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The Isolation of *Staphylococcus aureus* Tea Tree Oil-Reduced Susceptibility Mutants

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Abstract

Tea tree oil-reduced susceptibility (TTORS) mutants of two *Staphylococcus aureus* laboratory strains were isolated utilizing TTO gradient plates. Attempts to isolate TTORS mutants employing agar plates containing single TTO concentrations failed. All TTORS mutants demonstrated a small colony variant (SCV) phenotype and produced cells with a smaller diameter, as determined by scanning electron microscopy. The addition of SCV auxotrophic supplements to media did not lead to an increase in TTORS mutant colony size. TTORS mutant revertants (RV) were also isolated from the TTORS mutants following growth in drug free media and all RV strains demonstrated phenotypes similar to their respective parent strains. Transmission electron microscopy revealed that a SH1000 TTORS mutant demonstrated a thinner cell wall and novel septal invaginations compared to parent strain SH1000. In addition, comparative genomic sequencing did not reveal any mutations in a SH1000 TTORS mutant previously linked to well-characterized SCV genotypes. This study demonstrates that TTO can select for a unique SCV phenotype.

Keywords

Staphylococcus aureus; tea tree oil; reduced susceptibility; small colony variants; electron microscopy; genomics

INTRODUCTION

Tea tree oil (TTO) extracted from *Melaleuca alternifolia* is a complex hydrocarbon mixture that exhibits broad-spectrum antimicrobial activity (Carson *et al.*, 2006). Preliminary trials

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Conflict of Interest

All authors declare no financial/commercial conflicts of interest

suggest that TTO formulations may be effective in the treatment and management of acne and fungal infections and could be useful in bacterial pathogen decolonization protocols (Carson *et al.*, 2006). The bactericidal activity of TTO is primarily attributed to its ability to permeabilize the membrane, but it also affects cell wall structure and function (Carson *et al.*, 2002; Cuaron *et al.*, 2012; Cox *et al.*, 1998; Cox *et al.*, 2000; Gustafson *et al.*, 1998). There is strong evidence that the components responsible for the oil's cidal activity are the monoterpene alcohols, in particular terpinen-4-ol and α -terpineol (Carson *et al.*, 2006). Monoterpenes are known to integrate from the aqueous phase into membrane structures, resulting in increased membrane permeability which disrupts membrane-localized protein function (Sikkema *et al.*, 1995).

A number of research groups have investigated TTO reduced susceptibility (TTORS) bacterial mutants and phenotypic states that reduce the antimicrobial action of TTO. In 2000, Nelson utilized both clinical and laboratory *S. aureus* strains to select TTO-resistant mutants. One of these TTO-resistant mutants demonstrated a TTO MIC of 16% (v/v), which was 64 times higher than the parent strain (MIC = 0.25%). Another group demonstrated that the repeated exposure of *S. aureus* strains to sublethal TTO concentrations led to the development of "TTO-habituated" strains which expressed higher TTO MICs (McMahon *et al.*, 2008). Household disinfectant reduced susceptibility *S. aureus* mutants demonstrated altered cell wall metabolism (Lamichhane-Khadka *et al.*, 2008) and a TTORS phenotype (Davis *et al.*, 2005). *S. aureus* grown in the presence of salicylate which activates an intrinsic multiple antimicrobial mechanism (Riordan *et al.*, 2007) also demonstrated TTORS (Gustafson *et al.*, 2001).

Small colony variants (SCV) of *Staphylococcus aureus* demonstrate auxotrophy for hemin, menadione, thiamine or thymidine, slow growth and reduced susceptibility to antimicrobials (Proctor *et al.*, 2006). SCVs demonstrate defects in their electron transport systems or display thymidine deficiency due to a number of mutations (Proctor *et al.*, 2006).

We now report the characterization of *S. aureus* mutants expressing a novel TTORS-SCV phenotype selected on TTO containing gradient plates.

MATERIALS AND METHODS

Chemicals, TTO, and bacterial strains utilized

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO). The TTO utilized in this study purchased from Aura Cacia (Urbana IA, *M. alternifolia* product code A191139) contained 44.1% terpinen-4-ol and 3.9% α -terpineol (v/v) and complied with the TTO International Standard (ISO 4730). *S. aureus* strain SH1000 (Horsburgh *et al.*, 2002) and methicillin-resistant strain COL (Kornblum *et al.*, 1986) were utilized for all experiments. Mueller Hinton broth (MHB), Luria broth (LB) and bacteriological grade agar were purchased from Becton Dickinson and Company (Sparks, MD). All overnight liquid cultures were grown at 37°C with shaking at 200 rpm for 24 h and working MH agar (MHA) culture stock plates were stored at 4°C.

TTO reduced-susceptibility (TTORS) mutant and TTO-susceptible revertant isolation and colony size determination

Initially, aliquots of overnight SH1000 and COL cultures were adjusted to an OD_{580nm} of 1.0, serially diluted and then spread onto freshly produced MHA plates containing 0.5 % v/v Tween 20 and 0 – 1.0 % v/v TTO, in 0.1 % increments. After 48 – 72 h at 37°C, single colonies were picked from the 0.3, 0.4, 0.5 and 0.6 % v/v TTO containing plates which were then passed through drug free MHA media 2 times. The TTO MICs for these suspected TTORS mutants were then determined and compared to parent strains. For the gradient plate TTORS mutant isolation technique, initially 40 mL of MHA (50°C) was poured into a square plate (90 mm X 90 mm) raised on one side by 6 mm and allowed to solidify overnight. Forty ml of MHA containing Tween 20 (0.5% v/v) and TTO (1% v/v) was then poured onto the slanted MHA layer and the plates were allowed to solidify lying flat and then dried open face at 37°C for 2 h. Immediately following the drying step, overnight MHB cultures of SH1000 and COL were diluted to an OD_{580nm} of 1.0 and subcultured onto the entire surface of the gradient plate with sterile cotton swabs. Plates were then incubated at 37°C for 48 h to 72 h. Single colonies growing towards the high end of the TTO gradient were then passaged on drug free MHA 2 times and then subcultured onto fresh MHA to produce working cultures used to determine TTO MICs. Mutants demonstrating the TTORS phenotype (increased TTO MICs and MBCs, Table 1) were then once again plated on fresh MHA and resulting colonies were picked to make overnight cultures and freezer stocks (50% v/v glycerol final concentration, –80°C).

For TTO-susceptible revertant (RV) isolation, SH1000-TTORS-1 or COL-TTORS-3 cultures (37°C, 200 rpm) were initiated with a 2% (v/v) inoculum from a diluted overnight culture (A_{580nm} = 1.0) and each day a 2% inoculum from the former culture was placed into fresh LB for a total of 14 passages over as many days. Luria broth plates (LBA) plates were then inoculated with diluted 7 and 14 day-old passaged SH1000-TTORS-1 or COL-TTORS-3 cultures with a sterile cotton swab. Dilution plates with ~ 100–300 well-isolated colonies were then replica-plated onto a drug free LBA and LBA containing Tween 20 (0.5% v/v) and 0.65% v/v TTO. Colonies appearing on the drug free plate that failed to appear in the same location on the TTO containing plate were considered RV. The RVs of TTORS strains investigated (SH1000-RV-1 and -2, COL-RV-1 and -2, Table 1), were then subcultured on fresh LBA plates and allowed to grow for 24 h. TTO MICs were then performed with individual colonies from these plates. RVs that expressed greater TTO susceptibility compared to their TTORS parent were then picked to make frozen glycerol stock cultures.

Colony diameters were measured utilizing a caliper after growth on LBA plates incubated for 24 h at 37°C.

Antimicrobial susceptibility and auxotrophy testing

Overnight MHB cultures of all strains were used to initiate growth for cultures required for the following experiments. TTO, α -terpineol and terpinen-4-ol MICs were performed by adding 1 ml of diluted overnight culture (final OD_{580nm} = 0.01) into 1 mL of MHB containing Tween 20 (0.5% v/v) and various concentrations of TTO, α -terpineol or

terpinen-4-ol (0.05% to 0.65% in 0.05% or 0.01% v/v increments) and after 24 h incubation, the MICs were determined. Alcohol MICs were performed with MHB containing filter sterilized (0.22 μ m) (Nalge Nunc International, Rochester, NY) ethanol or isopropanol (5% to 20% in 1 % v/v increments). MBCs were determined by streaking 100 μ L aliquots of the MIC tube and all tubes containing higher drug concentrations onto MHA and scoring for the lowest antimicrobial concentration with no observable growth following 24 h incubation (37°C).

Auxotrophy for hemin, menadione and thymidine were determined by spreading serial diluted cultures with a cotton swab onto LBA and LBA containing hemin (1 μ g/mL), menadione (10 μ g/mL) or thymidine (100 μ g/mL). Colony size (n = 20) was then measured on these plates after 24 h incubation (37°C).

Scanning and transmission electron microscopy

Fifty microliter aliquots of overnight MHB cultures (adjusted to an $OD_{580nm} = 1.0$ with fresh MHB) were deposited onto sterile 12 mm diameter glass coverslips and allowed to stand for 30–60 s before gentle immersion of individual coverslips into 2 mL of 2.5% glutaraldehyde-0.1 M imidazole HCl (pH 7.2) solution. For scanning electron microscopy (SEM) imaging, cells on the coverslips were washed free of glutaraldehyde fixative solution with 0.1 M imidazole buffer and gradually dehydrated in graded ethanol solutions (50, 80, and then 100 %), a 1:1 mixture of absolute ethanol and hexamethyldisilazane (HMDS, from Electron Microscopy Sciences, Hatfield, PA) and a final immersion in 100 % HMDS before air drying. Coverslips with a layer of dry sample were then mounted on aluminum specimen stubs with carbon adhesive tabs, sputter coated with a thin layer of gold and examined in a model S-3400N scanning electron microscope (Hitachi High Technologies, Pleasanton, CA) operated in the high vacuum-secondary electron imaging mode. Digital images were then acquired at an instrumental magnification of 15 000 X. The cell diameter of 100 cells from each strain was then determined (Table 1).

For transmission electron microscopy (TEM) 0.15 mL aliquots of 25% glutaraldehyde were added to 1.35 mL of $OD_{580nm} = 1.0$ adjusted cultures and the mixtures were rapidly vortexed before sedimenting the cells in a microcentrifuge. The cell pellets were then washed with 0.1 M imidazole HCl buffer (pH 7.2), post-fixed with 2% osmium tetroxide-0.1 M imidazole buffer (pH 6.8) for 4 h and then washed free of osmium solution with ddH_2O . These samples were then stained en bloc in an aqueous solution of 2% uranyl acetate solution at 60°C in sealed vials, followed by dehydration in a graded series of ethanol solutions (50, 80 and 100 %) and gradual infiltration with mixtures of Spurr's resin (Electron Microscopy Sciences, Hatfield, PA) diluted with acetone (25, 50, 75 and 100% resin over 4 days). The individual pellets were then embedded in resin and cured at 60°C for 24 h. Embedded cell pellets were thin sectioned with a diamond knife using a model EM UC6 ultramicrotome (Leica Microsystems, Bannockburn, IL) and sections on copper specimen grids were then stained with uranyl acetate and lead citrate solutions. All specimens were then examined with a side-mount CCD camera system (AMT, Waltham, MA) integrated with a model H-7650 transmission electron microscope (Hitachi High Technologies, Pleasanton, CA) operated in the bright field imaging at an instrumental

magnification up to 100 000 X for ultrastructural details. A single cell wall width was then determined for 100 individual cells at various magnifications of each strain investigated.

Comparative genomic sequencing, *acpP* sequencing and fatty acid analysis

Genomic comparisons were performed using comparative genomic sequencing (CGS), a two phase tiling microarray-based service provided by NimbleGen Systems Inc. (Madison, WI) (<http://www.nimblegen.com/products/cgs/index.html>) that can identify up to 95% of all SNPs and InDels and confirms mutations identified. A complete description of the genomic tiling microarray design and the CGS comparison methodology has been described (Lannergard *et al.*, 2011). The CGS experiments utilized *S. aureus* strain NCTC 8325 as a reference genome (GenBank accession number NC_007795), which SH1000 is a derivative of (Horsburgh *et al.*, 2002).

Chromosomal DNA was isolated (Riordan *et al.*, 2007) from MHB cultures (12 h, 37°C and 200 rpm) initiated with single colonies of all SH1000 strains in Table 1. This DNA was then utilized to amplify the entire acyl carrier protein gene (*acpP*) with forward (acpP-F, GGA GGT GAA TCG ACG TGG) and reverse primers (acpP-R, GTC GAC AAT ACT GAC GAC CCA G). All PCR mixtures were then loaded into a 1% agarose gels and the *acpP* amplicons were gel purified using a Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison, WI). The PCR isolates were then sequenced utilizing an ABI/Hitachi 3730 DNA Analyzer using POP-7 polymer and BigDye (R) v3.1 reagents (Life Technologies, Foster City, CA).

Fatty acid analysis was performed in triplicate samples at Microbial ID, Inc. (Newark, DE). A complete description of methodology and analysis has been described (Assih *et al.*, 2002). Briefly, overnight cultures of SH1000, SH1000-TTORS-1 and SH1000-RV-1 were subcultured onto MHA plates which were incubated for 24 h at 37 °C and then sent to Microbial ID Inc. Fatty acid methyl esters of these samples were then extracted and the samples were analyzed with gas chromatography.

RESULTS AND DISCUSSION

Confluent growth of SH1000 and COL inocula was observed on all MHA plates containing at or below 0.2% v/v TTO, and no growth was observed on plates containing 0.7% v/v TTO concentrations or greater. Single colonies of suspected SH1000 and COL TTORS mutants appeared on the 0.30, 0.40, 0.50 and 0.60% TTO (v/v) containing MHA, yet none of these colonies passaged through drug free media demonstrated increased TTO MICs compared to their respective parent strains. These results do not support the work of Nelson (2000), who reported selection of TTO-resistant *S. aureus* mutants on single TTO concentrations, followed by sub-culturing mutants in media containing TTO concentrations higher than the original mutant selection concentration.

Three suspected TTORS mutants of both SH1000 and COL isolated from the highest TTO concentration area of the gradient plates did however express elevated TTO MICs and MBCs compared to their respective parent strains (Table 1). Since the gradient plate procedure exposed *S. aureus* populations to a TTO gradient, as opposed to a single TTO

concentration, it had a greater possibility of producing an appropriate TTORS mutant selection TTO concentration.

All of the TTO gradient plate-selected SH1000 and COL TTORS mutants also demonstrated reduced colony diameters (Table 1), slower growth (data not shown) and smaller cell diameters as determined by SEM micrograph analysis (Table 1). SH1000-RV-1 and -2 and COL-RV-1 and -2 demonstrated TTO MICs and MBCs, as well as colony and cell sizes, comparable to their respective parent strains (Table 1).

Most SCV phenotypes can be reversed by hemin, menadione or thiamine supplementation, while thymidine addition is required for the growth of thymidine-dependent SCVs (Proctor *et al.*, 2006). The supplementation of media with hemin, menadione, or thymidine did not lead to an increase in the colony size of the SH1000 and COL TTORS mutants. Triclosan selected SCV mutants also did not demonstrate increased cell colony size when grown in the presence of these supplements (Bayston *et al.*, 2007).

The SCV phenotype is supported by mutations in genes required for the biosynthesis of the electron transport system such as *hemB*, *hemH*, *menD*, and *ctaA* (Proctor *et al.*, 2006). Deletion in genes required for the production of succinate dehydrogenase (*sdhCAB*) also leads to a SCV phenotype (Gaupp *et al.*, 2010). SCV mutants selected with the antibiotic fusidic acid demonstrate mutations in *rplF* which encodes the ribosomal protein L6 as well as *men* or *hem* mutations (Lannergard *et al.*, 2011). Comparative genomic sequencing revealed that SH1000-TTORS-1 harbored 2 intragenic and 19 intergenic mutations (Table 2), yet none of these mutations have been reported on in the SCV literature. In *S. aureus* the essential gene *acpP* (Chaudhuri *et al.*, 2009) is located at the end of the five gene *fapR* operon, similar to that reported on in *Bacillus subtilis* (Martinez *et al.*, 2010). *acpP* encodes a 76 amino acid protein which is a critical component of fatty acid synthase II required for fatty acid biosynthesis (Chan and Vogel, 2010). In *E. coli*, *acpP* is post-translationally modified on a highly conserved serine S36 residue by the addition of a 4'-phosphopantetheine group on which fatty acid chain elongation then occurs (Rock and Cronan, 1996). The A34D ACP alteration observed in SH1000-TTORS-1 is only two amino acids away from the highly conserved serine. *acpP* amplicon sequencing revealed that all SH1000 TTORS mutants contained the exact same *acpP* mutation. SH1000-RV-1 *acpP* sequencing revealed a perfect wild-type reversion, while the *acpP* mutation observed in the TTORS mutants remained in SH1000-RV-2. This evidence suggests that since the *acpP* mutation is conserved in a TTORS revertant that this mutation alone does not support to the TTORS phenotype. Fatty acid analysis was unrevealing and did not demonstrate any significant alteration in the percentage of fatty acid species found in SH1000, SH1000-TTORS-1 and SH1000-RV-1 (data not shown). It is of interest to note that in *Escherichia coli*, reduced ACP activity leads to a decrease in growth rate (Keating *et al.*, 1995).

Comparison of cell wall thickness utilizing TEM micrographs (Fig. 1A) revealed that SH1000-TTORS-1 possessed a cell wall that was significantly thinner ($29.0 \text{ nm} \pm 12.7$) than either SH1000 ($82.5 \text{ nm} \pm 11.7$) or SH1000-RV-1 ($41.2 \text{ nm} \pm 11.0$) ($n = 100$ cells measured, two tailed t-test - $p < 0.05$, compared to SH1000). In addition, while SH1000-RV-1 regained some cell wall thickness it did not demonstrate the degree of cell wall thickness observed in

parent strain SH1000. Furthermore, SH1000-TTORS-1 also demonstrated the presence of a septal-membrane invagination defect (Fig. 1B) not readily observed in the SH1000 or SH1000-RV-1 micrographs investigated. Septal alterations also occurred in *S. aureus* exposed to methicillin (Wilkinson and Nadakavukaren, 1983).

Besides demonstrating reduced susceptibility to TTO, SH1000-TTORS-1 also demonstrated increased terpinen-4-ol, α -terpineol and alcohol MICs and MBCs compared to SH1000 and SH1000-RV-1 (Table 3). Household disinfectant reduced susceptibility *S. aureus* mutants with altered cell wall metabolism (Lamichhane-Khadka *et al.*, 2008) can also exhibit reduced susceptibility to TTO, ethanol and α -terpineol (Davis *et al.*, 2005).

CONCLUSIONS

In this report we describe a protocol to select for *S. aureus* mutants that express reduced susceptibility to TTO. TTORS mutants express a unique small colony variant phenotype, aberrant cell wall formation and harbor multiple mutations. This study also supports the notion that laboratory selection for fully TTO-resistant microbes is probably not possible, as has been previously suggested (Hammer *et al.*, 2008).

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Figure 1.
Figure 1a. Cell wall thickness of strains investigated (40,000 X). 1b. Septal membrane invaginations observed in SH1000-TTORS-1 (40,000 X).

Table 1

Strains, TTO MICs and MBCs, and colony and cell sizes.

Strain	Parent strain	TTO MIC ^a	TTO MBC ^a	Colony diameters ^b	Cell diameters ^c
SH1000		0.27 ± 0.02	0.35 ± 0.01	1.23 ± 0.07	667.50 ± 6.02
SH1000-TTORS-1	SH1000	0.38 ± 0.01 ^d	0.50 ± 0.01 ^d	0.34 ± 0.05 ^d	614.10 ± 4.38 ^d
SH1000-TTORS-2	SH1000	0.34 ± 0.01 ^d	0.45 ± 0.01 ^d	0.36 ± 0.04 ^d	
SH1000-TTORS-3	SH1000	0.34 ± 0.01 ^d	0.45 ± 0.01 ^d	0.37 ± 0.04 ^d	
SH1000-RV-1	SH1000-TTORS-1	0.26 ± 0.01	0.35 ± 0.01	1.24 ± 0.10	632.10 ± 5.18
SH1000-RV-2	SH1000-TTORS-1	0.24 ± 0.01	0.35 ± 0.01	1.24 ± 0.07	
COL		0.17 ± 0.02	0.35 ± 0.01	0.44 ± 0.05	663.80 ± 5.00
COL-TTORS-1	COL	0.25 ± 0.01 ^d	0.40 ± 0.01 ^d	0.30 ± 0.03 ^d	
COL-TTORS-3	COL	0.29 ± 0.03 ^d	0.45 ± 0.01 ^d	0.29 ± 0.02 ^d	631.30 ± 6.36 ^d
COL-TTORS-8	COL	0.27 ± 0.02 ^d	0.40 ± 0.01 ^d	0.28 ± 0.04 ^d	
COL-RV-1	COL-TTORS-3	0.16 ± 0.01	0.35 ± 0.01	0.43 ± 0.04	663.30 ± 3.04
COL-RV-2	COL-TTORS-3	0.19 ± 0.03	0.35 ± 0.01	0.42 ± 0.03	

^a% v/v ± S.D., n = 3

^bmm ± S.D., n = 30

^cnm ± S.D., n = 100

^dTwo tailed t-test - p 0.05, compared to respective parent strain SH1000 or COL.

Table 2Mutations identified in SH1000-TTOR^{RS}-1

NCTC 8325 Locus ^a	Function ^a	SNP ^b Position	Amino acid change
SH1000-TTORS-1			
<u>Intragenic</u>			
SAOUHSC_01201 (<i>acpP</i>)	acyl carrier protein	C ¹¹⁵¹⁶⁴⁵ → A ¹¹⁵¹⁶⁴⁵	A34D
SAOUHSC_01390	transposase for IS1272	C ¹³³³¹⁴¹ → T ¹³³³¹⁴¹	H86Y
<u>Intergenic</u>			
SAOUHSC_01687/SAOUHSC_01688 (<i>lepA</i>)	hypothetical protein GTP-binding protein LepA	T ¹⁵⁹⁷⁷⁵⁶ → C ¹⁵⁹⁷⁷⁵⁶ A ¹⁵⁹⁷⁷⁶⁰ → T ¹⁵⁹⁷⁷⁶⁰ T ¹⁵⁹⁷⁷⁷⁷ → C ¹⁵⁹⁷⁷⁷⁷	
SAOUHSC_01864/SAOUHSC_01865 (<i>trmB</i>)	metallo-beta-lactamase family protein tRNA (guanine-N(7)-)-methyltransferase	T ¹⁷⁷¹⁷⁶⁵ → A ¹⁷⁷¹⁷⁶⁵ C ¹⁷⁷¹⁷⁸⁵ → A ¹⁷⁷¹⁷⁸⁵	
SAOUHSC_01904 (<i>crcB</i>)/SAOUHSC_01905	camphor resistance protein CrcB transposase for IS1272	A ¹⁸¹³⁶²⁵ → G ¹⁸¹³⁶²⁵	
SAOUHSC_02144/SAOUHSC_02145	YolD-like family protein putative exported protein	T ²⁰¹⁸⁶⁴⁸ → C ²⁰¹⁸⁶⁴⁸	
SAOUHSC_02339/SAOUHSC_02340 (<i>atpC</i>)	hypothetical protein F ₀ F ₁ ATP synthase subunit epsilon	A ²¹⁶⁷²³⁰ → C ²¹⁶⁷²³⁰	
SAOUHSC_02412/SAOUHSC_02416	hypothetical protein/hypothetical protein	G ²²⁴⁴⁴⁹⁵ → A ²²⁴⁴⁴⁹⁵	
SAOUHSC_02436/SAOUHSC_02437	aerobactin biosynthesis protein, IucA/IucC family transposase for IS1272	A ²²⁶⁴¹³⁹ → G ²²⁶⁴¹³⁹	
SAOUHSC_02448/SAOUHSC_02449	hydrolase 6-phospho-beta-galactosidase	A ²²⁷²⁹³⁶ → T ²²⁷²⁹³⁶	
SAOUHSC_02512 (<i>rplC</i>)/SAOUHSC_02515	50S ribosomal protein L3 putative membrane protein	C ²³¹⁸²⁹⁰ → A ²³¹⁸²⁹⁰	
SAOUHSC_02638/SAOUHSC_A02505	membrane protein hypothetical protein	A ²⁴²⁵²¹⁰ → G ²⁴²⁵²¹⁰	
SAOUHSC_02750/SAOUHSC_02751 (<i>pnbA</i>)	APC family amino acid-polyamine- organocation transporter para-nitrobenzyl esterase	A ²⁵²⁷⁶³⁶ → T ²⁵²⁷⁶³⁶	
SAOUHSC_02770 (<i>dapF</i>)/SAOUHSC_02771	diaminopimelate epimerase hypothetical protein	G ²⁵⁴⁷⁵⁹⁶ → C ²⁵⁴⁷⁵⁹⁶	
SAOUHSC_02836/SAOUHSC_02837	acetyltransferase, GNAT family hypothetical protein	T ²⁶¹³³⁹⁷ → C ²⁶¹³³⁹⁷ C ²⁶¹³⁴⁰³ → T ²⁶¹³⁴⁰³ A ²⁶¹³⁴²⁰ → T ²⁶¹³⁴²⁰ T ²⁶¹³⁴⁸¹ → A ²⁶¹³⁴⁸¹	

^aDetermined using the published NCTC 8325 genome sequence (NCBI Genbank accession: NC_007795).^bSingle nucleotide polymorphism.

Table 3

Antimicrobial MICs and MBCs

Strain		Terpinen-4-ol ^a	α -Terpineol ^a	Ethanol ^a	Isopropanol ^a
SH1000	MIC	0.13 $\pm$ 0	0.38 $\pm$ 0	9.67 $\pm$ 0.6	8.0 $\pm$ 0
	MBC	0.20 $\pm$ 0	0.47 $\pm$ 0.1	13.7 $\pm$ 0.6	12.0 $\pm$ 0
SH1000-TTORS-1	MIC	0.23 $\pm$ 0 ^b	0.55 $\pm$ 0 ^b	13.0 $\pm$ 0 ^b	10.0 $\pm$ 0 ^b
	MBC	0.30 $\pm$ 0 ^b	0.65 $\pm$ 0 ^b	18.0 $\pm$ 0 ^b	16.0 $\pm$ 0 ^b
SH1000-RV-1	MIC	0.12 $\pm$ 0.1	0.38 $\pm$ 0	9.75 $\pm$ 0.2	8.0 $\pm$ 0
	MBC	0.20 $\pm$ 0	0.48 $\pm$ 0.2	14.4 $\pm$ 0.3	12.0 $\pm$ 0

^a % v/v $\pm$ S.D., n = 3^b Two tailed t-test - p 0.05, compared to SH1000.