MAJOR ARTICLE

Serine/Threonine Phosphatase Stp1 Contributes to Reduced Susceptibility to Vancomycin and Virulence in *Staphylococcus aureus*

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(See the editorial commentary by Cheung and Duclos, on pages 1625–7.)

The genetic mechanisms that contribute to reduced susceptibility to vancomycin in *Staphylococcus aureus* are complex and heterogeneous. In addition, debate is emerging as to the true effect of reduced susceptibility to vancomycin on staphylococcal virulence. To investigate this, comparative genomics was performed on a collection of vancomycin-exposed isogenic *S. aureus* pairs (14 strains in total). Previously described mutations were observed in genes such as *vraG*, *agrA*, *yvqF*, and *rpoB*; however, a new mechanism was identified involving a serine/threonine phosphatase, Stp1. After constructing an *stp1* deletion mutant, we showed that *stp1* is important in vancomycin susceptibility and cell wall biosynthesis. Gene expression studies showed that *stp1* also regulates virulence genes, including a hemolysin, superantigen-like protein, and phenol-soluble modulin, and that the deletion mutant is attenuated in virulence in vivo. Stp1 provides a new link between vancomycin susceptibility and virulence in *S. aureus*.

Staphylococcus aureus is a well-known human pathogen that causes both hospital- and community-acquired infections. The severity of disease caused by *S. aureus* is vast, ranging from superficial skin infections to severe and often complex infections such as osteomyelitis and endocarditis. With the emergence of methicillin-resistant *S. aureus*, the reliance on last-line antistaphylococcal antibiotics such as vancomycin has increased dramatically. Fortunately, vancomycin resistance remains rare. However, *S. aureus* strains with reduced susceptibility to vancomycin, or vancomycin-intermediate *S. aureus* (VISA), are reported more commonly [1, 2] and have been associated with prolonged bacteremia and vancomycin treatment failures [3–5].

The genetic pathway in the evolution of reduced susceptibility to vancomycin in *S. aureus* remains heterogeneous, with no consistent genetic marker for resistance identified [6–8]. Although a number of genes have been shown to be involved in reduced vancomycin susceptibility, such as *vraSR* [9], *graSR* [6, 10], and *agr* [11], it appears that VISA arises due to the accumulation of mutations in multiple genetic pathways [7]. For example, Mwangi et al used whole genome sequencing of a series of clinical strains derived from the same patient who was treated with vancomycin and showed that it was a cumulative mutation process that correlated with a stepwise decrease in vancomycin susceptibility [7]. Despite the diversity of genes involved, common themes exist, including genes that regulate cell wall biosynthesis and cellular stress response [12]. A deepening of our

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understanding of the array of genes involved in the evolution of VISA will provide greater insights into possible preventative or therapeutic strategies to fight this problematic organism.

Debate exists over the pathogenic consequences of reduced susceptibility to vancomycin in *S. aureus*. Initial clinical studies reported a high complication rate and mortality in those infected with VISA strains [3, 13]. However, more contemporary studies do not support these findings and rather highlight the importance of adjusting for other factors that contribute to poor outcomes such as severity of illness and comorbidities [14]. In fact, laboratory studies point toward reduced virulence in VISA strains, which could be explained by mutations in virulence regulator genes such as *agr* and changes to the bacterial cell surface involving capsule and protein A [15]. Furthermore, we have previously shown in an invertebrate model that *S. aureus* strains with reduced susceptibility to vancomycin were attenuated in virulence, independent of growth [16].

In this study, we explored not only the genetic mechanism behind VISA formation but also the potential impact of these mutations on *S. aureus* virulence. Whole genome sequencing was performed on 14 strains making up 5 pairs or series, whereby the first isolate was vancomycin-susceptible and the subsequent isolates were isogenic vancomycin-nonsusceptible daughter strains that arose as a result of vancomycin therapy. We identified mutations in a number of genes previously defined in VISA, including *vraG*, *agrA*, *dltA*, *rpoB*, and *yvqF*. Most importantly, we describe a new gene that is involved in reduced susceptibility to vancomycin, *stp1*. This gene encodes for a serine/threonine phosphatase, and deletion of the *stp1* gene resulted in an increase in the minimum inhibitory concentration (MIC) of vancomycin and a cellular phenotype that resembles VISA strains. We also demonstrate the role of Stp1 in *S. aureus* virulence through gene expression studies and a murine bacteremia model.

Table 1. Characteristics of Vancomycin-Exposed Strains of *Staphylococcus aureus*

Isolate	Clinical Syndrome	MLST	MIC (μg/mL) Vn	MIC (μg/mL) Dp
Pair 1				
A5937	Bacteremia, endocarditis	5	1.5	0.12
A5940			4	0.25
Pair 2				
A6224	Bacteremia	5	2	0.25
A6226			3	2
Pair 3				
A6300	Bacteremia, prosthetic joint infection	5	2	0.25
A6298			4	1
Series 1				
A9635	Bacteremia, vertebral osteomyelitis	1892	1	0.5
A9636			1	0.5
A9637			2	1
A9638			3	2
A9639			4	4
Series 2[a]				
A8117	Laboratory-derived series	5	1	…
A8118			4	…
A8392			8	…

Abbreviations: Dp, daptomycin; MIC, minimum inhibitory concentration; MLST, multilocus sequence type; Vn, vancomycin.

[a] A8117, A8118, and A8392 correspond to previously described strains RN9120, RN9120-V, and RN9120-VISA, respectively [11, 16].

METHODS

Bacterial Strains and Susceptibility Testing

Bacterial strains used in this study are shown in Table 1. Four clinical strain pairs or series collected from patients in North America who had failed vancomycin therapy were included in the analysis [11, 16]. All strains within a pair or series were determined as isogenic using pulsed-field gel electrophoresis as described [11]. To assess for differences between in vivo and in vitro evolution of resistance, a laboratory-derived series, which was generated previously [17], was included for genetic analysis (Table 1). MICs for vancomycin were determined by broth dilution, according to the Clinical and Laboratory Standards Institute, and by Etest (bioMérieux), according to the manufacturer's specifications. To assess for vancomycin heteroresistance, population analysis profiles (PAPs) were performed as described previously [18]. In brief, serial dilutions of overnight cultures were plated onto heart infusion agar (Oxoid) at vancomycin concentrations ranging from 0 to 8 μg/mL. Colony-forming units (CFUs) were counted after 48 hours of incubation in aerobic conditions at 37°C.

Genome Sequencing

Whole genome sequencing of the vancomycin-susceptible parent genomes was performed using 454 FLX pyrosequencing (Roche) with an average 24-fold coverage for each genome (range, 14- to 39-fold). Vancomycin-exposed daughter genomes (hVISA/VISA) were sequenced to higher coverage (>100-fold) using the Illumina sequencing platform. Genomes were assembled in Newbler (Roche) and open reading frames identified and annotated using both ab initio (Genemark, Glimmer3, Metagene, and ZCURVEb models) and evidence-based approaches (BLASTX against nonredundant *S. aureus* protein database). Single-nucleotide polymorphisms (SNPs) were determined between vancomycin-exposed clinical pairs using the variant ascertainment algorithm polymorphism discovery algorithm (Broad Institute) [19]. Select mutations were independently confirmed by polymerase chain reaction (PCR)

and sequencing. Multilocus sequence type (MLST) typing was performed for all strains (http://saureus.mlst.net/). Sequences of parent strains were deposited in the National Center for Biotechnology Information GenBank under the accession numbers ACKE00000000 (A6224), ACKC00000000 (A5937), ACKF00000000 (A6300), ACYO00000000 (A8117), and ACKI00000000 (A9635). Genome sequences and SNP data for all isolates can be viewed at the Broad Institute *S. aureus* Drug Resistance Project group database (http://broadinstitute.org/annotation/genome/staphylococcus_aureus_drug_resistance).

Genetic Manipulation

An *stp1* (SA1062) in-frame deletion mutant was generated using the *Escherichia coli*/*S. aureus* shuttle vector pKOR1 [20]. In brief, approximately 1 kb of DNA flanking up- and downstream of *stp1* was PCR-amplified from clinical *S. aureus* isolate A5937 using primers AP1/AP2 and AP3/AP4, respectively (see Supplementary Table 1). The products were ligated and subcloned into pKOR1 using BP Clonase (Invitrogen) per the manufacturer's instructions. The final construct was electroporated into vancomycin-susceptible clinical isolate A5937, and deletion of the *stp1* gene was confirmed using DNA sequencing, Southern blot analysis, and reverse-transcription (RT) PCR. Complementation was performed by subcloning the intact *stp1* gene into pALC2073 using primers AP153/AP154 (see Supplementary Table 1) and transforming the *stp1* deletion mutant using chloramphenicol (10 μg/mL) selection [21]. Tetracycline at a concentration of 200 ng/mL was used to induce expression of the *stp1* gene, and complementation was confirmed using RT-PCR (Supplementary Figure 1).

Microarray Analysis

Total RNA was extracted during the exponential growth phase (Optical Density$_{600}$ of 0.5). Cells were first incubated for 15 minutes in TE buffer supplemented with lysostaphin (Sigma-Aldrich) to a final concentration of 400 μg/mL. RNA was prepared using the RNeasy Mini Kit (Qiagen). Microarray transcriptional analysis was performed using The Institute of Genome Research (TIGR) version 9.0 *S. aureus* slides as described previously [22]. RNA extraction and microarray hybridizations were performed in triplicate, including 1 dye swap analysis. Slides were imaged using a Genetic Microsystems 418 scanner and analyzed with Imagene 5.2 software using the Bioarray Software Environment (BASE) [23], with normalization being performed within and between arrays using Linear Models for Microarray (LIMMA) and Statistics for Microarray (SMA) (Bioconductor) [24]. *P* values, adjusted for multiple comparisons, and fold ratios were determined using moderated *t* tests [25]. Fold changes of >1.5 with an adjusted *P* value of <.05 were considered significant.

Quantitative RT-PCR (qRT-PCR) of select genes was performed to validate the microarray data. Five micrograms of RNA from 2 biological replicates was reverse transcribed using Superscript II (Invitrogen) per the manufacturer's instructions, except 1 μg of random primers was used. Primers used for qRT-PCR are listed in Supplementary Table 1. The qRT-PCR reactions were performed in triplicate, as described [26], using a Mastercycler ep realplex real-time PCR machine (Eppendorf). Normalization using 16S ribosomal RNA and fold-change calculations were performed as described previously [27].

Electron Microscopy

Electron microscopy was performed as described previously [28]. Cells were sectioned using an Ultracut T microtome (Leica), then stained with lead citrate and 2% uranyl acetate. Cells were visualized using an H-7500 transmission electron microscope (Hitachi). Four separate points on 25 equatorially cut cells were measured for each strain in a blinded fashion, with significance determined by Student *t* test at a significance level of $P \leq .05$.

Murine Infection Model

Wild-type 6-week-old female C57BL/6 mice were injected via the tail vein with approximately 1.0×10^8 CFU of the desired bacterial strain in 0.1 mL of sterile saline. A subset of mice was euthanized 120 hours after infection, and histopathology was performed on the liver at these time points. Slides were analyzed using an Olympus B×51 microscope and imaged using an Olympus DP70 camera. The remaining mice were monitored at least 3 times daily, with those showing signs of stress euthanized by CO_2 inhalation. Kaplan-Meier curves and log-rank tests were performed using STATA 6 with a significance level of $P \leq .05$. All animal experiments were performed in accordance with the Animal Research Platform Ethics Committee, Monash University, Australia.

RESULTS AND DISCUSSION

Characteristics of Vancomycin-Exposed *S. aureus* Isolates

Characteristics of the clinical isolates used in this study are described in Table 1. The strains were isolated from patients with complicated *S. aureus* bacteremia including endocarditis, osteomyelitis, and foreign-body infections [11, 16, 29]. All patients failed vancomycin therapy, and this was associated with reduced susceptibility to vancomycin (VISA). Despite no exposure to daptomycin, all vancomycin-nonsusceptible isolates had elevated MICs to daptomycin, an observation that has previously been reported [30]. Four of the 5 parent strains were MLST type 5, which is one of the more common hospital-associated *S. aureus* sequence types in North America [31].

Vancomycin Exposure Leads to a Diverse Range of Mutations in *S. aureus*

To characterize the genetic mechanisms behind VISA formation and its relationship with virulence, we performed whole

genome sequencing and comparative genomics using our vancomycin-exposed strains. In total, 14 *S. aureus* genomes were sequenced, including 3 clinical pairs, 1 clinical series (consisting of 5 isolates), and 1 laboratory-derived series (consisting of 3 isolates). The average genome size was 2.8 Mb (range, 2.72–2.87 Mb) consisting of an average of 2653 predicted protein-encoding genes (range, 2594–2755). An average of 7 coding region mutations (range, 5–13) were identified in each of the vancomycin-exposed pairs or series (Table 2). The majority were SNPs (mean, 6 [range, 3–7]), with an average of 2 deletions and 1 insertion in each of the pairs. All of the point mutations, excluding 4, resulted in a change in amino acid sequence.

The mutational profile of each of the vancomycin-exposed pairs varied (Table 2), highlighting the heterogeneity of genetic changes associated with vancomycin exposure and VISA formation. Despite the absence of a consistent gene mutation to explain the phenotype, mutations in genes of similar functional categories were observed, including those involved in cell wall metabolism, regulation, and protein synthesis (Table 2). We observed a number of mutations that have been associated with reduced susceptibility to vancomycin, including the ABC transporter permease *vraG* [32]; *yvqF* (SA1702) [7], which is a member of the *vraSR* operon; and the DNA-dependent RNA polymerase subunit β, *rpoB*, which has recently been shown to be important in the development of reduced susceptibility to both vancomycin and daptomycin [7, 33]. However, the point mutations and subsequent amino acid changes identified in this study were different from those described previously (Table 2), further highlighting the diversity of mutations that can result due to vancomycin exposure. Compared with that described by Mwangi et al [7], we did not observe a clear stepwise accumulation in mutations in our vancomycin-exposed series (Table 2). We observed mutations in genes in early strains that were not conserved throughout the series. For example, an SNP in *yvqF* emerged in an early vancomycin-exposed isolate (A9638) but was absent from a later isolate (A9639; Table 2), which suggests that the emergence of VISA may be due to the selection of resistant subpopulations within a strain, as opposed to the stepwise accumulation of mutations that are the result of prolonged vancomycin exposure.

Given our previous findings of attenuated virulence of *S. aureus* strains with reduced susceptibility to vancomycin [16], we were particularly interested in gene mutations that not only contribute to reduced vancomycin susceptibility but also alter staphylococcal virulence. An ideal example of this is a mutation within the *agr* operon, which is known to be an important virulence regulator in *S. aureus* as well as being associated with VISA development in the presence of vancomycin exposure [11]. We identified a single nucleotide insertion in the response regulator gene *agrA* within the clinical VISA isolate A5940, which resulted in gene truncation (Table 2). It has been shown that this strain has *agr* dysfunction due to a lack of δ-hemolysin activity [11]. We also identified point mutations in *walK* (previously *yycG*), which is a sensor kinase of an essential 2-component regulatory system that is emerging as a likely contributor to VISA formation [34, 35]. None of our clinical pairs or series had a *walKR* mutation, but the laboratory-derived series (A8117, A8118, and A8392) had 2 nonsynonymous point mutations as well as an amino acid deletion in *walK* (Table 2). The deletion (Gln371) and its role in vancomycin susceptibility have been described recently [35]. It has also been shown that WalKR has a role in *S. aureus* virulence [36, 37]. The operon directly regulates up to 5 virulence determinants in *S. aureus* that are important for bacterial adhesion to host surfaces [36]. Furthermore, a temperature-sensitive *walR* mutant has been shown to be attenuated in virulence in vivo [38].

In *Bacillus* spp, the *walKR* operon is negatively regulated by both YycH and YycI [39]. Mutation within *yycH* has been described previously in a VISA clinical series [7], but its direct effect on vancomycin susceptibility remains to be established. Here, we describe a truncation (~10% of wild-type) of *yycI* in 1 of the clinical VISA isolates (A6226; Table 2). We propose that this mutation may lead to increased WalKR activity, which may be contributing to the VISA phenotype in this strain. We also identified mutations in *tcaR*, a teicoplanin resistance–associated transcriptional regulator, and *dltA*, which is integral to the D-alanation of teichoic acids within the cell wall. Both these genes have been described previously to be associated with reduced susceptibility to glycopepetides as well as *S. aureus* virulence [40–42].

Serine/Threonine Phosphatase Stp1 Influences Vancomycin Susceptibility in *S. aureus*

Of great interest was a mutation in a serine/threonine phosphatase gene known as *stp1* that, along with its cognate kinase, encoded by *pknB*, forms a eukaryotic-like signal transduction system. The identified mutation was a single nucleotide deletion in 1 of the clinical pairs (A5937/A5940) that resulted in a frame shift truncation (~45% of wild-type). To further characterize the role of the *stp1* gene in VISA formation independent of other mutations, we created an *stp1* deletion mutant from the clinical vancomycin-susceptible parent strain (A5937) using allelic exchange methodology. An in-frame deletion of *stp1* resulted in a rise in MIC for vancomycin from 1.5 μg/mL to 3 μg/mL (Figure 1*A*), which is within the nonsusceptible range. Importantly, complementation of *stp1* returned the MIC for vancomycin to the level of the vancomycin-susceptible parent strain (Figure 1*A*), confirming the role of *stp1* in reduced vancomycin susceptibility in *S. aureus*. However, the MIC for vancomycin of the *stp1* mutant was not as high as the clinical VISA daughter isolate (A5940; MIC, 4 μg/mL), which suggests that other mutations apart from *stp1* also contribute to the reduced vancomycin susceptibility. In fact, an additional 5 genetic mutations exist

Table 2. Mutations Observed Between Isogenic *Staphylococcus aureus* Strain Pairs or Series Exposed to Vancomycin

ORF ID[a]	Gene	Product	Function	Protein Size[b]	Mutations[c]		
					Type	Change	Truncated Size[c]
Clinically derived pairs							
1. A5937/A5940							
SA1062	*stp1*	Serine/threonine phosphatase	Signal transduction	247	Deletion	Frameshift	113
SA1844	*agrA*	Accessory gene regulator A	Transcription	238	Insertion	Glu162Stop	161
SA0500	*rpoB*	RNA polymerase subunit β	Transcription	1183	SNP	His481Tyr	
SA1505	*lysP*	Lysine-specific permease	Amino acid metabolism/transport	315	SNP	Leu290Phe	
SA2093	*ssaA2*	Staphylococcal secretory antigen	Immune interaction	267	Insertion	Frameshift	190
SA0022		5′-nucleotidase	Nucleic acid metabolism/transport	773	SNP	Asp537Asp	
2. A6224/A6226							
SA0793	*dltA*	D-alanine-poly(phosphoribitol) ligase, subunit 1	Cell wall/outer membrane metabolism	485	SNP	Ser38Arg	
SA0020	*yycI*	Regulatory protein, WalKR operon	Cell wall/outer membrane metabolism	262	Deletion	Frameshift	51
SA0905	*atl*	Bifunctional autolysin	Cell wall/outer membrane metabolism	1255	SNP	Ser752Ser	
SA1246	*arlS*	Histidine-kinase	Signal transduction	451	Insertion	Frameshift	38
SA1022	*mraW*	S-adenosyl-methyltransferase	Amino acid metabolism/transport	311	Deletion	Frameshift	143
SA1721	*pcrA*	ATP-dependent helicase	DNA replication and repair	730	SNP	Glu455Glu	
SA0467	*tilS*	tRNA(ile)-lysidine synthetase	Nucleic acid metabolism/transport	431	SNP	Met128Ile	
SA0587		PsaA adhesin homologue	Inorganic ion transport/metabolism	309	Deletion	Frameshift	225
SA1528		Universal stress protein	Stress response	137	SNP	Leu105Leu	
SA0837		2-isopropylmalate synthase	Amino acid metabolism/transport	381	Insertion	Frameshift	296
SA1778		Hypothetical protein	Unknown	114	Deletion	Frameshift	76
SA0349		Hypothetical protein	Unknown	495	SNP	Pro474Ser	
SA0668		Hypothetical protein	Unknown	157	SNP	Val41Ile	
3. A6300/A6298							
SA2024	*rpsK*	30S ribosomal protein S11	Translation	129	SNP	Arg127Gly	
SA1404	*rpsU*	30S ribosomal protein S21	Translation	67	Deletion	Frameshift	51
SA0926	*purD*	Phosphorybosylamine-glycine ligase	Nucleotide metabolism/transport	415	SNP	Val389Ala	
SA1491	*hemL*	Glutamate-1-semialdehyde-2,1-aminomutase	Secondary metabolism biosynthesis	425	SNP	Gly48Asp	
SA2480	*drp35*	Lactonase	Carbohydrate metabolism and transport	324	SNP	Asn83Ser	
SA1398		Diacylglycerol kinase, putative	Lipid metabolism	114	SNP	Glu66Lys	
SA0949		Hypothetical protein	Unknown	179	Deletion	No start codon	
SA0556		Hypothetical protein	Unknown	210	SNP	Phe147Phe	

Table 2 continued.

ORF ID[a]	Gene	Product	Function	Protein Size[b]	Mutations[c]		
					Type	Change	Truncated Size[c]
Clinically derived series							
A9635/A9636							
SA1438	*greA*	Transcription elongation factor	Transcription	158	1. SNP 2. Insertion	Arg29Pro, 30Ser, 31Cys	160
A9637							
SA1404	*rpsU*	30S ribosomal protein S21	Translation	67	Deletion	Frameshift	53
SA1478		Hypothetical protein	Unknown	94	SNP	Glu24Lys	
A9638							
SA0617	*vraG*	ABC transporter permease	ABC transporter, defense	633	SNP	Ala580Val	
SA1702	*yvqF*	Protein of the VraSR operon	Cell wall/outer membrane metabolism	233	SNP	Asn74Asp	
SA2047	*rplC*	50S ribosomal protein L3	Translation	220	SNP	Gly7Val	
SA1404	*rpsU*	30S ribosomal protein S21	Translation	67	Deletion	Frameshift	53
SA1861	*ilvC*	Ketol-acid reductoisomerase	Amino acid metabolism and transport	334	Deletion	30Gln/31Gly	332
A9639							
SA1067	*vraG*	ABC transporter permease	ABC transporter, defense	633	SNP	Ala580Val	
SA1870	*rsbW*	Ser-protein kinase, anti-σ β factor	Transcriptional regulation	159	Deletion	88Ser/89Phe	157
SA1404	*rpsU*	Ribosomal protein S21	Translation	67	Deletion	Frameshift	53
SA1861	*ilvC*	Ketol-acid reductoisomerase	Amino acid metabolism/transport	334	Deletion	30Gln/31Gly	332
Laboratory-derived series							
A8117/A8118							
SA0018	*walK*	Sensor protein kinase	Cell wall/outer membrane metabolism	608	1. SNP, 2. SNP	Arg263Cys, Ser273Asn	
SA2147	*tcaR*	Teichoplanin-resistance associated HTH-type transcriptional regulator	Transcription	151	SNP-1, SNP-2	Ile69Ser, Lys95Asn	
SA1936	*luxS*	S-ribosylhomocysteinase	Amino acid metabolism/transport, quorum sensing	156	SNP	Lys15Glu	
A8392							
SA0018	*walK*	Sensor protein kinase	Cell wall/outer membrane metabolism	608	Deletion	371Gln	607
SA1702	*yvqF*	Protein of the VraSR operon	Cell wall/outer membrane metabolism	233	SNP	Leu57Phe	
SA0500	*rpoB*	RNA polymerase subunit β	Transcription	1183	SNP	Ser1052Leu	
SA2147	*tcaR*	Teicoplanin resistance associated HTH-type transcriptional regulator	Transcription	151	1. SNP, 2. SNP	Ile69Ser, Lys95Asn	
SA0375	*guaB*	Inosine-5-monophosphate dehydrogenase	Nucleotide metabolism/transport	488	SNP	Glu417Glu	

Abbreviations: ABC, ATP binding casette; ATP, adenosine triphosphate; ID, identification; ORF, open reading frame; SNP, single-nucleotide polymorphism.

[a] ORF ID derived from N315 genome annotation.

[b] Sizes of the gene or protein based on the susceptible strain of *S. aureus*.

[c] Size of the truncated/extended protein in the nonsusceptible strain of *S. aureus*.

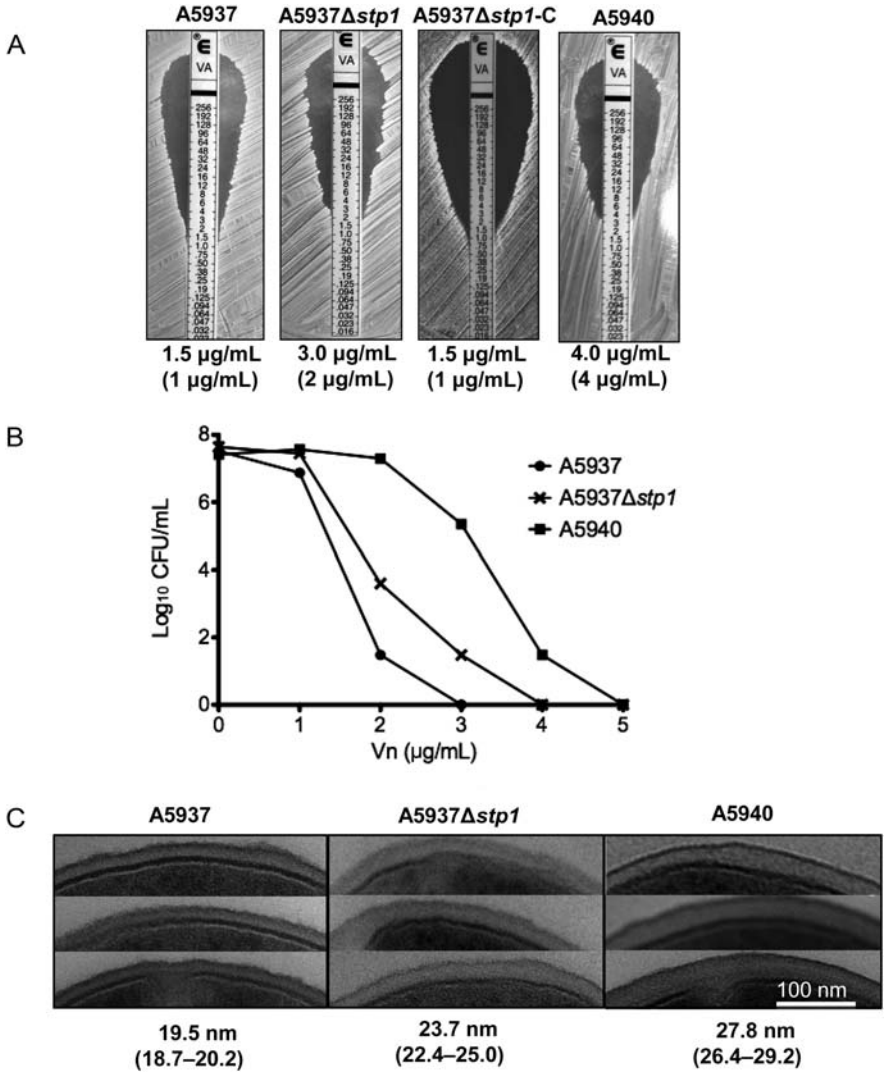

Figure 1. Deletion of *stp1* results in reduced vancomycin susceptibility in *Staphylococcus aureus*. (*A*) Vancomycin minimum inhibitory concentrations (MICs) were determined by Etest for A5937, A5937Δ*stp1*, the complemented strain A5937Δ*stp1*-C, and the vancomycin nonsusceptible isolate A5940. Values in parentheses represent MICs based on broth dilution. (*B*) Determination of vancomycin heteroresistance was performed using population analysis profiling. The *stp1* deletion mutant had subpopulations that grew in the nonsusceptible range (>2 μg/mL); this was not seen in the parent strain (A5937) but was more pronounced in the clinical vancomycin-intermediate *S. aureus* daughter strain (A5940). CFU, colony-forming unit. (*C*) Transmission electron microscopy was performed to determine cell wall thickness. Three representative cells from each strain are shown, and values represent mean cell wall thickness (95% confidence interval). A5940 had the thickest cell wall, followed by the A5937Δ*stp1* and finally A5937.

between the pair, including mutations in *rpoB*, *lysP*, and *agrA* (Table 2), which have all been described as being associated with VISA. These data were supported by the findings of the PAP analysis, which showed that the *stp1* mutant had a heterogeneous population of cells with MICs of vancomycin into the nonsusceptible range (Figure 1*B*). This change, however, was not as pronounced as the change for the clinical VISA daughter strain (Figure 1*B*). Taken together, these data confirm the importance of *stp1* in reduced vancomycin susceptibility in *S. aureus*. Furthermore, they correlate with a recent study by Renzoni et al, which assessed the impact of *stp1* mutation on reduced susceptibility to a different glycopeptide antibiotic, teicoplanin [43]. Using a laboratory-derived strain that was exposed to teicoplanin, they showed that *stp1* mutation resulted in a reduction in teicoplanin susceptibility [43]. Although they were unable to show clear differences in MIC for vancomycin using their *stp1* mutant strain, they did show subtle growth differences in the presence of vancomycin using a highly sensitive spot population analysis method [43]. Their data also supported recent work that has shown that *stp1* is an important regulator of cell wall biosynthesis and that deletion of the *stp1* gene leads to an increase in cell wall thickness [28, 43], which is a consistent phenotype of VISA isolates.

Stp1 Affects Vancomycin Susceptibility by Regulating Cell Wall Metabolism

In order to determine the mechanism by which Stp1 affects vancomycin susceptibility, we assessed the transcriptional profile of the *stp1* deletion mutant. Using microarray analysis, we identified upregulation of genes that could potentially contribute to cell wall thickening (Table 3). The first of these genes is *uppS*, encoding undecaprenyl pyrophosphate synthase, which is involved in the production of undecaprenyl phosphate, an important lipid carrier in the biosynthesis of peptidoglycan and cell wall teichoic acids [44]. The second gene with altered regulation is *sceD*, encoding a lytic transglycolase, which has been shown to have peptidoglycan hydrolase activity and involvement in cell wall turnover [45]. Given these findings and those in recently published reports [28, 43], we performed TEM analysis on our *stp1* mutant (Figure 1*C*). This confirmed a thickened cell wall in our *stp1* deletion mutant compared to its parent strain ($P < .001$; Figure 1*C*), and, as seen with the vancomycin susceptibility testing, the cell wall was not as thick as the clinical VISA isolate (A5940), further supporting the importance of multiple mutations in the observed VISA phenotype. It has previously been shown that disorganization of the peptidoglycan and an increase in false vancomycin binding sites prevent the penetration of vancomycin to its active site on the cytoplasmic membrane [8, 46, 47].

Stp1 Affects *S. aureus* Virulence

As mentioned previously, we have evidence, using a nonmammalian model system, that VISA isolates may be less virulent than their vancomycin-susceptible parent strains [16]. In

Table 3. Differentially Expressed Genes in the *stp1* Deletion Mutant[a] Compared to Its Parent Strain[b]

ORF ID[c]	Gene	Description	Fold Change[d]
Downregulated genes			
Virulence			
SACOL1187	PSM β2	Phenol-soluble modulin	1.9
SAS065	*hld*	δ-hemolysin	1.9
SA0387	*set11*	Superantigen-like protein	1.55
Unknown function			
SAP007		Hypothetical protein	2.1
SAS059		Hypothetical protein	1.64
Upregulated genes			
Cell wall metabolism			
SA1103	*uppS*	Undecaprenyl pyrophosphate synthetase	1.77
SA1898	*sceD*	Transglycosylase	1.86
Other cellular function			
SACOL1053	*yfbB*	Acyl-CoA thioester hydrolase	2.37
SA2186	*nasF*	Uroporphyrin-III C-methyl transferase	2.16
SA1082	*rimM*	16S ribosomal RNA processing protein	1.69
SA1184	*citB*	Aconitate hydratase	1.62
SA2180	*nreB*	Sensor histidine kinase	1.61
SA0587	*mntC*	Manganese ABC transporter permease	1.53
SA1421		Putative metal-dependent phosphohydralase	1.52
Unknown function			
SACOL1331		Hypothetical protein	2.9
SACOL2365		Hypothetical protein	2.15
SA0287		Hypothetical protein	2.09
SA0289		Hypothetical protein	1.96
SAR0592		Hypothetical protein	1.58
SA0336		Hypothetical protein	1.56

Abbreviations: ABC, adenosine triphosphate binding casette; ID, identification; ORF, open reading frame; PSM, phenol-soluble modulin.

[a] The cotranscribed kinase, *pknB,* showed no significant change in expression between the *stp1* deletion mutant and its parent strain (A5937).

[b] Differential expression was confirmed using quantitative reverse-transcriptase polymerase chain reaction normalized to 16S ribosomal RNA expression for *hld* (downregulated 4.07 [±1.0]-fold), PSM β2 (downregulated 2.31 [±0.7]-fold), and *sceD* (upregulated 1.51 [±0.2]-fold).

[c] ORF IDs derived from N315 (SA/SAP/SAS), COL (SACOL), and methicillin-resistant *Staphylococcus aureus* MRSA252 (SAR) genome annotations.

[d] Genes with fold change of ≥1.5 with adjusted *P* value of ≤.05 were included.

addition, it has recently been shown that *stp1* is important for toxin production and virulence in *S. aureus* [48]. In support of these findings, our gene expression analysis of the *stp1* mutant relative to its parent strain showed downregulation of a number of genes involved in *S. aureus* virulence, most notably *hld*, which encodes δ-hemolysin, a toxin that assists in the destruction of leukocytes and a number of other host cell types [49]. Importantly, the *hld* gene is embedded within RNAIII, which is the effector molecule of *agr*, suggesting a possible relationship between Stp1 and Agr. In addition to this, we observed downregulation of other genes that are either directly or indirectly regulated by *agr*, including a superantigen-like protein, encoded by *set11*, and the phenol-soluble modulin β2 gene (SACOL1187), which encodes a protein that belongs to a family of short peptides that are emerging as important virulence determinants, particularly in community-associated *S. aureus* [50]. To mimic the bacteremia that these strains caused in our patient, we tested the vancomycin-susceptible parent strain (A5937), its VISA daughter strain (A5940), and the *stp1* deletion mutant (A5937Δ*stp1*) in a murine bacteremia model. We found that the *stp1* deletion mutant and the VISA daughter strain were significantly attenuated in virulence compared with the parent strain (Figure 2*A*). Furthermore, hepatic abscess formation was observed in A5937-infected mice at 5

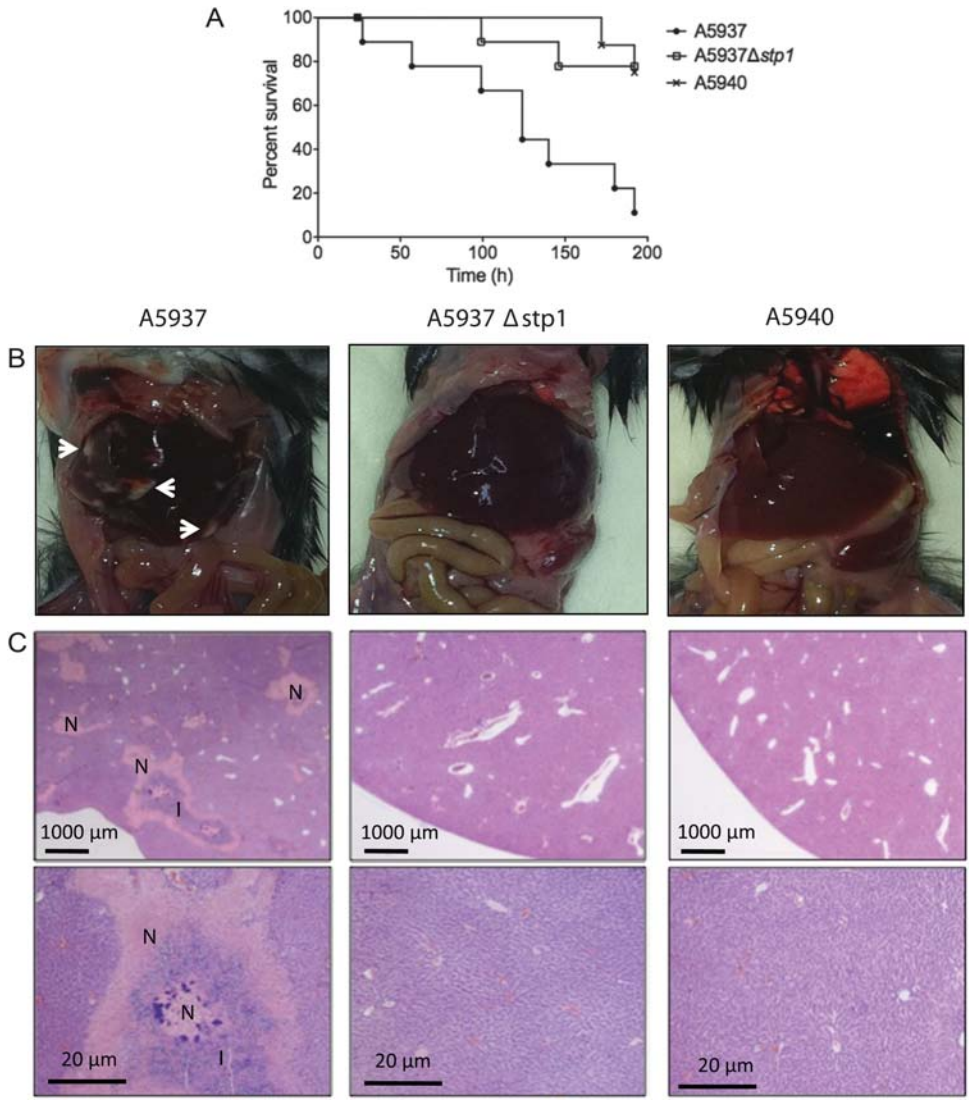

Figure 2. Deletion of *stp1* results in attenuated virulence in *Staphylococcus aureus*. (*A*) Six-week-old C57BL/6J mice were injected with ~1.0×10^8 bacterial cells. Kaplan-Meier curves indicate time to euthanasia for each strain. A5937 produced significantly more killing compared with both A5937Δ*stp1* and A5940 ($P = .01$; log-rank test). (*B*) Macroscopic and (*C*) histopathological analyses showed that the clinical vancomycin-susceptible parent strain (A5937) caused hepatic abscess formation and tissue necrosis, whereas this was not seen with the *stp1* deletion mutant (A5937Δ*stp1*) and the clinical vancomycin-intermediate *S. aureus* daughter strain (A5940). White arrows point to abscess formation in the liver; N represents severe necrosis; and I represents inflammatory infiltrate. Tissues were stained with hematoxylin and eosin.

days after infection, whereas this was not present with the *stp1* deletion mutant or the VISA daughter strain (Figure 2*B*). This was supported by histopathological analysis, which showed severe necrosis and abscess formation only in the A5937-infected mice (Figure 2*C*). These data not only illustrate the importance of the *stp1* gene in staphylococcal virulence but also support our previously generated data using the invertebrate *Galleria mellonella* model, which showed attenuated virulence in clinical VISA isolates when compared with their isogenic vancomycin-susceptible parent strains [16].

Conclusion

Comparative genomics of carefully selected strains is a useful tool when trying to understand the genetic mechanisms behind VISA formation. Our data, along with a number of previous studies, have shown that mutations in a range of biological pathways can contribute to reduced vancomycin susceptibility in *S. aureus*. We have also characterized a new mechanism of reduced vancomycin susceptibility, the serine/threonine phosphatase *stp1*. Using gene expression analyses, we have shown that *stp1* regulates genes that alter cell wall biosynthesis, providing a possible mechanism for its effect on vancomycin susceptibility. We have also shown that this gene has a role in staphylococcal virulence in mammals, which provides support to the hypothesis that virulence may actually be attenuated in *S. aureus* strains with reduced susceptibility to vancomycin. These data provide important insights into the pathogenic consequences of antibiotic resistance in *S. aureus* and may assist with the development of novel strategies for the prevention or treatment of infections due to this problematic organism.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. R. C. M. has served as a consultant to Cubist, Forest, Merck, Novartis, Ortho, Johnson & Johnson, Pfizer, Theravance, and Wyeth. G. M. E. has served on scientific advisory boards for Cubist, Bayer Schering, Johnson & Johnson Pharmaceutical Research and Development, Novartis, Pfizer, Shionogi, and Theravance; has received research training support from Cubist and research contracts from Novexel, Pfizer, and Theravance; has received speaking honoraria from Novartis; and serves on the board of directors of the National Foundation for Infectious Diseases. A. Y. P. has been to 1 advisory board meeting for Abbott Molecular and Ortho-McNeil-Janssen and has received a speaker's honorarium from AstraZeneca and Merck Sharp & Dohme for 1 presentation each. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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