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NOTE

A novel mutation in the *vraS* gene of *Staphylococcus aureus* contributes to reduce susceptibility against daptomycin

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Staphylococcus aureus is a Gram-positive bacterium that infects the skin and soft tissue in humans.[1,2] The spread of drug-resistant pathogens, such as methicillin-resistant *S. aureus* strains (MRSA), is a major clinical problem resulting in treatment failure.[3–5] The development of novel antibiotics to effectively treat resistant strains is urgently desired. Daptomycin, a cyclic lipopeptide antibiotic, was recently approved for the treatment of MRSA infection.[6] The bactericidal action of daptomycin results from disruption of the bacterial membrane integrity depending on the presence of calcium.[6] Clinical observation of *S. aureus* with reduced susceptibility to daptomycin, however, was recently reported.[7] To better understand the clinical limitations of daptomycin, it is important to identify the gene mutations involved in daptomycin resistance. For this purpose, we screened strains of *S. aureus* with reduced susceptibility to daptomycin and identified a mutation that contributes to daptomycin susceptibility. Identification by the usual genetic methods, however, was not possible due to the low level reduction of daptomycin susceptibility, thus we attempted to isolate mutants exhibiting both reduced daptomycin sensitivity and temperature sensitivity.[8] Here we demonstrated that a single mutation (E276K) in the *vraS* gene was responsible for both phenotypes.

To obtain mutants of *S. aureus* with reduced susceptibility to daptomycin, we prepared a full growth culture of a wild-type *S. aureus*, RN4220, in Luria Bertani medium (tryptone 10 g l^{-1}, yeast extract 5 g l^{-1} and NaCl 10 g l^{-1}) containing 0.2% ethylmethanesulfonate at 30 °C. The culture was diluted 100-fold with tryptic soy broth (TSB) and further incubated at 30 °C for 9 h. The resulting outgrowth was then spread onto TSB agar plates containing daptomycin (6.4 μg-ml^{-1}) and incubated at 30 °C for 2 days. Among 60 resistant colonies, two strains (D19 and D52) showed a temperature-sensitive phenotype that grew at 30 °C, but not at 43 °C. These mutants exhibited a twofold increase in the daptomycin MIC compared with the parent strain RN4220. To identify the mutated gene responsible for the temperature-sensitive phenotype, we screened a genomic library using a previously described method.[8] Transformants that grew at 43 °C were isolated, followed by plasmid extraction and sequencing of the inserted genome fragment. The plasmids complemented both phenotypes: temperature sensitivity and reduced daptomycin susceptibility. We found that a region containing the *vraS* gene was responsible for the complementation. We determined the mutated position in the *vraS* gene of the mutant genome by sequencing (primers; 5′-GCGACCTACATATTGACT-3′, 5′-GCCTTCACCAACTACTTC-3′ and 5′-ACCACCATTAGACCAACA-3′) and revealed that both mutants had the same mutation (g826a) in the *vraS* gene, which results in a single amino acid substitution (E276K).

To further confirm that this mutation in the *vraS* gene accounted for both reduced daptomycin susceptibility and temperature sensitivity, we performed a phage transduction analysis as described previously.[9] As a donor strain for phage transduction, we used a strain harboring an erythromycin-resistant marker inserted in the *SA1705* gene (M1705),[10] located 2.7 kb from the *vraS* gene. For the first phage transduction, the wild-type *vraS* gene was introduced into the mutant D52 (Dap^r,TS). Phages from the donor were used to infect the mutant D52, and transductants were selected based on the erythromycin-resistant phenotype. Among 20 transductants, 8 strains restored daptomycin sensitivity and temperature resistance, whereas the remaining transductants exhibited both reduced daptomycin susceptibility and temperature-sensitivity phenotypes. We next used *S. aureus* RN4220 with the wild-type *vraS* gene as the recipient strain in the second phage transduction experiment. A transductant from the first phage transduction with reduced daptomycin susceptibility and temperature-sensitive phenotypes was used as a donor strain. The findings indicated that the temperature-sensitive and reduced daptomycin-sensitive phenotypes were associated with the mutation in the *vraS* gene (Supplementary Table 1). On the basis of these results, we concluded that a single mutation in the *vraS* gene (g826a) was responsible for both the temperature-sensitive and reduced daptomycin-sensitive phenotypes. We used transductants isolated by the second phage transduction experiment for further analysis.

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Table 1 MIC of antimicrobial agents against second phage transductants

Genotype of vraS	MIC ($\mu g\,ml^{-1}$)						
	Daptomycin	*Vancomycin*	*Chloramphenicol*	*Linezolid*	*Ciprofloxacin*	*Tetracycline*	*Lysocin E*
Wild type	0.25	0.8	4	1.6	0.4	0.1	4
Mutant (E276K)	1	1.6	4	1.6	0.4	0.1	4

These MICs were determined by Clinical and Laboratory Standards Institute standard method using CAMHB (cation-adjusted Mueller–Hinton broth; Ca^{++} 50 mg l^{-1})[16] and using two different transductants for each genotype, and the same values were obtained.

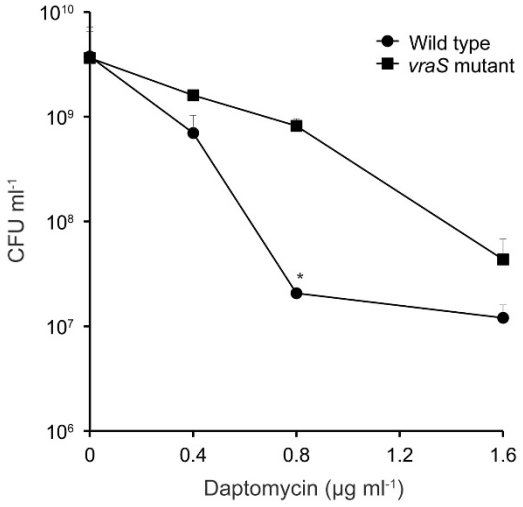

Figure 1 Bactericidal effect of daptomycin against the *Staphylococcus aureus* mutant. *S. aureus* was incubated in cation-adjusted Muellar–Hinton Broth (CAMHB: Ca^{++} 50 mg l^{-1}) containing daptomycin (0, 0.4, 0.8 and 1.6 μg ml^{-1}) at 30 °C for 1 h, and then the culture aliquots were collected and plated on LB10 plates according to standard method.[15] Cell viability was determined by counting the colony forming units per milliliter. Circle: wild-type *vraS* strain of the second phage transductant, square: *vraS* mutant of the second phage transductant. Statistically significant differences were analyzed by Student's one-tailed *t*-test (*$P<0.01$) from three independent experiments. The error bars are standard deviation.

The MIC of daptomycin against the *vraS* mutants was fourfold higher than that against the wild-type *vraS* strain (Table 1).

We next compared the bactericidal effects of daptomycin on the wild-type *vraS* strain and the *vraS* mutant strain. The number of surviving cells of the wild-type *vraS* strain was significantly reduced by two orders of magnitude after exposure to daptomycin at 0.8 μg ml^{-1}, whereas reduction of the *vraS* mutation (g826a) was less than an order of magnitude (Figure 1). This finding suggests that this single mutation in the *vraS* gene induced resistance against the bactericidal activity of daptomycin. We further examined the sensitivity of the *vraS* gene mutant to other antibiotics. The MIC value of vancomycin against the *vraS* gene mutants was twofold higher, whereas the sensitivity of the *vraS* gene mutants to chloramphenicol, linezolid, ciprofloxacin and tetracycline was indistinguishable (Table 1). These results suggest that the single point mutation of the *vraS* gene in *S. aureus* reduced the susceptibility to both daptomycin and vancomycin. To our knowledge, this is the first report that a single mutation (g826a) in the *vraS* gene is involved in reduction of the daptomycin and vancomycin susceptibility, and temperature sensitivity.

VraS functions as a membrane sensor in a two-component system that responds to cell wall stress induced by antibiotics such as vancomycin.[11] Previous studies reported that disruption of the *vraS* gene in *S. aureus* increased susceptibility to daptomycin and overexpression of the *vraS* gene restored resistance to daptomycin.[12,13] In addition, two amino acid substitutions in the *vraS* gene were recently reported to cause both vancomycin and daptomycin resistance.[14] In this study, we demonstrated that a novel single mutation in the *vraS* gene contributed to reduction of the daptomycin and vancomycin susceptibility of *S. aureus*. These findings imply that *S. aureus* with reduced daptomycin susceptibility has decreased susceptibility to vancomycin. In addition, the *vraS* gene mutant did not change susceptibility to lysocin E, which has membrane-damaging effects in *S. aureus* by a different mechanism from daptomycin (Table 1).[8] This finding suggests that lysocin E does not show cross-resistance with antibiotics such as vancomycin and daptomycin.

In conclusion, a single novel mutation (g826a) in the *vraS* gene is involved in a mechanism of reduced daptomycin susceptibility. We demonstrated that this mutation causes reduced vancomycin sensitivity. This single mutation (g826a) in the *vraS* gene may cause excess activation of the VraRS signaling pathway, although the precise mechanism requires further investigation.

CONFLICT OF INTEREST

KS is a consultant for Genome Pharmaceutical Institute. The remaining authors declare no conflict of interest.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)