**Small-Colony Variants of *Staphylococcus aureus* Persist in the Presence of Sub-lethal Doses of Hydrogen Peroxide**

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Small-colony variants (SCVs) of *Staphylococcus aureus* are notorious for tolerating therapeutic doses of antimicrobials and persisting within host cells. However, the determinants of SCV formation are poorly understood. Understanding how *S. aureus* SCVs develop and endure is crucial, as *S. aureus* infections are responsible for more deaths in the United States each year than the annual number of deaths in the U.S. due to complications of AIDS. *S. aureus* strains exposed to sub-lethal concentrations of hydrogen peroxide (H2O2), a major component of immunity, exhibited a concentration-specific increase in the frequency of SCVs exhibiting gentamycin resistance. Pathways associated with the SOS response, including utilization of DNA double-strand break repair proteins recombination exonuclease A and B (RexAB), recombinase A (RecA), and polymerase V (Pol V), mediated the survival of bacteria in the forms of SCVs. Exposure to hydrogen peroxideselected for the SCV phenotype, increased stability of the phenotype, and reduced the rate of reversion from SCV to wild-type colonies. SCVs also displayed increased catalase production compared to wild-type bacteria. As a whole, these findings clarify a potential mechanism for development and persistence of SCVs in human tissues. Improved understanding of SCVs will contribute to resolving these infections.

**Introduction**

*Staphylococcus aureus* is a major public health threat in the United States. Hospital and community acquired infections caused over 80,000 severe invasive infections in 2011 and more deaths than AIDS in the same year1. Agriculturally, *S. aureus* infections are a leading cause of bovine mastitis, which can severely reduce milk production by dairy cattle2. *S. aureus* strains are notorious for developing antimicrobial resistance or acquiring genes that confer resistance3. An example of this is the gene *mecA*, which encodes an alternative penicillin-binding protein (PBP 2a), and results in the methicillin-resistant *S. aureus* (MRSA) phenotype4.

Chronic and recurring infections are characterized by the presence of small colony variants (SCVs). SCVs grow at a reduced rate compared to wild-type strains and are typically auxotrophic for hemin, menadione, or thymidine due to mutations in their respective operons or genes, meaning they do not grow properly without supplementation of the specific compound5. SCVs that result from mutations in the heme or menaquinone biosynthetic pathways have dysfunctional electron-transport chains and are therefore resistant to aminoglycoside antibiotics such as gentamycin and frequently demonstrate resistance to other classes of antimicrobials5. Occasionally, gentamycin-resistant SCVs arise spontaneously and randomly but are unstable and often revert to the wild type5. In addition to the multi-antibiotic resistance phenotype, SCVs often have enhanced virulence factors such as elevated rates of host cell invasion, ability to survive in the host cell, production of a more robust capsule, and formation of particularly stubborn biofilms5. *S. aureus* SCVs are well-rounded pathogens with enhanced invasion, survival, and resistance abilities.

The puzzle of SCV persistence lies in a fundamental quality of these bacteria. The immune system attacks pathogens using reactive oxygen species such as superoxide (O2-) and hydrogen peroxide (H2O2),which are produced by neutrophils. Hydrogen peroxide destroys bacteria primarily by inducing DNA damage5. *S. aureus* defends itself from neutrophil attack by using superoxide dismutase to neutralize superoxide, utilizing catalase to convert hydrogen peroxide to water, and by incorporating the carotenoid staphyloxanthin into the cell membrane5. However, *S. aureus* SCVs produced reduced levels of staphyloxanthin and heme auxotroph SCVs produce less catalase than the wild type5. An important question concerning the nature of these microbes is how reactive oxygen species impact the development of SCVs without eradicating the bacteria.

**Recent Progress**

Culturing *S. aureus* in the presence of hydrogen peroxide resulted in a dose-dependent increase in the size of gentamicin-resistant SCVs. The change was up to 50 fold greater in the presence of hydrogen peroxide compared to *S. aureus* cultured without oxidants5. This effect was observed in both methicillin-susceptible and methicillin-resistant *S. aureus* strains. Small colony variants also develop in the presence of oxidants when the bacteria are not growing. The *S. aureus* population remained in lag phase until the hydrogen peroxide concentration fell below 400 μM5. The bacteria then replicated at a rate similar to that of *S. aureus* in the absence of oxidant.

The SOS response is stimulated in *S. aureus* exposed to hydrogen peroxide. Mutations have been observed in stressed *Escherichia coli* cells during the SOS response as a result of DNA double-strand break repair. The authors hypothesized the SOS response in *S. aureus* may prompt conversion to the SCV phenotype4. Wild type strains were cultured and compared to transposon mutants deficient in genes associated with DNA repair and the SOS response, including recombinase A (*recA)*, DNA polymerases IV and V which are error prone (*dinB* and *umuC*), and another recombinase gene (*rexAB*). The mutants lacking *recA* and *rexAB* demonstrated increased sensitivity to hydrogen peroxide implicating both genes in the tolerance of oxidative stress. These conditions lead to a 10-fold increase in the incidence of SCVs5. Mutants lacking *umuC* also demonstrated a reduced rate of SCV development, but when the mutant was complemented with a functional *umuC* gene the SCV rate was restored5.

SCVs that developed in the presence of hydrogen peroxide demonstrated reduced reversion rates compared to strains lacking the selective pressure of hydrogen peroxide. SCVs that developed spontaneously and were then exposed to hydrogen peroxide also demonstrated increased stability. Heme auxotrophs produced reduced amounts of catalase to defend against hydrogen peroxide damage, but auxotrophic SCVs produced more catalase than wild-type strains5.

**Discussion**

Mutations in *S. aureus* occur at an increased rate in response to hydrogen peroxide, which is produced by neutrophils and is a major component of immunity. The SOS response, ensuing mutations, and repair machinery increased the mutation rate to increase the odds of bacteria developing beneficial mutations and surviving stress. The resistance to oxidative stress and increased catalase production allows SCVs to neutralize hydrogen peroxide and enhances survival. Other qualities, such as persistence in host tissues, survival within host cells, formation of biofilms, and antibiotic tolerance also contribute to SCV virulence.

The mutant *S. aureus* strain engineered to lack *umuC* demonstrated increased sensitivity to hydrogen peroxide than the wild type, indicating the enzyme it encodes, DNA polymerase V, facilitates repair of DNA damage caused by hydrogen peroxide. Strains demonstrating reduced staphyloxanthin production secreted increased amounts of catalase in what could be a compensatory defensive mechanism. The most frequently observed SCV type was a menadione auxotroph that produced catalase; the catalase deficient heme auxotroph was observed less often, suggesting hydrogen peroxide selects for catalase producing SCVs.

SCVs demonstrate resistance to many classes of antibiotics, especially aminoglycosides. Inducing reversion from SCV to the wild-type phenotype is a possible treatment option for eradicating infections. Sub-inhibitory ciprofloxacin can prompt reversion to the wild type, at which point the infection could be attacked with another antibiotic.

Future research is necessary to explore the mechanism of SCV formation, especially how this relates to preventing SCVs from occurring and developing resistance to antimicrobial substances.

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