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Mechanism of Reduced Vancomycin Susceptibility Conferred by *walK* Mutation in Community-Acquired Methicillin-Resistant *Staphylococcus aureus* Strain MW2

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Point mutations with unclear molecular mechanisms are often associated with vancomycin resistance in *Staphylococcus aureus*. Here, we observed that the *walK* (G223D) mutation caused decreased expression of genes associated with cell wall metabolism, decreased autolytic activity, thickened cell walls, and reduced vancomycin susceptibility. A phosphorylation assay showed that WalK (G223D) exhibited reduced autophosphorylation, which led to reduced phosphorylation of WalR. An electrophoretic mobility shift assay indicated that WalK (G223D)-phosphorylated WalR had a reduced capacity to bind to the *atlA* promoter.

With the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin has been the leading therapy for serious MRSA infections. However, *S. aureus* strains with reduced susceptibilities to vancomycin (vancomycin-intermediate *S. aureus* [VISA] and heterogenous VISA [hVISA]) have emerged (1). Mutations identified in a couple of two-component regulatory systems, such as *vraRS* (2), *graRS* (3, 4), and *walKR* (5, 6), were shown to contribute to the development of VISA (5, 7). However, the molecular mechanisms have been incompletely defined (8). WalK is a sensor kinase of the WalKR two-component regulatory system (9–11), and *walK* mutations across the spectrum of the domains that contribute to two-component regulatory function have been found in many clinical VISA strains isolated from various countries and laboratory-derived VISA (5, 6). Nevertheless, the same mutations are not found in both clinical and laboratory-derived VISA strains. Thus, a key question is whether the mechanism of laboratory-derived VISA strains is analogous to that of clinical VISA strains.

The laboratory-derived VISA strain, designated SV-1, was selected by serial passage of the susceptible *S. aureus* strain MW2 through progressively increasing concentrations of vancomycin according to a previously described protocol (5). To identify the genetic changes that confer vancomycin resistance, whole-genome sequencing of wild-type MW2 and SV-1 was completed at the National Center for Gene Research using the paired-end sequencing of Solexa. Five mutations were identified (Table 1) and further confirmed by PCR and sequencing (see the supplemental material). Among the mutations, a single-nucleotide polymorphism within *walK* (conferring the G223D amino acid change) in SV-1 was also found in the clinical VISA strain JKD6008 (6), supporting the validity of our approach in identifying clinically relevant resistance mechanisms and suggesting that this point mutation probably plays an important role in reduced vancomycin susceptibilities in clinical *S. aureus* isolates.

To determine the effect of the *walK* mutation, allelic replacement was performed to generate the *walK* mutant using the vector pBTs, which was derived from pBT2 and pKOR1 (12, 13). To construct pBTs, the segment containing antisense *secY*, which can inhibit colony formation on agar plates, was cloned into pBT2, and the fragment containing the *walK* point mutation was cloned into pBTs (see the supplemental material). Antibiotic susceptibilities of the *walK* mutant were evaluated by determining the MICs of vancomycin and daptomycin using

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TABLE 1 Whole-genome sequence comparison of SV-1 and MW2[a]

Mutation	Effect of mutation	Locus	Gene	Function
T to G	L307V	MW1069	*ftsZ*	Bacterial cell division, essential
G to A	G223D	MW0019	*walK*	Response regulator, essential
C to T	L294F	MW0498	*rpoC*	RNA polymerase β′ subunit
CACCATTCAC to C	Frame shift deletion	MW1156	*rpsO*	30S ribosomal protein S15
T to TGTTTGTATTAAT	Frame shift insertion	MW0709	*llM*	Lipophilic protein affecting lysis rate and methicillin resistance level protein

[a] Vancomycin-susceptible MRSA strain used as a sequence control.

TABLE 2 Antibiotic susceptibilities of *Staphylococcus aureus* used in this study

Strain	MIC[a] (mg/liter) of indicated antibiotic in:		
	BHI[b] medium, Vancomycin	Mueller-Hinton broth	
		Vancomycin	Daptomycin
MW2	2	1	1
SV-1	16	16	4
walK mutant	4	2	2

[a] MICs were determined by broth microdilution method. The results were read after 24 h of incubation at 37°C.
[b] BHI, brain heart infusion.

microbroth MIC according to CLSI criteria (14). Significant increases in the vancomycin and daptomycin MICs were detected in the *walK* mutant compared with those of the parent strain MW2 (Table 2). Other recognized VISA phenotypes, such as a thickened cell wall (Fig. 1A) and decreased autolytic activity (Fig. 1B), were observed by electron microscope evaluation of cell wall thicknesses (15) and Triton X-100-stimulated autolysis (16) in the *walK* mutant. Population analysis was performed as described previously (17), and the results showed reduced vancomycin susceptibility in the *walK* mutant (Fig. 1C). It has been recognized that *atlA*, *ssaA*, *isaA*, and *lytM* are involved in cell wall metabolism and are potential members of the WalK/WalR regulon (18) and are upregulated by *walKR* (19). To determine whether the expression of these genes changed in the *walK* mutant, total RNAs were isolated, and real-time reverse transcriptase PCR was performed as described previously (20). The results indicate that the expression of *atlA*, *ssaA*, *isaA*, and *lytM* decreased in the *walK* mutant (Fig. 1D), implying the coupling of cell wall metabolism to autolytic activity and antibiotic resistance.

It has been reported that the *walK* mutation in strain JKD6008 caused reduced vancomycin susceptibility (6), but the mechanism of how a single nucleotide substitution led to vancomycin resistance was not known. To illustrate the molecular mechanism linking the emergence of vancomycin resistance to point mutations, we overexpressed WalK, WalK (G223D), and WalR by cloning the full-length *walR* open reading frame (ORF) and partial *walK* ORF (isolated cytoplasmic histidine kinase domain, residues 220 to 609) into pET28a(+) (Novagen) (see the supplemental material). To determine whether the kinase activity of WalK (G223D) changed, WalK and WalK (G223D) autophosphorylation was examined as described previously (21). Autophosphorylation of WalK (G223D) was decreased compared with that of WalK, as demonstrated by incubating the proteins with [λ-^{32}P]ATP and then subjecting them to SDS-PAGE and autoradiography (Fig. 2A, lanes 1 to 6). When incubated with WalK or WalK (G223D), WalR was phosphorylated by the kinases upon addition of [λ-^{32}P]ATP. The phosphorylation levels of WalR by WalK (G223D) were lower than those by WalK at approximately 1 and 5 min and then reached equilibrium at approximately 10 min (Fig. 2A, lanes 7 to 14). A previous report showed that WalR can regulate the expression of *lytM*, *ssaA*, and *isaA* by binding to their promoters (19). In our experiments, WalK or WalK (G223D) was incubated with increasing amounts of WalR and the *atlA* promoter labeled with biotin at room temperature, and the reaction was initiated by the addition of ATP. The mixtures were electrophoresed in 8% native polyacrylamide gel 1 min after the reaction, and bands were detected by the BrightStar BioDetect kit (Ambion). Our electrophoretic mobility shift assay (EMSA) result

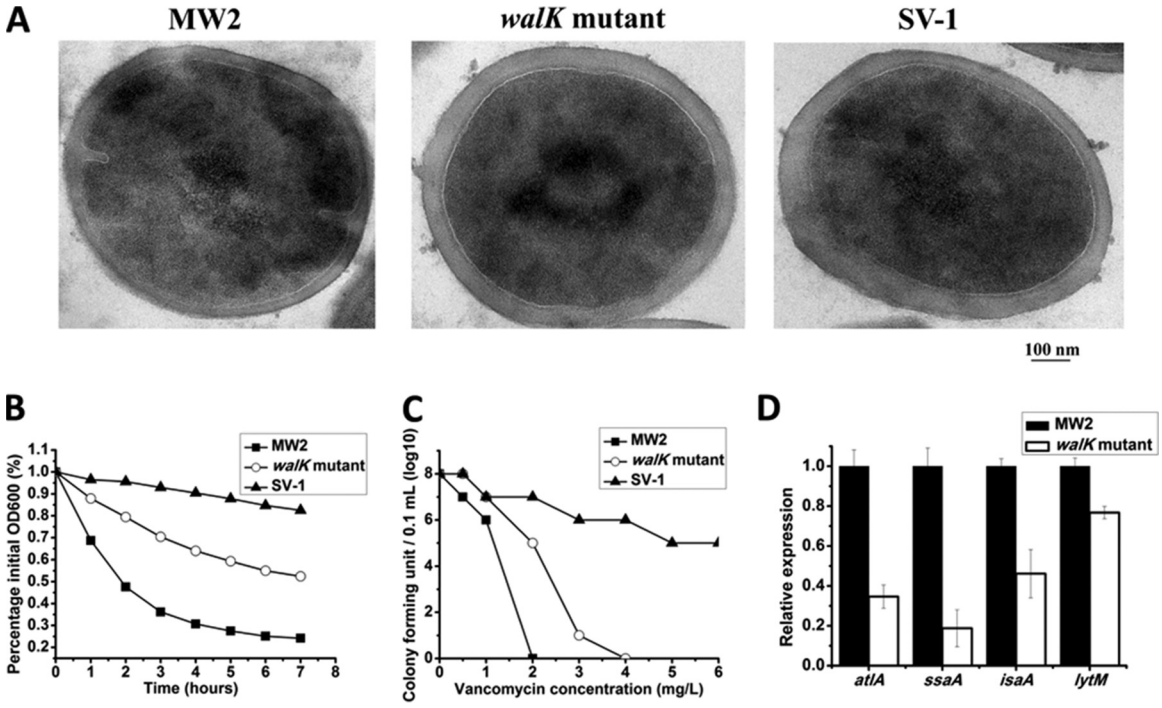

FIG 1 Phenotypic and gene expression analyses of *S. aureus* strain MW2, the *walK* mutant, and SV-1. (A) Transmission electron microscopy was performed to evaluate cell wall thicknesses. SV-1 was a laboratory-derived vancomycin-intermediate *S. aureus* isolate from strain MW2. The *walK* mutant was generated from MW2 by substitution of *walK* with that of SV-1. (B) Triton X-100-stimulated autolysis. (C) Vancomycin population analysis profiles of MW2, the *walK* mutant, and SV-1. (D) Genes involved in cell wall degradation, such as *atlA*, *ssaA*, *isaA*, and *lytM* have decreased expression in the *walK* mutant compared with that of MW2.

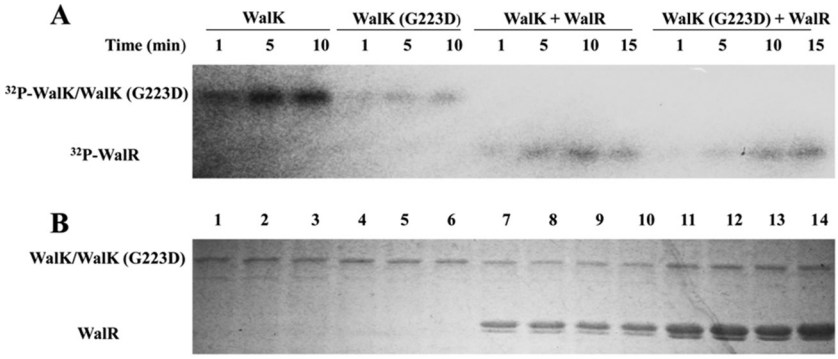

FIG 2 Phosphorylation of WalK, WalK (G223D), and WalR. (A) *In vitro* phosphorylation of WalK, WalK (G223D), and WalR. WalK was incubated with [λ-^{32}P]ATP for 1 (lane 1), 5 (lane 2), and 10 min (lane 3) at room temperature. WalK (G223D) was incubated with [λ-^{32}P]ATP for 1 (lane 4), 5 (lane 5), and 10 min (lane 6) at room temperature. WalK and WalR were incubated for various time intervals following the addition of [λ-^{32}P]ATP. Lane 7: 1 min; lane 8: 5 min; lane 9: 10 min; lane 10: 15 min. WalK (G223D) and WalR were incubated for various time intervals following the addition of [λ-^{32}P]ATP. Lane 11: 1 min; lane 12: 5 min; lane 13: 10 min; lane 14: 15 min. (B) SDS-PAGE analysis of proteins in the phosphorylation assay above.

showed that WalR can bind specifically to the *atlA* promoter, and WalK (G223D)-phosphorylated WalR exhibited a reduced capacity to bind to the *atlA* promoter region (Fig. 3). These results indicate that WalK (G223D) exhibited decreased autophosphorylation activity and led to the weaker ability of WalR to bind to the *atlA* promoter. Among the mutations detected in a range of functional domains of *walK*, the G223D mutation in SV-1 is found at a highly conserved residue of the HAMP domain, which connects extracellular sensory domains with intracellular signaling domains. The HAMP domain relays conformational changes between the two domains (22). As a result, we speculate that the G223D mutation causes the functional change of the HAMP domain and then affects the kinase activity of WalK.

In conclusion, our data indicate that a *walK* mutation led to reduced autophosphorylation of WalK and reduced phosphorylation of WalR, which caused the decreased expression of genes associated with cell wall metabolism, decreased autolytic activity, thickened cell walls, and reduced vancomycin susceptibility. This study suggests a molecular mechanism linking the emergence of antibiotic resistance to point mutations in two-component regulatory systems or transcriptional regulators.

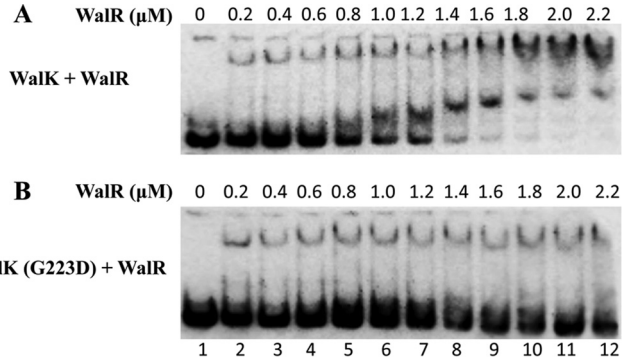

FIG 3 Measurement of binding ability of WalR to the *atlA* promoter by EMSA. WalR was incubated with WalK or WalK (G223D) and the *atlA* promoter, and the reaction mixtures were electrophoresed after the addition of 20 μM ATP at room temperature for 1 min. The bottom band was free DNA. Lane 1 was the free DNA probe; Lanes 2 to 12 were the DNA probe with increasing amounts of WalR. (A) Incubated with WalK. (B) Incubated with WalK (G223D).

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