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## Antimicrobial Effect and Mode of Action of Terpeneless Cold Pressed Valencia Orange Essential Oil on Methicillin-Resistant *Staphylococcus aureus*

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### Abstract

**Aims**—The objective of this study was to evaluate the antistaphylococcal effect and elucidate the mechanism of action of orange essential oil against antibiotic resistant *Staphylococcus aureus* strains.

**Methods and Results**—Inhibitory effect of commercial orange essential oil (EO) against six *S. aureus* strains was tested by disc diffusion and agar dilution methods. The mechanism of EO action on MRSA was analyzed by transcriptional profiling. Morphological changes of EO treated *S. aureus* were examined by transmission electron microscopy. Results showed that 0.1% of cold pressed terpeneless Valencia orange oil (CPV) induced the cell wall stress stimulon consistent with inhibition of cell wall synthesis. Transmission electron microscopic observation revealed cell lysis and suggested a cell wall-lysis related mechanism of CPV.

**Conclusions**—CPV inhibits the growth of *S. aureus*, causes gene expression changes consistent with inhibition of cell wall synthesis and triggers cell lysis.

**Significance and Impact of the Study**—Multiple antibiotics resistance is becoming a serious problem in the management of *S. aureus* infections. In this study the altered expression of cell wall associated genes and subsequent cell lysis in MRSA caused by CPV suggests that it may be a potential antimicrobial agent to control antibiotic resistant *S. aureus*.

### Keywords

*Staphylococcus aureus*; MRSA; Natural antimicrobial; Mechanism of Essential Oil

### Introduction

In recent years methicillin-resistant *S. aureus* (MRSA) has appeared more in communities outside the hospital settings and has emerged as a major public health concern worldwide (Kennedy et al. 2008, DeLeo et al. 2010). Since infections caused by MRSA are increasing,

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as are rates of antibiotic therapy failures, new measures to treat and prevent this infectious pathogen are becoming inevitable (Pirri et al. 2009). One such approach to counter the antibiotic resistance emphasizes the search of biologically active pharmacophores possessing novel modes of action from natural resources (Saxena and Kumar 2002, Saleem et al. 2010). Natural products have been investigated and utilized to alleviate disease since early human history. Before the “synthetic era”, 80% of all medicines were obtained from roots, barks, leaves, flowers, seeds and fruits (McChesney et al. 2007).

Numerous studies have discovered promising novel antimicrobial candidates from plant derived essential oils (EOs). EOs are particularly interesting since some oils have been used by native groups for curative purposes in the past (Saravolatz et al. 1982, Burt 2004). Also, research data indicate that many EOs have antimicrobial activity. For instance, tea tree oil obtained from *Melaleuca alternifolia* has been shown to be active against a wide range of microorganisms (Gustafson et al. 1998, Hammer et al. 2006). In previous studies the antimicrobial activities of other EOs have also been investigated and their actions against various pathogens, including clinical MRSA isolates, have been demonstrated (Cox et al. 1998, Elsom and Hide 1999, Hammer et al. 1999, May et al. 2000, Takarada et al. 2002, Edwards-Jones et al. 2004, Brady et al. 2006, Prabuseenivasan et al. 2006, Chao et al. 2008, Doran et al. 2009, Tohidpour et al. 2010). There are also several clinical studies and case reports noting the successful use of EOs in treating MRSA nasal carriage and wound infections (Caelli et al. 2000, Sherry et al. 2001, Dryden et al. 2004, Sherry and Warnke 2004).

Fisher and Phillips (Fisher and Phillips 2006) studied the effectiveness of citrus EOs and their components citral, limonene, and linalool against a number of common foodborne pathogens *Listeria monocytogenes*, *S. aureus*, *Bacillus cereus*, *Escherichia coli* O157, and *Campylobacter jejuni* both *in vitro* and on food models. Previous studies in our laboratory have demonstrated the inhibition of *Salmonella* (O’Bryan et al. 2008), *Escherichia coli* O157: H7 (Nannapaneni et al. 2008), *Listeria* (Shannon et al. 2011) and *Campylobacter* (Nannapaneni et al. 2009) by citrus derived cold pressed Valencia orange oil, terpeneless Valencia orange oil, cold pressed orange terpenes, high purity orange terpenes, d-limonene, and terpenes from orange essence. However, these oils were not tested specifically against antibiotic resistant *S. aureus*. Therefore, the objective of this study was to evaluate the inhibitory activity and mechanism of action of orange essential oil on *S. aureus* to determine their potential for use as antistaphylococcal agents against MRSA.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

The following *S. aureus* strains were used in this study: methicillin-susceptible strain SH1000 (Horsburgh et al. 2002), methicillin-resistant strains COL (Sabath et al. 1974), 13136 p<sup>-</sup>m<sup>+</sup> (Brown and Reynolds 1980), and N315 (Kuroda et al. 2001), and methicillin- and vancomycin intermediate-resistant strains 13136 p<sup>-</sup>m<sup>+</sup> V<sub>20</sub> (Pfeltz et al. 2000), and Mu50 (Kuroda et al. 2001). Cultures were propagated in tryptic soya broth (TSB) (Difco Laboratories, Inc. Detroit, MI). A loop of bacteria from a tryptic soya agar (TSA) (Difco Laboratories, Inc.) was inoculated into a 10 mL tube of sterile TSB and subsequently incubated for 18 h at 37 °C, after which a 100 µL aliquot was transferred into a fresh sterile 10 mL of TSB, which was incubated an additional 18 h before use.

### Orange essential oils

All essential oils were obtained as commercially available products of *Citrus sinensis* (L.) Osbeck from Firmenich Citrus Center, Safety Harbor, FL, USA and were stored per

manufacturer's recommendations at 4 °C prior to use. Oils tested included terpeness cold pressed Valencia orange oil (CPV), Valencia orange oil, cold pressed orange terpenes, high purity orange terpenes, d-limonene, terpenes from orange essence, 5-fold concentrated Valencia orange oil, and cold pressed citronellal.

### **Disc diffusion assay for screening the inhibitory effect of EOs**

Disc diffusion assay was carried out by the method described by O'Bryan et al. (O'Bryan et al. 2008). Overnight cultures of the *S. aureus* were streaked on sterile TSA (Difco Laboratories, Inc.) by dipping a sterile cotton swab into the culture. The swab was used to streak the agar plate to produce a lawn of growth by streaking the plate in 3 different directions. The orange EOs (10 µL) were aseptically pipetted onto sterile 6-mm paper discs (Becton Dickson, Franklin Lakes, NJ) and subsequently the paper discs were aseptically placed on the agar. Diameters of zones of inhibitions were measured in mm after 24 h of incubation at 37 °C. The assays were carried out on three independent experiments conducted in duplicate.

### **Minimum inhibitory concentration assay (MIC)**

The MIC of CPV for *S. aureus* strains was performed by modified agar dilution method. A final concentration of 0.5% (v/v) Tween-80 (Sigma, St. Louis, MO) was incorporated into the agar medium to enhance oil solubility. Different concentrations of oil were added to the sterile TSA at 48 °C. Plates were dried at room temperature for 12 h prior to spot inoculation with 5 µL aliquots of culture containing approximately 5 Log CFU per spot of each organism in triplicate. Inoculated plates were incubated at 37 °C for 24 h and the MICs were determined as the lowest concentration of oil inhibiting visible growth of organisms on the agar plate. Experiments were carried out in three independent experiments in duplicate. Inhibition of bacterial growth in the plates containing test oil was judged by comparison with visible growth in control plates.

### **Growth inhibitory concentration (GIC) studies**

Overnight grown cultures were used to inoculate (1% v/v) 20 ml TSB in 50 ml Erlenmeyer flasks and were grown at 37 °C with shaking at 200 rpm. Two different concentrations (½, and 1x of MIC) of EO were used in this study. After adding the EO to the log phase cultures (OD<sub>600</sub> approximately 0.4) growth was measured at 600 nm at regular intervals in a Beckman DU65 spectrophotometer. A final concentration of 0.5% (v/v) Tween-80 (Sigma) was used as a dispersing agent for EO.

### **RNA extraction and transcriptional profiling**

Based on the GIC study ½ x MIC concentration of EO was added to the log phase cells for 15 min of challenge. Control culture was not challenged with EO and was also incubated for 15 min. RNA extraction and microarray hybridization was carried out as described by Muthaiyan et al. (2008). Briefly, total bacterial RNA was extracted from 3 ml of culture which was mixed with 6 ml of bacterial RNA protect solution (Qiagen, Valencia, CA) and subsequently centrifuged to collect the cells. To extract the RNA, bacterial pellets were resuspended in 1 ml of Trizol (Invitrogen, Grand Island, NY) and the cells were broken using the FastPrep system (Qbiogene, Irvine, CA) at a speed setting of 6.0 for 40 seconds. Extracted RNA was purified using the RNeasy mini kit (Qiagen). cDNA was generated from DNase treated and purified RNA by using random hexamers (Invitrogen) as primers for reverse transcription. The primers were annealed (70 °C for 10 min, followed by 1 min incubation in ice) to total RNA (2.5 µg) and were extended with SuperScript III reverse transcriptase (Invitrogen) with 0.1 M dithiothreitol 12.5 m mol dNTP/aa-UTP (Ambion, Austin, TX) mix at 42 °C. Residual RNA was removed by alkaline treatment followed by

neutralization, and cDNA was purified with a QIAquick PCR purification kit (Qiagen). Purified aminoallyl-modified cDNA was subsequently labeled with Cy3 or Cy5 mono-functional NHS ester cyanogen dyes (GE Healthcare, Piscataway, NJ) according to the manufacturer's instruction. Labeled cDNA was purified using a QIAquick PCR purification kit (Qiagen) and the purified labeled cDNA was hybridized with *S. aureus* genome microarrays version 6.0 provided by the Pathogen Functional Genomics Resource Center (PFGRC).

### Microarray data analysis

Hybridization signals were scanned using an Axon4000B scanner with Acuity 6.0 software (Molecular Devices, Inc. U.S.) and scans were saved as TIFF images. Data analysis was performed by TM4 microarray software suite (Saeed et al. 2003). Scans were analyzed using TM4-Spotfinder software and the local background was subsequently subtracted. The data set was normalized by applying the LOWESS algorithm using TM4-MIDAS software. The normalized log<sub>2</sub> ratio of test/control signal for each spot was recorded. Significant changes of gene expression were identified with significance analysis of microarrays (SAM) software using one class mode (Tusher et al. 2001). The differentially regulated genes were further classified according to the functional categories described in the comprehensive microbial resource of TIGR (<http://cmr.tigr.org/tigr-scripts/CMR/shared/Genomes.cgi>). As per our standard transcriptional profiling protocol, to minimize the technical and biological variations and to ensure that the data obtained were of good quality three independent cultures were used to prepare RNA samples and each RNA preparation was used to make probes for at least two separate arrays for which the incorporated dye was reversed (Muthaiyan et al. 2008).

### Microarray data accession number

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE33465 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33465>) (Edgar et al. 2002).

### Electron microscopy

TSB grown exponential phase *S. aureus* COL was treated with ½ x MIC of the CPV for 30 min. Following the treatment, 2 mL of the culture was collected by centrifugation at 10,000 RPM for 10 min. The cell pellets were subsequently fixed in Karnovsky's fixative for 2 h under a weak vacuum. Samples were rinsed 3 times in 0.05 mol cacodylate buffer, pH 7.2, post fixed in 1% osmium tetroxide (aqueous), rinsed with distilled water and stained with 0.5% uranyl acetate overnight at 4°C. The sample was dehydrated in a graded ethanol series, followed by propylene oxide, and embedded in Spurr's medium. Ultra-thin sections were cut with a diamond knife on a MT2B Ultratome (Dupont Company, Newtown, CT). Sections were placed on 300 mesh copper grid, and stained with 2% aqueous uranyl acetate, followed by lead citrate. Grids were viewed at 80 kv with a JEM 100 CX transmission electron microscope (JEOL, Tokyo, Japan).

## RESULTS

### Inhibitory effect of citrus oils against *S. aureus*

Of the eight EOs tested terpeneless cold pressed Valencia orange oil (CPV) and cold pressed citronellal exhibited a high level inhibition against all *S. aureus* strains in disc diffusion assays (Table 1). The MICs of CPV for six *S. aureus* strains were determined by agar dilution method. CPV concentration at 0.18% caused complete inhibition for the strains 13136 p<sup>-</sup>m<sup>+</sup> and 13136 p<sup>-</sup>m<sup>+</sup>V<sub>20</sub>. However, for strains COL, Mu50, and N315 0.21% CPV

was required to inhibit the growth. Based on the MICs, growth inhibitory concentration was determined for CPV to choose the concentration and duration of treatment for transcriptional profiling studies. Two different concentrations ( $\frac{1}{2}$  x and 1 x of MIC) of CPV were used to determine the GIC. Both  $\frac{1}{2}$  x and 1 x of MIC concentrations caused significant growth inhibition for strains SH1000, COL, 13136 p<sup>-</sup>m<sup>+</sup>, Mu50, and N315. However, the VISA strain 13136 p<sup>-</sup>m<sup>+</sup>V<sub>20</sub> exhibited reduced susceptibility to  $\frac{1}{2}$  x MIC of CPV (Fig 1).

### Effect of CPV on the cell lysis related gene expression

*S. aureus* COL challenged with 0.1% CPV for 15 min showed upregulation of 431 genes and down regulation of 551 genes from a variety of functional categories (Supplementary Table S1 and S2). In the initial growth study, the CPV treated COL cells showed rapid lysis within 60 min of the treatment; also 24 fold induced expression of *cwrA* (SACOL2571) in the transcriptional profile supported the CPV induced cell wall damage. Therefore, we particularly focused on the altered expression pattern of the cell wall related genes to better understand the mechanism of the CPV action on *S. aureus*. In the transcriptional profiling analysis about 62 and 36 cell envelope-related genes were under- and over-expressed, respectively. Some of the well recognized cell wall stress stimulon member genes include penicillin binding protein *pbp1*, *pbp2(mecA)*, *pbp3*, and murein sacculus and peptidoglycan biosynthesis related *murB*, *murC*, *muD*, *murE*, *murG*, and autolysin related *atl*, *lytM* were downregulated (-3 to -2 fold). *pbp4* and capsular polysaccharide biosynthesis related genes (*cap*) were upregulated in the cell envelope related category (Table 2).

Related to the cell envelope, classified under Cellular Processes, approximately 54 and 49 genes were down- and up-regulated, respectively. D-alanine-activating enzyme/D-alanine-D-alanyl carrier protein ligase encoding *dltA*, *dltB*, *dltC*, *dltD*, cell division related *divIB*, *ftsA*, *ftsL*, *ftsW* and, universal stress resistance family protein encoding SACOL1753, SACOL1759, SACOL0552, drug resistance transporter EmrB/QacA subfamily encoding SACOL2347 were downregulated (-11 to -2 fold). Two component response regulators encoding *vraS*, *vraR*, *arlS*, *arlR* and transcriptional regulator *tcaR*, staphylococcal accessory regulator *sarA*, *sarV*, *sarY* were upregulated between 2 to 5 fold (Table 2).

Another prominent category of genes altered by CPV treatment is the Protein Fate, set of genes which includes chaperones and proteases. Some of the genes encoding the chaperones and proteases are known as marker genes for cell wall stress condition. In this Protein Fate category 24 genes were downregulated and 26 genes were upregulated by CPV treatment. Most of the genes encoding for degradation of proteins, peptides, and glycopeptides were down regulated between -4 to -2 fold. However, CPV induced the expression of *clpB* and *clpC* (chaperone/protease), *spsA* and *spsB* (type 1 signal peptidases A and B), and SACOL2683 (putative methionine sulfoxide reductase). In addition, expression of genes encoding the major heat shock proteins GroEL, GroES, DnaK, DnaJ, GrpE had increased between 2 to 5 fold (Table 2).

In addition to cell wall related genes, a variety of genes involved in DNA metabolism that play a role in DNA replication, recombination, and repair were down- and up-regulated by CPV challenge (Supplementary Table S1 and S2). Some of the known DNA metabolism related genes affected by the CPV treatment encode DNA repair protein RecN, R subunit of type I restriction-modification enzyme, M subunit type I restriction-modification enzyme, DNA gyrase, single-stranded-DNA-specific exonuclease RecJ, ATP-dependent DNA helicase PcrA, DNA polymerase, and DNA ligase.



## Electron microscopic analysis of CPV induced cell wall damage

Actively growing COL cells exhibited an ultra structure typical of *S. aureus*, with septa that were normal in appearance and a trilaminar cell wall (Fig 2A), whereas, CPV (0.1%) treatment for 30 min led to extensive cell lysis in COL cells (Fig 2B). In addition to cell lysis, loss of cellular electron dense material, coagulation of cytoplasmic constituents, deformed septum and lack of a distinct midline were observed. As a consequence of profound structural alterations and breaks in the cell walls several ghosts of lysed cells appeared after the CPV treatment (Fig 2B). Electron microscopic observation of cell wall lysis in CPV treated cells substantiates the results of CPV induced altered expression of cell wall- and cell division-related genes in *S. aureus*.

## DISCUSSION

The infections caused by MRSA and VISA due to acquisition of resistance towards current antimicrobials pose a serious challenge for therapy (Payne 2008). In an effort to explore the potential use of orange EO against antibiotic resistant *S. aureus*, in this study inhibitory effects and mode of action of CPV against MRSA and VISA strains were studied. The inhibitory effect of CPV against each *S. aureus* strain varied in the disc diffusion screening assay. The VISA strain 13136 p<sup>-</sup>m<sup>+</sup> V<sub>20</sub> exhibited greater inhibition (76.67 ± 4.08 mm) than other MRSA strains. Similar to our results, inhibitory effects of various EOs against MRSA and other bacteria have previously been confirmed by disc diffusion and agar dilution methods (Prabuseenivasan et al. 2006, Chao et al. 2008, Fisher and Phillips 2006, Dorman and Deans 2000, Burt and Reinders 2003, Abulrob et al. 2004, Nostro et al. 2004, Busatta et al. 2008, Viuda-Martos et al. 2008, Goñi et al. 2009, Patharakorn et al. 2010).

In previous reports antimicrobial properties of EOs and their components have been reviewed extensively (Burt 2004). However, only a few studies have reported the mechanism of antibacterial action of EOs in great detail (Cox et al. 1998, Cox et al. 2000, Fisher and Phillips 2006). Therefore, based on the significant inhibitory effect we observed in our study, we selected CPV for the further experiments to study the mode of action on MRSA.

Numerous studies have investigated the changes in gene expression patterns in response to antibiotics at the sub-inhibitory concentration to obtain an in depth understanding of mode of action of antimicrobials (Wecke and Mascher 2011). Accordingly, in our study *S. aureus* genome microarrays were used to capture the genomic response of CPV treated *S. aureus* cells. Transcriptional profiling revealed alteration of gene expression in a variety of functional categories including amino acid biosynthesis, cell envelope, cellular processes, central intermediary metabolism, DNA metabolism, protein synthesis, and signal transduction. Specifically, the observation of 24 fold induced expression of *cwrA* (SACOL2571) (equivalent locus SA2343 in strain N315) along with rapid lysis of *S. aureus* cells during the CPV treatment supported the CPV induced cell wall damage in *S. aureus*. In previous research high level upregulation of *cwrA* has been reported in a variety of transcriptomic studies examining cell wall inhibition (McAleese et al. 2006, Sobral et al. 2007, Balibar et al. 2009) and recently, Balibar et al. showed that *cwrA* was robustly induced by cell wall-targeting antibiotics: vancomycin, oxacillin, penicillin G, phosphomycin, imipenem, himeglusin and bacitracin, but not by antimicrobials with other mechanisms of action, including ciprofloxacin, erythromycin, chloramphenicol, triclosan, rifampicin, novobiocin and carbonyl cyanide 3-chlorophenylhydrazone (Balibar et al. 2010). Therefore, we focused on the cell wall related genes to elucidate the mechanisms of action of CPV. In support of our view in a similar study Cox et al. (2000) demonstrated that in *Escherichia coli* and *S. aureus* the antimicrobial activity of tea tree oil results from its ability to disrupt the permeability barrier of membrane structures. In a later study Carson et al. (2002)

reported that the mechanism of action of tea tree oil on *S. aureus* is not specific on cytoplasmic membrane but due to induction of the release of membrane-bound cell wall autolytic enzymes and eventual cell lysis.

In earlier studies, based on the results of transcriptional profiling experiments after the exposure of *S. aureus* to cell wall-active agents a set of cell wall-associated genes were identified and assigned as a “cell wall stimulon”. These genes have been used as marker genes for cell wall related gene response (Kuroda et al. 2003, Utaida et al. 2003, Wilkinson et al. 2005, Gardete et al. 2006). In our study we observed the altered expression of several cell wall stimulon member genes in CPV treated *S. aureus* cells and those genes are discussed in the following sections.

The PBPs are membrane-associated proteins that catalyze the final step of murein biosynthesis of cell-wall peptidoglycan in *S. aureus*. PBP1 (*pbp1*) is essential and important for cell division (Pereira et al. 2007) and PBP2a, encoded by *mecA*, is responsible for methicillin resistance in *S. aureus* (Pinho and Errington 2005). It has been reported that inactivation of *pbp3* caused a small but significant decrease in autolysis rates. Cells of abnormal size and shape and disoriented septa were reported when bacteria with inactivated *pbp3* were grown in the presence of cell wall active antibiotic methicillin (Pinho et al. 2000). In our study downregulation of these PBPs encoding genes could very well be involved in the observed lysis of CPV treated *S. aureus* cells. Additionally, we believe that the observed upregulation of PBP4 encoding *pbp4* and capsular polysaccharide related *cap* genes could be a protective response of *S. aureus* during the CPV induced cell wall damage. In support of our view it was recently demonstrated that PBP4 may play an important role in cell wall antibiotic resistance (Memmi et al. 2008, Navratna et al. 2010).

The *S. aureus* cell wall has been reported as a structure composed of highly cross-linked peptidoglycan, a complex structure composed of sugars and amino acids (murein), teichoicacids, and cell wall-associated proteins (Dmitriev et al. 2004). We observed the repression of these murein sacculus and peptidoglycan biosynthesis related *murB*, *murC*, *muD*, *murE*, *murG*, *murAB* genes upon the CPV treatment. Cell wall autolysis related *atl* and *lytM* genes were also downregulated by CPV treatment. Downregulation of *atl* and *lytM* has previously been reported in *S. aureus* exposed to cell wall-active agents and viewed as a response of the cell to preserve peptidoglycan when challenged with cell wall-active agents (Kuroda et al. 2003, Utaida et al. 2003, Wilkinson et al. 2005, Muthaiyan et al. 2008).

Along with cell envelope related group, genes belonging to *S. aureus* cellular processes were affected by CPV treatment. D-alanine-activating enzyme/D-alanine-D-alanyl carrier protein ligase encoding *dltA*, *dltB*, *dltC*, *dltD* were reportedly down regulated in CPV treated cells. *dltABCD* operon controls the alanylation of wall teichoicacids which are involved in the control of autolysin activity in *S. aureus*. Previous research by Peschel et al. (1999; 2000) demonstrated that mutation in *dlt* genes lead to failure of alanylation in the teichoic acids and consequently *S. aureus* cells become sensitive to human defensin HNP1–3, animal-derived protegrins, tachyplesins, and magainin II, and to the bacteria-derived peptides gallidermin and nisin, and cell wall antibiotics. Thus, the repression of *dlt* genes by the CPV could be viewed as one of the reasons for the rapid lysis of CPV treated cells.

Genes involved in cell division and stress resistance were also downregulated in the CPV treated cells. In previous transcriptional profiling studies cell division proteins encoding genes were shown to be induced by cell wall antibiotics but downregulated by membrane active compounds (Muthaiyan et al. 2008). Downregulation of these genes in response to CPV treatment indicates that CPV potentially acts on both cell walls as well as membranes of the *S. aureus* cells.

In *S. aureus* *vraSR* is a two-component system that positively regulates a number of genes involved in cell wall synthesis and *arlSR* is another two-component system involved in several cell wall activities including rate of autolysis as well as the attachment to a polymer (Fournier and Hooper 2000, Kuroda et al. 2003). Induction two component of these response regulators along with *arlSR* associated accessory regulators *sarA*, *sarV*, *sarY* in response to CPV treatment could be contemplated as a *S. aureus* protective response to the cell wall damage caused by the down regulation of cell wall synthesis associated genes.

Some of the known cell wall stress stimulon genes encoding the chaperones and proteases were affected during exposure to CPV. Electron microscopic analysis of CPV treated cells revealed that CPV acted on *S. aureus* cell walls and caused profound cell wall damage and cell lysis. Treatment of *S. aureus* cells with cell wall-active agents is considered to result in the accumulation of misfolded and damaged proteins (Utaiida et al. 2003, Wilkinson et al. 2005, Muthaiyan et al. 2008). Presumably in an attempt to counter the CPV mediated peptidoglycan biosynthesis inhibition and subsequent cell lysis *S. aureus* increased the chaperones to restore the lysed proteins. Therefore, we speculate that the genes associated with degradation of proteins and peptides *clpB* and *clpC* (chaperone/protease), *spsA* and *spsB* (type 1 signal peptidases A and B), and SACOL2683 (putative methionine sulfoxide reductase) and genes encoding the major heat shock proteins GroEL, GroES, DnaK, DnaJ, GrpE were induced in CPV treated cells.

In addition to cell wall related genes, DNA-replication, -recombination, and -repair related genes were also apparently down- and up-regulated in CPV treated cells. In previous studies some of these genes have been identified as members of the cell wall stress stimulon. In *S. aureus* altered expression of genes involved in DNA metabolism has been recognized as a characteristic mode of cell-wall active agent's action and reported as a part of SOS response (Maiques et al. 2006). Also, in *E. coli* it has been reported that transcription of genes encoding DNA polymerase and DNA repair, is induced during the inhibition of cell wall synthesis caused by  $\beta$ -lactam antibiotics (Perez-Capilla et al. 2005). Interestingly, in our study approximately 31 genes related to DNA replication and repair were repressed and 20 genes were upregulated upon CPV treatment. Therefore, we speculate that along with cell wall damage CPV may possibly repress the *S. aureus* SOS system that normally used by the bacterial cells to survive during the adverse condition.

The electron microscopic observation of cell morphology of CPV treated cells confirmed the transcriptional response of CPV treated *S. aureus* cells exhibiting down regulation of cell envelope related genes and corroborated the cell wall active and lytic effect of CPV on *S. aureus*. Electron micrographs illustrated the cell wall and membrane damage and loss of cytoplasmic materials and several cell-wall ghosts that accompanied in CPV treatment of *S. aureus* cells. Similar cytoplasmic losses have also been reported in tea tree oil treated *S. aureus* cells (Carson et al. 2002). Apparently the down regulation of the autolysis related gene observed in the transcriptional profiling may serve as a protective response of cells from the CPV mediated cell wall lysis observed in the electron microscope.

Results of this *in vitro* study indicate that CPV effectively inhibits the *S. aureus* by affecting the cell wall. While the MRSA is becoming a significant public health problem, the findings of the present study are promising and reveal the potential of CPV as an alternative natural therapeutic antimicrobial agent against MRSA. However, prior to the use of CPV for MRSA decolonization issues of safety and toxicity will need to be addressed.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.



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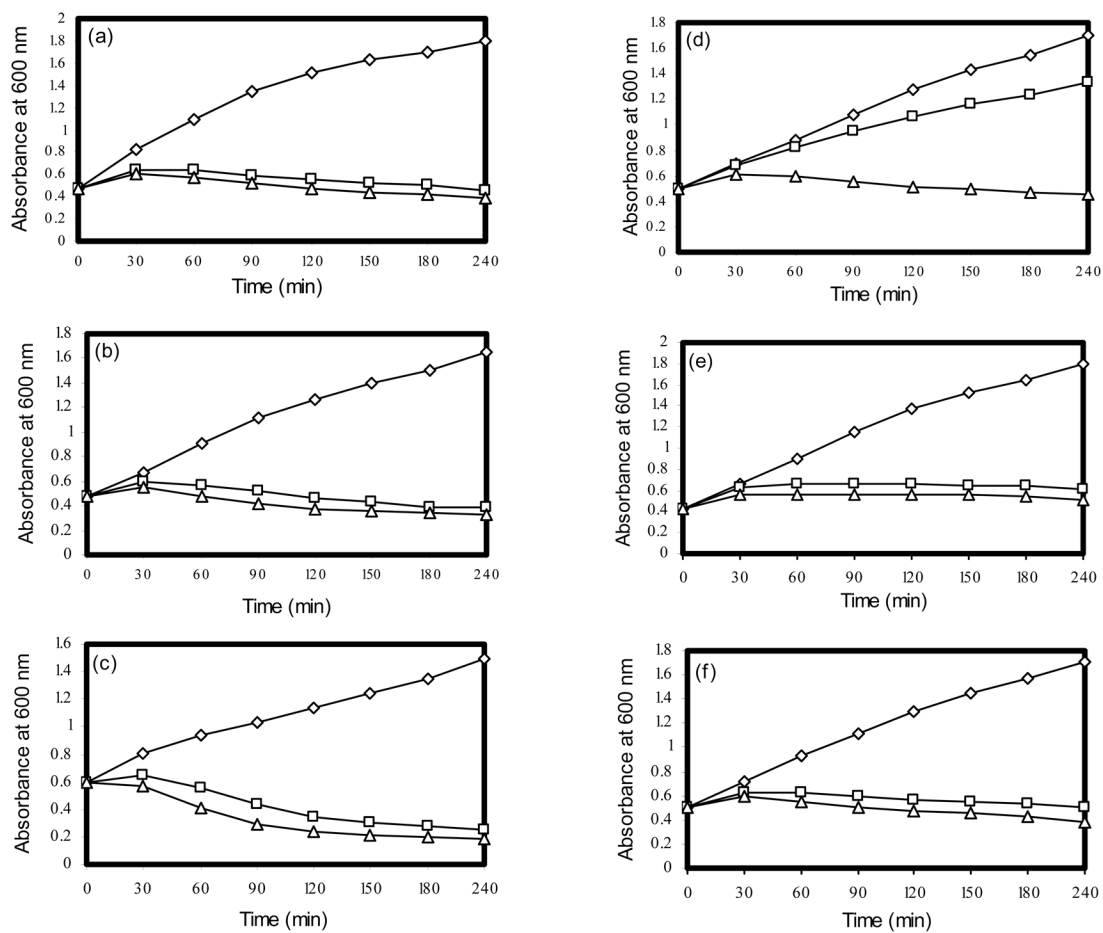
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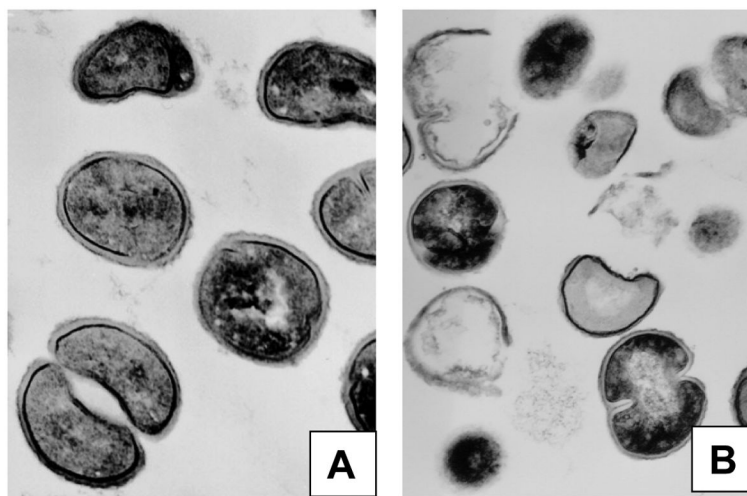
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**Figure 1.** Growth inhibitory effect of CPV on *S. aureus* strain SH1000 (a), COL (b), 13136 p<sup>-</sup>m<sup>+</sup> (c), 13136 p<sup>-</sup>m<sup>+</sup>V<sub>20</sub> (d), Mu50 (e), and N315 (f). ◇, control; △, 1x MIC; □, 1/2 x MIC.





**Figure 2.** Electron micrograph of  $\frac{1}{2}$  x MIC of CPV treated *S. aureus* COL cells. A, control; B, cells after 30 min of CPV treatment. Magnification,  $\times 30,000$ , 1 mm = 33.33 nm.

**Table 1**

Inhibitory effect of terpeneless cold pressed Valencia orange oil (CPV) and citronellal against *S. aureus* strains determined by a disk-diffusion assay

<i>S. aureus</i> strain	Inhibition Zone (mm) <sup>a</sup>	
	CPV	Citronellal
SH1000	31.50 ± 3.02	9.20 ± 0.84
COL	65.83 ± 3.76	19.83 ± 1.33
13136 p <sup>-</sup> m <sup>+</sup>	65.67 ± 4.59	18.83 ± 1.33
13136 p <sup>-</sup> m <sup>+</sup> V <sub>20</sub>	76.67 ± 4.08	11.00 ± 1.26
N315	65.83 ± 3.76	11.17 ± 0.98
Mu50	32.50 ± 2.74	8.33 ± 0.82

<sup>a</sup>Inhibition zones are average values of three independent trials ± the standard deviation (SD, n=6) of the mean.

Table 2

Altered expression of genes associated with cell lysis in CPV treated *S. aureus* COL cells

Locus ID	Gene <sup>a</sup>	Gene/Protein Name	Sub-functional Category	Fold Change
<b>Downregulated Genes</b>				
SACOL0938	dlfD	DlfD protein	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-10.41
SACOL0054	NA	Mur ligase family protein, authentic frameshift	Biosynthesis and degradation of murein sacculus and peptidoglycan	-9.61
SACOL0937	dlfC	D-alanyl carrier protein	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-5.99
SACOL0936	dlfB	DlfB protein	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-5.41
SACOL0247	lrgA	holin-like protein LrgA	Biosynthesis and degradation of murein sacculus and peptidoglycan	-4.64
SACOL0052	NA	glycosyl transferase, group 1 family protein	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-4.19
SACOL1195	mraY	phospho-N-acetylmuramoyl-pentapeptide-transferase	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-3.71
SACOL0697	tagX	teichoic acid biosynthesis protein X	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-3.51
SACOL0801	murB	UDP-N-acetylenolpyruvoylglucosamine reductase	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-2.97
SACOL1023	murE	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase	Biosynthesis and degradation of murein sacculus and peptidoglycan	-2.89
SACOL1194	pbp1	penicillin-binding protein 1	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-2.5
SACOL1196	murD	UDP-N-acetylmuramoylalanine--D-glutamate ligase	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-2.45
SACOL2074	NA	D-alanine--D-alanine ligase	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-2.39
SACOL1687	NA	N-acetylmuramoyl-L-alanine amidase, family 3	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-2.38
SACOL0543	glmU	UDP-N-acetylglucosamine pyrophosphorylase	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-2.21
SACOL2103	NA	UDP-N-acetylglucosamine 2-epimerase	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-2.21
SACOL0033	mecA	penicillin-binding protein 2	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-2.15
SACOL1329	femC	glutamine synthetase FemC	Biosynthesis and degradation of murein sacculus and peptidoglycan	-2.15
SACOL0242	NA	teichoic acid biosynthesis protein, putative	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-2.12
SACOL1134	ktfB	lipopolysaccharide core biosynthesis protein KdtB	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-2.09
SACOL1609	pbp3	penicillin-binding protein 3	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-2.01
SACOL1453	murG	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	Biosynthesis and degradation of murein sacculus and peptidoglycan	-1.98
SACOL0263	lytM	peptidoglycan hydrolase	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-1.96

Locus ID	Gene <sup>a</sup>	Gene/Protein Name	Sub-functional Category	Fold Change
SACOL0695	NA	tagG protein, teichoic acid ABC transporter protein, putative		-1.87
SACOL1066	NA	fnt protein		-1.83
SACOL1062	atl	bifunctional autolysin	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-1.78
SACOL1424	NA	phosphate ABC transporter, phosphate-binding protein	Other	-1.74
SACOL1790	murC	UDP-N-acetylmuramate--alanine ligase	Biosynthesis and degradation of murein sacculus and peptidoglycan	-1.58
SACOL1951	NA	Mur ligase family protein		-1.57
<b>Upregulated Genes</b>				
SACOL2571	cwrA	conserved hypothetical protein	Conserved	24.05
SACOL1434	NA	alanine racemase family protein	Biosynthesis and degradation of murein sacculus and peptidoglycan	21.26
SACOL0147	cap5L	capsular polysaccharide biosynthesis protein Cap5L		4.22
SACOL0150	cap5O	capsular polysaccharide biosynthesis protein Cap5O		4.02
SACOL0148	cap5M	capsular polysaccharide biosynthesis galactosyltransferase Cap5M	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	3.98
SACOL0151	cap5P	UDP-N-acetylglucosamine 2-epimerase Cap5P		3.61
SACOL0149	cap5N	capsular polysaccharide biosynthesis protein Cap5N		3.55
SACOL1161	murI	glutamate racemase	Biosynthesis and degradation of murein sacculus and peptidoglycan	3.11
SACOL0146	cap5K	capsular polysaccharide biosynthesis protein Cap5K		2.95
SACOL0144	cap5I	capsular polysaccharide biosynthesis protein Cap5I		2.83
SACOL0145	cap5J	capsular polysaccharide biosynthesis protein Cap5J	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	2.79
SACOL0140	cap5E	capsular polysaccharide biosynthesis protein Cap5E		2.67
SACOL0141	cap5F	capsular polysaccharide biosynthesis protein Cap5F		2.17
SACOL2092	murAA	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1	Biosynthesis and degradation of murein sacculus and peptidoglycan	2.1
SACOL0143	cap5H	capsular polysaccharide biosynthesis protein Cap5H	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	2.04
SACOL0699	pbp4	penicillin-binding protein 4	Biosynthesis and degradation of murein sacculus and peptidoglycan	2.04
SACOL0142	cap5G	UDP-N-acetylglucosamine 2-epimerase Cap5G		1.99
SACOL0136	cap5A	capsular polysaccharide biosynthesis protein Cap5A	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	1.68
SACOL0137	cap5B	capsular polysaccharide biosynthesis protein Cap5B		1.67
SACOL2116	murAB	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 2	Biosynthesis and degradation of murein sacculus and peptidoglycan	1.67

Locus ID	Gene <sup>a</sup>	Gene/Protein Name	Cellular processes	Sub-functional Category	Fold Change
<b>Downregulated Genes</b>					
SACOL0935	dlfA	D-alanine-activating enzyme/D-alanine-D-alanyl carrier protein ligase		Toxin production and resistance	-11.93
SACOL2295	NA	staphyloxanthin biosynthesis protein, putative		Pathogenesis	-8.55
SACOL1535	srrA	DNA-binding response regulator SrrA		Cell division	-8
SACOL0244	scdA	ScdA protein		Pathogenesis	-6.24
SACOL0270	NA	staphyloxanthin biosynthesis protein, putative		Pathogenesis	-5.55
SACOL0095	spa	immunoglobulin G binding protein A precursor		Adaptations to atypical conditions	-5.54
SACOL1437	NA	cold shock protein, CSD family		Other	-4.87
SACOL2126	luxS	autoinducer-2 production protein LuxS		Adaptations to atypical conditions	-4.15
SACOL0956	kapB	kinase-associated protein B		Cell division	-3.8
SACOL1193	NA	cell division protein FtsL		Cell adhesion	-3.6
SACOL0608	sdrC	sdrC protein		Toxin production and resistance	-3.53
SACOL1328	glnR	glutamine synthetase repressor		Pathogenesis	-3.35
SACOL2291	NA	staphyloxanthin biosynthesis protein		Adaptations to atypical conditions	-3.33
SACOL0552	NA	general stress protein I3		Pathogenesis	-3.32
SACOL1534	srrB	sensor histidine kinase SrrB		Toxin production and resistance	-3.27
SACOL0743	bacA	bacitracin resistance protein		Toxin production and resistance	-3.23
SACOL1396	fntC	fntC protein		Cell division	-3.18
SACOL2075	ftsW	cell division protein, FtsW/RodA/SpoVE family		Cell division	-3.12
SACOL1197	divIB	cell division protein		Pathogenesis	-2.91
SACOL0766	saeR	DNA-binding response regulator SaeR		Cell division	-2.87
SACOL1205	NA	cell-division initiation protein, putative		Detoxification	-2.86
SACOL0452	ahpC	alkyl hydroperoxide reductase, C subunit		Cell division	-2.8
SACOL1537	scpB	segregation and condensation protein B		Toxin production and resistance	-2.72
SACOL1624	era	GTP-binding protein Era		Adaptations to atypical conditions	-2.46
SACOL1538	scpA	segregation and condensation protein A		Toxin production and resistance	-2.31
SACOL1184	NA	exfoliative toxin, putative		Toxin production and resistance	-2.14
SACOL0746	norR	transcriptional regulator, MarR family		Adaptations to atypical conditions	-2.06
SACOL2731	NA	cold shock protein, CSD family		Adaptations to atypical conditions	-2.06
SACOL1383	mscL	large conductance mechanosensitive channel protein		Adaptations to atypical conditions	-1.96



Locus ID	Gene <sup>a</sup>	Gene/Protein Name	Sub-functional Category	Fold Change
SACOL2024	agrD	accessory gene regulator protein D	Pathogenesis	-1.91
SACOL0610	sdrE	sdrE protein	Cell adhesion	-1.89
SACOL1410	femA	femA protein	Toxin production and resistance	-1.76
SACOL0765	sacS	sensor histidine kinase SacS	Pathogenesis	-1.69
SACOL0245	lytS	sensor histidine kinase LytS	Cell division	-1.66
SACOL1198	ftsA	cell division protein FtsA	Cell division	-1.53
<b>Upregulated Genes</b>				
SACOL1943	vraS	sensor histidine kinase VraS	Toxin production and resistance	5.22
SACOL1450	arlS	sensor histidine kinase ArlS	Pathogenesis	4.74
SACOL2353	icaR	transcriptional regulator IcaR	Pathogenesis	4.18
SACOL0672	sarA	staphylococcal accessory regulator A	Toxin production and resistance	4.11
SACOL2157	NA	drug resistance transporter, EmrB/QacA subfamily	Toxin production and resistance	3.75
SACOL1451	arlR	DNA-binding response regulator ArlR	Pathogenesis	3.61
SACOL2289	sarY	staphylococcal accessory regulator Y	Pathogenesis	3.21
SACOL2258	sarV	staphylococcal accessory regulator V	Toxin production and resistance	2.87
SACOL1942	vraR	DNA-binding response regulator VraR	Toxin production and resistance	2.61
SACOL1003	NA	negative regulator of competence MecA, putative	DNA transformation	2.01
SACOL0608	sdrC	sdrC protein	Cell adhesion	1.82
<b>Downregulated Genes</b>				
SACOL1801	NA	peptidase, M20/M25/M40 family	Degradation proteins, peptides, and glycopeptides	-3.45
SACOL0806	pepT	peptidase T	Degradation proteins, peptides, and glycopeptides	-2.89
SACOL2385	NA	heat shock protein, Hsp20 family	Protein folding and stabilization	-2.77
SACOL1946	NA	methionine aminopeptidase, type I	Protein modification and repair	-2.62
SACOL1777	NA	serine protease HtrA, putative	Degradation proteins, peptides, and glycopeptides	-2.42
SACOL0581	secE	preprotein translocase, SecE subunit	Protein and peptide secretion and trafficking	-2.13
SACOL1588	NA	proline dipeptidase	Degradation proteins, peptides, and glycopeptides	-2.12
SACOL2038	NA	metalloendopeptidase, putative, glycoprotease family	Degradation proteins, peptides, and glycopeptides	-2.06
<b>Upregulated Genes</b>				
SACOL0979	clpB	ATP-dependent Clp protease, ATP-binding subunit ClpB	Degradation proteins, peptides, and glycopeptides	9.42
SACOL1433	NA	peptidase, M20/M25/M40 family	Degradation proteins, peptides, and glycopeptides	8.7

**Protein fate**

Locus ID	Gene <sup>a</sup>	Gene/Protein Name	Sub-functional Category	Fold Change
SACOL2125	NA	peptidase, M20/M25/M40 family		7.13
SACOL0570	clpC	ATP-dependent Clp protease, ATP-binding subunit ClpC, authentic frameshift		5.92
SACOL1636	dnaJ	dnaJ protein	Protein folding and stabilization	4.94
SACOL0833	clpP	ATP-dependent Clp protease, proteolytic subunit ClpP	Degradation proteins, peptides, and glycopeptides	3.72
SACOL1638	grpE	heat shock protein GrpE	Protein folding and stabilization	3.5
SACOL0968	spsA	signal peptidase IA, inactive	Protein and peptide secretion and trafficking	2.98
SACOL1637	dnaK	dnaK protein	Protein folding and stabilization	2.67
SACOL2438	NA	endopeptidase, putative	Degradation proteins, peptides, and glycopeptides	2.46
SACOL1459	NA	peptide methionine sulfoxide reductase, degenerate	Protein modification and repair	2.38
SACOL2016	groEL	chaperonin, 60 kDa	Protein folding and stabilization	2.36
SACOL0556	NA	chaperonin, 33 kDa	Protein folding and stabilization	2.17
SACOL0844	secG	preprotein translocase, SecG subunit	Protein and peptide secretion and trafficking	2.13
SACOL0969	spsB	signal peptidase IB	Protein and peptide secretion and trafficking	2.02
SACOL2017	groES	chaperonin, 10 kDa	Protein folding and stabilization	1.81

<sup>a</sup>NA – Gene symbol not available