A Mutation in the PP2C Phosphatase Gene in a *Staphylococcus aureus* USA300 Clinical Isolate with Reduced Susceptibility to Vancomycin and Daptomycin

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Methicillin-resistant *Staphylococcus aureus* (MRSA) strains with reduced susceptibility to vancomycin (MIC of 4 to 8 µg/ml) are referred to as vancomycin-intermediate *S. aureus* (VISA). In this study, we characterized two isogenic USA300 *S. aureus* isolates collected sequentially from a single patient with endocarditis where the *S. aureus* isolate changed from being susceptible to vancomycin (VSSA) (1 µg/ml) to VISA (8 µg/ml). In addition, the VISA isolate lost beta-lactamase activity and showed increased resistance to daptomycin and linezolid. The two strains did not differ in growth rate, but the VISA isolate had a thickened cell wall and was less autolytic. Transcriptome sequencing (RNA-seq) analysis comparing the two isolates grown to late exponential phase showed significant differences in transcription of cell surface protein genes (*spa*, SBI [second immunoglobulin-binding protein of *S. aureus*], and fibrinogen-binding proteins), regulatory genes (*agrBCA*, RNAPII, *sarT*, and *saeRS*), and others. Using whole-genome shotgun resequencing, we identified 6 insertion/deletion mutations between the VSSA and VISA isolates. A protein phosphatase 2C (PP2C) family phosphatase had a 6-bp (nonframeshift) insertion mutation in a highly conserved metal binding domain. Complementation of the clinical VISA isolate with a wild-type copy of the PP2C gene reduced the vancomycin and daptomycin MICs and increased autolytic activity, suggesting that this gene contributed to the reduced vancomycin susceptibility phenotype acquired *in vivo*. Creation of *de novo* mutants from the VSSA strain resulted in different mutations, demonstrating that reduced susceptibility to vancomycin in USA300 strains can occur via multiple routes, highlighting the complex nature of the VISA phenotype.

In humans, the bacterium *Staphylococcus aureus* is both a commensal and pathogenic organism that has the ability to cause infections in multiple tissue sites, including blood, skin and soft tissue, bone, and heart. The ubiquitous presence of *S. aureus* (14, 31), combined with its ability to acquire antibiotic resistance via multiple mechanisms, make this bacterium one of the most troublesome infectious agents worldwide (10). Methicillin-resistant *S. aureus* (MRSA) USA300, is a pandemic community-associated, as well as health care-associated, strain responsible for a variety of serious infections (18, 47). The complete genomes of two unique clinical MRSA USA300 isolates have been published, highlighting the genetic basis of the pathogenic nature of this strain (20, 35).

The recommended antibiotics for serious MRSA infections, such as bacteremia, endocarditis, osteomyelitis, and meningitis, are the glycopeptide vancomycin and the lipopeptide daptomycin (48). However, MRSA strains with different levels of resistance to these important antibiotics have been reported (37, 79), and clinical infections from MRSA with reduced to intermediate vancomycin susceptibility (MICs of >2 but <8 µg/ml) have been observed (26, 29, 32, 36, 50, 74). Even within this range, there appears to be yet another hierarchy; strains with vancomycin MICs of ~1.5 to 2 µg/ml, at the high end of susceptibility, can show a heterogeneous vancomycin-susceptible *S. aureus* (hVISA) phenotype where subpopulations (~1 × 10⁶) of cells have increased vancomycin resistance (37). The clinical significance of hVISA infections is still not fully understood and remains controversial (1, 57). However, infections from VISA strains (MICs of 4 to 8 µg/ml) are associated with prior vancomycin use and result in poorer clinical outcomes (26, 40, 49).

VISA strains acquire resistance to vancomycin through the ac-

cumulation of mutations in a variety of genes (37). Many of these genes encode regulatory proteins: the alternative sigma factor *rpoB* (17, 81), the two-component systems *vraSR*, *graSR*, and *walRK* (*vicK*) (15, 39, 55, 60, 62, 71), the regulatory protease *clpP* (71), the *saeRS* regulatory locus (52), the accessory gene regulator *agr* (67), and others (37). Most of the genes were identified after selection under antibiotic pressure in the laboratory, while few studies have reported mutations associated with *in vivo* vancomycin resistance in clinical strains (39, 58). Further work is needed to characterize the genetic basis of VISA in clinical strains as the bacterial genomic background and growth conditions within the host may influence the mutation spectrum. Additionally, some VISA strains have a reduced susceptibility to daptomycin (16, 56, 79), and clinical strains that have gained a *de novo* increase in daptomycin MIC during infection have been observed (54, 72, 78).

To address these issues, we used a whole-genome shotgun approach to identify mutations incurred in a previously described clinical USA300 MRSA isolate that went from being vancomycin

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susceptible (VSSA) (MIC = 1 µg/ml) to vancomycin intermediate (VISA) (MIC = 8 µg/ml) with decreased daptomycin susceptibility during patient infection and treatment (32). We further characterized this strain for typical VISA attributes, such as thickened cell wall and loss of autolytic activity, and performed a transcriptome sequencing (RNA-seq) experiment in the absence of antibiotic pressure.

MATERIALS AND METHODS

Bacterial growth and DNA isolation. USA300 clinical strains A1-VSSA and A2-VISA were provided by the Centers for Disease Control and Prevention (CDC). All genomic DNA preparations were performed using standard phenol-chloroform extraction methods as described previously in reference 9 with the following modifications: digestion with lysostaphin only, proteinase K digestion overnight, and no cetyltrimethylammonium bromide (CTAB) added.

Growth assays. Strains A1-VSSA and A2-VISA were grown overnight in brain heart infusion (BHI) broth containing 5% beef extract with shaking at 35°C and diluted back 1:1,000 to 10 ml fresh broth. Growth was monitored by optical density at 600 nm (OD₆₀₀) measurements at 30-min intervals for 10 h.

Antibiotic resistance testing. Vancomycin MICs were measured by Etest and reference broth microdilution by standard procedures (12, 13). β-Lactamase activity was confirmed by cefinase β-lactamase detection discs (BD, Franklin Lakes, NJ).

PAP-AUC. Heterogeneous and intermediate resistance to vancomycin was evaluated by population analysis profiling-area under the curve analysis (PAP-AUC) as previously described (69).

De novo mutant construction. For the creation of *de novo* VISA mutations in strain A1-VSSA, a liquid culture was grown to serve as a frozen “parental” stock. This culture was then grown on a nonselective solid medium, and 4 colonies were picked to start 4 parallel broth cultures in BHI with 3 µg/ml oxacillin. After the cultures had become turbid, cultures were transferred to BHI broth with 2 µg/ml vancomycin and subsequently to BHI broth with 4 µg/ml vancomycin. Aliquots (100-µl aliquots) were then spiral plated to BHI agar with 8 µg/ml vancomycin, and single colonies were transferred to fresh BHI plates containing 4 or 8 µg/ml vancomycin.

Autolysis assay. Strains were grown in BHI at 35°C with shaking to an OD₆₀₀ of ~0.7. Ten milliliters of cells was pelleted by centrifugation, washed 1× in 10 ml cold phosphate-buffered saline (PBS), and resuspended in 10 ml phosphate buffer (pH 7.2) with and without 0.5% Triton X-100 (Triton X). The cells were rocked gently at 30°C, and the OD₆₀₀ was taken every hour for 5 h.

Genome sequencing and sequence analysis. Shotgun sequencing was performed on the 454 Titanium and Junior platforms via standard 454 protocols. De Novo genome assemblies and Reference assemblies against finished *S. aureus* genomes were performed using CLC Workbench software (Aarhus, Denmark). Single-nucleotide polymorphism (SNP) and insertion-deletion analysis used the USA300 strain TCH1516 genome as a reference, with a minimum quality of the central base equal to 20, a minimum coverage of 4, and a minimum variant frequency of 85%. As a cross verification, we also identified all SNPs and indels between the strains assembled *de novo* and the TCH1516 reference using the nucmer program in the MUMmer package (19). Sequencing coverage and quality scores at each variant site identified by the above methods were interrogated by hand to compile the list of putative genomic differences between strains A1-VSSA and A2-VISA to be confirmed by PCR and Sanger sequencing. Multilocus sequence typing (MLST) sequence types were determined by BLAST analysis of the 7 *S. aureus* MLST genes for 100% identity and determination at <http://www.mlst.net/>.

PCR confirmation of genomic mutations. DNA used for whole-genome sequencing was used to amplify specific regions for confirmation of mutations identified in deep-sequence analysis (for primers, see Table S2 in the supplemental material). Clontech Advantage 2 DNA polymerase

was used for standard PCR amplification. PCR products were purified using a Qiagen PCR purification kit, and 500-ng samples were sent to Agencourt Bioscience for Sanger sequencing. Products were sequenced in both the forward and reverse directions and were considered “true” results if the result matched with at least 20 nucleotides on either side of the putative mutation.

Electron microscopy. For transmission electron microscopy (TEM), strains A1-VSSA and A2-VISA were grown in liquid BHI medium with 0.5% beef extract from a single colony in a 10-ml volume. One sample of A2-VISA was also grown in the presence of 1 µg/ml vancomycin. The cells were grown at 35°C with shaking for approximately 5 h to mid-exponential phase (OD₆₀₀ of ~0.7 to 0.8). A volume of 5 ml of each culture was pelleted by centrifugation and suspended in 900 µl of 2.5% glutaraldehyde in 0.1 M cacodylate buffer and stored at 4°C. The cells were then postfixed with 1% osmium tetroxide in the same buffer. After dehydration, cells were infiltrated with and embedded in Eponate 12 resin. Ultrathin sections were cut at 70 nm, stained with uranyl acetate and lead citrate, and then examined on a Hitachi H7500 TEM equipped with a Gatan BioScan charge-coupled-device (CCD) camera. Images were taken of bacterial cells that were roughly symmetrical around the cell division septum. Measurements were taken using ImageJ software as follows. Cell images at ×150,000 magnification were divided into 4 equal quadrants using the cell septum as the initial bisecting line. Then, an overlay of radial lines starting at the cell center was created such that the lines bisected the cell wall at least 4 times per quadrant in a smoothly arcing region. Sixteen cell wall measurements were taken for 15 different cells per sample, and averages and standard deviations were calculated in Excel. For complemented strains, the same approach was used, but 9 cells were measured for each sample.

RNA isolation and RNA-seq. For RNA isolation, strains A1-VSSA and A2-VISA were grown in 10 ml of BHI to an OD₆₀₀ of ~0.7. Three independent samples were collected for each strain. The cells were briefly pelleted by centrifugation and immediately resuspended in the RNA-Wiz solution of the Ambion RiboPure bacteria kit (<5 min between spinning and resuspension in RNA-Wiz). RNA quality was assessed on an Agilent BioAnalyzer using the 2100 expert mRNA pico chip. Samples were digested with Ambion Turbo DNase, and rRNA was removed from samples using the Ambion MicroExpress kit two times. RNA concentration was measured using a NanoDrop spectrophotometer and the Invitrogen Qbit fluorometer. cDNA was made using ~700 ng mRNA and 20 ng random hexamers as described in reference 63. Sequencing libraries were made using cDNA sheared to 300 nucleotides (nt) using a E210 acoustic focusing instrument (Covaris, Woburn, MA) using standard protocols, and samples were run on an Illumina HiSeq 2000 with 100-nt paired-end protocol. CLC Genomics Workbench was used for data analysis. Sequence files were trimmed, and reads with <50 nucleotides were removed. Sequences were mapped to the *S. aureus* TCH1516 genome (NCBI sequence NC_010079) using default parameters. Expression analysis was also performed in CLC Genomics Workbench. The data were normalized using quantile normalization and then square root transformed. Baggerley’s test on proportions was used to determine *P* values for differential gene expression on normalized-transformed values. Score distribution was performed using the log₂ transformed mean normalized expression values and conducted with Excel.

Complementation of strain A2-VISA. To complement strain A2-VISA with a wild-type copy of the putative PP2C (protein phosphatase 2C) protein phosphatase (locus tag number USA300HOU_1156), the gene was amplified by PCR and inserted into plasmid pOS1-Plgt (7). Briefly, PP2C was amplified using strain A1-VISA genomic DNA with primers A2_PP2C_comp_1 and A2_PP2C_comp_2 (see Table S3 in the supplemental material). These primers created an NdeI and XhoI restriction site for in-frame insertion downstream of the Plgt promoter in pOS1-Plgt. Insertion of the PCR product with standard ligation techniques and growth in *Escherichia coli* with ampicillin was verified by PCR using primers pOS1_plgt_up and pOS1_plgt_down and primers PP2C_insert_1 and

PP2C_insert_3 and Sanger sequencing (Table S3). The PP2C(pOS1-P_{lgt}) construct and the vector alone were electroporated into strain A2-VISA as follows. A colony picked from an overnight agar plate culture was used to inoculate BHI broth containing 0.5% beef extract and was grown to an OD₆₀₀ of 0.2 to 0.3. The cells were washed twice in a 0.2-μm filter flask with cold 0.5 M sucrose. The cells were refiltered in a 0.2-μm filter flask and washed with an additional 25 ml of cold 0.5 M sucrose, pelleted by centrifugation at 6,000 × g for 5 min, and suspended in 2.0 ml of cold 0.5 M sucrose. Extra 200-μl aliquots were stored at −80°C. Approximately 200-ng amounts of the vectors listed above were mixed with 45 μl cells and incubated on ice for 15 min. The cells were electroporated in 0.1-cm cuvettes at 2.3 kV/25 μF/100 Ω and suspended in 1 ml warm BHI broth containing 16 g/liter casein. The cells were allowed to recover for 90 min in a 37°C incubator with 5% CO₂ and then plated on BHI-chloramphenicol plates (10 μg/ml) containing 16 g/liter casein and incubated overnight at 35°C. Transformants were checked by miniprepations with lysostaphin preincubation and confirmed by PCR using the primers listed in Table S3.

Quantitative reverse transcriptase PCR (qRT-PCR). New RNA samples of A1-VSSA and A2-VISA (three biological replicates each) were isolated as described previously for RNA-seq, treated with Ambion Turbo DNase, and ethanol precipitated (no ribosome depletion was performed). cDNA was made using the Roche transcript first-strand cDNA kit. cDNA was run on the LightCycler 480 II using the LightCycler 480 SYBR green I master mix (20-μl volumes). All experimental samples were run in sets of 5 samples (3 of which were technical replicates) with 3 no-RT controls and 3 no-template controls. Fold changes were calculated using the $\Delta\Delta C_T$ method (23) using the *dnaK* gene (USA300HOU_1581) as the constitutively expressed reference gene. Threshold cycle (C_T) values for the 5 replicates did not have a standard deviation of more than 1 C_T . Pearson correlation was done in Excel comparing fold changes of genes *agrC* (USA300_2035), *SBI* (second immunoglobulin-binding protein of *S. aureus*) (USA300_2401), *RNAIII* (USA300_nc0020), *spa* (USA300_0122), *cfx* (USA300_1159), and PP2C (USA300_1156). Primers are listed in Table S4 in the supplemental material.

RESULTS

Whole-genome shotgun sequencing of two clinical USA300 strains reveals multiple insertion and deletion mutations incurred in the transition from VSSA to VISA. Hageman et al. described a USA300 MRSA strain that developed intermediate resistance to vancomycin and nonsusceptibility to daptomycin in a patient with endocarditis after exposure to both antibiotics (32). The isolates were collected sequentially, exhibited different antibiotic susceptibility patterns, and were typed as USA300 by pulsed-field gel electrophoresis (PFGE). We obtained two of these isolates, designated *S. aureus* A1-VSSA and *S. aureus* A2-VISA, for genetic analysis. The first strain isolated from the patient, A1-VSSA, was susceptible to vancomycin, daptomycin, and linezolid, whereas the subsequently isolated strain, A2-VISA, was intermediate resistant to vancomycin, not susceptible to daptomycin, and resistant to linezolid as measured by reference broth microdilution (Table 1). Additionally, A1-VSSA was beta-lactamase positive and A2-VISA was beta-lactamase negative by the cefinase β-lactamase detection disc assay. The vancomycin phenotypes of both strains were confirmed by PAP-AUC (Fig. 1). A2-VISA had an AUC ratio of 0.92 to Mu50, the prototypical VISA, indicating the full VISA phenotype and no heteroresistance (AUC ratio of the isolate to hVISA strain MU3 of ≥0.90) was observed in A1-VSSA (Fig. 1).

A1-VSSA and A2-VISA were sequenced using the Roche/454 Junior instrument and the raw data were trimmed to remove the terminal 30 nucleotides from both the 5' and 3' ends, low quality and ambiguous nucleotides, and any reads under 250 nucleotides

TABLE 1 Antibiotic MICs and beta-lactamase activity for isogenic USA300 strains A1-VSSA and A2-VISA

Antibiotic	Strain A1-VSSA		Strain A2-VISA	
	MIC (μg/ml)	Resistance ^a	MIC (μg/ml)	Resistance
Vancomycin^b	1	S	8	I
Penicillin	>2	R	1	R
Daptomycin	<0.5	S	4	NS
Cefoxitin	>16	R	>16	R
Chloramphenicol	8	S	8	S
Clindamycin	≤0.25	S	≤0.25	S
Erythromycin	>8	R	>8	R
Gentamicin	≤2	S	≤2	S
Levofloxacin	4	I	8	R
Linezolid	≤1	S	2	R
Oxacillin	>16	R	>16	R
Rifampin	≤0.5	S	≤0.5	S
Tetracycline	≤1	S	≤1	S
Trimethoprim-sulfamethoxazole	≤0.5/9.5	S	≤0.5/9.5	S
Beta-lactamase activity^c	Positive		Negative	

^a Antibiotic resistance interpretation abbreviations: R, resistant; I, intermediate; S, susceptible; NS, nonsusceptible.

^b The changes in resistance in the A1-VSSA and A2-VISA strains are shown in boldface font.

^c Beta-lactamase activity as measured by cefinase disk, where positive means there was beta-lactamase activity and negative means there was no beta-lactamase activity.

long. The average read size was used in analysis for each strain was ~400 nt. *De novo* assembly yielded 77 and 113 contigs for A1-VSSA and A2-VISA, respectively, composed of over 40 million total bases, with average read lengths of over 350 bp and a GC content of 32%. BLAST analysis of the 7 MLST alleles (<http://www.mlst.net/>) (25) and *spa* repeat pattern alignment (Ridom SpaServer; www.spaserver.ridom.de) confirmed that both isolates were sequence type 8 (ST8) and *spa* t008 (33).

In order to determine the best reference, the 454 data were remapped against finished genomes of two vancomycin-susceptible USA300 strains, TCH1516 (NC_010079) and FPR3757 (NC_007793) (21, 35). The sequencing data for A1-VSSA and A2-VISA (experimental strains) covered the entire genomes of both USA300 reference sequences (average genome-wide nucleotide coverage of 14× and 13×, respectively). Both experimental strains contained the entire sequences of the two plasmids from TCH1516 (pUSA01HOU and pUSA300HOUR) (35, 44). Sequencing coverage for the smaller plasmid was between 200 and 1,000×, while the larger plasmid was covered about 90× for A1-VSSA and ~200× for A2-VISA.

There were at least 70 putative SNPs and one indel that A1-VSSA and A2-VISA shared in common compared to either reference strain. Because our strains were more related to TCH1516, this genome was used as the reference strain for further comparative analysis. The complete list of variants discovered in the experimental strains and TCH1516 are listed in Table S1 in the supplemental material. Additionally, we noted that two particular regions were divergent in the experimental strains and strain TCH1516. First, an approximately 600-bp region of the chromosome overlapping two tandem lipoproteins (USA300HOU_0109 and USA300HOU_0110) was missing in both experimental strains. Second, a putative noncoding RNA region annotated as

FIG 1 PAP-AUC shows the VISA phenotype in clinical USA300 strains. The figure shows the results from one representative experiment of five experiments. (A) Population analysis profile of USA300 clinical strains A1-VSSA and A2-VISA compared to VISA strain Mu50 and hVISA strain Mu3. (B) PAP-AUC ratio of the strains in panel A to hVISA strain Mu3.

sprA (USA300HOU_nc0013) was identical in the experimental strains, but different from TCH1516 (note that this feature is not annotated in the FPR3757 genome, but the sequence exists as intergenic and is 100% identical to the TCH1516 sequence). Also, both experimental strains have a 5-nucleotide deletion in the *set* gene, which encodes a putative superantigen protein.

Six indels were identified in A2-VISA that were not present in A1-VSSA, suggesting that one or more of these mutations may be responsible in whole or in part for the VISA phenotype and daptomycin nonsusceptibility (Fig. 2), under the hypotheses that A1-VSSA and A2-VISA were isogenic strains derived from the same founder population. There was a single nucleotide frameshift deletion in the *blaZ* gene (pUSA300HOUmr0011) on the large plasmid (NC_010063), which supports the loss of beta-lactamase activity in A2-VISA. Multinucleotide indels were confirmed in the housekeeping sigma factor *rpoD*, the tetrahydrofolate synthase gene *folC*, an M20D subfamily peptidase, a PP2C protein phosphatase, and the peptidylprolyl isomerase *prsA2* (see Fig. 2 for locus tags and base changes). Two of these were in-frame mutations (PP2C and *folC*) that would presumably not entirely eliminate translation of these genes, and three were frameshift mutations (*rpoD*, *prsA2*, and M20D peptidase). The *folC* mutation would putatively result in the deletion of two amino acids, isoleucine and aspartate. The insertion mutation in PP2C was a replication of 6 nucleotides, resulting in duplication of the amino acids glutamate and aspartate (ED) (Fig. 2). It is likely that the M20D peptidase and PrsA2 protein would be nonfunctional, since the frameshifts occurred in the middle of the open reading frames. However, since the *rpoD* deletion affected only the last 4 amino acids at the C terminus of the protein, it is unlikely that this mutation is deleterious, as this is putatively an important sigma factor for *S. aureus*,

FIG 2 Nucleotide and amino acid changes in strain A2-VISA compared to isogenic vancomycin-susceptible strain A1-VSSA and reference strain TCH1516. (A) Seven-nucleotide deletion in the *rpoD* gene (USA300HOU_1563), which encodes a subunit of the housekeeping RNA polymerase of *S. aureus*. This mutation results in the Asp365 frameshift at the very 3' end of the gene (Phe366 change to Ile and deletion of the final two amino acids). (B) Six-nucleotide nonframeshift mutation in the tetrahydrofolate synthase gene *folC* (USA300HOU_1654), resulting in the deletion of Ile285 and Asp286. (C) Two-nucleotide deletion in a putative M20D peptidase (USA300HOU_2309) resulting in the Asp365 frameshift. (D) Eleven-nucleotide deletion in peptidyl prolyl isomerase *prsA2*, resulting in a Lys209 frameshift mutation. (E) One-nucleotide deletion mutation in the beta-lactamase gene *blaZ* (USA300HOU_pUSAHOUmr0011) on the large plasmid of USA300 NC_010063. (F and G) Six-nucleotide insertion replication of the AAGATG sequence in the PP2C protein phosphatase gene (black lines under the codons) (F), resulting in the duplication of aspartate and glutamate residues 18 and 19 (G) (shown in the black box).

FIG 3 Transmission electron micrographs (TEM). TEM showing differences in cell wall thickness between vancomycin-susceptible strain A1-VSSA (A) compared to VISA strain A2-VISA grown without vancomycin (B) and with 1 µg/ml vancomycin (C). The cell wall thickness measurements are given in Table 2. Scale bars, 200 nm.

and in a growth curve experiment, the A2-VISA strain grew at a rate identical to that of A1-VSSA (data not shown).

Changes in cell wall thickness and autolysis in A2-VISA strain. To determine whether A2-VISA had typical VISA characteristics such as a thickened cell wall and low autolytic activity (37), we performed TEM and autolysis assays. TEM showed differences in cell wall thickness between A1-VSSA and A2-VISA grown with and without vancomycin (1 µg/ml) (Fig. 3 and Table 2). The laboratory strain Newman (24), a non-USA300 strain was also examined (not shown in Fig. 3). The overall cell size as measured by septum length was not significantly different between any of the strains, but cell wall thickness was significantly larger in A2-VISA grown both with and without vancomycin (~40 nm for Newman and A1-VSSA versus ~50 nm for A2-VISA) ($P < 0.001$), suggesting that the A2-VISA isolate expresses the VISA thickened cell wall morphology constitutively. Autolysis assays both with and without Triton X (Fig. 4) showed that A2-VISA is substantially less autolytic than A1-VSSA, which is a common trait of VISA strains (6, 45). We concluded that the A1-VSSA strain, as a result of one or several of the mutations listed above, acquired a typical VISA phenotype *in vivo*.

Complementation of the PP2C protein phosphatase in A2-VISA restores vancomycin susceptibility to hVISA levels and daptomycin susceptibility. To determine the roles of the PP2C phosphatase in vancomycin and/or daptomycin susceptibility, we moved a wild-type PP2C phosphatase gene *in trans* via the complementation vector pOS1-Plgt into strain A2-VISA. The complemented strain [A2:PP2C(pOS1-Plgt)] had a reduced vancomycin MIC (3 to 4 µg/ml) by Etest, but the vancomycin MIC of the

vector-only strain [A2(pOS1-Plgt)] was similar to that of A2-VISA (6 to 8 µg/ml) (Fig. 5 and Table 3). PAP-AUC was performed on the complemented and vector-only strains to confirm the VISA phenotype. The PP2C complemented strain had a lower PAP-AUC profile, closer to the hVISA strain Mu3, while the vector-only strain had a profile identical to the original A2-VISA strain (Fig. 6). Additionally, a partial reduction of daptomycin susceptibility in the complemented strain was observed by Etest (Table 3).

TABLE 2 Cell wall thicknesses measured by transmission electron microscopy

Strain	Septum length (µm) ^a	Cell wall thickness (µm) ^a
Newman	758.9 ± 60.7	39.2 ± 4.9
A1-VSSA	768.8 ± 58.5	40.1 ± 4.7
A2-VISA	794.2 ± 56.8	51.9 ± 6.0 ^c
A2-VISA + VAN ^b	723.1 ± 48.1	50.7 ± 6.9 ^c

^a The measurements (in micrometers) are averages ± standard deviations from 15 cells per sample (16 measurements per cell).

^b The A2-VISA strain was grown in 1 µg/ml vancomycin (VAN).

^c The values for both A2-VISA strains were significantly different ($P < 0.001$) from the values for Newman and A1-VISA strains by Student's 2-tailed *t* test (unequal variance).

FIG 4 Comparison of autolysis with and without Triton X-100 (Triton X). (A and B) Autolysis assays comparing the change in OD₆₀₀ over time at 30°C between the vancomycin-susceptible strain A1-VSSA and VISA strain A2-VISA in PBS (A) and PBS plus Triton X (B). The figure shows the results from one representative experiment of three experiments.

FIG 5 PAP-AUC shows partial complementation of vancomycin susceptibility with PP2C protein phosphatase. The figure shows the results from one representative experiment of four experiments. (A) Population analysis profile of complemented A2-PP2C and vector-only A2-pOS1-Plgt strains compared to clinical strains A1-VSSA, A2-VISA, and hVISA strain Mu3. (B) PAP-AUC ratios to hVISA strain Mu3. The values for the complemented strain and the strain with an empty vector were significantly different ($P < 0.001$) by the two-tailed Student's t test. There was no significant difference between the values for the A2-VISA strain versus the vector-only strain.

These results suggest that the insertion mutation in the PP2C phosphatase in A2-VISA may play a role in the reduced vancomycin and daptomycin susceptibility acquired *in vivo*.

To verify that the PP2C gene was being transcribed in the complemented strain, we performed SYBR green qRT-PCR and observed that the complemented strain expressed the PP2C gene $\sim 11\times$ higher than the vector-only strain (data not shown). Although the complemented strain is a merodiploid, with the mutated gene on the chromosome also likely being transcribed, we believe that the significant increase in PP2C expression is a result of the strong *Plgt* promoter driving transcription of the wild-type allele.

To test whether complementation of the PP2C mutation altered other VISA characteristics, we performed autolysis assays and TEM on A2:PP2C(pOS1-Plgt). Figure 6 shows that the com-

FIG 6 Autolysis assay showing partial complementation in strain A2:PP2C(pOS1-Plgt). (A and B) Autolysis assays comparing the change in OD₆₀₀ over time at 30°C between the vancomycin-susceptible strain A1-VSSA, VISA strain A2-VISA, empty vector strain A2-pOS1-Plgt, and the complemented strain A2-PP2C in PBS (A) and PBS plus Triton X (B). The graphs show the results from one representative experiment of three experiments.

plemented strain had autolytic activity similar to A1-VSSA, whereas the vector-only strain maintained the lower autolysis rate of the original A2-VISA isolate. Interestingly, in both autolysis experiments (Fig. 4 and 6), A2-VISA had either a steady or increased optical density for the first hour when incubated with Triton-X, whereas with PBS only, all strains had similar rates of decreases in the OD₆₀₀ for the first 3 h. This suggests that autolysis in *S. aureus* can be different in VISA strains, depending on the specific autolytic environment.

TEM analysis showed that the complemented strain A2:PP2C(pOS1-Plgt) did not have a reduced cell wall thickness compared to A2(pOS1-Plgt) or the original A2-VISA strain (average cell wall thicknesses of ~ 55 nm, 57 nm, and 52 nm, respectively; data not shown). Therefore, it is unlikely that the PP2C phosphatase is involved directly or through regulation of other genes in the physical structuring of the thickened VISA cell wall.

RNA-seq analysis shows differences in the transcriptional profiles of A2-VISA and A1-VSSA. To determine whether the A2-VISA strain with the mutated PP2C phosphatase gene had a different transcriptional pattern compared to A1-VSSA, we performed direct sequencing of transcriptional libraries using next-generation sequencing instruments (RNA-seq). RNA was harvested from A1-VSSA and A2-VISA grown in rich broth to late exponential phase (OD₆₀₀ of ~ 0.7) with no antibiotic pressure and reverse transcribed to cDNA after which it was used as a template for sequencing using the Illumina technology platform.

TABLE 3 Etest MICs for original and complemented strains for vancomycin and daptomycin

Strain	Antibiotic MIC (μg/ml) ^a	
	Vancomycin	Daptomycin
A1-VSSA	1.5	0.19
A2-VISA	6–8	2.0
A2(pOS1-Plgt)	6–8	2.0
A2:PP2C(pOS1-Plgt)	3	0.33

^a At least 3 separate experiments were conducted for each strain.

Three biological replicates were performed for each strain, and the total read counts are shown in Table S5 in the supplemental material.

The distribution of gene expression scores for A1-VSSA and A2-VISA are shown in Fig. S1 in the supplemental material. We used the USA300 TCH1516 genome as the scaffold for mapping the RNA sequences against 2,805 open reading frames. Both strains show a similar distribution of RNA abundance after quantile normalization, where the majority of transcripts were between 2^4 and 2^8 reads per kilobase per million mapped reads. The most abundant transcripts for both strains were essential genes such as DNA-binding protein HU, ribosomal proteins, elongation factors, and ATP synthase subunits, showing that our methods were robust. Of note, RNA-seq experiments served as further verification of the mutations harbored in A2-VISA, where the mapping clearly illustrated expressed transcripts with the indels identified in genome sequencing, such as the insertion in PP2C phosphatase, and the deletion in *folC* (see Fig. S1B and S1C in the supplemental material). This shows that despite these mutations, the genes are actively expressed in A2-VISA. We also performed SYBR green qRT-PCR on 6 genes (*agrC*, *spa*, *SBI*, *cfxE*, *RNAIII*, and PP2C phosphatase; see Materials and Methods) and compared their relative expression to the housekeeping gene *dnaK* (USA300_1581) to confirm a subset of RNA-seq data (data not shown). Pearson correlations comparing either fold changes (positive correlation) or mean RNAseq expression scores versus threshold cycle (C_T) values (negative correlation) had r values of 0.9 and -0.8 .

RNA-seq gene expression analysis identified 41 genes significantly more abundant and 40 genes less abundant in A2-VISA compared to A1-VSSA (Tables 4 and 5). The lists and the fold changes indicated in Tables 4 and 5 are based on square root-transformed expression values, not straight RPKM (reads per kilobase of exon model per million mapped reads) numbers, and thus are very conservative and likely represent underestimates.

As has been seen in other VISA strains (22, 39, 70), the immunoglobulin-binding protein A (*spa*) was downregulated in A2-VISA (~ 8 -fold). Other cell-surface-associated proteins were differentially regulated in A2-VISA as well, showing that this isolate, even in the absence of antibiotic pressure, was modifying the cell surface quite differently from A1-VSSA. Most notably, the gene for the alternative immunoglobulin-binding protein (SBI [second immunoglobulin-binding protein of *S. aureus*]), which has been shown to be involved in immune evasion (73, 83, 84), and two fibrinogen-binding proteins were upregulated (~ 3 - to 10-fold), while the iron-regulated cell surface receptor *isdA* and the fibrinogen-binding protein *sdrD* were downregulated (~ 2.4 and 3.8, respectively). Also, various regulatory proteins are more abundant in A2-VISA, especially the accessory gene regulators *agrA*, *agrC*, and *agrB* (~ 5 -fold each), which have been shown to contribute to the virulence of USA300 (11) and the regulatory *RNAIII* (~ 15 -fold), which has been implicated in the suppression of *spa* expression (27).

Differences in expression of metabolism-related genes can also be noted, where A2-VISA expressed several genes involved in nitrate metabolism at higher levels and various carbohydrate and amino acid transport genes at lower levels. Interestingly, two of the genes identified as having deletion mutations, *prsA2* and the M20D peptidase, were more abundant in A2-VISA (~ 2 -fold).

A final observation regarding gene expression is the decreased expression of several iron-related genes in A2-VISA; namely, the

siderophore transporter proteins *sirA* and *sirB*, another putative iron transporter (USA300HOU_0232), and the iron-regulated surface protein *isdA* (mentioned previously). Overall, the differences in gene expression in A2-VISA show that this strain is constitutively expressing genes involved in cell surface modeling and metabolic behavior such that its structure and behavior would likely interact with the immune system during infection in a very different way from A1-VSSA.

Unique mutations identified in *in vitro*-generated mutants of the A1-VSSA isolate with increased vancomycin MIC. To investigate the spectrum of genetic changes leading to the VISA phenotype that the A1-VSSA could produce *ex vivo*, we grew A1-VSSA in the presence of 4 $\mu\text{g/ml}$ vancomycin and then selected isolates that grew on agar plates with vancomycin (4, 6, and 8 $\mu\text{g/ml}$). We isolated putative mutants that grew on BHI agar with vancomycin (8 $\mu\text{g/ml}$) from 2 different starter broth cultures and designated these *de novo* mutants DN-A1X and DN-A1Z (DN stands for *de novo*). The vancomycin MICs for DN-A1X and DN-A1Z were 3 and 4 $\mu\text{g/ml}$, respectively, compared to 1.5 $\mu\text{g/ml}$ for the A1-VSSA parental strain by Etest. The daptomycin MICs for DN-A1X and DN-A1Z were 0.5 and 1.5 $\mu\text{g/ml}$, respectively, both higher than the MIC for the parental strain, which was 0.19 $\mu\text{g/ml}$. The vancomycin MIC by broth microdilution for both mutants was 2 $\mu\text{g/ml}$ and the daptomycin MICs were 1 and 2 $\mu\text{g/ml}$, compared to 0.49 $\mu\text{g/ml}$ for both antibiotics for the parental A1-VSSA strain.

DN-A1X and DN-A1Z, as well as the original inoculum strain A1-VSSA were sequenced on the 454 Junior to identify possible mutations that arose in the process and that may have contributed to the elevated MICs (average nucleotide coverage of $13\times$, $12\times$, and $10\times$, respectively). Strain DN-A1X had two SNPs that differed from the parental strain. The first was in the gene for the beta subunit of the RNA polymerase *rpoB* (USA300HOU_0536) that would cause a Pro475Ser amino acid replacement. This missense mutation occurred in a region of the gene called the rifampin resistance determining region (RRDR), where several other missense mutations have been identified in VISA/hVISA strains (81). The second mutation was Pro174Ser replacement in a hypothetical gene (USA300HOU_1882) that is located just upstream of, and potentially in an operon with, the regulatory protein *vraS*. Strain DN-A1Z had one identifiable SNP that would cause an amino acid change (Asp235Asn) in the sensor histidine kinase *vicK* (also known as *walK* or *yycG*), a signaling protein that has been implicated in the VISA phenotype (42, 71). Therefore, we concluded that the A1-VSSA USA300 genetic background was not limited to PP2C to escape vancomycin pressure in the laboratory.

DISCUSSION

Decreased susceptibility to the important cell wall and cell membrane active antibiotics vancomycin and daptomycin in *Staphylococcus aureus* has proven to have complex genetics. Importantly, *S. aureus* appears to incur mutations during growth within the host *in vivo*, while the patient is undergoing antibiotic therapy, causing selection of those bacteria most able to avoid clearance. It is still not known whether the mutations observed *in vitro* are different from what occurs *in vivo* or whether certain baseline mutations are prerequisite for eventual reduced susceptibility. Many genes have been implicated in the development of VISA (37), and the number of mutations identified in A2-VISA suggests that one or

TABLE 4 Genes with increased expression in strain A2-VISA relative to A1-VSSA during late-exponential-phase growth in rich broth by RNA-seq gene expression analysis

Category and gene and/or protein	Locus tag ^a	Fold change in expression ^b	P value ^c	COG functional category ^d
Cell surface				
Glycosyltransferase	0727	2.0	0.008	M
Fibrinogen-binding protein	1094	3.0	0.008	
Fibrinogen-binding protein	1095	10.2	0.00002	
<i>ssaA</i> : secretory antigen SsaA	2285	2.1	0.03	R
Immunoglobulin G-binding protein SBI	2401	3.0	0.002	
Secretory antigen	2561	2.0	0.05	R
Energy production				
<i>betB</i> : betaine-aldehyde dehydrogenase	2606	2.6	0.02	C
Nitrate metabolism				
<i>narK</i> : major facilitator superfamily nitrate:nitrite antiporter	2369	2.3	<0.00001	P
<i>narI</i> : nitrate reductase gamma subunit	2376	2.8	0.0003	C
<i>narJ</i> : nitrate reductase delta subunit	2377	3.2	0.00002	C
<i>narH</i> : nitrate reductase beta subunit	2378	2.0	0.003	C
Protein/posttranslational modification				
<i>prsA2</i> : peptidylprolyl isomerase	1833	2.3	<0.00001	O
M20D subfamily peptidase	2309	2.1	0.02	R
Reductase				
Aldo/ketoreductase	2193	2.6	0.04	R
Regulation of signal transduction				
<i>sarA</i> : staphylococcal accessory regulator A	0622	2.0	0.003	
<i>saeS</i> : sensor histidine kinase SaeS	0728	2.0	0.0001	T
<i>saeR</i> : response regulator SaeR	0729	2.0	0.002	TK
<i>agrB</i> : accessory gene regulator protein B	2032	5.0	0	OTK
<i>agrC</i> : accessory gene regulator protein C	2034	5.6	<0.00001	T
<i>agrA</i> : accessory gene regulator protein A	2035	4.7	<0.00001	TK
Transcriptional regulator	2373	2.5	0.00006	TK
Sensor histidine kinase	2374	2.6	0.004	T
<i>sarT</i> : staphylococcal accessory regulator T	2487	2.8	0.01	K
Miscellaneous RNAs (RNA to RNAlII)	nc0020	15.1	0.0001	
Virulence				
<i>nuc</i> : micrococcal nuclease	0823	4.8	0.00001	L
Leukocidin subunit	2011	2.2	0.04	
Unknown				
Hypothetical protein	0228	4.9	0.0005	
Hypothetical protein	0437	5.2	0.006	
Hypothetical protein	0456	4.6	0.04	
Hypothetical protein	0730	2.7	0.0003	
Hypothetical protein	0731	2.7	0.008	
Hypothetical protein	1007	2.3	0.005	
Hypothetical protein	1090	7.5	0.0006	
Hypothetical protein	1650	3.2	0.0008	
Hypothetical protein	1927	2.1	0.02	
Hypothetical protein	1946	3.8	<0.00001	
Hypothetical protein	2030	6.6	0.05	
Hypothetical protein	2088	2.1	0.02	
Hypothetical protein	2375	3.0	0.0001	
Hypothetical protein	2551	9.8	0.0005	
Hypothetical protein	2625	2.2	0.03	

^a Locus tag numbers from genome NC_010079 (*Staphylococcus aureus* USA300 strain TCH1516).^b Fold change in expression in strain A2-VISA compared to expression in strain A1-VSSA during late-exponential-phase growth in rich broth.^c The P values were calculated using Baggerly's test in CLC Genomics Workbench.^d COG stands for Clusters of Orthologous Groups (<http://www.ncbi.nlm.nih.gov/COG/grace/generin.cgi>) (76, 77).

TABLE 5 Genes with lower expression in strain A2-VISA relative to A1-VSSA during late-exponential-phase growth in rich broth by RNA-seq gene expression analysis

Category and gene and/or protein	Locus tag ^a	Fold change in expression ^b	P value ^c	COG functional category ^d
Amino acid transport and metabolism				
<i>argH</i> : argininosuccinate lyase	0919	−3.0	0.0002	E
<i>argG</i> : argininosuccinate synthase	0920	−3.5	0.00001	E
<i>oppB1</i> : oligopeptide ABC transporter membrane protein	0944	−2.2	0.03	EP
<i>ilvA1</i> : threonine dehydratase	1375	−3.2	0.0009	E
<i>ald1</i> : alanine dehydrogenase	1376	−4.1	0.0007	E
<i>arcB3</i> : ornithine carbamoyltransferase	2634	−3.5	0.02	E
<i>arcA3</i> : arginine deiminase	2635	−3.5	0.02	E
<i>hisD</i> : histidinol dehydrogenase	2679	−3.0	0.01	E
<i>hisG</i> : ATP phosphoribosyltransferase catalytic subunit	2680	−3.3	0.03	E
<i>hisZ</i> : ATP phosphoribosyltransferase regulatory subunit	2681	−3.0	0.03	E
Carbohydrate transport and metabolism				
Major facilitator superfamily tetracycline:cation symporter	0149	−4.1	0.0008	G
Maltose/maltodextrin ABC transporter ATP-binding protein	0220	−3.0	0.008	G
ABC transporter substrate-binding protein	0221	−2.6	0.01	G
Maltose/maltodextrin ABC transporter membrane protein	0222	−2.8	0.0004	G
Maltose/maltodextrin ABC transporter membrane protein	0223	−2.1	0.03	G
<i>ptsG</i> : PTS ^c family glucose/glucoside (Glc) porter component IIAABC	2530	−2.5	0.00005	G
<i>gntP</i> : GntP family gluconate:proton (H ⁺) symporter	2492	−2.4	0.007	GE
<i>gntK</i> : gluconokinase	2493	−2.9	0.0005	G
Cell surface				
<i>spsA</i> : immunoglobulin G-binding protein A	0122	−8.1	<0.00001	
<i>sasD</i> : cell wall surface anchor protein	0146	−2.5	0.0002	
Acid phosphatase	0327	−2.0	0.05	R
<i>sdrD</i> : Ser-Asp-rich fibrinogen/bone sialoprotein-binding protein SdrD	0556	−3.8	0.0005	R
Glycosyltransferase	0984	−2.6	0.01	M
<i>isdA</i> : iron (Fe ²⁺)-regulated surface determinant protein IsdA	1064	−2.4	0.04	M
Energy production				
<i>pfl</i> : formate acetyltransferase	0233	−2.1	0.0007	C
NhaC family Na ⁺ /H ⁺ antiporter	2306	−2.0	0.006	C
Inorganic ion transport and metabolism				
<i>sirB</i> : iron (Fe ³⁺) ABC transporter membrane protein	0125	−2.7	0.004	P
<i>sirA</i> : iron (Fe ³⁺) ABC transporter-binding protein	0126	−3.5	0.002	P
Iron (Fe ³⁺) ABC transporter membrane/binding protein	0232	−2.4	0.01	P
Nucleotide transport and metabolism				
<i>deoD1</i> : purine nucleoside phosphorylase	0148	−4.3	<0.00001	F
<i>deoC1</i> : deoxyribose-phosphate aldolase	0150	−2.3	<0.00001	F
<i>purK</i> : phosphoribosylaminoimidazole carboxylase ATPase subunit	1010	−2.5	0.05	F
Regulation of signal transduction				
<i>sarH1</i> : staphylococcal accessory regulator H1	0123	−2.3	0.0004	
<i>gntR</i> : gluconate operon transcriptional repressor	2494	−3.0	0.001	K
APC (amino acid-polyamine-organocation transporter) family	1374	−2.6	0.008	E
General transport				
ABC transporter ATP-binding protein	2503	−4.6	<0.00001	V
ABC transporter ATP-binding protein	2504	−3.6	0.0001	
Unknown function				
Hypothetical protein	0245	−2.7	0.006	
Hypothetical protein	1786	−2.1	0.004	
Hypothetical protein	1787	−2.0	0.03	

^a Locus tag numbers from genome NC_010079 (*Staphylococcus aureus* USA300 strain TCH1516).^b Fold change in expression in strain A2-VISA compared to expression in strain A1-VSSA during late-exponential-phase growth in rich broth.^c The P values were calculated using Baggerly's test in CLC Genomics Workbench.^d COG stands for Clusters of Orthologous Groups (<http://www.ncbi.nlm.nih.gov/COG/grace/generin.cgi>) (76, 77).^e PTS, phosphotransferase system.

several of these mutations may be involved in the acquisition of this phenotype.

Eukaryotic-like serine-threonine kinases and protein phosphatases are important signaling molecules in many bacteria (64) and have been implicated as having a role in cell wall metabolism, autolysis, and glutamine synthesis in *S. aureus* (3, 61). The *S. aureus* PP2C phosphatase gene, also referred to as *stp1*, is located on multiple *S. aureus* genomes in tandem with the serine-threonine kinase *stk*, also called *PknB* (61) (note that both genes are annotated as hypothetical proteins in several *S. aureus* genomes), and the insertion mutation in the PP2C phosphatase gene in A2-VISA is in a highly conserved beta sheet motif and manganese binding (5, 64, 65). A recent study showed that the *stp1* phosphatase plays a role in increased resistance to glycopeptides (teicoplanin and vancomycin) in an *S. aureus* mutant created in the laboratory (66). Additionally, an *stp1* knockout mutant in *S. aureus* N315 had thickened cell walls (3) and was more resistant to lysostaphin, characteristic of VISA.

We observed a decrease in both vancomycin and daptomycin MIC when the A2-VISA strain was expressing a wild-type copy of the phosphatase gene under a constitutive promoter. Although the complemented strain did not show a decrease in cell wall thickness, we believe that this protein likely plays a partial role in cell signaling that causes the remodeling of the cell surface, which seems to be a defining feature of these strains. Curiously, an *stp1* knockout strain had reduced virulence in a mouse sepsis model due to lowered expression of virulence genes, specifically hemolysins (8). It would be interesting to know how a phosphatase knockout strain might survive *in vivo* during vancomycin therapy. Though it is possible that one of the other noted mutations within the VISA strain confers vancomycin resistance and that overexpression of *stp1* or *pp2C* via the plasmid merely overcomes this phenotype, our results suggest that *stp1* or *pp2C* plays a role in the reduced susceptibility to vancomycin.

Also of particular interest is the observation that the *prsA2* gene and the M20D peptidase gene with potentially deleterious frameshift mutations are upregulated at the transcriptional level in A2-VISA. The absence of activity from both of these proteins could have a major role in affecting the structure of cell surface proteins. M20D peptidases in bacteria are not well defined, but they are members of a class of metalloproteins involved in various cellular processes (30). PrsA is a lipid-anchored protein located on the outer face of the plasma membrane, found ubiquitously in Gram-positive species (68, 75), and contains a peptidyl-prolyl *cis-trans* isomerase (PPIase) domain. PPIases catalyze *cis-trans* isomerization of peptide bonds, and *S. aureus* PrsA has been shown to function as a prolyl isomerase (34, 80). PrsA is thought to assist folding of proteins destined for secretion, and a PrsA homolog in *Bacillus subtilis* has been shown to be directly involved in folding penicillin-binding proteins (41). Recently, Jousselin et al. (43) demonstrated enhanced expression of *prsA* in a panel of clinical and laboratory-derived *S. aureus* strains with intermediate resistance to the glycopeptides vancomycin and teicoplanin (GISA) and showed that both VraS and VraR, as well as the signal transfer between them, is necessary for glycopeptide induction of *prsA* transcription. In their study, disruption of *prsA* resulted in hypersensitivity to low-level exposure to glycopeptides in some but not all strains, suggesting that a requirement for *prsA* likely depends on the underlying mechanisms driving the GISA/VISA phenotype. Whether or not the mutation in *prsA2* in A2-VISA is directly

involved in the maintenance of the VISA phenotype or merely a compensatory mutation due to the enhanced expression is unclear.

Another question involves the fitness of VISA strains and whether certain compensatory mutations are needed in clinical isolates to contribute to bacterial growth during infection (2, 4). Unlike the plasmid-borne *vanA* vancomycin resistance mechanism that has a high fitness cost to *S. aureus* (28), the fitness effects of VISA mutations have not been studied extensively. The thickened cell wall and the transcriptional changes in metabolic and cell surface structure genes observed in the RNA-seq data show that A2-VISA is metabolically different from A1-VSSA. However, whether this results in an actual fitness cost is not evident, since the growth kinetics of this strain are identical to those of A1-VSSA, at least under aerobic growth in rich broth. It should be noted that A2-VISA does appear to have a fitness defect when growing on agar plates, further underlining the difficulty of defining fitness defects, since their manifestation is determined by the context of their environment (2). Compensatory mutations that counteract the fitness costs of fusidic acid resistance have been observed to occur *in vivo* in *S. aureus* (46, 59), and it is very possible that similar classes of compensatory mutations occur during vancomycin therapy. It is of particular interest that in *E. coli*, specific combinations of resistance mutations involving the DNA gyrase gene *gyrA* and a transcriptional regulator *marR* caused lowered fitness, but adding an additional mutation in the topoisomerase gene *parC* not only increased drug resistance but also increased fitness (53). Another study showed that a specific mutation in *gyrA* in *Campylobacter jejuni* caused fluoroquinolone resistance but also increased fitness *in vivo* in the absence of antibiotic pressure (51). These observations are significant, because A1-VSSA and A2-VISA have mutations in *gyrA* and *parC* that substitute amino acid residues that have also been implicated in fluoroquinolone resistance in *S. aureus* (82), and it may be that this genetic background increases fitness in VISA strains. Unlike the common strains Newman and N315, the hVISA strain Mu3 and the VISA strain Mu50 have the exact GyrA (Ser84Leu) and ParC (Ser80Phe) fluoroquinolone mutations characterized in reference 82. In the case of A1-VSSA and A2-VISA, the ParC mutation is a tyrosine replacement at serine 80, as it is in USA300 strain FPR3757, but not in strain TCH1516, which has the wild-type serine. We propose that strains that acquire a VISA phenotype *in vivo* often either incur or have compensatory fitness mutations that may promote their survival and selection during antibiotic therapy in addition to those that increase antibiotic resistance.

Our data illustrate a possible role for serine-threonine phosphatase involvement in the development of both VISA and reduced susceptibility to daptomycin in strain USA300 *in vivo* but show that additional genetic changes probably contribute to the relatively high level of vancomycin resistance in A2-VISA. Overall, *S. aureus* is protean in its ability to change itself in response to antibiotic assault, and it will remain a challenge to clarify these patterns to a level where the trajectory of resistance acquisition might be predicted.

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