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Correlation between Reduced Daptomycin Susceptibility and Vancomycin Resistance in Vancomycin-Intermediate *Staphylococcus aureus*

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We present here findings of a strong positive correlation between reduced daptomycin susceptibility and vancomycin resistance in vancomycin-intermediate *Staphylococcus aureus* (VISA). This correlation is related to cell wall thickening, suggesting that, similar to the case with vancomycin resistance in VISA, the physical barrier of a thickened cell wall may contribute to daptomycin resistance in *S. aureus*.

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections have become a general occurrence in hospitals, and the situation is worrying, since the pathogen is resistant to many antibiotics, including vancomycin, which was considered the last resort for treatment of MRSA infection (14). Several new antibiotics have been developed to counter this threat, and among them, linezolid, quinupristin-dalfopristin, and daptomycin are reported to be potential hopefuls for treatment of infection caused by multidrug-resistant MRSA, including vancomycin-intermediate *S. aureus* (VISA) (1). Presently, daptomycin has been approved in the United States for use in the treatment of complicated skin and skin structure infections caused by *S. aureus*, beta-hemolytic streptococci, and *Enterococcus faecalis* (3).

Daptomycin, a cyclic polypeptide, is a semisynthetic lipopeptide antibiotic derived from *Streptomyces roseosporus* (20). It exhibits its bactericidal function by penetrating the bacterial cell wall to bind cytoplasmic membranes, causing a rapid depolarization of the membranes, which results in the loss of membrane potential. This will lead to inhibition of protein, DNA, and RNA synthesis, and finally bacterial cell death (19). Due to its unique mechanism of action, it has been generally assumed that daptomycin-resistant organisms are difficult to generate. However, recently Mangili et al. reported a case of daptomycin treatment failure, where the patient's MRSA developed daptomycin resistance, leading to therapeutic failure for high-grade MRSA bacteremia (17). In addition, the patient also did not respond to vancomycin treatment. Nevertheless, no mechanism of resistance towards daptomycin has been identified so far, and there are no known transferable elements that confer resistance to daptomycin. We report here our findings of a strong positive correlation between reduced susceptibilities of VISA to daptomycin and vancomycin, and the correlation is related to cell wall thickening.

Through our previous study on vancomycin resistance in VISA strains, we found that a thickened cell wall is a common characteristic for VISA strains, serving as a physical barrier against the penetration of vancomycin molecules, resulting in vancomycin resistance (6, 7, 9). Since daptomycin is quite big in molecular size (molecular weight, 1,620.67), comparable to vancomycin (molecular weight, 1,485.7), we suspected that daptomycin might not be able to penetrate the cell wall smoothly if the bacterial cell wall becomes as thick as that of VISA strains. If that is the case, daptomycin might be blocked by the thickened cell wall before reaching the cytoplasmic membrane, resulting in ineffective bactericidal function on the target cells. To clarify the questions raised, two approaches were chosen to evaluate the bactericidal activity of daptomycin against MRSA strains with different cell wall thicknesses and vancomycin susceptibilities. The correlation between cell wall thickness and daptomycin susceptibility was also analyzed. First, 16 isogenic triple sets of VISA phenotype-associated *S. aureus* strains (VISA clinical strains, their respective vancomycin-susceptible derivatives generated by serial passage of parent strains on drug-free medium, and in vitro-developed vancomycin-resistant revertants generated from respective vancomycin-susceptible derivatives by one-step vancomycin selection) along with some control strains were employed. These strains were well characterized in their glycopeptide susceptibility, cell wall thickness, and some other biological features (8, 9). Hence, they would be ideal tools for magnifying the changes associated with the vancomycin-resistant phenotype and for investigating the correlation of the above phenotypes with daptomycin susceptibility, together with the correlation between cell wall thickness and daptomycin susceptibility. Second, susceptibility tests of daptomycin and vancomycin for all 53 strains used in this study were carried out simultaneously to minimize systematic error in the experiments. MIC determination was carried out with both broth microdilution and agar dilution methods, and results were read after 24 and 48 h of incubation. In addition to Mueller-Hinton (MH) medium, brain heart infusion (BHI) medium was also used in the susceptibility tests for optimal expression of the VISA phenotype (10, 18). A gradient gel assay was also carried out to determine the antibiotic susceptibilities, since it can measure minor changes with continuous values, allowing appropriate statistical analysis to be performed. Media supplemented with 50 mg of calcium per liter were used for daptomycin susceptibility tests throughout this study as suggested elsewhere (5).

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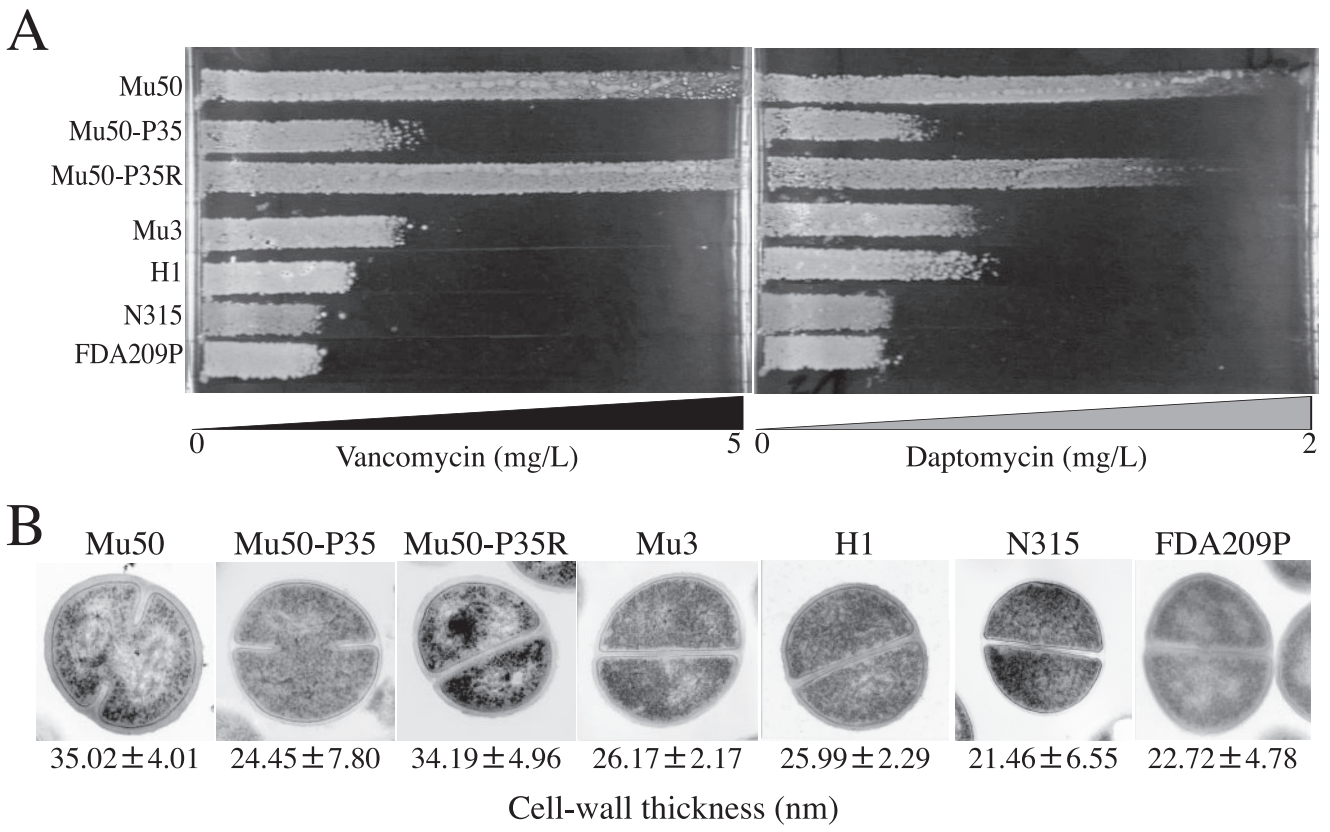

FIG. 1. Comparison of vancomycin and daptomycin susceptibilities for a variety of *S. aureus* strains with different cell wall thicknesses. (A) Vancomycin and daptomycin susceptibilities were determined using an antibiotic gradient gel assay with BHI agar that contained top concentrations of vancomycin (5 mg/liter) and daptomycin (2 mg/liter). (B) Representative transmission electron microscopy and cell wall thickness of tested strains. Magnification, ×20,700. The values given under each panel are the means and standard deviations of cell wall thickness calculated from 30 cells for each strain. The photos and data are from our previous study (9).

In our initial experiment, daptomycin and vancomycin susceptibilities were compared using an antibiotic gradient gel assay for a variety of *S. aureus* strains which have different cell wall thicknesses and levels of susceptibility towards vancomycin. Strains FDA 209P, N315, and H1 are vancomycin-susceptible clinical isolates with vancomycin MICs of 0.5, 1, and 2 mg/liter, respectively, and strain Mu3 is hetero-VISA with a vancomycin MIC of 2 mg/liter (12). Strain Mu50 is a type strain of VISA with a vancomycin MIC of 8 mg/liter (15). Mu50-P35 is a vancomycin-susceptible passage derivative of Mu50 with a vancomycin MIC of 2 mg/liter, and Mu50-P35R (vancomycin MIC, 8 mg/liter) is a VISA phenotype revertant obtained by one-step selection of Mu50-P35 on 4 mg/liter vancomycin (9). Figure 1A shows the results of the vancomycin and daptomycin gradient gel assay. As expected, the resistance patterns (growth length along the gradient gel) for each strain for both daptomycin and vancomycin were similar. Strains with higher resistance to vancomycin, i.e., strains Mu50 and Mu50-P35R, had lower susceptibility to daptomycin, while vancomycin-susceptible strains like N315 and FDA 209P were also susceptible to daptomycin. Mu3, whose vancomycin resistance level is between those of Mu50 and N315, had a growth length which is between those of the other two strains on the daptomycin gradient gel. Moreover, electron microscopy results showed that cell wall thickness might be related to daptomycin susceptibility, as shown in Fig. 1B.

To ascertain whether the observed similarity of resistance patterns of each strain for vancomycin and daptomycin can be attributed to similar resistance mechanisms, a large-scale study was performed with 53 well-established *S. aureus* strains, which included 16 isogenic triple sets of VISA, vancomycin-susceptible and -resistant in vivo mutants, and control strains (9). As expected, susceptibilities of vancomycin and daptomycin were well correlated (see Table S1 at http://www.staphylococcus.org/en/cui/dap2006/Table_S1.pdf). The range of daptomycin MIC for VISA was 2 to 5 (at 24 h) and 2 to 7 mg/liter (at 48 h) when MH broth was used as the growth medium, while it was 0.5 to 2 (at 24 h) and 1 to 3 mg/liter (at 48 h) for vancomycin-susceptible *S. aureus* (VSSA). When BHI medium was used as the growth medium, the range for VISA and VSSA increased to 2 to 7 and 1 to 3 mg/liter at 24 h and 2 to 10 and 1 to 4 mg/liter at 48 h, respectively. These results are consistent with the results reported by Petersen et al. (18). However, there are several significant findings which are different from previous reports on this subject (1, 2, 5, 18). First, a strongly positive correlation between vancomycin and daptomycin susceptibility was observed with *S. aureus*. The level of reduced daptomycin susceptibility was higher for the strains which have higher

TABLE 1. Linear regression coefficients among vancomycin and daptomycin susceptibilities and cell wall thicknesses[a]

Variable[b]	Linear regression coefficient[c]											
	Cell wall thickness (nm) (46[d])	DPT length (cm) (52)	VCM length (cm) (52)	VCM MIC				DPT MIC			
				BHI broth (53)		MH broth (53)		BHI broth (53)		MH broth (53)	
				24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Cell wall thickness (nm)		0.883	0.862	0.861	0.903	0.816	0.829	0.652	0.782	0.653	0.679
DPT length (cm)			0.814	0.784	0.811	0.743	0.802	0.589	0.714	0.569	0.674
VCM length (cm)				0.892	0.895	0.808	0.839	0.715	0.807	0.644	0.634
VCM MIC, BHIb, 24 h					0.955	0.899	0.924	0.676	0.779	0.576	0.605
VCM MIC, BHIb, 48 h						0.895	0.921	0.747	0.814	0.673	0.708
VCM MIC, MHb, 24 h							0.894	0.686	0.776	0.624	0.658
VCM MIC, MHb, 48 h								0.681	0.778	0.631	0.699
DPT MIC, BHIb, 24 h									0.927	0.774	0.733
DPT MIC, BHIb, 48 h										0.762	0.807
DPT MIC, MHb, 24 h											0.829
DPT MIC, MHb, 48 h											

[a] Linear regression analysis was performed on the values of vancomycin and daptomycin susceptibility and cell wall thickness. The susceptibility tests were performed with various culture conditions and methods (see the text). The values of cell wall thickness are from our previous report (9). *P* values are <0.0001 for all tests.

[b] Abbreviation: VCM, vancomycin; DPT, daptomycin; BHIb, brain heart infusion broth; MHb, Mueller-Hinton broth; MIC, minimum inhibitory concentration (mg/liter). DPT length, VCM length, etc., refer to length of bacterial growth on the antibiotic gradient gel. The MICs were read at 24 and 48 h of incubation.

[c] No. of tested samples is given parenthetically after each variable.

[d] For cell wall thickness and length of growth with DPT, no. of samples is 45.

levels of resistance to vancomycin, irrespective of culture medium, incubation time, and susceptibility test method. Second, the shift of vancomycin susceptibility among the isogenic triple sets of VISA, VSSA, and VISA revertants was coupled by changes in daptomycin susceptibility in the same direction without any exception in all tested sets. All vancomycin-susceptible derivatives had lower daptomycin MICs than their isogenic vancomycin-resistant counterparts (see Table S1 at the URL given above). Third, the reduction of daptomycin susceptibility showed a good correlation with the increment of cell wall thickness. These results provided support for our initial hypothesis that *S. aureus* has similar mechanisms for resistance to both daptomycin and vancomycin. The results of regression analysis for all tested strains in the study are summarized in Table 1, and the correlation between daptomycin and vancomycin susceptibilities, as well as that between daptomycin susceptibility and cell wall thickness, is illustrated in Fig. 2. Data show that the level of daptomycin susceptibility correlated strongly and positively with that of vancomycin susceptibility ($r = 0.814$; $P < 0.0001$) and with cell wall thickness ($r = 0.883$; $P < 0.0001$). Recently, Cha et al. reported a pharmacodynamic study of the first isolate of vancomycin-resistant *S. aureus* (known as Michigan VRSA) and showed that this strain was highly susceptible to daptomycin, with a MIC of 0.25 mg/liter (5), even though it had a vancomycin MIC of 1,024 mg/liter. The nonexistence of a correlation between vancomycin and daptomycin susceptibility demonstrated in this strain does not contradict our results (see below). Unlike VISA strains, which resist vancomycin by cell wall thickening (6, 7, 11), VRSA resists vancomycin by acquisition of a *vanA* gene transposon which carries a unique set of genes for vancomycin resistance. In the presence of the *vanA* gene transposon Tn*1546*, *S. aureus* can modify vancomycin-binding targets to prevent their cell wall components from being bound to vancomycin (4).

Nevertheless, more detailed studies have to be carried out, since it is known that the mechanisms of action of daptomycin

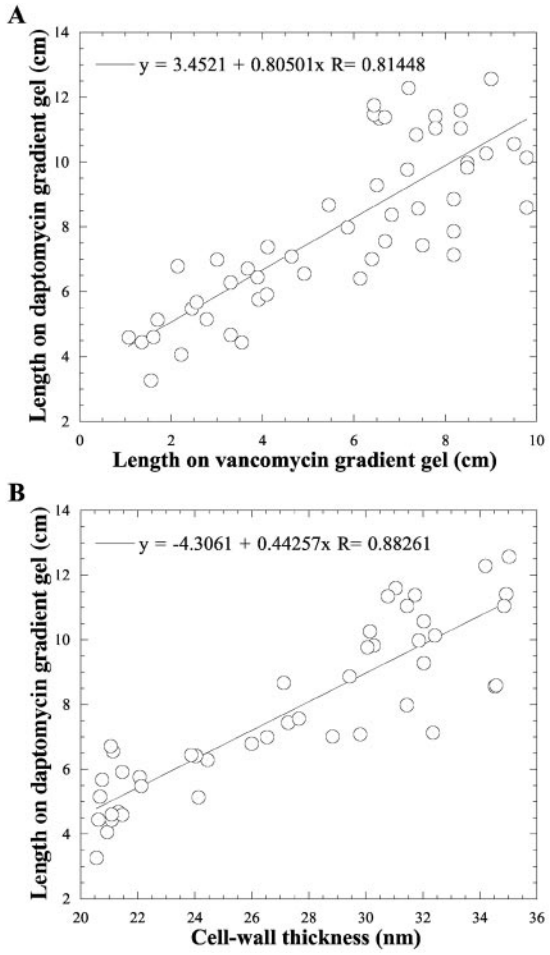

FIG. 2. Regression analysis of correlation between vancomycin and daptomycin susceptibilities and between cell wall thickness and daptomycin susceptibility. A significant correlation was seen in both between vancomycin and daptomycin susceptibilities (A) and cell wall thickness and daptomycin susceptibility (B).

and vancomycin are different, even though these two antibiotics have to penetrate through the cell wall layers to reach the cytoplasmic membrane, which is a lethal target for their action. Vancomycin, unlike daptomycin, appears to exert its bactericidal effect by binding to D-alanyl-D-alanine residues of peptidoglycan and its precursor units that are present in the membrane, inhibiting cell wall peptidoglycan synthesis (11, 13). However, the binding with cell wall peptidoglycan can also cause a remarkable decrease of vancomycin diffusion (clogging effect) through the cell wall when the cell wall become as thick as that of VISA strains (6, 7, 11). Recently we demonstrated that the cooperative effect of clogging and cell wall thickening enables VISA to obstruct vancomycin from its true target in the cytoplasmic membrane, resulting in vancomycin resistance in VISA strains (7). The strong positive correlation between vancomycin and daptomycin susceptibilities observed in the present study might imply the possibility of the existence of some machinery involved in cross-resistance between vancomycin and daptomycin in *S. aureus*. The possible explanation for this correlation may be that the thickened cell wall acts as a common obstacle to daptomycin and vancomycin penetration. Even though daptomycin does not bind peptidoglycan to form subsequent physical barriers within the cell wall (16), it might be hard for daptomycin, with a molecular weight over 1,620, to smoothly penetrate the cell wall when the cell wall becomes as thick as that of VISA. The data presented in this study suggest that development of new antibiotics with smaller molecular sizes than those of vancomycin and daptomycin may be a potential new way to overcome vancomycin- and daptomycin-resistant *S. aureus* infections.

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