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Identification of Point Mutations in Clinical *Staphylococcus aureus* Strains That Produce Small-Colony Variants Auxotrophic for Menadione

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Staphylococcus aureus **small-colony variants (SCVs) are implicated in chronic and relapsing infections that are difficult to diagnose and treat. Despite many years of study, the underlying molecular mechanisms and virulence effect of the small-colony phenotype remain incompletely understood. We sequenced the genomes of five *S. aureus* SCV strains recovered from human patients and discovered previously unidentified nonsynonymous point mutations in three genes encoding proteins in the menadione biosynthesis pathway. Analysis of genetic revertants and complementation with wild-type alleles confirmed that these mutations caused the SCV phenotype and decreased virulence for mice.**

Staphylococcus aureus is a leading cause of bacterial infections globally and has posed serious infection control problems since the emergence of methicillin-resistant *S. aureus* (MRSA) strains in 1961 (1). MRSA strains are now the leading cause of death by an infectious agent in the United States, with a mortality rate of approximately 20% (2). MRSA and methicillin-susceptible *S. aureus* (MSSA) strains cause a variety of diseases, including superficial skin and soft tissue infections and more severe diseases such as bacteremia, pneumonia, endocarditis, and osteomyelitis (3). *S. aureus* can also be a commensal organism, asymptomatically colonizing approximately 30% of healthy individuals (4), which can lead to subsequent infection (5). Strains of antibiotic-resistant *S. aureus* have spread worldwide, resulting in an endemic population of MRSA in many industrialized nations.

An ongoing diagnostic and treatment problem in *S. aureus* infections is due to a subpopulation of small-colony variants (SCVs) found in patients with persistent, antibiotic-resistant, and recurrent infections (6–8). Although SCVs were described >100 years ago (9), it was not until approximately 20 years ago that investigators began to study the biological and pathogenic traits of these organisms in earnest (7). SCVs have been recovered from patients with endocarditis, pneumonia, soft tissue infections, osteomyelitis, and severe bacteremia (reviewed in reference 10). The frequency of SCV recovery from clinical specimens ranges from 1 to 30% (reviewed in reference 11). A distinct characteristic of *S. aureus* SCV infections is their ability to persist in the presence of aggressive antimicrobial therapy. SCVs constitute a slow-growing, auxotrophic subpopulation with atypical colony morphology and physiology. For example, they produce small, nonpigmented, and nonhemolytic colonies with decreased coagulase activity and an unstable colony phenotype. In addition, they have altered drug resistance profiles, such as an increased resistance to aminoglycosides, and thus are particularly difficult to detect and treat (reviewed in references 11 and 12). SCV strains have significant clinical importance and in some cases have been shown to persist for >50 years (7). These naturally occurring variants have a survival advantage in their ability to persist within eukaryotic cells, thereby protecting them from host defenses and antibiotics (reviewed in reference 13).

The atypical morphology, slow growth, and abnormal biochemical characteristics of SCVs often lead to misidentification by clinical laboratories (14). An additional confounding attribute is the instability of the SCV phenotype, as the variants may revert to a normal phenotype (reviewed in reference 13). Three major categories of SCVs have been found in clinical isolates, namely, electron transport-defective strains that are auxotrophs for menadione, hemin, or thiamine. Auxotrophy for menadione and hemin makes the bacteria unable to synthesize menaquinone and cytochromes, respectively. This often results from mutations in genes coding for enzymes involved in the biosynthesis of these two molecules. Thiamine auxotrophs are rarely identified in human infections and are considered a subtype of menadione-dependent strains. As a consequence of auxotrophy, specific nutritional supplementation and prolonged culture enhance recovery from patient specimens (15). Many of the typical SCV characteristics (e.g., low growth rate, lack of pigmentation, increased aminoglycoside resistance, and decreased alpha-toxin production) can be linked to electron transport deficiencies (reviewed in reference 11). Most studies investigating SCVs have used laboratory strains containing stable mutations in the *menD* or *hemB* gene, resulting in menadione- and hemin-auxotrophic strains, respectively, the two most common SCV phenotypes (16–18).

Here we report genetic analyses of five SCV strains cultured from clinically important human infections. The strains are auxotrophic for menadione, and each strain has a single-nucleotide polymorphism (SNP) in one of the menadione biosynthesis genes, including *menC*, *menE*, or *menF*. Genetic complementation of the mutant alleles restored menadione auxotrophy, wild-type colony

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TABLE 1 *S. aureus* strains used in this study

Strain	Phenotype, auxotrophism	Diagnosis	Patient treatment(s)	Gene with SNP	Nucleotide change	Amino acid change
LAC	Wild type, none					
221_{SCV}	SCV, menadione	Abscess	Clindamycin	*menE*	G762A	L254F
221_{REV}	Wild type			Wild-type *menE*		
377_{SCV}	SCV, menadione	Bacteremia	Vancomycin			
			Piperacillin	*menC*	G625A	A209V
			Tazobactam			
			Gentamicin	*menF*	G382A	G128E
377_{COM}	Wild type			psk265*menC*[a] (wild type)	G625A	A209V
					G382A	G128E
453_{SCV}	SCV, menadione	Osteomyelitis	Vancomycin	*menC*	G514A	A172V
			Nafcillin			
			Gentamicin			
453_{COM}	Wild type			psk265*menC* (wild type)	G514A	A172V
726_{SCV}	SCV, menadione	Osteomyelitis	Ciprofloxacin, clindamycin	*menC*	G625A	A209V
				menF	G382A	G128E
726_{COM}	Wild type			psk265*menC* (wild type)	G625A	A209V
					G382A	G128E
927_{SCV}	SCV, menadione	Abscess	Clindamycin, trimethoprim-sulfamethoxazole	*menF*	A594G	I198M
927_{REV}				Wild-type *menF*		

[a] psk265 contains the wild-type *menC* allele from strain LAC (*menC* accession number SAU300_1735 from the NCBI whole-genome database).

morphology and growth characteristics, and strain virulence for mice.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and growth curves. Clinical isolates were recovered from hospitalized patients in Houston, TX. Five strains with small-colony morphology were analyzed (Table 1). Strain LA County (LAC) was used as a reference strain because it is a well-characterized USA300 community-acquired organism whose genome has been sequenced (19). The SCV isolates were grown on Columbia sheep blood agar, Trypticase soy agar (TSA), and Mueller-Hinton agar for 24 to 72 h at 37°C in 5% CO_2. The revertant and plasmid-complemented strains were grown on the same media for 12 to 16 h at 37°C. Liquid cultures were grown in Trypticase soy broth (TSB) at 37°C with shaking at 250 rpm. The five SCV strains produce nonpigmented, slow-growing microcolonies. Menadione (Acros Organics) was used, when indicated, at 1 μg/ml, and chloramphenicol was added at 20 μg/ml.

For growth curve experiments, fresh colonies from each strain were picked from TSA plates and incubated overnight in 5 ml of TSB at 37°C with shaking. Cultures grown overnight were diluted to an optical density at 600 nm (OD_{600}) of 0.02 in 50 ml of fresh TSB and incubated at 37°C with shaking. The OD_{600} was measured hourly for 8 h.

Genome sequencing and data analysis. Methods for genome sequencing and data processing and analysis were described previously (20). The sequencing reads were compared to the LAC reference genome to identify genetic polymorphisms (19).

Targeted *men* gene sequencing. *S. aureus* strains were grown overnight on TSA. Genomic DNA was extracted by alkaline-boiling lysis (21). Sanger sequencing was used to determine the *men* gene alleles by using previously described methods (20). The primer pairs used (5′ to 3′) were menC1 (CAGCAAGCAAGCATTCACAT) and menC2 (TCCAGGTATC AGTGACGCAG), menE1 (GCGTTACATTCCCCGAAATA) and menE2 (AGATGATGTGGCAACAATGGGT), and menF1 (AGTGAAATGGAT GGCTACGG) and menF2 (AATTCGCTGCTGCTGTACCT). Electropherograms were visually inspected by using Sequencher 4.7 (Gene Codes).

Selection of fast-growing (wild-type) revertant colonies. Small colonies were picked from fresh TSA plates, grown in TSB with serial passage daily for 3 to 10 days, and then plated and screened for fast-growing colonies after 14 h of incubation at 37°C. Wild-type-sized colonies were picked, purified by restreaking, and stored in TSB with 60% glycerol at −80°C. The *menE* and *menF* genes were sequenced by Sanger sequencing using primer pair menE1 and menE2 and primer pair menF1 and menF2, respectively, with an Applied Biosystems 3730 capillary sequencer. Fast-growing colonies that were derived from strains 221 and 927 and were confirmed to have a wild-type (revertant) allele in *menE* or *menF* were designated strains 221_{REV} and 927_{REV}, respectively.

Construction of genetically complemented strains. *cis*-acting elements that are required for complete *menE* and *menC* gene expression, including an upstream putative ribosomal binding site and promoter region, were amplified with primers menE2 and ID4 (5′-ATGTCATCCGC TTCATAAAGG-3′). The 2.7-kb fragment of the PCR product was purified with the QIAquick gel extraction kit, ligated into the PCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA), and transformed into competent *Escherichia coli* TOP10 cells (Invitrogen). A staphylococcal origin of replication was introduced by cloning plasmid pSK265 into the unique BamHI site on PCR 2.1-TOPO, and the construct was moved into *S. aureus* RN4220 by electroporation (22). Phage 80α was used to transduce the constructs from RN4220 into strains 377_{SCV}, 453_{SCV}, and 726_{SCV}. The final constructs were designated 377_{COM}, 453_{COM}, and 726_{COM}.

Determination of gentamicin MIC. The MIC of gentamicin was determined with Etest strips (0.016 to 256 μg/ml gentamicin) (bioMérieux SA) according to the manufacturer's instructions.

Hemolysis assay. Hemolytic activity was determined spectrophotometrically by measuring the release of hemoglobin from rabbit red blood cells (RBCs) (23). Briefly, bacteria were grown overnight in TSB at 37°C with shaking at 250 rpm. The cells were pelleted by centrifugation, and the supernatant was discarded. The cells were suspended in phosphate-buffered saline (PBS) containing 1 mM CaCl and 0.5 mM Mg, incubated for 1 h, and collected by centrifugation, and the supernatant was filter sterilized. Rabbit RBCs were washed three times in PBS with 0.85% NaCl to remove free hemoglobin. The washed RBCs were diluted to 1.4% in PBS containing 0.2% (wt/vol) bovine serum albumin. The filtered bacterial supernatants were added to an equal volume of the 1.4% RBC solution and incubated for 1 h at 37°C. A PBS control was used for spontaneous lysis, and *Staphylococcus epidermidis* was used as a nonhemolytic control. The cells were then pelleted for 10 s at maximum speed in a desktop microcentrifuge. The released hemoglobin was measured as the A_{540} with

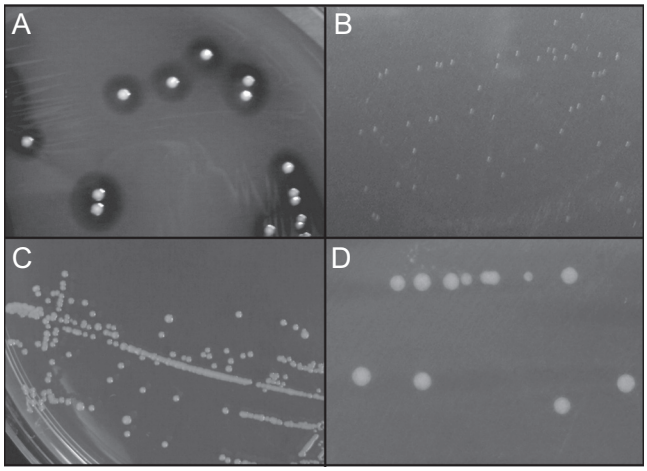

FIG 1 Colony morphology on TSA or blood agar plates. (A) *S. aureus* wild-type strain LAC grown on blood agar plates for 14 h; (B) pinpoint SCV colonies on TSA after 24 h of growth; (C) SCV colonies on TSA supplemented with 1 μg/ml menadione; (D) plasmid-complemented SCV strain 453_{COM} grown on TSA.

a spectrophotometer. Standard dilutions were made for comparison; deionized water was added to an equal volume of RBCs to represent 100% hemolysis (24). Additional standards were made for comparison (75%, 50%, 25%, 12.5%, and 6.25%). The results shown are the means ± standard deviations of at least three replicate experiments.

Mouse virulence experiments. Virulence of SCV (454_{SCV}) and isogenic complemented (453_{com}) strains were compared with a mouse model of bacteremia. Fifteen 5- to 6-week-old outbred immunocompetent female CD1 mice (Harlan Laboratories) were randomly assigned to a strain treatment group and inoculated intraperitoneally with 1×10^7 CFU. Bacterial doses were prepared at the time of inoculation from previously quantified frozen stocks, and the actual dose was confirmed by CFU analysis. Near mortality was determined by observation. Data were graphically displayed as a Kaplan-Meier survival curve and analyzed with the log-rank test (Prism 6; GraphPad Software Inc., La Jolla, CA). All mouse experiments were approved by the Institutional Animal Care and Use Committee, Houston Methodist Research Institute (protocol AUP-1010-0022).

RESULTS

Isolation of *S. aureus* SCVs. We screened ~1,000 *S. aureus* strains cultured from normally sterile sites for a SCV phenotype on blood agar plates. Five SCV strains were identified among these organisms (Table 1). Two methicillin-resistant strains (221_{SCV} and 927_{SCV}) caused abscesses in community-acquired infections occurring in patients with no underlying illness or risk factors (community-acquired MRSA [CA-MRSA]). The other three strains were from hospital-associated *S. aureus* (HA-SA) infections, two of which were MRSA strains (strains 377_{SCV} and 453_{SCV}) and one of which was an MSSA strain (strain 726_{SCV}). Both HA-MRSA-infected patients were treated with an antibiotic regimen that included gentamicin, an agent known to select for SCVs (25). The three patients with HA-SA infections had underlying medical conditions and had previously been hospitalized. All five strains were of the USA300 clonotype.

The five SCV strains formed colonies that were approximately 1/10 the diameter of normal *S. aureus* colonies after 24 h of incubation at 37°C (Fig. 1A and B). The colonies were white, versus the yellow-gold colonies of strain LAC and many other *S. aureus* strains (data not shown).

Identification of single-nucleotide polymorphisms in the *men* genes of the SCV strains. As a first step toward determining the genetic mechanism underlying the SCV phenotype in these five strains, the genome of each strain was sequenced. Inasmuch as mutations in *hemB*, *menB*, *menD*, or *thyA* have been reported to cause menadione, hemin, or thymine auxotrophy and SCV phenotypes in *S. aureus* (7, 17), we initially analyzed the genome sequences for polymorphisms in genes implicated in menadione (*men*), hemin (*hem*), or thymidine (*thy*) biosynthesis. Relative to the reference genome of strain LAC (19), all five SCV variants had nonsynonymous (amino-acid-altering) SNPs in one of the *men* genes. In contrast, no polymorphisms that have been associated with the SCV phenotype were present in the *hem* or *thy* gene (Table 1). Three strains each had a different SNP in one of the nine genes implicated in menadione biosynthesis. Strain 221_{SCV} had an SNP in *menE* at nucleotide 762, resulting in a L254F replacement. Strain 453_{SCV} had an SNP in *menC* at nucleotide 514 that resulted in an A172V change, and the *menF* gene in strain 927_{SCV} had an SNP that produced an I198M replacement. Strains 377_{SCV} and 726_{SCV} each had one SNP in *menC* that resulted in an A209V change and a second SNP in *menF* that produced a G128E replacement (Table 1). No other polymorphisms that could be readily hypothesized to cause SCV variants and menadione, hemin, or thymine auxotrophy were identified in the five genomes.

We next tested the hypothesis that the identified single-amino-acid replacements caused the SCV phenotype. SCVs are known to be unstable, so we first screened for wild-type revertants by growth on TSB after serial *in vitro* passage for 3 to 10 days (see Materials and Methods). Two strains (221_{SCV} and 927_{SCV}) produced growth revertants (designated 221_{REV} and 927_{REV}) at rates of 1.76×10^{-6} and 1.21×10^{-6} per cell per generation, respectively, as assessed by fluctuation analysis (22). These rates are consistent with expected values for the reversion of a point mutation and are similar to other data in the literature reported for reversion of SCV strains (26, 27). Sanger DNA sequencing confirmed that the revertant strains had a wild-type allele of the cognate *men* gene.

As *in vitro* passage yielded revertant colonies only for two of the five SCV strains, we introduced the wild-type allele of either the *menE* or *menC* gene into the remaining three strains to create 377_{COM}, 726_{COM}, and 453_{COM}. Sanger sequencing confirmed the presence of a wild-type copy of the cognate mutant gene.

Although strains 377_{SCV} and 726_{SCV} had SNPs in both *menC* and *menF*, we began our analysis by complementing the *menC* gene. The SNP in the *menC* gene changes an amino acid located directly between two invariant amino acids responsible for Mg cofactor binding (28).

Strain 453_{SCV} contains an SNP in the *menC* gene that changes an amino acid located within the absolutely conserved DAN (Asp-Ala-Asn) motif found in the *o*-succinylbenzoate synthase family of proteins, of which MenC is a member. The alanine residue of this motif is required to maintain the position of the Mg^+ cofactor-binding pocket (28). In strain 453_{SCV}, this critical alanine is replaced by valine (A172V), which may explain the stability of the SCV phenotype in strain 453_{SCV}. MenE belongs to the *o*-succinylbenzoate–coenzyme A (CoA) ligase family of proteins. Members of this family are responsible for catalyzing two reactions and share conserved catalytic domain structures (29). The SNP in the *menE* gene of strain 221_{SCV} results in a L254F replacement located

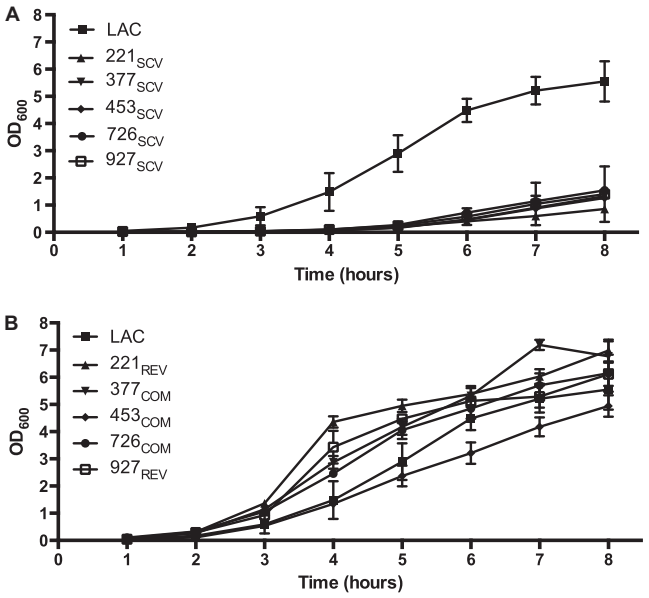

FIG 2 Growth curves of SCVs and revertant or genetically complemented strains. (A) Growth curves of control strain LAC and the five clinical SCV strains; (B) growth curves of the plasmid-complemented strains and revertant strains 927_{REV} and 221_{REV}. Growth curves were performed with TSB. Data are means ± standard deviations of at least 3 independent experiments.

directly adjacent to a conserved functional domain of the protein. This amino acid change may alter the catalytic site, leading to menadione auxotrophism and the observed SCV phenotype.

Using the five clinical SCV strains and their wild-type revertant or plasmid-complemented counterparts, we sought to characterize the functional consequences of these naturally occurring mutations. Our results demonstrate that correction of the *men* gene mutation causes reversion of the small-colony phenotype and restores wild-type growth, hemolytic activity, and antibiotic susceptibility.

Growth of bacteria. To test the hypothesis that the SCV phenotype in these five strains was related to menadione auxotrophy, we grew the strains on TSA supplemented with menadione. The results (Fig. 1C) showed that wild-type colony morphology was restored by growth in the presence of exogenous menadione. To further test this hypothesis, we grew plasmid-complemented cells on TSA lacking supplemental menadione. Consistent with our hypothesis, genetic complementation of SCV strains with a plasmid containing a wild-type *men* gene restored wild-type colony morphology, including size and gold pigmentation (Fig. 1D). Similarly, growth of SCV strains in liquid medium (TSB) supplemented with 1 μg/ml menadione or with a plasmid containing the cognate wild-type *men* gene restored growth to a wild-type phenotype (Fig. 2A and B).

Hemolysis of red blood cells. A key characteristic phenotype of *S. aureus* SCVs is a reduced level of alpha-toxin production, which is believed to enhance persistence in nonprofessional phagocytes (24). Consistent with other SCV strains reported in the literature, these five strains had greatly reduced alpha-toxin production, as assayed by hemolysis of red blood cells (Fig. 3). To establish that this reduction in hemolysis was due to the mutations in the *men* genes, bacteria were grown in TSB supplemented with 1 μg/ml menadione (Fig. 3, black bars). The addition of exogenous menadione significantly increased hemolysis of red blood cells compared to the SCV strains grown in the absence of menadione ($P < 0.05$). The natural revertant strains 221_{REV} and 927_{REV} and the plasmid-complemented strains 377_{COM}, 453_{COM}, and 726_{COM} (Fig. 3, white bars) caused significantly more RBC hemolysis ($P < 0.0001$) than their SCV parental strains. These results show that reversion of this mutation restores the amount of hemolysis to that of a wild-type strain, indicating that the *men* gene mutations are responsible for the small-colony phenotypes.

Gentamicin resistance. SCV *S. aureus* strains have altered antibiotic resistance profiles that are thought to be caused by interruptions in electron transport and low growth rates. Menadione-auxotrophic SCVs have defective electron transport chains that result in the decreased uptake of aminoglycosides such as gentamicin, thereby causing increased antibiotic resistance (30). Gentamicin-resistant SCVs are important pathogens repeatedly recovered from cystic fibrosis patients (31) and patients with chronic staphylococcal bone infections (32). Consistent with previous reports, our five clinical strains had increased resistance to gentamicin compared to the control strain LAC (Fig. 4). Geneti-

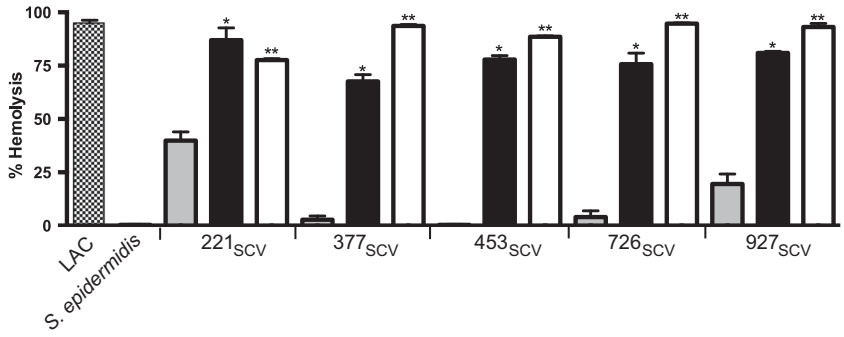

FIG 3 Hemolysis of red blood cells. Hemolysis of rabbit erythrocytes was measured spectrophotometrically and compared with lysed erythrocyte standards to calculate percent hemolysis. The tests were performed by using each SCV strain (gray bars), the SCV strains grown in media supplemented with 1 μg/ml menadione (black bars), and the SCV strains with wild-type *men* genes (white bars). LAC was included as a wild-type positive control; *S. epidermidis* does not produce alpha-toxin (negative control). A single asterisk indicates a significant difference in red blood cell hemolysis between the menadione-supplemented strain and the matching SCV strain. A double asterisk indicates a significant difference in percent hemolysis between the complemented or revertant strain and the corresponding SCV strain ($P < 0.05$ by *t* test). Data are the means ± standard deviations of at least 3 independent experiments.

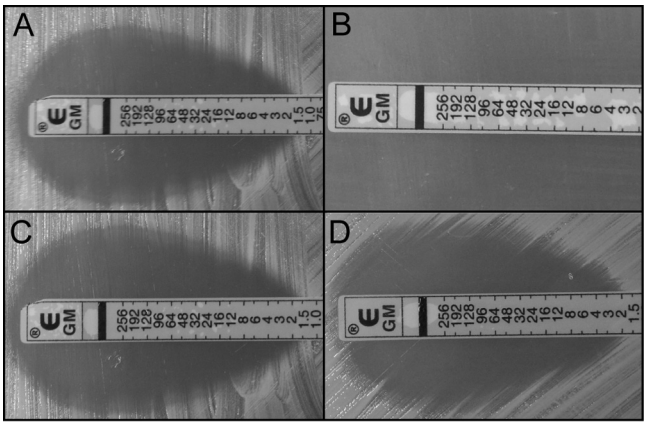

FIG 4 Gentamicin susceptibility of various strains. (A) USA300 LAC; (B) SCV strain 776_{SCV}; (C) plasmid-complemented strain 453_{COM}; (D) natural revertant strain 927_{REV}.

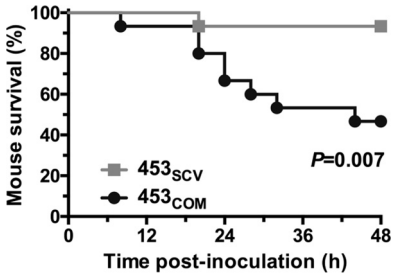

FIG 5 Virulence in mice. Fifteen outbred CD1 mice were infected intraperitoneally with 1×10^7 CFU of each indicated strain. Data are shown as Kaplan-Meier survival over time, with *P* values calculated with the log-rank test.

cally complemented strains 377_{COM}, 453_{COM}, and 726_{COM} and natural revertant strains 221_{REV} and 927_{REV} had MICs comparable to that of the wild-type strain (Fig. 4C and D). Additionally, menadione added exogenously to the SCV strains resulted in an MIC of gentamicin that was identical to that for the wild-type strain (data not shown). Thus, these results are consistent with the idea that mutations in the menadione biosynthesis genes responsible for the SCV phenotype also result in increased gentamicin resistance.

SCV strain 453_{SCV} is less virulent for mice than genetically complemented strain 453_{COM} in an invasive infection model. To test the hypothesis that *S. aureus* SCV strains have altered virulence, we compared strain 453_{SCV} with the isogenic genetically complemented strain 453_{COM} in a mouse model of bacteremia. The use of strain 453 in these experiments was an arbitrary choice. Consistent with the hypothesis of altered virulence, strain 453_{SCV} was significantly less virulent than the genetically complemented strain 453_{COM} (Fig. 5).

DISCUSSION

In this work, we identified the genetic basis of the SCV phenotype observed among five naturally occurring clinical strains of *S. aureus* cultured from normally sterile sites. These five strains were isolated from community-acquired and hospital-associated infections, included both MRSA and MSSA, and caused multiple infection types. All five strains had classic SCV phenotypes, including pinpoint colonies, lack of pigmentation, slow growth, reduced alpha-toxin production, and increased antibiotic resistance. Full-genome sequencing led to the discovery that these strains contained mutations in the *men* genes *menC*, *menE*, and *menF*. We showed a reversal of the SCV phenotype through restoration of a wild-type copy of the mutant *men* gene containing the point mutations or mutational reversion or through the addition of exogenous menadione. In aggregate, the data provide compelling evidence that mutations in *menC*, *menE*, or *menF* are the direct cause of menadione-auxotrophic SCVs in these clinical isolates of *S. aureus*.

Traditional experiments focused on identifying compounds that restored wild-type growth to small colonies, leading to the discovery that many SCVs are auxotrophic for menadione or hemin (7, 9, 33). The failure of SCVs to produce menadione or hemin results in interrupted electron transport and decreased ATP synthesis. Many of the phenotypes associated with SCVs, such as slow growth, decreased pigmentation, and resistance to aminoglycosides, can be linked to disrupted respiration and energy production (34, 35). In the majority of Gram-positive bacteria, menaquinone is the only quinone in the electron transport chain, making the enzymes that synthesize this compound promising drug targets for multidrug-resistant pathogens such as *S. aureus* and *Mycobacterium tuberculosis* (34). In this regard, we note that growth inhibition has been reported for compounds that target the gene products of *menA* (34, 36), *menD* (16), *menE* (18), and *menB* (37). The initial findings for this new class of antimicrobial molecules highlight the importance of the work presented here. Given the relative lack of success over the last 10 years in identifying new compounds to treat multidrug-resistant pathogens such as *S. aureus* (38), the menaquinone biosynthesis pathway may prove to be a fruitful area of focus to selectively target prokaryotes while preventing side effects to eukaryotes, which lack menaquinone.

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