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walK and *clpP* Mutations Confer Reduced Vancomycin Susceptibility in *Staphylococcus aureus*▽

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Vancomycin-intermediate *Staphylococcus aureus* (VISA) is generated from vancomycin-susceptible *Staphylococcus aureus* by multiple spontaneous mutations. We previously reported that sequential acquisition of mutations in the two-component regulatory systems *vraSR* and *graRS* was responsible for the VISA phenotype of strain Mu50. Here we report on the identification of a novel set of regulator mutations, a deletion mutation in two-component regulatory system *walRK* (synonyms, *vicRK* and *yycFG*), and a truncating mutation in a proteolytic regulatory gene, *clpP*, responsible for the raised vancomycin resistance in a laboratory-derived VISA strain, LR5P1-V3. The contributory effect of the two mutations to vancomycin resistance was confirmed by introducing the *walK* and *clpP* mutations into the vancomycin-susceptible parent strain N315LR5P1 by a gene replacement procedure. The vancomycin MIC of N315LR5P1 was raised from 1 to 2 mg/liter by the introduction of the *walK* or *clpP* mutation, but it was raised to 4 mg/liter by the introduction of both the *walK* and *clpP* mutations. The vancomycin MIC value of the double mutant was equivalent to that of strain LR5P1-V3. Like VISA clinical strains, LR5P1-V3 and the double mutant strain LR5P1*walK*clpP exhibited a thickened cell wall, slow growth, and decreased autolytic activity. Transcriptional profiles of the mutants with gene replacements demonstrated that introduction of both the *walK* and *clpP* mutations could alter expression of dozens or hundreds of genes, including those involved in cell envelope and cellular processes, intermediary metabolism, and information pathway. A mutation prevalence study performed on 39 worldwide clinical VISA strains showed that 61.5, 7.7, 10.3, and 20.5% of VISA strains harbored mutations in *walRK*, *clpP*, *graRS*, and *vraSR*, respectively. The mutation of *walRK* was most frequently carried by VISA strains. Together, these results suggested that the mutations of *walK* and *clpP* identified in LR5P1-V3 constitute a new combination of genetic events causing vancomycin resistance in *Staphylococcus aureus*.**

Staphylococcus aureus has become one of the most frequent causes of a wide range of both hospital- and community-acquired infections that range from superficial skin and soft tissue infections to life-threatening toxic shock, pneumonia, endocarditis, and septicemia. The spectacular adaptive capacity of this pathogen has resulted in the worldwide emergence and spread of clonal strains that have acquired resistance to the majority of currently available antimicrobial agents, including vancomycin. Vancomycin used to be the first-line antibiotic for the therapy of infections due to methicillin-resistant *S. aureus* (MRSA). However, increased use of vancomycin led to the emergence of vancomycin-intermediate *S. aureus* (VISA) in 1996 (33).

The genetic basis for vancomycin resistance in VISA remains largely unknown. Ever since the emergence of VISA in 1996, a number of investigations have been carried out to discover the molecular mechanism involved in the generation of VISA. More than a dozen genes have been reported to be associated with glycopeptide resistance, including *pbpB* (58), *pbpD* (25, 60), *sigB* (2, 13, 46, 52), *ddh* (4, 49), *tcaA* (44), and *walRK* (37). We have also reported on 12 other genes (*mgrA*, *msrA2*, *msrR*, *malR*, *lysC*, *graA*, *graB*, *graC*, *graD*, *graE*, *murZ*, and *rsbU*) whose overexpression raised vancomycin resistance in *S. aureus* (13). Most of these reports have provided evidence for the correlation between gene transcriptional levels with altered glycopeptide susceptibilities of tested cells. Several sets of up- and downregulated genes associated with glycopeptide resistance, including global regulators, were also reported (13, 41, 51, 63). However, none of these reports identified the mutations responsible for the altered vancomycin susceptibility.

Recently, a number of mutations were found in some VISA clinical strains, such as Mu50 (42, 54, 55), JH9 (53), and JKD6008 (35, 36), by comparison of their genomes with those of their clinically relevant glycopeptide-susceptible parent strains. However, except for the mutations in *vraSR* and *graRS*, the role of the mutations found in these strains has not been established. The *vraS* I5N mutation has been proven to confer heterogeneous vancomycin resistance when it was introduced into a vancomycin-susceptible MRSA strain (38).

The *graR* N197S mutation was shown to convert the heterogeneous resistant VISA (hVISA) phenotype (MIC ≤ 2 mg/liter) of strain Mu3 into the VISA phenotype (MIC ≥ 4 mg/liter) when it was introduced into Mu3 (54). Furthermore, the plasmid-mediated overexpression of *graR* N197S but not that of intact *graR* could raise the vancomycin MIC of vancomycin-

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susceptible strain N315 (13). In addition, when we sequentially introduced the *vraS* and *graR* mutations into VSSA strain Mu50Ω (MICs of both vancomycin and teicoplanin = 0.5 mg/liter), a mutant expressing a VISA phenotype indistinguishable from that of Mu50 (vancomycin MIC = 6 mg/liter, teicoplanin MIC = 12 mg/liter) was obtained (16). These results clearly demonstrated that point mutations in the two regulatory genes of *vraS* and *graR* were sufficient, at least in a strain having the Mu50 genetic background, for expression of the VISA phenotype. The contribution of the *graRS* regulator mutation to raising vancomycin resistance in another VISA clinical strain has also been confirmed by Howden et al. by introducing the *graS* T136I mutation found in VISA strain JKD6008 into a vancomycin-susceptible *S. aureus* strain (36). It was noticed, however, that the *vraSR* and *graRS* mutations are not frequently found in VISA clinical strains (36, 39). This implies that there are genetic mechanisms other than *vraSR-graRS* mutations that achieve a VISA phenotype. In this study, we demonstrated that another set of regulator mutations comprises an alternative pathway to the VISA phenotype, i.e., *walK*, encoding a histidine kinase of *walRK* two-component regulatory system (TCRS), and *clpP*, encoding a proteolytic regulatory protein. We also found that *walK* mutations were carried by as many as 24 of 39 (61.5%) VISA clinical strains isolated from various countries.

MATERIALS AND METHODS

Bacterial strains and growth conditions. N315LR5P1 is a laboratory MRSA strain in which *mecI* gene function has been inactivated by a 62-bp deletion of nucleotide positions 130 to 191 and its penicillinase (PCase) plasmid has been eliminated (1, 30). It is a derivative of pre-MRSA strain N315, whose *mecI* gene is intact and methicillin resistance is not fully expressed (43). N315LR5P1 represents the genotype of hospital-acquired MRSA (HA-MRSA) in Japan whose *mecI* gene function is inactivated by mutations. The absence of the PCase plasmid from this strain makes it more pertinent as a model to trace the development of vancomycin-intermediate resistance in Japanese HA-MRSA strains, since the hVISA and VISA representative strains Mu3 and Mu50, respectively, lacked the PCase plasmid. N315LR5P1 was classified as sequence type 5-staphylococcal cassette chromosome *mec* type IIa, and its *agr* type is II. LR5P1-V3 is an N315LR5P1-derived VISA strain generated by serial passage of N315LR5P1 (vancomycin and teicoplanin MICs = 1 mg/liter) on brain heart infusion (BHI) plates containing vancomycin with gradually increasing concentrations of 1 to 4 mg/liter as described previously (65). All the strains were grown in BHI broth (Difco, Detroit, MI) at 37°C with aeration (shaking at 20 rpm without a CO_2 supply), if not otherwise indicated. For each experiment, an overnight culture was diluted 100-fold in prewarmed fresh BHI broth and further incubated with aeration to ensure exponential growth condition before sampling. The cell growth was monitored by measuring the optical density of the culture at 578 nm (OD_{578}) with a spectrophotometer (Phamacia LKB Biotechnology, Inc., Uppsala, Sweden).

Antimicrobial susceptibility testing. The MICs for selected antimicrobials were determined for the constructed mutants and parent strains using Etest strips (AB Biodisk, Sweden). A sterile cotton swab was immersed in a 0.5 McFarland standard of tested bacterial culture and streaked on Mueller-Hinton (MH) agar and BHI agar plates. Antimicrobial strips were then applied 10 min after bacterial inoculation. Plates were then incubated at 37°C, and the MICs were read after 24 to 48 h of incubation. MICs of vancomycin and teicoplanin were also determined by both broth microdilution and agar dilution methods for vancomycin concentrations of 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 mg/liter and for teicoplanin at concentrations up to 16 mg/liter in 1-mg/liter increments. For daptomycin MIC determination, the test medium was supplemented with 50 mg/liter Ca^{2+} according to Clinical and Laboratory Standards Institute (CLSI) criteria (8).

Analysis of teicoplanin- and vancomycin-resistant subpopulations (population analysis). To know how many subpopulations of each strain in a fixed number of cells (usually about 10^7 colonies) are resistant to various given concentrations of vancomycin and teicoplanin, population analysis was carried out as described previously (32). Briefly, overnight cultures of tested strains in BHI broth were adjusted to an OD_{578} of 0.3 (about 1.0×10^8 CFU). A series of 10-fold dilutions of these cell suspensions was then prepared, and 0.1 ml of each suspension was spread on BHI agar plates containing various concentrations of teicoplanin or vancomycin. Plates were incubated at 37°C for 48 h, and the number of cell colonies was counted. The number of resistant cells in 0.1 ml of the starting cell suspension was plotted against each corresponding teicoplanin or vancomycin concentration.

Whole-genome-sequence comparison of N315LR5P1 and LR5P1-V3. Whole-genome-sequence comparison of VISA strain LR5P1-V3 and its vancomycin-susceptible parent strain, N315LR5P1, was performed using the array-based service (comparative genome sequencing [CGS]) provided by Roche NimbleGen, Inc. (Madison, WI), as described previously (17). Briefly, LR5P1-V3 (test) and N315LR5P1 (control) genome DNA samples were separately cleaved to form pools of low-molecular-weight fragments and labeled with the fluorescent dyes Cy3 and Cy5, respectively. The labeled samples were then competitively hybridized to two NimbleGen CGS whole-genome tiling arrays, which were generated with the *S. aureus* N315 genome sequence as a reference. The resulting hybridization signals were analyzed using NimbleScan (version 2.5) software, and the signal ratios of control versus test samples for all probes were plotted against the N315 genomic position. The locations of probes along the genome which have a significant ratio shift between test and control probes for both strands represent regions of possible sequence differences, including single nucleotide polymorphisms, deletions, sites of insertion, endpoints of inversion, or translocation. These locations were checked by PCR and resequencing of both test and control genomic DNA.

Generation of gene replacement mutants where an intended chromosome mutation(s) was introduced. To introduce chromosomal mutations identified in a drug-resistant mutant into its drug-susceptible parent strain, pKOR1-mediated gene replacement was performed as described previously (16, 54). Briefly, the gene in frame containing the intended nucleotide sequence was amplified from sequence donor strain LR5P1-V3 containing *walK* or *clpP* mutations using primers that contain *attB1* (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-) and *attB2* (5′-GGGGACCACTTTGTACAAGAAAGCTGGG-) sites on the respective up- and downstream sequences. This fragment was then cloned into pKOR1. Following cloning and *ccdB* selection in *Escherichia coli*, the constructed plasmid was then introduced into strain N315LR5P1 by electroporation using a GenePulser Xcell apparatus (Bio-Rad Laboratories, Inc., Hercules, CA) with the parameters described previously (43). Overnight culture of plasmid-carrying clones at 43°C selects for single-crossover mutants that carry both mutated and wild-type nucleotides. Single-crossover mutants were then cultured in drug-free broth to facilitate plasmid excision and subjected to anhydrotetracycline induction, whereby only non-plasmid-carrying mutants could survive. To check for successful introduction of the mutations, the resulting mutants were checked for the target sequences via sequencing. The colonies carrying the expected mutation that survived were set up as mutants, and those without the expected mutation were used as reverted controls as described previously (16, 17). By using this strategy, the chromosomal mutations, a deletion point mutation of *walK*, and a truncating mutation of *clpP* identified in drug-resistant strain LR5P1-V3 were introduced into the chromosome of drug-susceptible parent strain N315LR5P1.

Growth curve and autolysis assay. Growth curve and autolysis experiments were carried out in succession. Overnight cultures of tested strains were diluted 1/1,000 in 10 ml fresh BHI broth and grown at 37°C with shaking at 25 rpm in a photorecording incubator (TN-2612; Advantec, Tokyo, Japan). The OD was monitored automatically every 2 min, and cells were grown to an OD_{600} of 1.2. Following this, the cells were placed on ice for 10 min and pelleted by centrifugation at 7,500 rpm for 5 min for autolytic activity determination. The cell pellets were then washed twice with chilled 0.05 M Tris-Cl buffer (pH 7.2) and suspended in 10 ml Tris-Cl (0.05 M, pH 7.2) containing 0.05% Triton X-100. The suspensions were then incubated in the same photorecording incubator at 37°C with continuous shaking at 25 rpm. The decrease of the OD was monitored, with recording of the OD values every 2 min for 24 h. For growth curve and doubling time determinations, the values of OD versus time of each strain were plotted. Doubling times were calculated as follows: $[(t_2 - t_1) \times \log 2]/(\log OD_{600} \text{ at } t_2 - \log OD_{600} \text{ at } t_1)$, where t_1 is the first sampling time and t_2 is the second.

Nucleotide sequencing and mutation determination. Chromosomal DNAs of 39 VISA strains (14), including 23 VISA strains from the network on antimicrobial resistance in *S. aureus* (NARSA; http://www.narsa.net/content/default.jsp), were extracted using phenol-chloroform purification methods after digestion of cells with 2.0 μg/ml lysostaphin (Wako, Japan), and the nucleotide sequences of *vraSR*, *graRS*, *walRK*, and *clpP* (from 150 bp upstream to 100 bp downstream

TABLE 1. Antibiotic susceptibilities and doubling times of *S. aureus* strains used in this study

Strain	MIC (mg/liter)[a]													Doubling time (min)
	BHI medium		MH medium											
	VCM	TEIC	VCM	TEIC	LZD	DPC	IPM	BA	TC	MINO	CP	MUP	LVFX	
N315LR5P1	1.5 (1, 1)	1.5 (1, 1)	0.75 (1, 1)	0.75 (1, 1)	1.5	0.5	>32	48	0.38	0.25	8	0.38	0.19	35.62
LR5P1-V3	4 (8, 8)	12 (8, 8)	3 (4, 4)	4 (4, 8)	0.75	1	>32	96	0.064	0.032	6	0.125	0.19	44.16
LR5P1*walK*	1.5 (1, 1)	2 (1, 1)	0.75 (1, 1)	0.75 (1, 1)	1.5	0.38	>32	64	0.38	0.25	8	0.38	0.19	35.90
LR5P1*walK**	3 (4, 4)	6 (4, 4)	2 (2, 2)	3 (2, 2)	1.5	1	>32	64	0.38	0.25	8	0.38	0.19	37.20
LR5P1*clpP*	1.5 (1, 1)	1.5 (1, 1)	0.75 (1, 1)	0.75 (1, 1)	1.5	0.25	>32	48	0.38	0.25	8	0.38	0.19	36.30
LR5P1*clpP**	2 (2, 2)	3 (4, 4)	1.5 (2, 2)	2 (2, 2)	0.75	0.5	>32	48	0.094	0.032	6	0.125	0.19	43.23
LR5P1*walK***clpP*	2 (1, 1)	3 (1, 1)	1 (1, 1)	2 (1, 1)	1	0.5	>32	64	0.25	0.25	8	0.38	0.19	38.25
LR5P1*walK***clpP**	4 (4, 4)	12 (8, 8)	2.5 (4, 4)	4 (4, 4)	0.75	1	>32	64	0.047	0.032	6	0.125	0.19	45.29

[a] MICs were determined by Etest (Sysmex bioMérieux Co., Ltd.), broth microdilution, and agar dilution methods. The results were read after 24 h incubation at 37°C. Since some strains had slow growth, vancomycin and teicoplanin MICs were read after 48 h incubation to improve accuracy. The data in parentheses indicate MICs determined by broth microdilution, agar dilution. Abbreviations: VCM, vancomycin; TEIC, teicoplanin; LZD, linezolid; DPC, daptomycin; IPM, imipenem; BA, bacitracin; TC, tetracycline; MINO, minocycline; CP, chloramphenicol; MUP, mupirocin; LVFX, levofloxacin.

of the open reading frames [ORFs]) were determined using an ABI 3730 DNA analyzer (Applied Biosystems).

Electron microscope evaluation of cell wall thickness. Preparation of cells of the tested *S. aureus* strains for transmission electron microscopy and examination of the cells by transmission electron microscopy were performed as described previously (15). Morphometric evaluation of cell wall thickness was performed using photographic images at a final magnification of ×30,000. Thirty cells of each tested strain with nearly equatorial cut surfaces were measured for the evaluation of cell wall thickness, and results were expressed as mean values ± standard deviations (SDs).

RNA preparation and microarray analysis. The preparation of microarray chips with the whole ORF of the *S. aureus* N315 chromosome and transcriptional profile analysis of the constructed mutants versus controls were carried out as described previously (13). Bacterial culture, RNA extraction, cDNA labeling, hybridization, and data analysis for microarray analysis were carried out according to protocols described previously (13, 17).

Statistical analysis of data. The statistical significance of the data were evaluated with Student's *t* test.

Microarray data accession number. Transcriptional profiles of *walK* and/or *clpP* mutant-related strains may be found in CIBEX under accession number CBX144.

RESULTS AND DISCUSSION

Whole-genome-sequence comparison between N315LR5P1 and LR5P1-V3. To study the genetic mechanism of vancomycin resistance in *S. aureus*, we generated an *in vitro* VISA strain, LR5P1-V3, by stepwise vancomycin selection. The strain was derived from N315LR5P1, a teicoplanin- and vancomycin-susceptible laboratory MRSA strain (1, 30). The raised vancomycin MICs of LR5P1-V3 were 4 mg/liter in MH medium and 8 mg/liter in BHI medium, compared to 1 mg/liter each for N315LR5P1 (Table 1). Besides decreased susceptibility to vancomycin, the strain showed typical VISA phenotypes; i.e., thickened cell wall, slow growth, and decreased autolytic activity (14) (see Fig. 3 and 4). Sequencing of the *vraSR* and *graRS* genes of LR5P1-V3 revealed no mutation, indicating that LR5P1-V3 has a genetic mechanism distinct from that of Mu50 for the raised vancomycin resistance. This prompted us to carry out whole-genome-sequence comparison between strains LR5P1-V3 and N315LR5P1 to identify genes responsible for the raised vancomycin resistance.

We employed the NimbleGen tiling array to compare the whole-genome sequences of the two strains as reported previously (17). Two mutations in separate genes were identified in LR5P1-V3 compared to the sequence in parent strain N315LR5P1. The first, a deletion mutation, was found in two-component regulatory system *walRK*, where 3 nucleotides (CAA) from positions 1111 to 1113 were deleted from the *walK* gene, which encodes a histidine kinase, WalK. The mutation, designated *walK**, which did not cause a shift in the reading frame, resulted in the loss of a single amino acid, glutamine (Q), from the intact WalK protein (Fig. 1A). The other mutation was a deletion of *clpP*, where a 144-bp DNA fragment located at base positions −17 to 127 of *clpP* was deleted. Amino acid sequence alignment with the sequence in the conserved domain database (45) was carried out and found that the functional core motif was located at the N terminus of ClpP. Since the deletion contained the translation initiation codon ATG and a part of the promoter region of *clpP* and some possible initial codons existed, we predict that the gene function probably remained but was inactivated (Fig. 1B). The mutation was designated *clpP**.

Evaluation of effects of *walK* and *clpP* mutations on glycopeptide resistance. To evaluate the roles of the *walK* and *clpP* mutations in raising LR5P1-V3 vancomycin resistance, the mutations were introduced one by one into the chromosome of parent strain N315LR5P1 by a gene replacement method (16, 54). The obtained mutants were then studied for phenotypic changes, including susceptibility to vancomycin and teicoplanin.

(i) Effect of *walK* mutation. We first introduced the *walK* deletion mutation, *walK**, into the N315LR5P1 chromosome, obtaining the strain with the gene replacement, LR5P1*walK**, and a revertant strain, LR5P1*walK*. The latter strain had the intact *walK* gene that was recovered from the gene replacement procedure (see Materials and Methods). Revertants were obtained in every gene replacement experiment and used as isogenic control strains throughout this study. Antibiotic susceptibilities for the constructed mutants were evaluated by determination of the MICs of various antibiotics, including vancomycin and teicoplanin. In order to accurately evaluate the susceptibility changes, MIC determinations for glycopeptides were carried out by Etest, broth microdilution, and agar dilution methods. A significant increase in vancomycin and teicoplanin MICs was detected between parent strain N315LR5P1 and mutant strain LR5P1*walK** in both BHI medium and MH medium, while

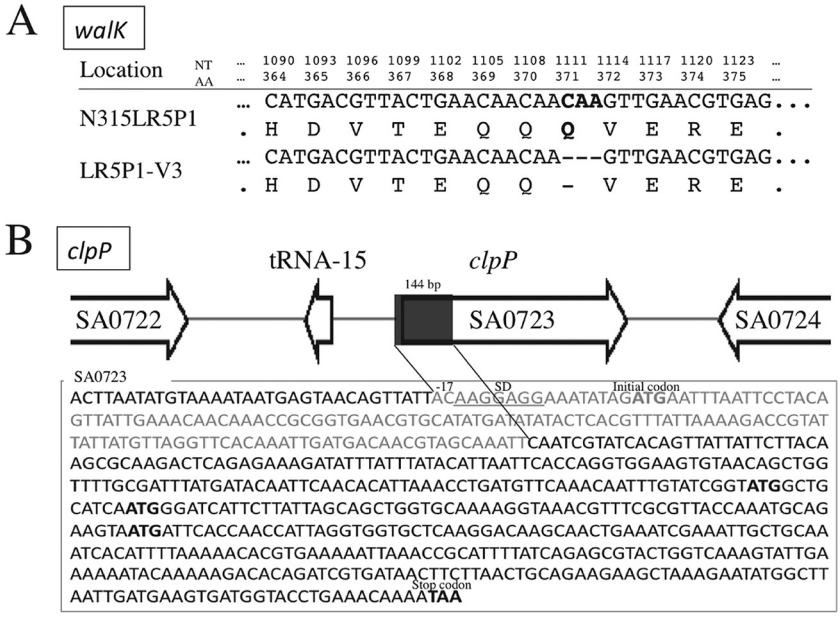

FIG. 1. VISA strain LR5P1-V3 harbors two mutations on its chromosome. Whole-genome-sequence comparison of VISA LR5P1-V3 and its vancomycin-susceptible parent strain, N315LR5P1, revealed two mutations: a deletion mutation in *walK* and a truncating mutation in *clpP* of the LR5P1-V3 chromosome. (A) *walK* deletion mutation. Three nucleotides (NT; CAA, positions 1111 to 1113) of *walK* were deleted, resulting in an amino acid (AA) deletion of Q371. (B) *clpP* truncating mutation. A total of 144 nucleotides, located at positions −17 to 127 of *clpP*, were deleted in LR5P1-V3, which results in a *clpP* truncation. Putative initial codons for translation are in boldface.

no such difference was observed between the parent and revertant strain LR5P1*walK* (Table 1). However, the level of glycopeptide resistance of LR5P1*walK** still fell short of that of LR5P1-V3, indicating that the *walK** mutation alone was not enough to express the same level of glycopeptide resistance as that of LR5P1-V3.

(ii) Effect of *clpP* mutation. Since the *walK** mutation was not sufficient for N315LR5P1 to attain a level of glycopeptide resistance similar to that of LR5P1-V3, we suspected that the *clpP* mutation detected in our genome comparison study is important in contributing toward the glycopeptide resistance detected in LR5P1-V3. The *clpP* mutation was first tested for an independent effect by introducing *clpP** into N315LR5P1, obtaining strain LR5P1*clpP**. Antibiotic susceptibility tests showed that the mutant had increased vancomycin and teicoplanin MICs compared to those of parent strain N315LR5P1 and revertant strain LR5P1*clpP* (Table 1). However, this increase in glycopeptide resistance was smaller than that as a result of the effect of the single *walK* mutation (Table 1). In other words, on its own, the *clpP** mutation does raise glycopeptide resistance, but the other mutation was clearly needed for N315LR5P1 to attain the level of glycopeptide resistance of LR5P1-V3.

(iii) Combination effect of *walK and *clpP** on glycopeptide resistance.** With the results of the experiments described above, we speculated that both the *walK** and *clpP** mutations are required for achieving LR5P1-V3 glycopeptide resistance. To test this hypothesis, the *clpP** mutation was additionally introduced into the chromosome of LR5P1*walK**, obtaining the double mutant strain LR5P1*walK*clpP**. As expected, the glycopeptide MIC values of the double mutant strain attained levels of glycopeptide resistance comparable to that of LR5P1-V3, though interestingly, revertant strain LR5P1*walK*clpP* had a reduced level of glycopeptide resistance compared to that of LR5P1*walK** (Table 1). LR5P1-V3 possessed a characteristic resistance pattern with a much higher teicoplanin MIC (12 mg/liter) than vancomycin MIC (4 mg/liter) in BHI medium (Table 1). This feature of resistance was also retained by the double mutant strain (Table 1).

Though LR5P1-V3 recorded an MIC of 4 mg/liter for vancomycin, which is the criterion of VISA (9), its population analysis profile (PAP) showed a profile which is typical of heterogeneous-type resistance (Fig. 2A). The single mutant strains LR5P1*walK** and LR5P1*clpP** had PAPs that were between those of N315LR5P1 and LR5P1-V3. LR5P1*walK** possessed much larger resistant subpopulations than LR5P1*clpP** for each concentration of vancomycin and teicoplanin. In agreement with the MIC results, the PAP of double mutant strain LR5P1*walK*clpP** was similar to that of LR5P1-V3. The PAP of LR5P1*walK*clpP** against teicoplanin was almost identical to that of LR5P1-V3, even at the higher teicoplanin concentrations of 8 to 12 mg/liter (Fig. 2B). Taken together, the glycopeptide resistance of LR5P1-V3 was shown to be caused by the combined effects of the two mutations, *walK** and *clpP**.

(iv) Truncation mutation of *clpP* confers susceptibility to protein synthesis inhibitor antibiotics. Table 1 shows that not only the susceptibilities to glycopeptides but also those to other antibiotics were affected by the introduction of the mutations. Daptomycin and bacitracin MICs were slightly increased in LR5P1-V3. This seemed to be associated with the introduction of *walK** but not of *clpP**, as far as this can be judged from the data presented in Table 1. More significant changes were observed with protein synthesis inhibitor antibiotics. MICs of linezolid, tetracycline, minocycline, chloramphenicol, and mupirocin for the mutants were significantly decreased with

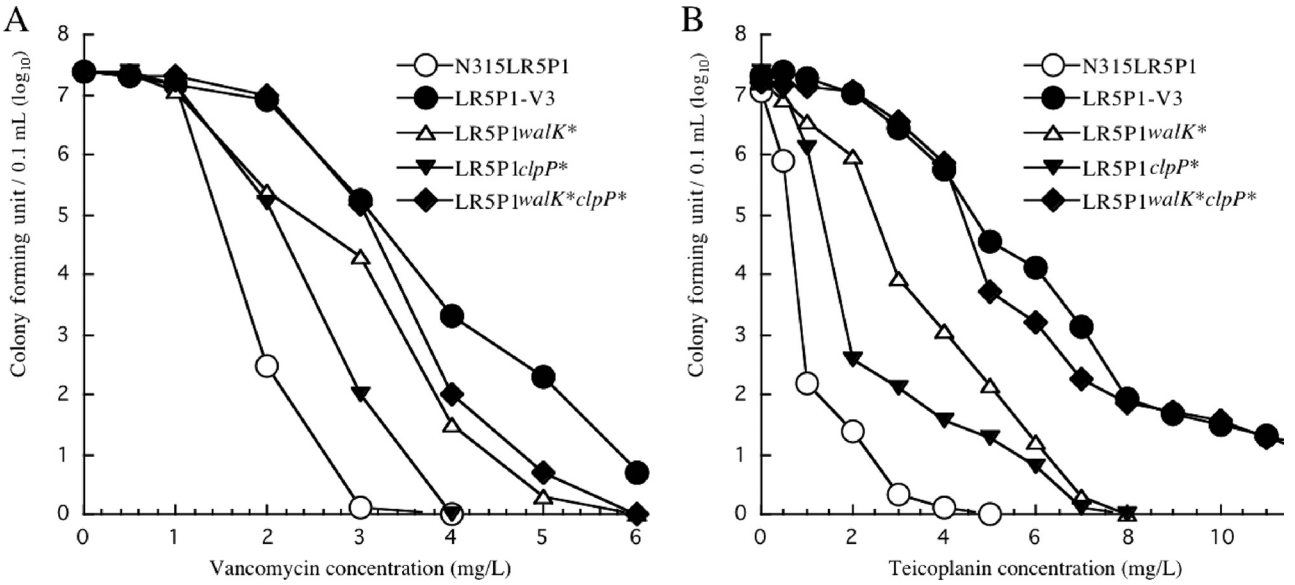

FIG. 2. Analysis of vancomycin- and teicoplanin-resistant subpopulations of gene replacement mutants and their isogenic controls. The data presented are representative of data from three experiments. The numbers of colonies on plates containing various concentrations of vancomycin (A) and teicoplanin (B) were counted after 48 h of incubation at 37°C.

the introduction of the *clpP** mutation (Table 1). The reason for these changes, which were probably due to the loss of *clpP* gene function, is currently unknown. However, a possible explanation for the increased susceptibility to protein synthesis inhibitor antibiotics is that a loss or decrease of ClpP function results in an impaired survival ability of the *clpP** mutants through the anaerobic respiratory pathway. ClpP is essential for the growth and survival of *S. aureus* through the anaerobic respiratory pathway (48), and full expression of the anaerobic respiratory pathway contributes to resistance to protein synthesis inhibitor antibiotics (50).

walK* **and *clpP**** **mutations caused cell wall thickening.** A thickened cell wall has been reported to be a common phenotypic feature of clinical VISA strains (11, 14, 31). The feature is closely associated with the peptidoglycan-clogging theory that explains vancomycin resistance by the difficulty and delay of passage of vancomycin molecules across the thickened peptidoglycan layers (12). We considered that either one of the mutations, *walK** or *clpP**, would cause thickening of the host cell wall. This hypothesis was tested by transmission electron microscopy of the mutant strains (Fig. 3). The glycopeptide-susceptible parent strain N315LR5P1 had a cell wall thickness of 19.69 ± 4.23 nm. Introduction of the *walK** mutation in N315LR5P1 caused the cell wall to be 1.63 times thickener. On the other hand, introduction of the *clpP** mutation caused the cell wall to be 1.48 times thicker (Fig. 3), while the revertant mutants had the same cell wall thickness as the parent strain (data not show). The double mutant LR5P1*walK***clpP** had a cell wall 1.67 times thicker than that of N315LR5P1. The cell wall of the double mutant strain was the thickest among the strains with gene replacements. However, the cell wall of LR5P1-V3 was still thicker than that of the double mutant strain (36.05 versus 32.94 nm). The reason for this discrepancy is unknown at the moment.

Slow growth and decreased autolysis of the *walK* and *clpP* mutants. Next, the growth rates of the mutant strains were compared with the growth rate of the parent strain. The doubling time and growth curve of mutant strain LR5P1*walK** were similar to those of parent strain N315LR5P1 (Table 1; Fig. 4A). On the other hand, introduction of the *clpP** mutation into N315LR5P1, whether singly or in combination with the *walK** mutation, significantly prolonged the doubling time of the cell, as shown in Table 1. Therefore, the slow-growth phenotype of LR5P1-V3 was found to be due to the effect of the *clpP** mutation. Slow growth has repeatedly been reported in VISA strains and is considered one of the important features of the VISA phenotype (10, 14, 18, 27, 59). Nevertheless, the contribution of slow growth to *S. aureus* glycopeptide resistance remains controversial. We recently studied a laboratory-derived hVISA strain with decreased susceptibility to both vancomycin and daptomycin (6). The strain had mutations in *rplV* and *rplC* (which code for 50S ribosomal proteins L22 and L3, respectively), which were considered to be the reason for the remarkably prolonged growth rate of the strain. However, this slow growth was not associated with decreased susceptibility to vancomycin and daptomycin (17). Therefore, the slow growth of LR5P1-V3 is not necessarily a factor contributing to the raised glycopeptide resistance. In this study, the slow growth and raised glycopeptide resistance were inseparable features of the *clpP** mutation; however, the direct contribution of slow growth to vancomycin resistance could still not be evaluated comprehensively.

Decreased autolytic activity is one of the features of VISA (3, 29). Our present study also demonstrated a correlation between the levels of autolytic activity and glycopeptide resistance (Fig. 4B). Glycopeptide-susceptible parent strain N315LR5P1 and LR5P1*walK* had the highest autolytic activity, while LR5P1-V3 had the lowest. The mutants LR5P1*walK**

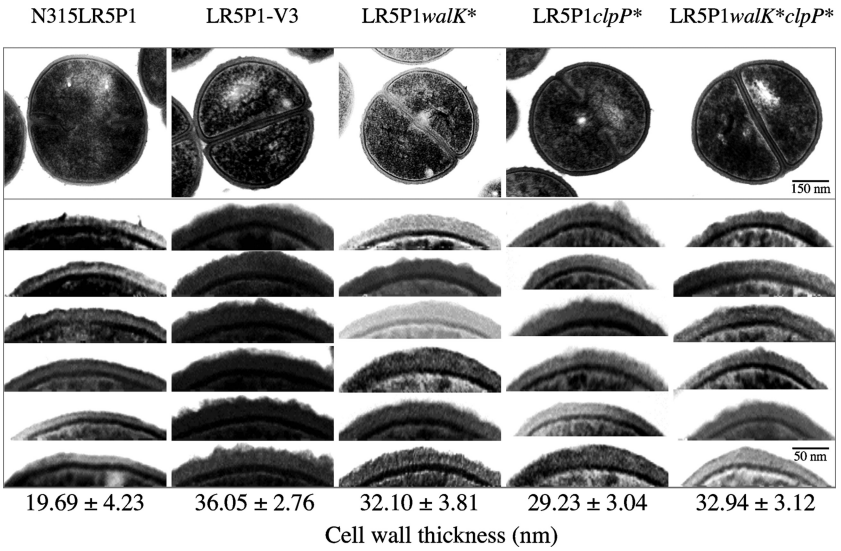

FIG. 3. Transmission electron microscopy of the gene replacement mutants and their control strains. Transmission electron microscopy was carried out on gene replacement mutants LR5P1*walK** and LR5P1*clpP**, which were generated from N315LR5P1 by substitution of its *walK* and *clpP* genes with those of LR5P1-V3, respectively. The LR5P1*walK*clpP** mutant was generated from LR5P1*walK** by substitution of its *clpP* gene with that of LR5P1-V3. The values given under each picture are the means and standard deviations of each strain's cell wall thickness. Note that all gene replacement cells had thick cell walls compared to those of the parent strain. Magnifications, ×30,000.

and LR5P1*clpP** had autolytic activities slightly lower than those of N315LR5P1 and LR5P1*walK*. The double mutant strain LR5P1*walK*clpP** exhibited a significantly reduced autolytic activity comparable to that of LR5P1-V3.

Microarray transcriptional analysis. In the above experiments, the *walK** and *clpP** mutations identified in the laboratory VISA LR5P1-V3 strain were shown to contribute to vancomycin resistance; however, the impact of these mutations on the regulatory function toward vancomycin resistance remains to be clarified. On the basis of this consideration, we carried out genome-scale transcriptional profiling, comparing the mutants from gene replacement or drug selection with their parent strains. Microarray analysis was carried out for a total of four combinations of strains: LR5P1*walK** versus N315LR5P1, LR5P1*clpP** versus N315LR5P1, LR5P1*walK*clpP** versus N315LR5P1, and LR5P1-V3 versus N315LR5P1. All pairs consisted of the vancomycin-resistant mutant and its vancomycin-susceptible parent strain. Analyses of the first two pairs revealed the effect of the individual *walK** and *clpP** mutations, while analyses of the last two elucidate the combination

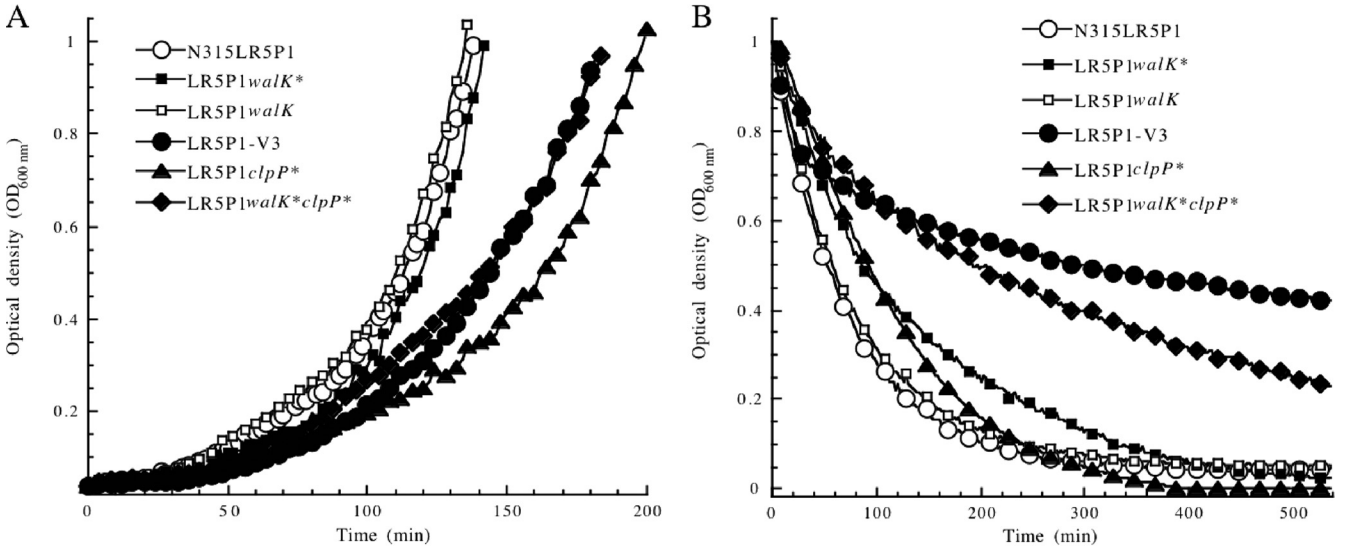

FIG. 4. Growth curves (A) and autolytic activities (B) of gene replacement mutants and their control strains. Note that strains with the *clpP** mutation (LR5P1-V3, LR5P1*clpP**, and LR5P1*walK*clpP**) had significantly slower growth than the parent strain, and strains with dual deletion mutations of *clpP** and *walK** (LR5P1-V3 and LR5P1*walK*clpP**) had autolytic activities remarkably different from the activity of the parent strain. Data shown are means of duplicate determinations.

effect of the *walK** and *clpP** mutations. All transcriptional profiles and comparison data are available on the CIBEX site under accession number CBX144. Table 2 shows the extracted representative data. The results revealed significant genome-wide differences in expression patterns between the mutants and parent strains. As shown in Table 2, significant differences in gene expression of the four vancomycin-resistant mutants and vancomycin-susceptible parent strain N315LR5P1 were repression of the metabolic pathways of galactose (*lac* operon), succinate dehydrogenase, the *efeUMN* operon, and aminoacyl-tRNA biosynthesis and enhancement of the metabolic pathways of urease formation, the *cydAB* operon, the *agr* locus, and many ABC transporters. The enhanced expression of the *sirABC*, *ahp*, *clpC*, and *opuC* operons was commonly seen in mutants with *clpP** (Table 2). A calculation of genes with 2-fold alterations in the transcriptional level showed that, compared to the sequence of parent strain N315LR5P1, there were a total of 39 and 28 genes down- and upregulated, respectively, in mutant strain LR5P1*walK**, into which *walK** was introduced (see Tables S1 and S2 at http://www.jj.em-net.ne.jp/~longzhu/data/pub1/table_s1-s4.pdf), while there were 56 and 141 genes (Table S3 and S4) down- and upregulated, respectively, in mutant LR5P1*clpP**, into which the *clpP** mutation was introduced, revealing a broad regulatory impact of these mutations on bacterial physiology (see Tables S1 to S4).

S. aureus is one of the organisms known to exclusively use enzymes of the D-tagatose-6-phosphate pathway (encoded by the *lac* operon) to metabolize lactose and D-galactose (47). In the *lac* operon, the *lacABCD* genes comprise the D-tagatose-6-phosphate pathway and are cotranscribed with the *lacFEG* genes, which specify proteins for transport and metabolism of lactose and galactose in *S. aureus* (47). Although the impact of the downregulated *lac* operon on the vancomycin-resistant phenotype still remains to be clarified, the downregulated *lac* operon in vancomycin-resistant strains was also observed in our previous study that was carried out with an hVISA strain with an *rpoB* mutation-mediated heteroresistance (17), suggesting that decreased lactose/galactose metabolism is associated with vancomycin resistance in *S. aureus.* The mutual biological relationship of the vancomycin-resistant phenotype with the genes or operons that were differentially expressed in vancomycin-resistant strains remains to be studied.

Impact of *walRK* mutation on vancomycin-resistant phenotypes. *walRK* (*vicRK*) was originally identified as *yycFG* in *Bacillus subtilis* (23, 26) to be an essential two-component system for the viability of cell growth (23, 26). Recently, Dubrac et al. stressed the importance of the system in cell wall metabolism in *S. aureus* and proposed renaming the system *walRK* (20). Two reports have described the correlation of the *walRK* system with the phenotype of vancomycin intermediate resistance in *S. aureus*. Jansen et al. reported that *walRK* was highly upregulated due to an insertion mutation in the *walRK* promoter in a VISA clinical isolate (37). Mwangi et al. found a mutation in the *yycH* gene in a clinical VISA strain (53). The *yycH* product is reported to downregulate the *walRK* system in *B. subtilis* (62). Thus, both studies have suggested that the increment of vancomycin resistance was mediated by activation of the *walRK* system. Nevertheless, in our study, we did not find any significant change (i.e., a more or less than 2-fold change) in the expression of *walRK* in any of our resistant mutants (Table 2; see Table S1 and Table S2 at http://www.jj.em-net.ne.jp/~longzhu/data/pub1/table_s1-s4.pdf). The causes of raised resistance due to the *walK** mutation remain unknown. From the microarray data, we could neither estimate the biological function of *walK** nor confirm the activation or deactivation of WalRK due to introduction of *walK**, since there was only one gene (SA0710) downregulated by *walK** introduction out of nine genes that have been reported to be directly regulated by the *walRK* system (21, 22). Recently, Delaune et al. reported on the effect of *walRK* on cell morphology, showing that *walRK* depletion could raise the cell wall thickness of *S. aureus* (19). As the mutant into which *walK** was introduced, LR5P1*walK**, had a thickened cell wall, it might be reasonable to consider that *walK** might hamper the function of the *walRK* system, but its regulatory pathway toward cell wall thickening remains to be studied.

Impact of *clpP* mutation on vancomycin-resistant phenotypes. ClpP is a proteolytic subunit of the ATP-dependent Clp protease, which consists of an ATPase specificity factor (ClpA or ClpX in *E. coli*; ClpX, ClpC, or ClpE in *Bacillus subtilis*) and a proteolytic domain (ClpP) (28). The global regulatory impact of ClpP on the virulence, stress response, and physiology of *S. aureus* was well documented by Michel et al. by transcriptional profile analysis using a *clpP* deletion mutant (48). Under normal growth conditions, the *clpP* deletion mutant showed a growth defect affecting the expression of many regulatory genes, such as *agr*, *sigB*, *sarT*, and *arlRS. walRK* was also reported to be upregulated by *clpP* deletion. Deletion of *clpP* in NTCT8325 caused accelerated autolysis (48). However, our findings with N315LR5P1 were contradictory to the previous ones. Introduction of mutated *clpP*, either singly or with the *walK* mutation, decreased autolysis of N315LR5P1 (Fig. 4). This signifies that the physiological role of *clpP* may differ from strain to strain, depending on their genetic backgrounds, and this is supported by our microarray data. Comparing the transcriptional profile of LR5P1*clpP** with that of the NTCT8325 mutant with the *clpP* deletion that was published by Michel et al (48), we found that only 44 out of 137 genes that were differentially expressed according to the data of Michel et al. were also similarly up- or downregulated in LR5P1*clpP** (data not shown).

Microarray analysis of mutant LR5P1*clpP** into which *clpP** was introduced showed quite a number of differentially expressed genes compared to the number in the *walK** mutants (see Tables S3 and S4 at http://www.jj.em-net.ne.jp/~longzhu/data/pub1/table_s1-s4.pdf). Among the genes which were differentially expressed include those involved in cell envelope and cellular processes, intermediary metabolism, and various regulatory functions, e.g., *sarA*, *sarX*, *norR*, *malR*, the *agr* locus, *yuaC*, *ctsR*, *ccrA*, *sarH1*, *lytR*, *ydcH*, and *sarV*, revealing a strong regulatory impact of ClpP on the expression of genes. Regarding the role of regulation of *clpP** in raising vancomycin resistance, the explanations for differentially expressed genes in the *clpP** mutants are speculative at this point; however, we suspect that the *clpP** mutation alters metabolic pathways of the whole cell by reducing protein biosynthesis and accumulation of cell wall material, directing the cell toward vancomycin resistance. Consistent with this hypothesis, our microarray data showed lowered expression of almost all of aminoacyl-tRNA biosynthesis genes and enhancement of many ABC transporters, including the *opuC* operon, which is related to cell wall and

TABLE 2. Representatives of genes differentially expressed in mutants with *walK** and/or *clpP** compared to that in parent strain N315LR5P1

Function and ORF	Gene	Product	Ratio of signal intensity[a]			
			LR5P1*walK**/ N315LR5P1	LR5P1*clpP**/ N315LR5P1	LR5P1*walK*clpP**/ N315LR5P1	LR5P1-V3/ N315LR5P1
Galactose metabolism						
SA1991	*lacG*	6-Phospho-beta-galactosidase	0.44	0.24	0.20	0.20
SA1992	*lacE*	PTS[b] system lactose-specific IIBC component	0.32	0.14	0.11	0.10
SA1993	*lacF*	PTS system, lactose-specific IIA component	0.35	0.17	0.13	0.11
SA1994	*lacD*	Tagatose-1,6-diphosphate aldolase	0.42	0.21	0.18	0.15
SA1995	*lacC*	Tagatose-6-phosphate kinase	0.36	0.18	0.14	0.12
SA1996	*lacB*	Galactose-6-phosphate isomerase LacB subunit	0.41	0.19	0.17	0.14
SA1997	*lacA*	Galactose-6-phosphate isomerase LacA subunit	0.56	0.24	0.26	0.18
SA2288	*gtaB*	Cell envelope, UTP-glucose-1-phosphate uridylyltransferase	0.62	0.63	0.66	0.85
efeUMN operon						
SA0331	*efeM*	Putative lipoprotein	0.25	0.26	0.43	0.35
SA0332	*efeN*	Dyp-type peroxidase family protein	0.23	0.27	0.43	0.35
SA0333	*efeU*	Ferrous iron transport permease EfeU	0.24	0.28	0.37	0.34
Succinate dehydrogenase						
SA0963	*pycA*	Pyruvate carboxylase	0.65	0.41	0.43	0.39
SA0994	*sdhC*	Succinate dehydrogenase, cytochrome *b*-558 subunit	1.18	0.49	0.45	0.49
SA0995	*sdhA*	Succinate dehydrogenase flavoprotein subunit	1.01	0.38	0.35	0.40
SA0996	*sdhB*	Succinate dehydrogenase iron-sulfur subunit 8	0.97	0.44	0.42	0.47
Aminoacyl-tRNA biosynthesis						
SA0009	*serS*	Seryl-tRNA synthetase	0.93	1.07	1.06	0.94
SA0448	*metS*	Methionyl-tRNA synthetase	0.69	0.73	0.78	0.74
SA0475	*lysS*	Lysyl-tRNA synthetase	1.10	1.12	1.11	1.00
SA0486	*gltX*	Glutamyl-tRNA synthetase	1.37	1.71	1.81	1.55
SA0488	*cysS*	Cysteinyl-tRNA synthetase	0.94	0.85	0.96	0.91
SA0564	*argS*	Arginyl-tRNA synthetase	0.55	0.67	0.66	0.66
SA0855	*trpS*	Tryptophanyl-tRNA synthetase	0.57	0.66	0.69	0.65
SA0985	*pheS*	Phenylalanyl-tRNA synthetase subunit alpha	0.81	0.67	0.70	0.70
SA0986	*pheT*	Phenylalanyl-tRNA synthetase subunit beta	0.95	0.74	0.77	0.80
SA1036	*ileS*	Isoleucyl-tRNA synthetase	0.71	0.69	0.73	0.78
SA1106	*proS*	Prolyl-tRNA synthetase	1.18	0.95	0.96	0.94
SA1287	*asnC*	Asparaginyl-tRNA synthetase	0.79	0.78	0.93	0.79
SA1446	*alaS*	Alanyl-tRNA synthetase	0.81	0.65	0.70	0.64
SA1456	*aspS*	Aspartyl-tRNA synthetase	0.94	0.86	0.89	0.77
SA1457	*hisS*	Histidyl-tRNA synthetase	0.81	0.80	0.76	0.73
SA1488	*valS*	Valyl-tRNA synthetase	0.82	0.64	0.65	0.62
SA1506	*thrS*	Threonyl-tRNA synthetase	0.72	0.63	0.64	0.63
SA1550	*tyrS*	Tyrosyl-tRNA synthetase	0.59	0.62	0.72	0.56
SA1563	*ytpR*	Phenylalanyl-tRNA synthetase protein	0.86	0.81	0.84	0.70
SA1579	*leuS*	Leucyl-tRNA synthetase	0.73	0.64	0.68	0.64
SA1715	*gatB*	Aspartyl-/glutamyl-tRNA amidotransferase subunit B	1.14	0.92	0.96	0.77
SA1716	*gatA*	Aspartyl-/glutamyl-tRNA amidotransferase subunit A	0.89	0.77	0.76	0.64
SA1717	*gatC*	Aspartyl-/glutamyl-tRNA amidotransferase subunit C	0.87	0.76	0.77	0.61
Urease formation						
SA2081		Urea transporter	1.32	2.11	2.51	3.11
SA2082	*ureA*	Amino acid biosynthesis (urease subunit gamma)	1.45	2.70	2.93	4.38
SA2083	*ureB*	Amino acid biosynthesis (urease subunit beta)	1.49	2.74	2.95	4.23
SA2084	*ureC*	Urease subunit alpha	1.44	2.59	2.78	4.19
SA2085	*ureE*	Urease accessory protein UreE	1.49	2.59	2.77	3.99
SA2086	*ureF*	Urease accessory protein UreF	1.50	2.50	2.73	4.06
SA2087	*ureG*	Urease accessory protein UreG	1.46	2.39	2.52	3.48
SA2088	*ureD*	Urease accessory protein UreD	1.52	2.85	2.97	4.29
cydAB operon						
SA0937	*cydA*	Cytochrome *d* ubiquinol oxidase subunit I protein	2.37	3.61	3.25	3.35
SA0938	*cydB*	Cytochrome *d* ubiquinol oxidase subunit II protein	2.39	3.62	3.42	3.52
SA0939	*ktrC*	TrkA potassium uptake family protein	1.73	2.23	2.15	2.14
agr locus						
SA1842	*agrB*	Accessory gene regulator protein B	1.66	2.52	1.98	1.44
SAS066	*agrD*	Accessory gene regulator protein D	1.50	2.23	1.77	1.31
SA1843	*agrC*	Accessory gene regulator protein C	1.61	2.34	1.83	1.30
SA1844	*agrA*	Accessory gene regulator protein A	1.55	2.12	1.81	1.24
sirABC operon						
SA0110	*sirB*	Siderophore compound ABC transporter permease protein	1.04	2.55	2.02	1.85
SA0111	*sirA*	Iron compound ABC transporter	1.09	4.31	3.61	3.00
SA0112	*sbnA*	Pyridoxal-5′-phosphate-dependent enzyme, beta subunit	1.05	3.03	2.76	2.23

Continued on following page

TABLE 2—*Continued*

Function and ORF	Gene	Product	Ratio of signal intensity[a]			
			LR5P1*walK**/ N315LR5P1	LR5P1*clpP**/ N315LR5P1	LR5P1*walK*clpP**/ N315LR5P1	LR5P1-V3/ N315LR5P1
SA0113	*ocd*	Ornithine cyclodeaminase protein	1.36	3.42	3.16	2.53
SA0114	*sbnC*	IucA/IucC family siderophore biosynthesis protein	1.24	2.77	2.68	2.12
ahp operon and *nfrA*						
SA0365	*ahpF*	Alkyl hydroperoxide reductase subunit F	0.92	3.91	4.62	4.66
SA0366	*ahpC*	Alkyl hydroperoxide reductase subunit C	0.89	3.24	3.58	3.51
SA0367	*nfrA*	NADPH-dependent oxidoreductase	1.15	2.46	2.61	2.32
clpC operon						
SA0480	*ctsR*	Transcriptional regulator of class III stress genes	0.57	4.24	4.41	4.42
SA0481	*mcsA*	UvrB/UvrC motif-containing protein	0.53	3.98	4.31	4.43
SA0482	*mcsB*	ATP guanido phosphotransferase	0.53	4.19	4.52	4.53
SA0483	*clpC*	Putative stress response-related Clp ATPase	0.94	1.07	1.11	1.00
ABC transporters						
SA0109	*sirC*	Siderophore compound ABC transporter permease protein SirC	0.89	1.78	1.56	1.40
SA0110	*sirB*	Siderophore compound ABC transporter permease protein SirB	1.04	2.55	2.02	1.85
SA0111	*sirA*	Iron compound ABC transporter	1.09	4.31	3.61	3.00
SA0209	*yvfM*	Maltose/maltodextrin transport permease protein	1.47	1.77	1.45	1.23
SA0229	*appAA*	Nickel ABC transporter nickel-binding protein	1.73	2.80	2.79	2.02
SA0639	*cydC*	ABC transporter ATP-binding protein	1.50	1.98	1.89	1.85
SA0640	*cydD*	ABC transporter ATP-binding protein	1.77	2.05	2.05	1.98
SA0677	*opuBA*	Glycine betaine ABC transporter ATP-binding protein	1.28	1.85	1.54	1.74
SA0891		Hypothetical protein, similar to ferrichrome ABC transporter	1.16	1.79	1.67	1.17
SA1213	*opp-2C*	Putative oligopeptide transporter membrane permease	1.33	1.80	1.87	1.97
SA1214	*opp-2B*	Putative oligopeptide transporter membrane permease	1.48	2.28	2.02	2.32
SA1979	*yhfQ*	Iron ABC transporter binding protein,	0.82	1.67	1.94	2.35
SA2234	*opuCD*	Glycine betaine/carnitine/choline ABC transporter membrane	1.02	2.14	2.02	2.40
SA2235	*opuCC*	Glycine betaine/carnitine/choline ABC transporter binding	1.04	2.17	2.28	2.54
SA2236	*opuCB*	Glycine betaine/carnitine/choline ABC transporter *opuCB*	1.08	2.22	2.22	2.52
SA2237	*opuCA*	Glycine betaine/carnitine/choline ABC transporter ATP-bindi	1.21	1.86	2.16	2.24
clpP gene SA0723	*clpP*	ATP-dependent Clp protease proteolytic subunit	1.04	0.44	0.45	0.44
walRK system						
SA0017	*walR*	Two-component response regulator	0.86	1.34	1.29	1.14
SA0018	*walK*	Two-component sensor histidine kinase	0.87	1.26	1.25	1.10

[a] Ratios of signal intensity of strains with mutations to that of the isogenic parent strain.
[b] PTS, phosphotransferase.

membrane envelope biogenesis (Table 2). *tagE*, encoding a poly(glycerol-phosphate) alpha-glucosyltransferase that is one of the important enzymes in the pathway of teichoic acid biosynthesis, and some operons related to the aerobic respiratory chain, such as *cydAB* in all *clpP** mutants, were also highly expressed (Table 2; see Table S4 at http://www.jj.em-net.ne.jp/~longzhu/data/pub1/table_s1-s4.pdf). The strong influence of the truncated *clpP* mutation on transcription of regulators observed in this study suggests that ClpP proteolytic activity may serve as an important mechanism to control gene expression in *S. aureus*.

Prevalence of *vraSR*, *graRS*, *clpP*, and *walRK* mutations among VISA clinical strains. To test if *walK* and *clpP* mutations are naturally occurring genetic events, we carried out a study of the prevalence of *walRK* and *clpP* mutations among a worldwide collection of clinical VISA strains. A total of 39 clinical VISA strains isolated from various countries were subjected to nucleotide sequencing of *vraSR*, *graRS*, *clpP*, and *walRK*. The mutations of the first two operons are causative of the Mu50 VISA phenotype (16, 54). Eight VISA strains, including Mu50, were found to harbor mutations in *vraS*, while the *graR* mutation was identified in only four strains, i.e., Mu50, HIP5836, HIP10267, and HIP13057 (Table 3). The *clpP* mutations were rare and found only in strains 99/3759-V, HIP07920, and HIP09313. All *clpP* mutations were accompanied by single amino acid substitutions and not by huge deletions, as seen in LR5P1-V3. On the other hand, *walK* mutations were found in as many as 61.5% of the VISA strains (Table 3). None of the four tested genes was mutated in 9 of the 39 VISA strains (23.1%). Therefore, there might still be alternative genetic pathways for generation of the VISA phenotype in nature.

In conclusion, we proved that the combination of the *walK* and *clpP* mutations confers glycopeptide resistance on *S. aureus.* Very recently, we have reported on the involvement of the mutation of *rpoB*, encoding the RNA polymerase β subunit, in the mechanism of glycopeptide resistance in some VISA strains (17, 64). Therefore, besides the set of *vraS-graR* and *rpoB* mutations, the novel set of mutations in *walK-clpP* represents an alternative genetic pathway through which the VISA

TABLE 3. Mutations of the genes associated with vancomycin resistance in clinical VISA strains[a]

Strain	NARSA no.	Vancomycin MIC (mg/liter)	Isolation information			Amino acid substitution(s)[d]						
			Yr	Country	Reference or source	WalK	WalR	ClpP	GraS	GraR	VraS	VraR
N315[b]		1	1982	Japan	1							
Mu3[c]		2	1996	Japan	32						I5N	
Mu50		8	1996	Japan	33					N197S	I5N	
MI (HIP5827)		8	1997	USA	61	V494L						
NJ (HIP5836)		8	1997	USA	61	I28T, I341V				S79F	A260V	
PC (HIP06297)		8	1998	USA	60	A567D						
IL		8	2001	USA	5	D496N						
AMC11094		8	1997	South Korea	40							A113V
99/3759-V		8	1999	UK	34	V156Q		M1V				
99/3700-W		8	1999	UK	34	R222K, V366M, A468T						
LIM2		8	1995	France	57							
98141		8	1998	France	7							
28160		8	1998	South Africa	24							
BR 1		8	1998	Brazil	56	R222K, A468T						
BR 2		8	1998	Brazil	56	R222K, A468T						
BR 3		8	1998	Brazil	56	R222K, A468T						
BR 4		8	1998	Brazil	56	R222K, A468T						
BR 5-1		8	1998	Brazil	56	R222K, A468T						
SA MER-S6	NRS12	8	1999	France	NARSA							
HIP06854	NRS18	4	1998	USA	NARSA	T492K						
HIP07920	NRS21	4	1998	USA	NARSA			H83R				
HIP07930	NRS22	4	1999	USA	NARSA						G9V	
HIP08926	NRS23	4	2000	USA	NARSA	R222I, T492K						
HIP09143	NRS24	4	2000	USA	NARSA							
HIP09313	NRS26	4	2000	USA	NARSA	L10F, S437T		R152H			P327S	
HIP09433	NRS27	4	2000	USA	NARSA	V1454G					G9V	
HIP09662	NRS28	4	2000	USA	NARSA	Ins.433N, 434D[e]						
HIP09735	NRS29	4	2000	USA	NARSA	A468T						
HIP09740	NRS51	6	2000	USA	NARSA	V380I						
HIP09737	NRS52	4	2000	USA	NARSA	G275V						
LY-1999-01	NRS63	4	1998	Oman	NARSA	N48K, R222K, A468T						
LY-1999-03	NRS65	4	1998	Oman	NARSA	N48K, R222K, A468T						
HIP10540	NRS73	8	2000	USA	NARSA							
HIP10267	NRS74	4	2000	USA	NARSA					T11A		
C2000001227	NRS76	8	2000	USA	NARSA	A243T					A314V	
NRS118	NRS118	4	2002	USA	NARSA	F330S						
NRS126	NRS126	4	2000	USA	NARSA							
P1V44	NRS272	16	1999	Belgium	NARSA							
HIP12864	NRS402	8	2003	USA	NARSA							
HIP13057	NRS403	8	2004	USA	NARSA	R282C				E15K		
HIP13036	NRS404	8	2004	USA	NARSA						T104A	

[a] Sequence determination was carried out with both corresponding forward and reverse primers. Mutations found in 10 vancomycin-susceptible *S. aureus* strains whose whole genomes were sequenced (including N315, COL, JH1, MRSA252, MSSA472, NCTC8325, Newman, RF122, MW2, and TW20) are not described in this table.

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

[c] hVISA strain.

[d] Amino acid substitutions are given with reference to the amino acid sequence of N315 as follows: the first letter of the N315 sequence, the amino acid sequence position, and the last letter of the altered amino acid sequence.

[e] Two amino acids were inserted at positions 433 and 434.

phenotype is achieved in *S. aureus*. Investigation of the biological significance of these mutations is ongoing with the hope of obtaining an understanding of the entire scheme by which *S. aureus* achieves resistance to glycopeptide antibiotics.

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