# Ethanol-Selected *Staphylococcus aureus* Small Colony Variants

**Whitney E. Peterson**, Nathanial J. Torres, John E. Gustafson

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**Abstract**

*Staphylococcus aureus* has been shown to have the ability to display a small colony variant (SCV) phenotype, which is associated with persistent infections caused by acquisition of various degrees of resistance from antimicrobial therapy. *S. aureus* is targeted for death via antiseptic techniques, including the use of alcohols; little is known about the effects of these alcohols on the selective fitness of the species. This study displays the selection of ethanol-reduced susceptibility (ERS) SCV mutants with inhibitory concentrations of ethanol. Additionally, these mutants showed changes in susceptibility to various antibiotics and antimicrobials.

**Introduction**

*Staphylococcus aureus* is a gram-positive pathogen and a leading cause of hospital-acquired infections (1). Methicillin-resistant *S. aureus* (MRSA) are of particular concern, since these organisms typically display a multiple-antibiotic resistance phenotype and infections caused by these organisms therefore are difficult to treat (2, 3). *S. aureus* can also transition into the small colony variant (SCV) phenotype, which provides this organism an additional degree of protection from antimicrobials. In addition, SCVs are associated with persistent and reoccurring infections (8).

The use of alcohol-based hand rubs (ABHR) can decrease the transmission of *S. aureus* among individuals within healthcare settings (4). Alcohol functions as an antiseptic by denaturing bacterial membrane and protein structures (5, 6). Previously our laboratory published on the ethanol-induced stress response for *S. aureus*. In this study, MRSA strain LP9, isolated from a patient's urine in El Paso, Texas, and hetero-vancomycin-intermediate *S. aureus* (hVISA)

and MRSA strain MM66, isolated from a patient's sputum in Las Cruces, New Mexico, were exposed to 10% (v/v) ethanol resulting in the up-regulation of genes associated with protein quality control, oxidative stress, and toxin and anti-toxin systems while down-regulating genes involved with cell growth (transcription, translation, and nucleotide biosynthesis) (7, 10). This study also showed a down-regulation of genes associated with protein synthesis, osmotic stress, and lipid metabolism (7). A mutation in *graS,* which encodes a sensor histidine kinase, has been previously identified to be characteristic of the hVISA MM66 strain (13).

The objective of this project was to determine if ethanol can directly select for *S. aureus* ethanol reduced susceptibility (ERS) mutants. We now report that prolonged exposure to inhibitory concentrations of ethanol can select for *S. aureus* ERS mutants, regardless of genetic background, that also display the SCV phenotype. These SCV-ERS mutants also exhibited altered susceptibility to other antimicrobials.

## Methods

### *Strains used in this study*

*S. aureus* strains SH1000 (9), MRSA and VISA strain MM66 (10), and MRSA LP9 (11) were utilized in this study. MM66 was reported to have an increased level of susceptibility to ethanol, compared to LP9 (7).

### *Isolation of ethanol mutants*

Single colonies of SH1000, MM66, and LP9 were used to inoculate 3 mL of Mueller Hinton broth (MHB) which was incubated overnight (37°C, 200 rpm). These overnight cultures were used to inoculate (2 % v/v inoculum) fresh MHB containing ethanol in capped growth flasks

which were allowed to grow (37°C, 200 rpm) until visible growth appeared and the $OD_{580nm}$ was then recorded. All cultures were then passaged at each ethanol concentration (strain specific) investigated a total of five times. A single loop full of culture was then take from the final ethanol containing culture and used to streak MHA plates in order to isolate single colonies.

*Gradient plates*

Briefly, 40 mL of molten MHA (55°C) was poured onto petri plates (90 mm x 90 mm) elevated to 6 mm on one side and dried overnight. To create a second layer, 40 mL of MHA infused with the appropriate antimicrobial agent (Table II) and poured on top of the first layer and allowed to dry for 4 h. Diluted overnight MHB cultures ($OD_{580nm}$ = 0.1) were then used to streak the gradients using a sterile cotton swab (X 3) and the gradient plates were incubated for 48 h (37°C) before the distances grown in mm were measured (Table 2).

*Colony size measurements*

Overnight cultures, prepared as above, were serially diluted from $10^{-1}$ to $10^{-6}$. One hundred microliters from each serial dilution was then spread onto MHA plates using a sterile metal rod and plates were grown overnight (37°C). Colony diameters were then measured for each strain using a caliper.

*graS amplification and sequencing*

Genomic DNA was isolated using the Qiagen Genomic-tip 100/g columns (Qiagen, Germantown, MD) following the manufacturer's instruction. *graS* specific primers graS-F-GTG TCG TAT GAT TAT TAG AC and graS-R-GTC ACT TCC GAC ATG CGT TC were used to

PCR amplify the previously described *graS* mutation in MM66 (13). All PCR amplicons were then gel purified from a 1% agarose gel using the Monarch DNA Gel Extraction Kit (New England Biolabs, Ipswich, MA) and sent for sequencing.

## Results

SH1000 was passaged from 9% to 12% v/v ethanol for 55 days before surviving colonies were picked (Table 1). MM66 took 36 days of passaging to complete its 10 passages from 10% to 11% v/v ethanol (Table 1). A 2% inoculum from the last 11% ethanol MM66 culture failed to initiate growth in MHB containing 12% v/v ethanol after seven days of incubation. LP9 took 29 days to be passaged through 10% to 11% v/v ethanol, and an additional 16 days to complete 5 passages at 12% ethanol (Table 1).

Ethanol-selected mutants from all three strains all grew further on the ethanol gradients and produced smaller colonies (except SH125, Table 2) compared to their respective parent strains and therefore displayed an SCV-ERS phenotype (Table 2).

The SH1000 SCV-ERS mutants grew to further distances on the isopropanol and vancomycin gradients but were unable to grow on a TTO gradient. SH121, SH123, and SH124 showed reduced growth distances on triclosan gradient. All the SH1000 SCV-ERS mutants, except SH122, showed reduced growth on oxacillin gradients (Table 2).

All MM66 SCV-ERS mutants exhibited reduced growth distances on isopropanol, TTO, triclosan, and vancomycin gradients in comparison to MM66. However, all of the MM66 SCV-ERS mutants grew further distances on oxacillin gradients compared to their parent strain (Table 2). The amplification and sequencing of *graS*, characteristic of hVISA, indicated that all of the MM66 ERS mutants still harbored the *graS* mutation previously reported in MM66.

LP122, LP124, and LP125 grew further distances on the isopropanol gradient, while LP121 and LP123 grew shorter distances than LP9. LP122, LP123, and LP125 grew further distances on the TTO gradient, while LP121 and LP124 grew shorter distances on the same gradient. LP122, LP124, and LP125 displayed increased distances grown on the triclosan gradients in comparison to the parent strain, while LP121 and LP123 demonstrated reduced growth distances on the triclosan gradients. All LP9 SCV-ERS mutants also grew further distances on an oxacillin gradient, except for LP124. All LP9 mutants also grew further distances on the vancomycin gradient examined compared to LP9 (Table 2).

## Conclusions

Prolonged exposure to inhibitory concentrations of ethanol selected for *S. aureus* SCV-ERS mutants that also exhibited altered susceptibility to other cell wall targeting antimicrobial agents. Although MM66 mutants did not show visible growth in 12% ethanol, this is likely due to its lower MIC in comparison to LP9. Because MM66 still maintained the *graS* mutation, which has been shown to contribute to its hVISA phenotype, the reduced-susceptibility to vancomycin was likely due to other factors or transcriptional changes. For further studies, metabolomic and proteomic assays of the SCV-ERS mutants can be analyzed for mutations that correlate to other SCV *S. aureus* strains. This study reinforces the necessity for all healthcare-associated workers to fully comply with ABHR protocols for hand sanitization and provides incentive to investigate ethanol susceptibilities of *S. aureus* clinical isolates.

**Table 1.** Ethanol selection *in-vitro*

| Strain | Ethanol passage (number of days – $OD_{580}$) 1 | 2 | 3 | 4 | 5 | Total days of selection |
|---|---|---|---|---|---|---|
| SH1000 | | | | | | |
| 9% | 4 - 2.250 | 2 - 1.052 | 1 - 2.672 | 1 - 1.494 | 1 - 1.183 | 9 |
| 10% | 3 - 1.454 | 2 - 2.137 | 1 - 1.127 | 1 - 1.127 | 1 - 1.398 | 17 |
| 11% | 4 - 1.476 | 5 - 1.099 | 2 - 1.166 | 3 - 1.304 | 3 - 1.280 | 34 |
| 12% | 5 - 0.525 | 4 - 1.125 | 5 - 0.771 | 4 - 1.110 | 3 - 1.250 | 55 |
| MM66 | | | | | | |
| 10% | 4 - 1.197 | 4 - 1.079 | 3 - 1.016 | 3 - 1.048 | 2 - 1.048 | 16 |
| 11% | 5 - 0.527 | 5 - 1.204 | 3 - 1.109 | 5 - 1.085 | 2 - 1.067 | 36 |
| LP9 | | | | | | |
| 10% | 3 - 1.180 | 5 - 0.232 | 2 - 1.528 | 2 - 1.698 | 1 - 1.218 | 13 |
| 11% | 5 - 0.599 | 4 - 1.402 | 2 - 1.069 | 3 - 1.343 | 2 - 1.343 | 29 |
| 12% | 3 - 1.108 | 5 - 1.044 | 3 - 1.375 | 2 - 1.304 | 3 - 1.459 | 45 |

**Table 2.** Gradient plate analysis and colony size.

| Strain | Ethanol | Isopropanol | Tea tree oil | Triclosan | Oxacillin | Vancomycin | Colony Size (N=10) |
|---|---|---|---|---|---|---|---|
| | 0 → 20% | 0 → 20% | 0 → 0.7% | 0 → 0.5 mg $l^{-1}$ | 0 → 0.2 mg $l^{-1}$ | 0 → 1 mg $l^{-1}$ | mm ± SD |
| SH1000 | 61.7 ± 5.8 | 42.0 ± 2.0 | 35.7 ± 1.2 | 90.0 ± 0 | 68.3 ± 1.5 | 22.7 ± 1.2 | 0.82 ± 0.05 |
| SH121 | 90.0 ± 0* | 57.7 ± 1.2* | 0 ± 0* | 51.0 ± 1.7* | 60.3 ± 1.5* | 43.0 ± 1.0* | 0.51 ± 0.03* |
| SH122 | 90.0 ± 0* | 56.0 ± 1.0* | 0 ± 0* | 90.0 ± 0 | 79.7 ± 0.6* | 90.0 ± 0* | 0.51 ± 0.06* |
| SH123 | 90.0 ± 0* | 55.0 ± 2.0* | 0 ± 0* | 42.0 ± 2.0* | 18.3 ± 1.2* | 90.0 ± 0* | 0.44 ± 0.05* |
| SH124 | 90.0 ± 0* | 57.3 ± 2.5* | 0 ± 0* | 35.7 ± 1.2* | 49.3 ± 1.2* | 37.7 ± 0.6* | 0.31 ± 0.04* |
| SH125 | 90.0 ± 0* | 64.7 ± 2.5* | 0 ± 0* | 90.0 ± 0 | 20.3 ± 2.1* | 90.0 ± 0* | 0.82 ± 0.04 |
| | 0 → 20% | 0 → 20% | 0 → 0.7% | 0 → 0.1 mg $l^{-1}$ | 0 → 12 mg $l^{-1}$ | 0 → 1 mg $l^{-1}$ | |
| MM66 | 54.3 ± 1.5 | 56.3 ± 3.2 | 27.0 ± 4.4 | 47.3 ± 1.5 | 0 ± 0 | 90.0 ± 0 | 0.61 ± 0.03 |
| MM111 | 90.0 ± 0* | 23.3 ± 5.5* | 5.3 ± 0.6* | 38.7 ± 0.6* | 32.7 ± 3.2* | 21.0 ± 2.7* | 0.39 ± 0.03* |
| MM112 | 90.0 ± 0* | 22.3 ± 6.1* | 6.0 ± 1.0* | 39.7 ± 0.6* | 38.3 ± 4.0* | 18.0 ± 1.7* | 0.43 ± 0.03* |
| MM113 | 90.0 ± 0* | 24.3 ± 6.4* | 5.7 ± 1.2* | 39.7 ± 2.1* | 36.0 ± 1.7* | 16.3 ± 1.2* | 0.23 ± 0.03* |
| MM114 | 90.0 ± 0* | 21.7 ± 5.7* | 3.7 ± 1.5* | 39.0 ± 2.7* | 33.7 ± 4.2* | 19.3 ± 0.6* | 0.52 ± 0.05* |
| MM115 | 90.0 ± 0* | 22.0 ± 5.8* | 3.3 ± 1.5* | 40.3 ± 0.6* | 29.3 ± 2.1* | 25.3 ± 1.2* | 0.26 ± 0.03* |
| | 0 → 25% | 0 → 20% | 0 → 0.7% | 0 → 0.1 mg $l^{-1}$ | 0 → 4 mg $l^{-1}$ | 0 → 1 mg $l^{-1}$ | |
| LP9 | 33.7 ± 4.2 | 62.7 ± 0.6 | 23.0 ± 1.7 | 33.0 ± 1.0 | 19.3 ± 0.6 | 19.3 ± 1.2 | 0.85 ± 0.05 |
| LP121 | 63.3 ± 1.2* | 63.3 ± 2.1 | 8.7 ± 2.3* | 32.7 ± 1.5 | 33.3 ± 0.6* | 90.0 ± 0* | 0.51 ± 0.05* |
| LP122 | 68.0 ± 1.0* | 60.3 ± 0.6* | 40.0 ± 2.0* | 37.3 ± 2.5* | 90.0 ± 0* | 26.7 ± 2.9* | 0.26 ± 0.03* |
| LP123 | 69.0 ± 1.0* | 63.0 ± 1.0 | 30.7 ± 2.1* | 17.0 ± 2.0* | 90.0 ± 0* | 39.7 ± 2.3* | 0.75 ± 0.03* |
| LP124 | 90.0 ± 0* | 86.7 ± 5.8* | 15.7 ± 2.1* | 37.7 ± 2.1* | 15.0 ± 2.0 | 90.0 ± 0* | 0.28 ± 0.02* |
| LP125 | 90.0 ± 0* | 90.0 ± 0* | 28.0 ± 1.0* | 38.0 ± 2.7* | 33.3 ± 1.2* | 90.0 ± 0* | 0.46 ± 0.05* |

Numbers represent distances grown (mm ± SD, n=3)
*$p < 0.05$ in comparison to parent strains SH1000, MM66, or LP9

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