**Staphylococcus aureus** Adapts to Oxidative Stress by Producing H$_2$O$_2$-Resistant Small-Colony Variants via the SOS Response

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The development of chronic and recurrent *Staphylococcus aureus* infections is associated with the emergence of slow-growing mutants known as small-colony variants (SCVs), which are highly tolerant of antibiotics and can survive inside host cells. However, the host and bacterial factors which underpin SCV emergence during infection are poorly understood. Here, we demonstrate that exposure of *S. aureus* to sublethal concentrations of H$_2$O$_2$ leads to a specific, dose-dependent increase in the population frequency of gentamicin-resistant SCVs. Time course analyses revealed that H$_2$O$_2$ exposure caused bacteriostasis in wild-type cells during which time SCVs appeared spontaneously within the *S. aureus* population. This occurred via a mutagenic DNA repair pathway that included DNA double-strand break repair proteins RexAB, recombinase A, and polymerase V. In addition to triggering SCV emergence by increasing the mutation rate, H$_2$O$_2$ also selected for the SCV phenotype, leading to increased phenotypic stability and further enhancing the size of the SCV subpopulation by reducing the rate of SCV reversion to the wild type. Subsequent analyses revealed that SCVs were significantly more resistant to the toxic effects of H$_2$O$_2$ than wild-type bacteria. With the exception of heme auxotrophs, gentamicin-resistant SCVs displayed greater catalase activity than wild-type bacteria, which contributed to their resistance to H$_2$O$_2$. Taken together, these data reveal a mechanism by which *S. aureus* adapts to oxidative stress via the production of a subpopulation of H$_2$O$_2$-resistant SCVs with enhanced catalase production.

*Staphylococcus aureus* is a frequent cause of chronic and recurrent infections, which often involve the emergence of slow-growing mutants known as small-colony variants (SCVs) (1–14). The majority of SCVs isolated from clinical samples are auxotrophic for hemin, menadione, or thymidine due to mutations in the *hem* or *men* operons or in *thyA*, respectively (2, 4, 15–19). However, SCVs with mutations conferring resistance to fusidic acid or which arise via mutation in succinate dehydrogenase have also been identified, and there also appear to be isolates with a transient SCV phenotype, which are likely not mutants (12, 20, 21). SCVs with mutations in heme or menaquinone biosynthetic pathways have defective electron-transport chains, which confers resistance to aminoglycoside antibiotics such as gentamicin (1, 6, 8, 9).

Previous work has shown that gentamicin-resistant SCVs emerge in replicating populations in the absence of environmental stress via stochastic mutations but frequently revert to the wild type (WT) via the acquisition of suppressor mutations (15, 17, 22). However, while a few factors have been identified that select for the SCV phenotype, there is also evidence that environmental stimuli can trigger the emergence of SCVs in *S. aureus* populations, although the mechanism(s) by which this occurs is unknown (12, 23, 24).

In addition to aminoglycoside resistance, SCVs that arise via the loss of the electron transport chain are more tolerant than wild-type bacteria of other classes of bactericidal antibiotics (8, 9, 25–29). Furthermore, SCVs exhibit other phenotypic characteristics which may promote survival in host tissues, including elevated rates of host cell invasion and intracellular survival, enhanced capsule production, and robust biofilm formation (5, 12, 13, 30–32). Several of these phenotypes are ascribed to a combination of decreased Agr activity and enhanced SigB activity, which results in strong expression of surface proteins and an absence of cytolsin production (16, 33–35).

However, there is one aspect of the biology of electron-transport chain defective SCVs that appears to be at odds with a role in chronic infection: an apparently reduced level of defense against oxidative stress. This is important because the generation of reactive oxygen species (ROS) such as O$_2^-$ and H$_2$O$_2$ by neutrophils is a crucial host defense mechanism against *S. aureus* (36, 37). To combat ROS, *S. aureus* uses a number of defensive molecules, including catalase (KatA), superoxide dismutases (SodA/UM), and the golden pigment staphyloxanthin (36–44). Despite the importance of these defenses for wild-type *S. aureus* survival in the host, SCVs have been reported to produce significantly reduced levels of staphyloxanthin and heme auxotrophs are deficient in catalase, which would be expected to make them more susceptible to ROS generated by neutrophils and thus clearance from host tissues (9, 19, 28, 36, 45). Therefore, the aim of this work was to determine the effect of ROS on the emergence and persistence of electron-
transport chain defective SCVs within \textit{S. aureus} populations and establish the degree to which SCVs are sensitive to oxidative stress.

**MATERIALS AND METHODS**

Bacterial strains and culture conditions. Strains used in the present study are listed in Table 1. \textit{S. aureus} was cultured in tryptic soy broth at 37°C with shaking as described previously (22). Broth cultures were inoculated with bacteria from stationary phase (10^6 CFU ml^{-1}), followed immediately by antibiotics or ciprofloxacin and incubated for 16 h at 37°C with shaking at 180 rpm. Bacteria were used in stationary phase since this is when pigmentation of the wild type is greatest (36). Transposon mutants were cultured in the presence of erythromycin (10 \mu g ml^{-1}), but subsequent assays were performed in the absence of the antibiotic to reduce off-target effects. CFU counts were determined by serial dilution and plating of aliquots onto tryptic soy agar (TSA) or Columbia blood agar (CBA) with or without gentamicin (2 \mu g ml^{-1}). SCVs were defined as gentamicin-resistant (MIC > 2 \mu g ml^{-1}) bacteria that produced small, slow-growing, nonhemolytic or weakly hemolytic, and nonpigmented or weakly pigmented colonies on blood agar. We did not study other types of SCVs, such as those resistant to sulfonamides or fusidic acid. DNA from transposon mutants was transfected into wild-type SH1000 by transduction with phi1 as described previously, and transductants bearing the inserted transposon were selected for on TSA containing erythromycin (10 \mu g ml^{-1}) (46).

USA300-derived \textit{hemB} and \textit{menD} mutants (35) were complemented by cloning the appropriate gene, under the control of the native promoter of the relevant operon, into integrative plasmid pCL55 (47). For \textit{hemB}, the promoter region (48) was amplified by using the primer pair Hem For (CCCCTTCGCTTCACCAAGATATTTCACTTGACC) and Hem Rev (GGAGATCGCTTTTATTACCTTAAATTATCTAAATAGC), while \textit{menD}, the 331 bases upstream of \textit{menF}, the first gene in the men operon, were amplified by using the primer pair Men For (CCCCCTTCGTCCTTCAATGATACAAAAATCTTTTAAATC) and Men Rev (ACTCGGACTGCAATTTCTTATATTTCTGATATGGGACTTATATATCAC). Amplicons containing promoter regions were fused with coding sequences by using the Gibson assembly protocol (NEB). DNA overhangs were built into primers (indicated in boldface) to facilitate recombination. The \textit{hemB} gene was amplified by using the primer pair Hem For (GGAGGATGAGACAATGTAATGAAATGATGACATG) and Hem Rev (TACGGAGCTGCAATTTCAATATATTTCACTTGACC), while \textit{menD} was amplified by using the primer pair Men For (GGAGATCGCTTTTATATTTGGAGGAGATGACATG) and Men Rev (ACTCGGACTGCAATTTCTTATATTTCTGATATGGGACTTATATATCAC). The vector, pCL55, was amplified by using primers with overhangs to facilitate Gibson assembly. For \textit{hemB} constructs, pCL55 was amplified by using the primer pair pCL55 Hem For (AGATAATCAGGTGAATTTTACAGGAGCATATGTGACATG) and pCL55 Hem Rev (AATTCAATATATATATTTGAGGAGGAGCATATGTGACATG), for \textit{menD} constructs, the primer pair pCL55 Men For (CATGAGACATATTAGAATTTGGAGGAGCATATGTGACATG) and pCL55 Men Rev (AGAGCTTTTTTATATTTGGAGGAGCATATGTGACATG) was used.

The SH1000 \textit{umuC:Tn} mutant was complemented with the \textit{umuC} coding sequence (including the promoter region) using pCN34 (49). The \textit{umuC} gene and the promoter region was amplified by using the primer pair \textit{umuC} For (AAAGGATCCCGGTCGTTATATTTTCACTTGACC) and \textit{umuC} Rev (AAAGGATCCCGGTCGTTATATTTTCACTTGACC), which included BamHI restriction sites (underlined) to enable ligation into BamHI-digested pCN34. Vector without the \textit{umuC} coding sequence served as a control. The successful generation of constructs was confirmed by DNA sequencing. Vectors were constructed in \textit{Escherichia coli} strain DC10B and transformed directly into \textit{S. aureus} strains (50). In the case of pCL55, plasmid integration was confirmed by PCR. DC10B was cultured in LB broth containing ampicillin (100 \mu g ml^{-1}) where necessary to select for plasmid maintenance (50). \textit{S. aureus} strains containing plasmids were cultured in the presence of 10 \mu g of chloramphenicol ml^{-1} (pCL55) or 90 \mu g of kanamycin ml^{-1} (pCN34) and washed in phosphate-buffered saline (PBS) to remove antibiotics, and experiments were performed in the absence of antibiotics to avoid off-target effects.

**Hydrogen peroxide quantification.** The concentration of H$_2$O$_2$ in culture medium was determined by using a Pierce quantitative peroxide assay kit according to the manufacturer’s instructions.

**SCV stability assays.** The stability of SCV isolates was determined as described previously (22). SCV colonies (n = 30 to 50) on TSA plates containing 2 \mu g of gentamicin ml^{-1} were subcultured by streaking them onto antibiotic-free TSA using a sterile pipette tip, followed by incubation at 37°C for 48 h. Subsequently, subcultured bacteria were scored for reversion. If all colonies in the subcultured streak retained the SCV phenotype, then that SCV was scored as stable. If all of the colonies had the WT phenotype, the streak was scored as unstable. SCV’s that generated a mixture of SCV and WT phenotype were categorized as partially stable (22).

**Phenotype-switching assay.** To understand the relative contributions of phenotype-switching and replication to determining the size of the SCV population, we used a previously described assay (22). Briefly, inocula of 10^6 CFU tetracycline-sensitive wild-type SH1000 \textit{S. aureus} and 10 CFU SH1000t tetracycline-resistant SCVs were cultured in the absence of presence of antibiotics. The total CFU were quantified by growing serial dilutions on TSA plates. SCVs were isolated on TSA plates containing gentamicin, as described above. Subsequently, 100 SCV colonies were picked and patched onto TSA plates containing tetracycline to determine the percentage of SCVs that were resistant to the antibiotic. This assay determines the percentage of the final SCV population that arose from wild-type or SCV bacteria in the inoculum. Previous work has shown that the tetracycline-resistant strain does not suffer a fitness cost under the conditions used (22).

**SCV reversion assay.** Individual SCV colonies were picked from TSA plates containing gentamicin (2 \mu g ml^{-1}) and resuspended in 150 \mu l of PBS. Aliquots (50 \mu l) of each bacterial suspension were then spread over TSA plates containing pararaut (0.1 mM), ciprofloxacin (0.05 \mu g ml^{-1}), or neither before incubation for 24 h at 37°C. Subsequently, plates were examined for the presence of colonies of wild-type bacteria (large, pigmented colonies).

**Mutation rate analyses.** \textit{S. aureus} strains were cultured in 3 ml of TSB after inoculation from agar plates. Cultures were diluted to 10^6 CFU ml^{-1} in 30 parallel 1-ml cultures (this was the smallest inoculum that allowed bacterial growth in the presence of H$_2$O$_2$ and grown to stationary phase at 37°C with shaking. Total CFU counts were determined in 10 randomly selected cultures by plating of serial dilutions onto TSA without antibiotics. Each culture was then plated onto TSA containing rifampin (100 \mu g ml^{-1}), followed by incubation for 24 h at 37°C. The number of resistant colonies was counted, and mutation rates with confidence intervals were calculated by using the maximum-likelihood setting of the FALCOR mutation rate calculator (51, 52). The statistical significances of differences between the mutation rate in the absence and presence of H$_2$O$_2$ were determined by using a Student t test as described in equation 5 of FALCOR (51, 52).

**Hydrogen peroxide killing assays.** \textit{S. aureus} cells in late exponential phase (when pigmentation is strongest) were washed by sequential rounds of centrifugation and resuspension in PBS before subsequent adjustment to a final concentration of ~10^6 CFU ml^{-1} in PBS. Bacterial suspensions (10 \mu l) were added to the wells of a microtiter plate, and H$_2$O$_2$ was added to 30 mM for SH1000-derived isolates or 25 mM for USA300-derived strains (this concentration was chosen because preliminary assays indicated that they were the lowest required to achieve >1-log killing of the wild-type over 1 h [data not shown]). The microtiter plate was incubated at 37°C in the dark for 15 to 60 min. Surviving bacteria were enumerated by serial dilution in PBS and plating onto CBA (which naturally contains catalase to neutralize residual H$_2$O$_2$).

**Catalase activity assay.** \textit{S. aureus} was grown and washed as described above for hydrogen peroxide killing assays before 10^7 CFU were added to 1 ml of PBS containing 100 \mu M H$_2$O$_2$. The concentration of H$_2$O$_2$ was measured over time by using a Pierce quantitative peroxide assay kit in
TABLE 1  Bacterial strains used in this study

| Bacterial strain                        | Relevant characteristics                                                                 | Source or reference |
|----------------------------------------|-----------------------------------------------------------------------------------------|---------------------|
| **E. coli**                            |                                                                                         |                     |
| DC10B                                  | DNA cytosine methyltransferase deficient                                                 |                     |
| DC10B/pCN34                            | DC10B transformed with pCN34                                                             |                     |
| DC10B/pCL55                            | DC10B transformed with pCL55                                                             |                     |
| DC10B/phenB                            | DC10B transformed with pCL55 containing the promoter of the **hem** operon fused to the coding sequence of **hem** |                     |
| DC10B/pmenD                            | DC10B transformed with pCL55 containing the promoter of the **men** operon fused to the coding sequence of **men** |                     |
| DC10B/pumuC                            | DC10B transformed with pCN34 containing the promoter and coding region of **umuC**       |                     |
| **S. aureus**                          |                                                                                         |                     |
| SH1000                                 | Functional *rsbU* derivative of NCTC 8325-4                                            |                     |
| SCV2                                   | SH1000-derived Gm* SCV without auxotrophy for Men, Hem, Thy, CO₂ or fatty acids; isolated in the absence of oxidants | This study          |
| SCV4                                   | SH1000-derived Gm* SCV without auxotrophy for Men, Hem, Thy, CO₂ or fatty acids; isolated in the absence of oxidants |                     |
| SCV9                                   | SH1000-derived Gm* SCV with auxotrophy for Hem; isolated in the absence of oxidants    |                     |
| SCV13                                  | SH1000-derived Gm* SCV with auxotrophy for CO₂ isolated in the absence of oxidants     |                     |
| SCV14                                  | SH1000-derived Gm* SCV with auxotrophy for Men; isolated in the absence of oxidants; single nucleotide deletion in **menB**, leading to premature stop codon after 28 amino acids |                     |
| SCV15                                  | SH1000-derived Gm* SCV with auxotrophy for fatty acids; isolated in the absence of oxidants |                     |
| SCV17                                  | SH1000-derived Gm* SCV with auxotrophy for Men; isolated in the absence of oxidants; single nucleotide deletion in **menB**, leading to premature stop codon after 28 amino acids |                     |
| SCV20                                  | SH1000-derived Gm* SCV with auxotrophy for CO₂ isolated in the absence of oxidants     |                     |
| SCV21                                  | SH1000-derived Gm* SCV without auxotrophy for Men, Hem, Thy, CO₂, or fatty acids; isolated in the absence of oxidants |                     |
| SCV1036                                | SH1000-derived Gm* SCV with auxotrophy for Men; isolated in the absence of oxidants     |                     |
| SCV1045                                | Single nucleotide deletion in **menA**, leading to a premature stop codon after 184 amino acids |                     |
| SCV1047                                | SH1000-derived Gm* SCV with auxotrophy for Men; isolated in the absence of oxidants; single nucleotide polymorphism in **menF**, resulting in A367D substitution of a highly conserved alanine |                     |
| SCV1057                                | SH1000-derived Gm* SCV with auxotrophy for Men; isolated in the absence of oxidants; single nucleotide deletion in **menE**, leading to a premature stop codon after 205 amino acids |                     |
| SCV1058                                | SH1000-derived Gm* SCV with auxotrophy for Men; isolated in the absence of oxidants; single nucleotide deletion in **menB**, leading to a premature stop codon after 113 amino acids |                     |
| SCV1060                                | SH1000-derived Gm* SCV with auxotrophy for Men; isolated in the presence of H₂O₂; single nucleotide polymorphism in **menE**, leading to a premature stop codon after 165 amino acids |                     |
| SCV1072                                | SH1000-derived Gm* SCV with auxotrophy for Men; isolated in the presence of paraquat; single nucleotide polymorphism in **menE**, leading to premature stop codon after 373 amino acids |                     |
| SCV1072 katA::Tn                       | SCV1072 transduced with DNA from NE1366, resulting in inactivation of catalase; Ery⁺     |                     |
| SCV1077                                | SH1000-derived Gm* SCV with auxotrophy for Men; isolated in the presence of paraquat; single nucleotide polymorphism in **menF**, resulting in T79K substitution |                     |
| SCV1080                                | SH1000-derived Gm* SCV with auxotrophy for Men; isolated in the presence of paraquat; single nucleotide polymorphism in **aroB**, resulting in H241Q substitution of a highly conserved histidine likely involved in metal binding |                     |
| SH1000t                                | SH1000-derived Tc⁺ strain geh::pTM304                                                   |                     |
| MJH502                                 | SH1000 sigB::Tc                                                                         |                     |
| SH331                                  | SH1000 rexA::Tn                                                                         |                     |
| SH445                                  | SH1000 **umuC**:Tn                                                                       |                     |
| SH805                                  | SH1000 **rexA**:Tn                                                                       |                     |
| SH1102                                 | SH1000 **rexB**:Tn                                                                       |                     |
| SH1136                                 | SH1000 transduced with DNA from NE1366, resulting in inactivation of catalase; Ery⁺     |                     |
| SH1186                                 | SH1000 **dinB**:Tn                                                                       |                     |
| SH445/pCN34                            | SH1000 **umuC**:Tn transformed with pCN34                                              |                     |
| SH445/pumuC                            | SH1000 **umuC**:Tn transformed with pumuC                                               |                     |
| USA300 LAC                             | LAC strain of the USA300 CA-MRSA lineage                                               |                     |
| USA300 hemB                            | USA300 in which **hemB** has been deleted                                               |                     |
| USA300 hemB geh::pCL55                 | USA300 **hemB** mutant with pCL55 integrated into the geh locus                          |                     |
| USA300 hemB geh::phenB                 | USA300 **hemB** mutant with phenB integrated into the geh locus, restoring wild-type phenotype |                     |
| USA300 menD                            | USA300 in which **menD** has been deleted                                               |                     |
| USA300 menD geh::pCL55                 | USA300 **menD** mutant with pCL55 integrated into the geh locus                          |                     |
| USA300 menD geh::phenD                 | USA300 **menD** mutant with phenB integrated into the geh locus, restoring wild-type phenotype |                     |
| USA300 JE2                              | USA300 cured of plasmids                                                                |                     |

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TABLE 1 (Continued)

| Bacterial strain | Relevant characteristics | Source or reference |
|------------------|--------------------------|---------------------|
| NE351            | USA300 JE2 rexA::Tn      |                     |
| NE445            | USA300 JE2 ummC::Tn      |                     |
| NE805            | USA300 JE2 rexA::Tn      |                     |
| NE1012           | USA300 JE2 rexB::Tn      |                     |
| NE1366           | USA300 JE2 katA::Tn      |                     |
| NE1866           | USA300 JE2 dinB::Tn      |                     |
| CX003SCV         | Clinical Men-auxotroph SCV | This study        |
| CX003WT          | Revertant of CX003SCV with wild-type phenotype |                     |
| CX004SCV         | Clinical Men auxotroph SCV |                     |
| CX004WT          | Revertant of CX004SCV with wild-type phenotype |                     |
| CX005SCV         | Clinical Men auxotroph SCV |                     |
| CX005WT          | Revertant of CX005SCV with wild-type phenotype |                     |
| CX006SCVM        | Clinical Men auxotroph SCV |                     |
| CX006SCVH        | Clinical Hem auxotroph SCV |                     |
| CX006WT          | Revertant of CX006SCVM with wild-type phenotype |                     |
| CX009SCV         | Clinical Hem auxotroph SCV |                     |
| CX009WT          | Revertant of CX009SCV with wild-type phenotype |                     |
| Wood             | Wild-type                | NCTC 7121           |
| MRSA252          | Wild-type                | 86                  |

a Gm\textsuperscript{r}, gentamicin resistance; Tc\textsuperscript{r}, tetracycline resistance; Ery\textsuperscript{r}, erythromycin resistance; Hem, hemin; Men, menadione; Thy, thymidine.

accordance with the manufacturer’s instructions and the use of a standard plot.

Whole-genome sequencing. DNA was extracted from wild-type SH1000 and derived SCVs using lysostaphin and phenol-chloroform extraction (46). Purified DNA was sheared into fragments of ~150 bp and sequenced using an Illumina MiSeq DNA sequencer. The sequences obtained yielded >100-fold coverage.

RESULTS

Culture of *S. aureus* in the presence of hydrogen peroxide leads to a specific and dose-dependent increase in the size of the SCV subpopulation. To determine the effect of oxidative stress on the size of the gentamicin-resistant SCV subpopulation, *S. aureus* was cultured in the presence of increasing concentrations of H\textsubscript{2}O\textsubscript{2}, paraquat, or diamide, which have previously been shown to trigger distinct changes in the staphylococcal proteome (53).

We used an inoculum size (~10\textsuperscript{5} ml\textsuperscript{-1}) that was predicted to not contain SCVs due to their low frequency in the population. Therefore, SCVs that appeared in the cultures were generated by the acquisition of mutations in wild-type cells (22).

Increasing concentrations of H\textsubscript{2}O\textsubscript{2}, paraquat, or paraquat, but not diamide, led to dose-dependent increases in the size of the gentamicin-resistant SCV subpopulation, which was up to 50-fold greater than in the absence of oxidative stress (Fig. 1A, B, and C). Similar effects of H\textsubscript{2}O\textsubscript{2} (1 mM) and paraquat (5 mM) on the gentamicin-resistant SCV subpopulation were also observed for genetically diverse *S. aureus* strains USA300 LAC, Wood, and MRSA252 (Fig. 1D).

Paraquat generates superoxide radicals, which *S. aureus* can convert to H\textsubscript{2}O\textsubscript{2} via superoxide dismutases (43). To determine whether the effect of paraquat on SCV numbers was due to H\textsubscript{2}O\textsubscript{2} or superoxide production, *S. aureus* was cultured with paraquat in the presence or absence of purified bovine catalase (10 μg ml\textsuperscript{-1}). The presence of exogenous catalase abrogated the effect of paraquat on SCV subpopulation expansion, indicating that H\textsubscript{2}O\textsubscript{2} production, rather than superoxide, was responsible for the increased SCV frequency (Fig. 1E). However, it is possible that superoxide enhances H\textsubscript{2}O\textsubscript{2}-mediated damage by increasing free iron levels in the cell (54).

To investigate the nature of the recovered SCVs, representative colonies from independent cultures containing H\textsubscript{2}O\textsubscript{2} or paraquat (n = 6) were examined, and each was found to have a typical SCV phenotype, with reduced pigmentation, and were classified as menadione auxotrophs (Fig. 1F). Whole-genome sequencing of a selection of each of the independently isolated menadione-auxotrophs (two from TSB only, three from TSB plus H\textsubscript{2}O\textsubscript{2}, and three from TSB plus paraquat) revealed mutations in genes in the menaquinone biosynthetic pathway (*aroB* and *menABDEF*) (Table 1) (55). These mutations were similar to those reported previously in clinical isolates and confirm that the SCV phenotype was due to genetic changes rather than to epigenetic effects or the physiological response of the bacterium to oxidative stress (15, 17).

SCVs emerge during H\textsubscript{2}O\textsubscript{2}-induced bacteriostasis. To understand how H\textsubscript{2}O\textsubscript{2} modulates the size of the SCV subpopulation, we monitored the population dynamics of *S. aureus* during growth in the absence or presence of H\textsubscript{2}O\textsubscript{2}. As reported previously, wild-type *S. aureus* grew rapidly in the absence of H\textsubscript{2}O\textsubscript{2} and produced a small SCV subpopulation during the early exponential phase (Fig. 2A) (22). In contrast, there was no change in total CFU counts in the presence of 1 mM H\textsubscript{2}O\textsubscript{2}, resulting in an extended lag phase that lasted until the H\textsubscript{2}O\textsubscript{2} concentration was reduced to <400 μM (presumably due to the action of catalase and/or alkyl hydroperoxidase [40]). Once the H\textsubscript{2}O\textsubscript{2} concentration was reduced, *S. aureus* replication began at a similar rate to that seen in the absence of H\textsubscript{2}O\textsubscript{2} (Fig. 2A).

We hypothesized that the elevated rate of SCV emergence in the presence of H\textsubscript{2}O\textsubscript{2} was either due to switching of wild-type bacteria into SCVs or, despite the small inoculum size, the replication of a very few SCVs present in the inoculum. To test whether SCVs could replicate in the presence of H\textsubscript{2}O\textsubscript{2}, we inoculated broth containing 1 mM H\textsubscript{2}O\textsubscript{2} with a mixed population of phenotypically stable SCVs (including those auxotrophic for menadione, hemin, fatty acids, and those without identified auxotrophy) that
Hydrogen peroxide exposure leads to a specific, dose-dependent increase in the size of the SCV subpopulation. (A to C) *S. aureus* SH1000 was cultured in increasing concentrations of H$_2$O$_2$ (A), paraquat (B), or diamide (C) for 16 h, and the sizes of the total and SCV populations were determined. (D) The effect of H$_2$O$_2$ (1 mM) or paraquat (5 mM) on the size of the SCV subpopulations of strains USA300 LAC (LAC), Wood, and MRSA252 (252) were also determined. None of the oxidants used affected the size of the total population at the concentrations used (data not shown). (E) Wild-type *S. aureus* SH1000 was incubated in the absence (−) or presence (+) of paraquat and/or catalase, and the sizes of the total (open bars) and SCV populations (closed bars) were determined. (F) Colony morphology of wild-type *S. aureus* SH1000 (top left panel) and a representative menadione-auxotrophic SH1000-derived SCV (SCV1072) isolated from a culture containing paraquat (top right panel). The lack of pigment in the SCV seen on agar plates (top right panel) was also seen after liquid culture (bottom right panel). Culture of this SCV isolate in the presence of menadione restored pigmentation, indicating deficiencies in menaquinone production (bottom right panel). Values which are significantly different (*P < 0.05 [Student t test]) from oxidant-free conditions are indicated (*). These data represent the mean averages of 12 independent cultures. Error bars represent the standard deviations of the mean.

represents the composition of SCVs found in cultures not exposed to oxidants and then monitored growth. Similar to wild-type bacteria, the growth of the SCV population was inhibited by 1 mM H$_2$O$_2$, leading to an extended lag phase relative to SCV growth in the absence of H$_2$O$_2$ (Fig. 2B). However, as seen for the wild-type population, once the concentration of H$_2$O$_2$ fell to ~400 µM, SCV replication began (Fig. 2B). Because menadione-auxotrophic SCVs were the predominant SCV type isolated from cultures exposed to H$_2$O$_2$, we undertook a similar experiment to that described in Fig. 2B using a stable menadione auxotroph isolated from a culture exposed to paraquat (SCV1072). As for the wild-type and the mixed SCV inoculum, SCV1072 did not initiate replication until the H$_2$O$_2$ concentration had fallen to ~400 µM (Fig. 2C). Therefore, SCV replication is not a viable explanation for the appearance of SCVs at early time points in cultures exposed to H$_2$O$_2$, when the oxidant is at concentrations inhibitory to staphylococcal growth.

SCV emergence in the presence of H$_2$O$_2$ is dependent upon mutagenic DNA repair. Because mutations have been shown to occur in stressed, nonreplicating *E. coli* cells via DNA double-strand break repair and the SOS response, which is strongly induced in *S. aureus* upon exposure to H$_2$O$_2$, we hypothesized that this may provide a mechanism for the emergence of SCVs under growth-inhibitory conditions (56–80). To test this, we utilized the NARSA transposon library to identify genes that were important for mutagenic DNA repair in the USA300 background (81). Wild-type and transposon mutants deficient in genes associated with DNA repair and the SOS response, including recombination A (recA::Tn), error-prone polymerases IV or V (dinB::Tn, umuC::Tn), and recAB (functionally equivalent to recBCD in *E. coli*), were grown in the absence or presence of H$_2$O$_2$. Several mutants, including recA and recAB mutants, displayed increased sensitivity to H$_2$O$_2$, confirming a role in repair of damage caused by oxidative stress (data not shown). However, this increased sensitivity required a lower concentration of H$_2$O$_2$ (0.05 mM) to be used in these experiments compared to that of Fig. 1 and 2. Nonetheless, even at these reduced concentrations, H$_2$O$_2$ resulted in an increase in SCV frequency of ~10-fold in wild-type *S. aureus* populations (Fig. 3A). In contrast, H$_2$O$_2$ exposure had no effect on the size of the SCV subpopulations of the umuC::Tn, recA::Tn, recA::Tn, or recB::Tn mutants relative to cultures without oxidant (Fig. 3A), indicating that double-strand break repair and the SOS response is required for SCV emergence during H$_2$O$_2$ exposure but not in its absence. In contrast, the mutant lacking functional dinB (which is not part of the *S. aureus* SOS regulon [62]) had only a slight defect in H$_2$O$_2$-induced SCV formation (Fig. 3A).

These findings were concordant with measurements of the mutation rate in *S. aureus* grown with or without H$_2$O$_2$, which showed that H$_2$O$_2$ exposure increased the mutation rate >5-fold in wild-type *S. aureus* but had no effect on the mutation rate of strains
defective for rexAB or polymerase V (Fig. 3B). There was a modest (<3-fold) increase in the mutation rate of the recA::Tn mutant, but this was still significantly reduced compared to the wild-type (Fig. 3B). In contrast, there was no decrease in H₂O₂-induced mutation in S. aureus lacking polymerase IV (dinB) (Fig. 3B).

To ensure that these findings also applied to the SH1000 genetic background, DNA from recA::Tn, rexA::Tn, rexB::Tn, dinB::Tn, and umuC::Tn was transduced into SH1000. Each of the DNA repair mutants behaved in a very similar manner to that described above for the USA300 mutants. Specifically, the mutants were defective for H₂O₂-induced SCV formation or mutation, with the exception of dinB::Tn (Fig. 3C and D). Complementation of the umuC coding sequence, under the control of the native promoter, to the umuC::Tn mutant restored H₂O₂-induced mutation and SCV formation, while the umuC::Tn mutant transformed with vector alone was defective for H₂O₂-induced mutation and SCV formation (Fig. 3C and D).

In E. coli, stress-induced mutation requires both the SOS regulon.
possibility used in Fig. 1 due to the increased sensitivity of the alternative sigma factor SigB (64, 65). However, the absence of SigB did not prevent an SCV transition to SCV phenotype via mutagenic DNA repair, which explains the emergence of SCVs in the presence of H2O2 or paraquat at any concentration shown previously that SCV replication is associated with a high frequency in the inoculum. Taken together, these data demonstrate that components of the SOS response trigger switching from the wild-type to SCV phenotype via mutagenic DNA repair, which explains the emergence of SCVs in the presence of H2O2.

H2O2 selects for phenotypically stable SCVs, enhancing population expansion via replication. The data presented in Fig. 2A demonstrate that the SCV subpopulation emerged in the presence of H2O2 and continued to expand after the concentration of the oxidant fell below the growth-inhibitory concentration. However, it was not clear whether SCV population expansion at growth-permissive concentrations of H2O2 was predominantly due to the replication of a few SCVs generated by mutagenic DNA repair or was due to a very high rate of phenotype-switching from the wild-type to the SCV phenotype.

To investigate this, culture medium with or without oxidants was inoculated with ~10^5 CFU tetracycline-sensitive (Tet+) wild-type S. aureus ml^-1 and ~10 CFU SCVs from tetracycline-resistant SH1000 (Tet-) ml^-1 and grown for 24 h. It should be noted that the mixed SCV subpopulation arose in cultures that had not been exposed to oxidants.

As expected, the size of the SCV subpopulation in cultures containing oxidants was greater than those without oxidants (Fig. 4A). In the absence of oxidative stress, the percentage of SCVs that were tetracycline resistant fell from 100% in the inoculum to ca. 20% in the mature culture, indicating that 80% of the final SCV population had arisen via phenotype switching from the tetramerine-sensitive wild-type population (Fig. 3F). Therefore, SigB does not appear to be required for oxidative-stress-induced mutation in S. aureus. It should be noted, however, that we were unable to test higher concentrations of paraquat or H2O2 at any concentration used in Fig. 1 due to the increased sensitivity of the sigB mutant to oxidative stress (66). Taken together, these data demonstrate that components of the SOS response trigger switching from the wild-type to SCV phenotype via mutagenic DNA repair, which explains the emergence of SCVs in the presence of H2O2.
found that SCVs that arose in cultures exposed to H₂O₂ or paraquat were significantly more stable than those that arose in broth only (Fig. 4C, D, and E). We then generated a pool of SCVs that had arisen spontaneously in cultures not exposed to oxidants and then grew them in the absence or presence of H₂O₂. Oxidative stress resulted in significantly increased SCV stability, demonstrating that H₂O₂ selects for SCV stability regardless of whether SCVs arose via the SOS response or spontaneously (Fig. 4F and G). Therefore, the exposure of SCVs to oxidants results in enhanced stability, which reduces reversion to the wild-type and thus enables SCV population expansion via replication.

**Nonoxidative SOS induction promotes SCV reversion to the wild type.** The data presented in Fig. 4 strongly suggest that H₂O₂ selects for phenotypically stable SCVs, which was surprising because activation of the SOS response would be expected to increase the frequency of suppressor mutations which promote SCV reversion to the wild-type phenotype. This suggests that the selective pressure exerted by oxidants on SCVs is great enough to overcome the increased mutation rate caused by induction of the SOS response.

However, we considered two alternative explanations for the enhanced stability of SCVs exposed to oxidative stress: that SCVs generated by the SOS response are inherently more stable than those that arise spontaneously or that the SOS response cannot trigger reversion of SCVs to wild-type bacteria.

To test these possibilities, we used the antibiotic ciprofloxacin, which induces a very similar DNA damage repair to that described upon H₂O₂ exposure (56, 62). Exposure of wild-type but not *umuC*::Tn mutant bacteria to a subinhibitory concentration of ciprofloxacin led to an increase in SCV frequency, confirming that induction of the SOS response promotes SCV emergence via mutagenic DNA repair (Fig. 5A). However, the SCVs triggered by ciprofloxacin were no more stable than those which emerged in the absence of the antibiotic (Fig. 4C and 5B). This demonstrates that SCVs generated via the SOS response are not inherently more stable than those that arise spontaneously during bacterial replication. We then tested whether the SOS response can promote SCV reversion to the wild type by exposing a panel of SCVs with various levels of stability to ciprofloxacin or paraquat. In five of the seven SCVs examined, ciprofloxacin exposure promoted the frequency of reversions, indicating that SOS induction can indeed promote SCV reversion to the wild-type phenotype. This suggests that the selective pressure exerted by oxidants on SCVs is great enough to overcome the increased mutation rate caused by induction of the SOS response.

**SCVs are less susceptible to H₂O₂ than parental strains.** Previous work has suggested that SCVs should be more susceptible to H₂O₂ than wild-type bacteria due to the lack of staphyloxanthin pigment and reduced catalase activity in heme auxotrophs (19, 36, 37, 45). However, since H₂O₂ selected for the SCV phenotype we considered the possibility that SCVs are in fact less sensitive to oxidative stress than wild-type bacteria. To test this, the survival of wild-type SH1000 and a *umuC* mutant were grown in the absence (CTL) or presence (C) of a subinhibitory concentration of ciprofloxacin for 16 h, and the frequency of gentamicin-resistant SCVs was determined. The data represent the mean average of 10 independent cultures, and error bars represent the standard deviations. Values which differ from those found in media lacking ciprofloxacin are highlighted (*). (B) Stability of SCV isolates that arose in wild-type SH1000 populations in the presence of ciprofloxacin (n = 6). The data are presented and analyzed as described in the legend to Fig. 4. (C) The frequency of SCV reversion to the wild type was determined in the absence (CTL) or presence of ciprofloxacin (Cipro) or paraquat (Para). Reversion rates that differ from those found in media without supplements are highlighted (*). (D) Stability of SCVs that arose in the *umuC* background in the absence of oxidants or ciprofloxacin (n = 8).

**FIG 5** Ciprofloxacin promotes bidirectional phenotype-switching. (A) Wild-type SH1000 and a *umuC* mutant were grown in the absence (CTL) or presence (C) of a subinhibitory concentration of ciprofloxacin for 16 h, and the frequency of gentamicin-resistant SCVs was determined. The data represent the mean average of 10 independent cultures, and error bars represent the standard deviations. Values which differ from those found in media lacking ciprofloxacin are highlighted (*). (B) Stability of SCV isolates that arose in wild-type SH1000 populations in the presence of ciprofloxacin (n = 6). The data are presented and analyzed as described in the legend to Fig. 4. (C) The frequency of SCV reversion to the wild type was determined in the absence (CTL) or presence of ciprofloxacin (Cipro) or paraquat (Para). Reversion rates that differ from those found in media without supplements are highlighted (*). (D) Stability of SCVs that arose in the *umuC* background in the absence of oxidants or ciprofloxacin (n = 8).
We also considered the possibility that gentamicin-resistant SCVs may consistently accumulate mutations which decrease susceptibility to \( \text{H}_2\text{O}_2 \). To test this, menadione-auxotrophic SCV isolates were cultured in the absence or presence of menadione, and their susceptibility to \( \text{H}_2\text{O}_2 \) killing was determined. Culture of menadione-auxotrophic SCVs in the presence of menadione produced bacteria that were as sensitive as the wild-type parental strain to \( \text{H}_2\text{O}_2 \), indicating that secondary mutations are not responsible for the elevated \( \text{H}_2\text{O}_2 \) resistance (Fig. 6D). It should be noted that menadione has been used as a redox cycling agent to generate superoxide within bacteria. However, the concentrations typically used in such studies are \(~1,000\)-fold greater than those used here, and there was no significant effect on the viability of the wild-type bacteria in the presence of \( \text{H}_2\text{O}_2 \) (2, 67). To further test whether reduced susceptibility to \( \text{H}_2\text{O}_2 \) was solely due to defects in the electron transport chain, \( \text{S. aureus} \) wild type was cultured in the presence of the \( \text{Pseudomonas} \) exoproduct HQNO, which blocks the electron transport chain of Gram-positive bacteria and confers an SCV phenotype upon \( \text{S. aureus} \) (68). Culture of \( \text{S. aureus} \) in the presence of HQNO produced bacteria that were resistant to \( \text{H}_2\text{O}_2 \) (Fig. 6E). However, the presence of HQNO alone did not alter \( \text{H}_2\text{O}_2 \) resistance of \( \text{S. aureus} \) which had been cultured in the absence of the exoproduct (Fig. 6E). Therefore, simply blocking the electron transport chain is not protective against \( \text{H}_2\text{O}_2 \). Rather, resistance is most likely due to the physiological adaptation of \( \text{S. aureus} \) to loss of the electron transport chain.

To ensure that these findings were of clinical relevance, we assessed the survival of \( \text{hemB} \) and \( \text{menD} \) deletion mutants constructed in the USA300 community-associated MRSA strain. In keeping with the data for SH1000, the survival of wild-type USA300 in the presence of \( \text{H}_2\text{O}_2 \) was significantly lower than that of isogenic \( \text{hemB} \) or \( \text{menD} \) mutants (Fig. 6F). Complementation of either mutant with the relevant coding sequence restored the wild-type phenotype (Fig. 6F).
type growth phenotype (data not shown) and resulted in decreased survival in \( \text{H}_2\text{O}_2 \), whereas vector alone did not affect growth or survival (Fig. 6G). Finally, increased \( \text{H}_2\text{O}_2 \) resistance was demonstrated in a clinical menadione-auxotrophic SCV isolate (CX003SCV), relative to a wild-type revertant (CX003WT) (Fig. 6H). Therefore, resistance to \( \text{H}_2\text{O}_2 \) is an inherent property of electron-transport chain-deficient SCVs that very likely contributes to their ability to persist within host tissues during chronic infections.

**Elevated catalase activity in SCVs partially explains enhanced \( \text{H}_2\text{O}_2 \) resistance.** In addition to staphyloxanthin, catalase is a major staphylococcal defense against \( \text{H}_2\text{O}_2 \), and has been shown to be expressed at higher levels in clinical SCVs than wild-type \( S. \text{aureus} \) (33, 40–42). However, heme-auxotrophic SCVs cannot generate functional catalase and SCVs isolated from the lungs of patients with cystic fibrosis have been reported to have reduced catalase activity (5, 45).

To resolve the question of catalase activity in electron-transport chain-deficient SCVs, we measured the ability of wild-type \( S. \text{aureus} \) SH1000, menadione-auxotrophic SCV isolate SCV1072 and heme-auxotrophic SCV9 to degrade \( \text{H}_2\text{O}_2 \). This revealed significantly elevated catalase activity in the menadione-auxotrophic SCV relative to the wild type, while the catalase activity of the heme-auxotrophic SCV was significantly impaired relative to wild type (Fig. 7A). Similarly, a clinical menadione-auxotrophic SCV isolate had significantly higher catalase activity than a revertant isolate with the wild-type phenotype (Fig. 7B). In keeping with these data, analyses of an additional four clinical SCV isolates and matching revertants revealed that menadione-auxotrophic SCVs degraded significantly more \( \text{H}_2\text{O}_2 \) than revertants, whereas heme auxotrophs exhibited defective catalase activity (Fig. 7C).

Next, we examined catalase activity in a panel of gentamicin-resistant SCVs with diverse auxotrophies or no identified auxotrophy isolated from broth cultures in the absence of oxidants. In every case, the catalase activity of the SCV was greater than that of the wild type, although significant variation was observed between isolates (Fig. 7D). Finally, to demonstrate that selection for SCVs does not itself select for elevated catalase activity, we measured catalase in isogenic menD and hemB mutants and the wild-type USA300 parent strain. Consistent with the previous data, this revealed that the menD deletion mutant had significantly higher levels of catalase activity than wild-type USA300, while the hemB deletion mutant was unable to degrade \( \text{H}_2\text{O}_2 \) (Fig. 7E) (45). Complementation of the hemB and menD mutants with the relevant wild-type coding sequence restored catalase activity to wild-type levels (Fig. 7F).
To confirm that the degradation of H2O2 was due to catalase, rather than alkyl-hydroperoxidase or other peroxidases, we transduced SH1000 WT and SCV1072 with DNA from a USA300 katA::Tn mutant (40). Both strains were completely devoid of catalase activity, confirming the role of catalase (KatA) in the H2O2 breakdown (Fig. 7G).

Next, we sought to determine whether catalase activity explained the enhanced resistance of menadione-auxotrophic SCVs to H2O2 (Fig. 6A and B). Wild-type SH1000, SCV1072, SH1000 katA::Tn, and SCV1072 katA::Tn were each exposed to 30 mM H2O2 for 1 h, and the survival was determined. Strains deficient in catalase showed increased sensitivity to H2O2 (Fig. 7H). However, SCV1072 katA::Tn was not as sensitive to H2O2 as SH1000 katA::Tn, indicating that elevated catalase activity only partially explains the resistance of menadione-auxotrophic SCVs to H2O2 (Fig. 7H).

Taken together, these data indicate that enhanced catalase activity is common to most electron transport chain-deficient SCVs, with the exception of those that cannot synthesize heme. However, additional factors beyond catalase contribute to the resistance of SCVs to H2O2, particularly in heme auxotrophs.

**DISCUSSION**

*S. aureus* is responsible for a raft of chronic and recurrent infections despite triggering a potent immune response and antibiotic therapy (69, 70). During the course of infection, *S. aureus* frequently acquires mutations which promote survival in host tissues, including those that confer a small-colony variant phenotype. The data presented in this report reveal that these mutations increase in frequency in response to one of the major ROS produced by neutrophils, H2O2, via the SOS response. These data support previous work showing that increases in the mutation rate following DNA damage are due to the action of specific repair machinery, rather than the DNA damage itself (57–60).

The ability of bacteria to transiently increase mutation rates in response to environmental stress increases the probability of beneficial (adaptive) mutations that enhance survival (58, 71). Certainly, the emergence of electron transport chain-deficient SCVs in response to oxidative stress appears to be beneficial to *S. aureus* due to their resistance to oxidative stress and enhanced catalase production, which may enhance survival and/or replication of wild-type bacteria via detoxification of H2O2 (Fig. 6 and 7). In addition, SCVs have a number of other phenotypic properties which might promote persistence in host tissues, including intracellular survival, strong biofilm formation, and a high degree of antibiotic tolerance (5, 12, 13, 30–32). Therefore, a single inactivating mutation in the menaquinone biosynthetic pathway has a profound effect on the phenotype of *S. aureus*, changing it from a fast-growing, toxin-producing pathogen to a much less pathogenic and slow-growing variant that is able to persist within host tissues for extended periods. However, the close correlation between mutation rate (as determined by mutations at the rpoB locus) and SCV emergence indicates that the *men* operon is probably not a mutation hot spot, at least with respect to H2O2-associated mutations.

In *E. coli*, stress-induced mutation involves the low-fidelity polymerases IV and V. Although the ability of a bacterium to increase the mutation rate is a beneficial tool, the principal function of these polymerases is the replication of damaged DNA in a process known as “trans-lesion synthesis,” the low-fidelity nature of the polymerase enabling it to bypass DNA lesions at the cost of a high-frequency of base pair mismatches (72). However, it is not clear whether the increased mutation rate associated with polymerases IV and V is simply a consequence of DNA repair or part of a coevolved mechanism to promote the mutation rate during times of DNA-damaging stress and thus increase the likelihood of beneficial mutations arising.

In *S. aureus*, trans-lesion synthesis appears to make a small contribution to *S. aureus* resistance to oxidative stress since the *umuC::Tn* mutant lacking polymerase V (but not *dinB/polymerase IV*) was slightly more sensitive to H2O2 than the wild type (data not shown). These data fit with previous work which shows that the expression of *umuC*, but not *dinB*, is increased in response to H2O2 (56). Therefore, in *S. aureus*, the expression of polymerase V appears to facilitate efficient repair of H2O2-mediated DNA damage. However, this does not rule out the possibility that polymerase V is part of a coevolved mechanism to increase the mutation rate in response to environmental stress. For example, *umuC* is one of the most strongly expressed genes in response to various genotoxic stresses, and this may result in greater polymerase V production than is strictly necessary to repair the damaged DNA (56, 62, 73).

Although H2O2 exposure led to large increases in SCV frequency, this was not solely due to an elevated mutation rate but also to the subsequent replication of emergent SCVs. H2O2 selected for the SCV phenotype, which may reflect the enhanced resistance of SCVs to H2O2 coupled with enhanced catalase production. Therefore, with the possible exception of heme auxotrophs, which lack catalase activity, gentamicin-resistant SCVs appear to be well equipped to persist in environments with a high burden of ROS. This correlates with the clinical evidence that SCVs are able to persist in host tissues, resisting clearance by immune cells that expose the pathogen to the oxidative burst (1–14, 36, 37).

The discovery of enhanced catalase activity in non-heme-auxotrophic SCVs is in keeping with a transcriptomic study of clinical SCV isolates, which reported enhanced *katA* expression (33). Also in keeping with previous work, proteomics analysis of clinical and *in vitro* selected heme-auxotrophic SCVS revealed reduced catalase than in corresponding wild-type bacteria (74). Therefore, it appears that loss of the electron transport chain results in enhanced expression of *katA*, leading to elevated catalase activity, except where heme biosynthesis is defective (33, 74). The reason why catalase activity is elevated in menadione auxotrophic SCVs is under investigation but may reflect the significantly altered metabolic profile of these mutants, which results in altered production of virulence factors and defense molecules such as staphyloxanthin (discussed below) (8, 9, 14, 19, 28, 30, 36, 45). It could, therefore, be hypothesized that enhanced catalase activity is a compensatory mechanism for the loss of staphyloxanthin, but this remains to be tested.

Although there appear to be a number of different pathways by which electron transport chain-deficient SCVs can arise (resulting in diverse auxotrophies), cultures exposed to H2O2 consistently generated menadione auxotrophs. Since menadione auxotrophs were no more resistant to H2O2 and produced similar levels of catalase to other SCVs (with the exception of heme auxotrophs), this is most likely explained by the increased likelihood of this variant arising relative to others. Specifically, menadione-auxotrophic SCVs can arise via inactivating mutations anywhere in the menaquinone biosynthetic pathway, whereas other types of SCV
might only arise via mutations in much smaller loci. In support of this hypothesis, in cultures not exposed to H$_2$O$_2$, menadione-auxotrophic SCVs were the most abundant (40%), followed by heme auxotrophs (35%). Therefore, it appears that H$_2$O$_2$ selects for catalase producing SCVs, of which menadione auxotrophs are the most abundant, over the catalase-deficient heme auxotroph.

While SCVs are resistant to oxidative stress and have many phenotypic properties which promote survival in host tissues, these come at the cost of slow growth and loss of exotoxin production (2, 4, 6, 9, 13, 35). Therefore, \emph{S. aureus} populations must provide a balance between fast-growing, toxin-producing wild-type bacteria which are essential for the establishment of infection and slow-growing non-toxin-producing SCVs, which are able to resist threats such as oxidative stress or antibiotics. Indeed, such a strategy parallels the formation of antibiotic tolerant persister cells (75, 76). Balaban et al. showed that persister cells arise stochastically during growth (type I) and that the frequency increases in response to specific environmental stresses such as subinhibitory concentrations of antibiotics (type II) (75, 77). The production of persister cells prior to antibiotic exposure is hypothesized to be a bet-hedging strategy to ensure the population against exposure to lethal concentrations of antimicrobials that would otherwise eradicate the entire population (75–79).

Although SCVs arise via mutation and persister cells via changes in the physiological state of cells, both events are stochastic in nature and the frequency of these events is influenced by genetic factors and there are, therefore, clear parallels in their emergence within populations (77). We have previously shown that SCVs emerge constitutively in replicating \emph{S. aureus} cultures (type I) and in this report demonstrate that a specific environmental stress enhances SCV emergence and population size via the action of specific gene products (type II) (22). Therefore, we hypothesize that SCVs comprise a bet-hedging strategy against lethal oxidative and antibiotic stress in a similar way to persisters ensuring populations against bactericidal antibiotics. A key part of such an assurance policy is the ability to restore the population of wild-type bacteria, which SCVs can do via the repair of mutations or acquisition of suppressor mutations that restore the function of mutated gene products (15, 17). In addition, activation of the SOS mutagenic repair pathway via subinhibitory ciprofloxacin (but not oxidative stress) can promote SCV reversion to the wild type.

The high resistance of SCVs to concentrations of H$_2$O$_2$ that are lethal to the wild type was a surprising finding given the reduced pigmentation (and catalase levels in the hemin auxotroph). The SCVs that arose under oxidative stress were gentamicin resistant and consistently auxotrophic for menadione, indicating loss of menaquinone biosynthesis and thus interruption of the electron transport chain (2, 9).

The ability of electron transport chain-deficient bacteria to resist H$_2$O$_2$ is in apparent contrast to previous work which showed that blockage of the electron transport chain of \emph{E. coli} using KCN, or disruption of the \emph{menA} gene, resulted in increased susceptibility to H$_2$O$_2$ (80). Loss of the electron transport chain in \emph{E. coli} led to a significant increase in reducing power inside the cell, which propagates the highly damaging Fenton reaction by reducing iron (80). Our experiments with HQNO demonstrate that SCV resistance to H$_2$O$_2$ is not simply a function of a defective electron transport chain. Rather, it is only when \emph{S. aureus} has been cultured in the absence of a functional electron transport chain that it is able to survive subsequent H$_2$O$_2$ challenge. Although this is partially due to catalase activity, additional factors promote the resistance of electron transport chain-deficient \emph{S. aureus} to H$_2$O$_2$. For example, \emph{S. aureus} can avoid redox stress during loss of the electron transport chain by switching to fermentative metabolism via the redox-regulatory element Rex (81–83). Metabolic and transcriptomic analyses of SCVs reveal a huge increase in lactate and alcohol dehydrogenase activity, and this maintains redox balance in the cell, preventing an accumulation of reducing power (14, 30, 83). Furthermore, it is possible that fermentative metabolism renders SCVs more resistant to H$_2$O$_2$ killing by reducing the need for iron-containing metabolic enzymes in the cytoplasm, as well as cytochromes. In support of this hypothesis, wild-type \emph{S. aureus} exposed to H$_2$O$_2$ increase expression of genes associated with fermentation and a \emph{Staphylococcus epidermidis} mutant lacking a functional TCA cycle displayed elevated resistance to H$_2$O$_2$ killing (54, 55, 84).

Taken together, the data presented here reveal an additional strategy by which \emph{S. aureus} can promote its survival under conditions of oxidative stress via the production of small-colony variants in response to H$_2$O$_2$ exposure. In addition to ensuring the population against potentially lethal oxidative stress, elevated SCV production is likely to promote persistent infection via reduced susceptibility to antibiotic therapy, increased biofilm formation, and enhanced intracellular persistence.

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