *mprF* mutations resulting in amino acid substitutions or deletion: A2 (T345I); B2 (L776S); C4 (459–466del); D2 (L826F). These same amino acid substitutions and deletion were also observed in the 4 *in vitro* revertant DAP-S strains. While WGS identified the presence of the same *mprF* mutations in both the DAP-NS and *in vitro* DAP-S revertant strains, new mutations were also detected in other genes and intergenic regions of *in vitro* DAP-S revertant strains: A2-S strain - 5 genes (*ppnK*, *prfC*, *moaB*, *lipA*, *kdpB2*); B2-S strain - 4 genes (*atl*, *sodA*, *argC*, *ahpF*); C4-S strain - 1 gene (*aadD*) and 1 point mutation in the intergenic region; and D2-S strain - 1 point mutation in the intergenic region (Table 2).

3.3. Comparison of cell-wall thickness

Transmission electron microscopy to assess cell-wall thickness of 4 sets strains (pre- and post-DAP therapy isolates and *in vitro* DAP-S revertant) showed that 3 of the 4 isolates developed increased thickness of the cell-wall after DAP therapy. After reversion to the

Table 1
Description of the strains used in this study.

Isolate source	Strain	Daptomycin exposure	Source
Patient A	A1	7 days	Post-DAP treatment clinical strain, Toho University Sakura medical center, 2013
	A2		Clinical DAP-NS strain, Toho University Sakura medical center, 2013
	A2-S revertant		*In vitro*-revertant DAP-S
Patient B	B1	13 days	Pre-DAP treatment clinical strain, Toho University Sakura medical center, 2013
	B2		Clinical DAP-NS strain, Toho University Sakura medical center, 2013
	B2-S revertant		*In vitro*-revertant DAP-S
Patient C	C1	16 days	Pre-DAP treatment clinical strain, Toho University Sakura medical center, 2014
	C4		Clinical DAP-NS strain, Toho University Sakura medical center, 2014
	C4-S revertant		*In vitro*-revertant DAP-S
Patient D	D1	21 days	Pre-DAP treatment clinical strain, Toho University Sakura medical center, 2014
	D2		Clinical DAP-NS strain, Toho University Sakura medical center, 2014
	D2-S revertant		*In vitro*-revertant DAP-S

Table 2
Gene mutations and predicted amino-acid changes.

Strain	MIC (μg/mL)				Number of mutation	Gene mutation											intergenic
	DAP		VAN			*mprF*	*ppnK*	*prfC*	*moaB*	*lipA*	*kdpB2*	*atl*	*sodA*	*argC*	*ahpF*	*aadD*	
A1	0.38	(S)	0.75	(S)	Reference	–	–	–	–	–	–	–	–	–	–	–	–
A2	2	(NS[a])	1	(S)	1	T345I	–	–	–	–	–	–	–	–	–	–	–
A2-S revertant	0.5	(S)	1	(S)	6	T345I	I183T	I349T	C121R	M92I	G481R	–	–	–	–	–	–
B1	0.38	(S)	1	(S)	Reference	–	–	–	–	–	–	–	–	–	–	–	–
B2	2	(NS)	2	(S)	3	L776S	–	–	–	–	–	–	–	–	–	–	1 point mutation, 1 insertion
B2-S revertant	0.5	(S)	0.75	(S)	7	L776S	–	–	–	–	–	G876S	K154E	T161S	L188F	–	Same as B2
C1	0.38	(S)	0.75	(S)	Reference	–	–	–	–	–	–	–	–	–	–	–	–
C4	2	(NS)	2	(S)	1	459–466del	–	–	–	–	–	–	–	–	–	–	–
C4-S revertant	0.5	(S)	1.5	(S)	3	459–466del	–	–	–	–	–	–	–	–	–	L179L[b]	1 point mutation
D1	0.25	(S)	1	(S)	Reference	–	–	–	–	–	–	–	–	–	–	–	–
D2	2	(NS)	1.5	(S)	3	L826F	–	–	–	–	–	–	–	–	–	–	2 point mutations
D2-S revertant	0.5	(S)	0.75	(S)	4	L826F	–	–	–	–	–	–	–	–	–	–	Same as D2 + 1 point mutation

MIC breakpoints for susceptibility, non-susceptibility, respectively: daptomycin, ≦1 μg/mL and >1 μg/mL; for susceptibility, intermediate resistance, and resistance, respectively: vancomycin, 2, 4–8 and 16 μg/mL according to CLSI.
[a] NS: non-susceptible.
[b] Synonymous mutation.

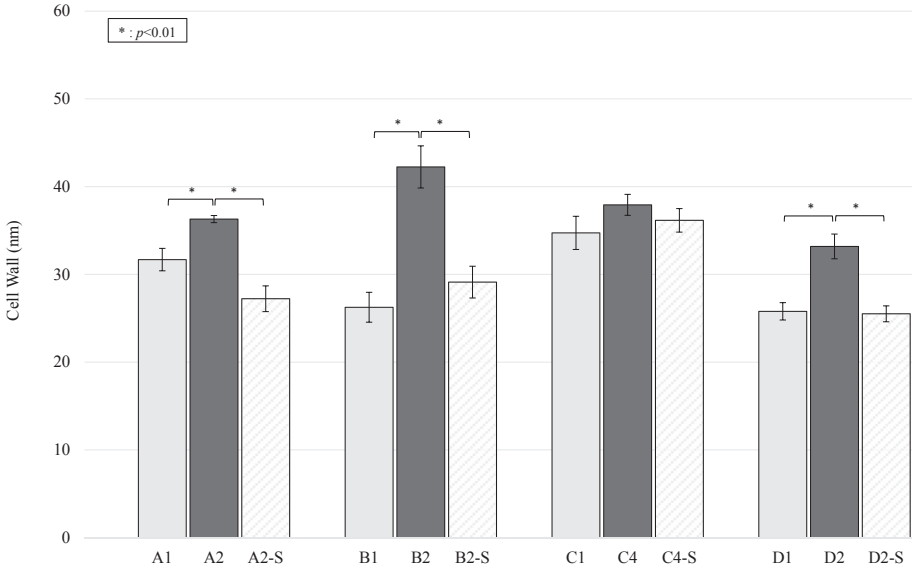

Fig. 1. Mean cell-wall thicknesses (Mean ± SEM) of DAP-S, DAP-NS and *in vitro* revertant DAP-S MRSA strains using transmission electron microscopy. The x-axis represents the mean of 16 measurements taken of 10 cells from each strain. A two-sided *t*-test was used to compare mean cell wall thickness differences.

susceptible strain, cell-wall thickness was decreased to the same level as DAP-S MRSA. One paired strain set (C4 vs C4-S revertant) showed no change in the cell-wall thickness (Fig. 1).

4. Discussion

Previous studies of DAP-NS *S. aureus* strains suggested that resistance is linked to changes in the cell membrane and cell wall [16,17]. The most commonly proposed mechanism of DAP resistance is bacterial cell membrane surface charge changes from negative to positive associated with mutations in the *mprF* gene, which prevents positively charged DAP-calcium complex insertion by electrostatic repulsion [18]. Several studies have reported that DAP-NS *S. aureus* strains frequently exhibit phenotypic changes that parallel those typically observed in vancomycin-intermediate *S. aureus* (VISA) isolates [19]. The most important change is a marked increase in the thickness of the cell wall. In VISA isolates, the degree of cell wall thickness has correlated with increase in DAP MICs [20]. Recent research is consistent with the understanding that multiple genetic mechanisms are involved in the development of DAP-NS *S. aureus* as none of the resistant strains in that study showed identical mutations except for strains which were derived from the same parent [21]. This study suggests that *S. aureus* evolves due to complex molecular changes involved in the development of DAP-NS. Nevertheless, the mechanisms leading to DAP resistance in *S. aureus* are not fully understood.

In this study, we investigated the factors explaining why MRSA, that develop resistance to DAP during treatment, reverted to DAP-S after treatment with DAP was discontinued. WGS of *in vitro* revertant DAP-S MRSA showed the presence of *mprF* mutations in all isolates tested. This would indicate that *in vitro* reversion of DAP-NS MRSA to DAP-S is independent of mutations in the *mprF* gene. This observation is consistent with previous findings reporting the presence of *mprF* mutations in clinical DAP-S MRSA [11,22].

In the study by Bayer et al., SNPs (I498N and Q692E) detected in DAP-S MRSA genomic regions outside of mutation hotspots raises the possibility that these mutations do not directly affect *mprF* function [22]. However, using WGS, a mutation (T345I) we detected in an *in vitro* revertant DAP-S strain (A2-S) was located within a mutation hotspot affecting L-PG synthesis and translocation function of the *mprF* gene [23]. This would suggest that, even with a *mprF* mutation within the bifunctional domain hotspot, a MRSA can be DAP-S. In addition, multiple gene mutations not detected in the parent DAP-NS MRSA strain were present in the *in vitro* revertant DAP-S strains. A mutation in *kdpB2*, which encodes a β-subunit (transmembrane protein) of the potassium-transport ATPase, was detected in an *in vitro* revertant DAP-S (A2-S) strain. It has been reported that increased expression of this transmembrane protein, which is a part of the cell membrane transport system, is the result of DAP exposure inducing *kdpB2* gene expression [24]. The *atl* gene mutation detected in an *in vitro* revertant DAP-S (B2-S) MRSA codes for a bifunctional autolysin involved in peptidoglycan hydrolysis and plays a role in cell wall turnover, cell separation, cell division and antibiotic-induced autolysis [25]. It has been reported that, decreased expression of the *atl* gene, resulting in lower levels of cell wall autolysin, leads to increased cell wall thickness and plays a role in less susceptibility to vancomycin in VISA [26]. In this study, we were not able to clarify how these gene mutations affect susceptibility to DAP although it cannot be ruled out that they play a role in reversion of DAP-NS to DAP-S. Additional research on the relationship between mutations involving *kdpB2* and *atl* and reversion of DAP-NS MRSA to DAP-S is necessary.

Results of cell wall thickness measurements to identify factors independent of *mprF* mutations accounting for reversion of DAP-NS MRSA to DAP-S showed that increased cell-wall thickness was associated with resistance to DAP while a decrease in cell wall thickness correlated with reversion to DAP-S in 3 of the 4 pairs of DAP-NS and DAP-S strains tested ($p < 0.01$). These observations suggest that cell-wall thickness may be a factor in reversion of DAP-NS strains to DAP susceptibility although there was no difference in cell-wall thickness pre- and post-DAP treatment in strain C between pre-DAP (C1) versus DAP-NS (C4) as well as DAP-NS (C4) and *in vitro* revertant DAP-S (C4-S). In C4 and C4-S strains, nucleotide deletion mutations (1375–1398) in the *mprF* gene resulted in amino acid deletions (459–466). Although a mutation in the *mprF* gene has not been firmly established to be linked to cell-wall thickness differences, a SNP (L826F) was previously reported to be associated with cell-wall thickening [27]; however, as there are few reports of deletions in *mprF* being associated with cell-wall thickness, this observation remains to be determined validated.

On the other hand, Roch et al. reported that there is a high biological fitness cost associated with DAP resistance as a result of mutations in the *mprF* gene and that, in the absence of selection pressure from DAP, reversion to the susceptible phenotype occurs due to better fitness [28]. Although we were not able to detect any genetic mutations to explain reversion to DAP susceptibility, selection pressure may account for changes in the overall bacterial population structure leading to reversion from DAP-NS to DAP-S MRSA.

5. Conclusion

Our results indicate that *in vitro* conversion of DAP-NS MRSA to DAP-S is independent of *mprF* gene mutations and may be partially explained by a change in cell wall thickness. However, as some strains showed no change in the cell wall, further studies are required to elucidate the different mechanisms of resistance to DAP, and factors for conversion of DAP-NS to DAP-S.

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Conflicts of interest

The authors have declared that no conflict of interest exists.
All the authors do not have any competing financial interests.

Ethical approval

This study was conducted with the approval of the ethics committee of Toho University Sakura Medical center (No. 2015-033).

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