Curr Microbiol (2011) 63:354–359
DOI 10.1007/s00284-011-9984-3

Effect of Farnesol on Structure and Composition of *Staphylococcus epidermidis* Biofilm Matrix

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Received: 11 May 2011 / Accepted: 1 July 2011 / Published online: 29 July 2011

Abstract *Staphylococcus epidermidis* is the most frequent cause of nosocomial sepsis and catheter-related infections in which biofilm formation is considered to be one of the main virulence mechanisms. Moreover, their increased resistance to conventional antibiotic therapy enhances the need to develop new therapeutical agents. Farnesol, a natural sesquiterpenoid present in many essential oils, has been described as impairing bacterial growth. The aim of this study was to evaluate the effect of farnesol on the structure and composition of biofilm matrix of *S. epidermidis*. Biofilms formed in the presence of farnesol (300 μM) contained less biomass, and displayed notable changes in the composition of the biofilm matrix. Changes in the spacial structure were also verified by confocal scanning laser microscopy (CSLM). The results obtained by the quantification of extracellular polymers and by wheat germ agglutinin (WGA) fluorescent detection of glycoproteins containing $\beta(1\rightarrow4)$-*N*-acetyl-D-glucosamine support the hypothesis that farnesol causes disruption of the cytoplasmic membrane and consequently release of cellular content.

Introduction

Staphylococcus epidermidis is a coagulase-negative staphylococcus, which has emerged in the recent years as one of the most important nosocomial and opportunistic pathogens [17]. Owing to its ability to attach to polymeric surfaces, *S. epidermidis* is a common pathogen in chronic medical device-associated infections [9, 11]. Nowadays, it is established that the natural mode of bacterial life is in multicellular complexes referred to as biofilms [16]. A biofilm can be defined as a surface-attached agglomeration of cells that are embedded in a heterogeneous matrix secreted by themselves [19, 20].

The matrix is one of the most distinctive features of a microbial biofilm. The composition of the matrix varies according to the nature of the organisms present. In general, the *S. epidermidis* biofilm matrix comprises several extracellular polymeric substances, such as polysaccharides, proteins, considerable amounts of extracellular teichoic acids and also extracellular DNA [17]. Two of the best characterized matrix polysaccharides in bacteria are alginate produced by *Pseudomonas aeruginosa*, and poly β-1,6-linked *N*-acetylglucosamine (PNAG) secreted by *S. epidermidis*. Synthesis of both polysaccharides has been related to bacterial virulence [1]. Essential for *S. epidermidis* cell accumulation is the expression of PNAG, also called polysaccharide intercellular adhesin (PIA), which mediates cell-to-cell adhesion [11, 17].

Bacterial pathogens have evolved numerous defence mechanisms against antimicrobial agents, and resistance to older and newly developed drugs is on the rise. Moreover, microbial biofilms being responsible for a number of diseases of chronic nature demonstrate extremely high resistance to antibiotics and host defence mechanisms [12, 18]. Farnesol, a natural sesquiterpenoid present in many essential oils, has been described to have antibacterial effect. Some authors have shown the antimicrobial effect of farnesol on *S. epidermidis* [7] and *S. aureus* [10] and it has been hypothesized that its mode of action is by compromising cell membrane integrity. Relatively to *S. epidermidis*, Gomes

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et al. (2009) demonstrated a high antimicrobial effect of farnesol against planktonic cells and a similar or higher effect than vancomycin against biofilm cells [8]. Since farnesol was defined as a potential alternative to antibiotics, namely those traditionally used to treat *S. epidermidis*-related infections, such as vancomycin, it seems to be relevant to study and to try to understand the mechanism of action of this antimicrobial agent on *S. epidermidis*.

Because we are generally concerned with *S. epidermidis* biofilm control, the main aim of this study was to evaluate the effect of farnesol on biofilm structure and matrix composition.

Materials and Methods

Bacterial Strains and Growth Conditions

In this study, a good biofilm-producing strain was used, *S. epidermidis* 1457. This strain is a clinical isolate and has been previously used in mutagenesis studies to determine the basis of biofilm formation [13]. Tryptic soy broth (TSB) and tryptic soy agar (TSA) were prepared according to the manufacturer's instructions. Strains were grown as previously described [2]. In brief, the strain was inoculated into 15 ml of TSB from TSA plates not older than 2 days and grown for 18 (±2) h at 37°C in an orbital shaker at 130 rpm. Cells were harvested by centrifugation (for 10 min at 9,500×*g* and 4°C), and resuspended in TSB adjusted to an optical density (640 nm) equivalent to 1×10^9 cells ml^{-1} and then used in the subsequent assays. Each stock solution of farnesol was prepared in methanol. It was confirmed that methanol, at the concentration used, had no effect on the growth of the *S. epidermidis* strain studied.

Biofilm Matrix Extraction

Biofims were formed in six well tissue culture plates containing 4 ml of *S. epidermidis* cell suspension (1×10^6 cells ml^{-1}) in TSB supplemented with 0.25% glucose per well to promote biofilm formation. Plates were incubated at 37°C with orbital shaking at 130 rpm for 24 h. At the end, planktonic cells were removed carefully, and the biofilm was washed twice with 4 ml of 0.9% NaCl. The biofilms were incubated in fresh nutrient medium containing farnesol (0, 30 and 300 μM) (Sigma) for 24 h.

The extraction of the biofilm extracellular material was performed using the cation exchange Dowex resin (50 × 8, Na^+ form, 20–50 mesh aldrich-fluka 44445), according to the procedure described by Frølund et al. [6]. Before extraction, the Dowex resin was washed with the extraction buffer [2 mM Na_3PO_4; 4 mM NaH_2PO_4; 9 mM NaCl and 1 mM KCl; pH 7.0]. Then, the biofilms previously scrapped off the six well plates were washed with phosphate buffer (0.01 M; pH 7.0) and centrifuged for 5 min, at 9,000×*g*. The extraction was performed using 2 g of washed Dowex resin and 10 ml of extraction buffer per gram of biofilm and stirring for 2 h at 400 rpm and −4°C. The extracellular polymers (supernatant) were obtained by centrifugation at 9,000×*g* for 20 min.

Proteins and Polysaccharides Quantification

The total protein content extracted from the matrix was determined by the colorimetric Bicinchoninic acid assay (Bicinchoninic Acid Kit for Protein Determination, Sigma, USA) using bovine serum albumin (BSA) as standard. The extracted polysaccharides were quantified by the phenol–sulphuric acid method of Dubois et al. [5], using glucose as standard. All experiments were carried out in triplicate and repeated three times.

Biofilm Dry-weight Measurements

After 24 h of farnesol exposure, biofilm dry-weight was assessed. The biofilm cells were filtered through pre-weighed filters (0.22 μm) and washed three times with ultrapure sterilized water. Filters were dried at 80°C until constant weight and cell dry weight were determined. This step was repeated at least four times. Biofilm dry-weights were assessed by the difference between the weight of the membrane with and without biomass.

Confocal Scanning Laser Microscopy

CSLM was performed as described before [3]. Biofilms were formed as described previously. Thereafter, farnesol at concentrations of 0 and 300 μM was added to the pre-formed biofilms. Plates were incubated 24 h at 37°C and at 130 rpm. Following incubation, the biofilms were washed twice with 0.9% NaCl and stained with DAPI and WGA (conjugated with Alexa Fluor 488-Molecular Probes) for the fluorescent detection of glycoproteins containing $\beta(1\rightarrow4)$-*N*-acetyl-D-glucosamine, or with live/dead staining, to determine cell viability. For live/dead staining, a negative control was employed to determine the baseline threshold for dead cells, by killing the biofilm with 96% ethanol for 4 h. The plates were incubated for 20 min at room temperature in the dark. After staining, the biofilms were gently rinsed with 0.9% NaCl. The biofilm images (1024 × 1024) were acquired in an Olympus™ FluoView FV1000 confocal scanning laser microscope. Biofilms were observed using a 60× water-immersion objective (60×/1.2 W). For each condition, three independent biofilms were used, and in each biofilm four different regions

of the surface were analysed. For biofilm maximum thickness determination, 20 different regions per surface were analysed, by determining the first and last layers of the biofilm, and calculating the maximum thickness of each region.

Acquisition of Resistance/tolerance Determination

S. epidermidis were grown planktonically in TSB with sub-inhibitory concentration of farnesol (30 μM) for 12 h. Then, the cells were harvested by centrifugation and adjusted to a cellular concentration of $\sim 1 \times 10^6$ cells ml^{-1} in fresh medium with and without farnesol (at an inhibitory concentration of 100 μM) for 24 h at 37°C at 130 rpm. Afterwards, cellular activity was assessed by colony-forming units (CFU). The initial cells, harvested after being in contact with a sub-inhibitory concentration of farnesol (30 μM), were again subjected to that sub-inhibitory concentration of farnesol and this process was repeated for five consecutive days. Controls were cells not exposed to farnesol.

All the experiments were carried out in triplicate and repeated three times.

Statistical Analysis

The data from all assays were compared using one-way analysis of variance (ANOVA) by applying Tukey's and Bonferroni tests with all calculations carried out using SPSS software (Statistical Package for the Social Sciences). Differences achieving a confidence level of 95% were considered significant.

Results

Fig. 1a presents the quantification of polysaccharides and proteins in the biofilm matrix of *S. epidermidis* 1457 strain. According to the results of this study, after 24 h of farnesol (300 μM) exposure, there was an increase in the exopolymers concentration present in the matrix of the biofilm ($P < 0.05$). On the other hand, the sub-inhibitory concentration of farnesol tested (30 μM) appears to inhibit the formation of biofilm matrix as there was a reduction in the amount of existing exopolymers in the matrix ($P < 0.05$). Farnesol at 300 μM caused a slight reduction on total biomass of biofilms of *S. epidermidis* 1457 strain (Fig. 1b).

WGA was employed to detect the presence of PNAG/PIA [3] because this lectin binds to the biofilm matrix of *S. epidermidis* [14], because of its ability to recognize the *N*-acetylglucosamine component of PNAG/PIA antigen, although it may recognize other components, such as peptidoglycan and teichoic acid, which also contain glucosamine and form part of biofilm matrix [4].

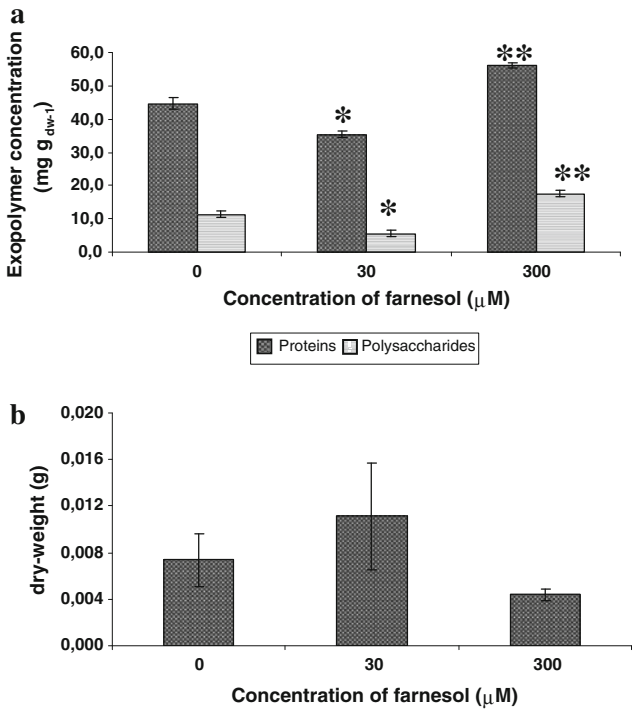

Fig. 1 Concentration of polysaccharides (glucose as standard) and proteins (BSA as standard) extracted (mg g_{dw-1}) by Dowex resin method from *S. epidermidis* strain 1457 biofilm matrix (**a**) and biofilm biomass expressed by dry weight (**b**). *Error bars* represent standard deviation. *Exopolymers concentration decreased significantly after treatment with 30 μM farnesol compared with non-treated biofilms ($P < 0.05$); **Concentration of exopolymers after treatment with 300 μM of farnesol significantly increased compared with non-treated biofilms ($P < 0.05$)

CSLM images showed that *S. epidermidis* 1457 strain formed a thick biofilm when grown in the absence of farnesol having a noticeable amount of PNAG/PIA. After addition of farnesol, a significant destruction of biofilm structure (Fig. 2a) and a clear reduction of biofilm thickness (Fig. 3) were observed. Furthermore, most cells inside the biofilm were either dead or with damaged cell wall, as determined by live/dead staining (Fig. 2b).

Although the mechanism of action of farnesol is not yet understood, it appears to have an antimicrobial effect against *S. epidermidis* cells [7]. It was previously shown that sometimes biofilm bacteria can be induced to increase their resistance to antimicrobials by continuing exposure to sub-inhibitory concentrations of antibiotics [3]. Therefore, it is important to study the ability of cells to acquire tolerance/resistance to this agent. For this purpose, planktonic cells were treated with a sub-inhibitory concentration of farnesol over several generations to determine whether after contact with this compound, the cells acquire tolerance/resistance. The results obtained in this study are shown in Fig. 4.

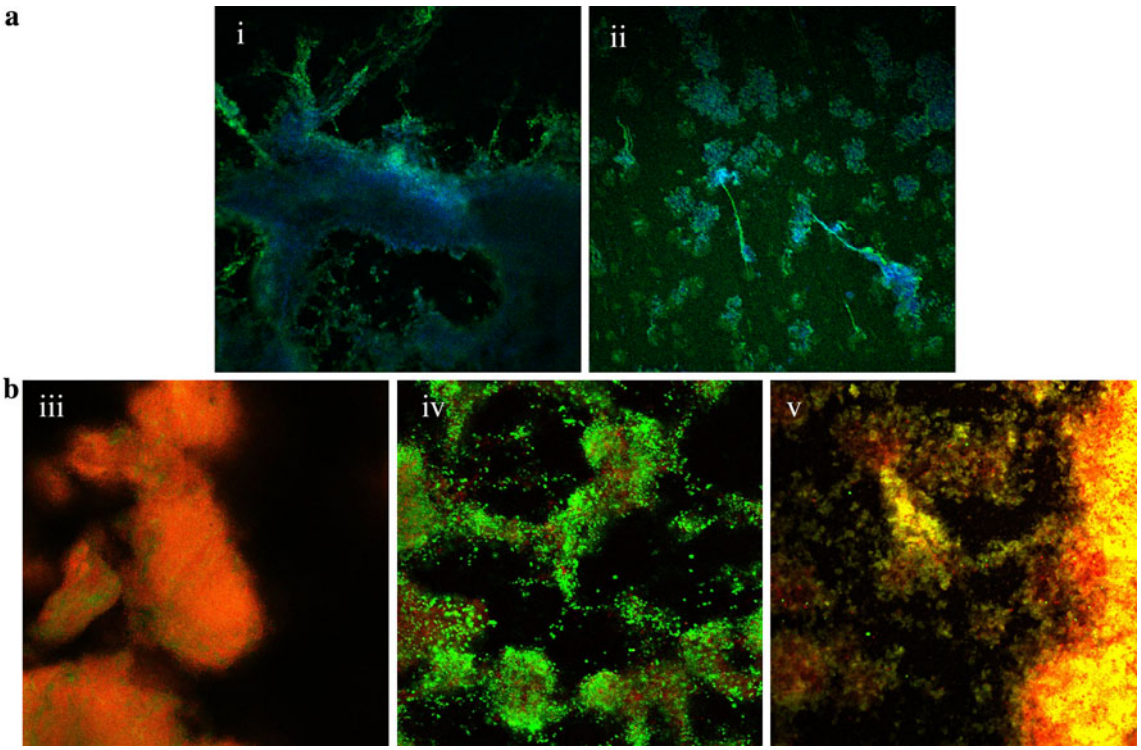

Fig. 2 **a** CLSM images of 24-h biofilm following 24-h exposure to 0 μM (*i*) and 300 μM (*ii*) farnesol, stained with DAPI and WGA. **b** Biofilms stained with live/dead: (*iii*) left image is a negative control (killed biofilm bacteria killed with 96% ethanol), (*iv*) biofilm following 24-h exposure to 0 μM farnesol and (*v*) biofilm exposed for 24-h to 300 μM farnesol

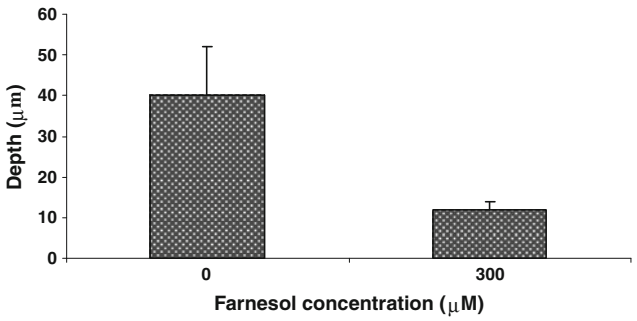

Fig. 3 Biofilm maximum depth average obtained by CSLM for biofilms without exposure to farnesol and after 24-h exposure to 300 μM of farnesol. *Statistically different from control (untreated cells) ($P < 0.05$)

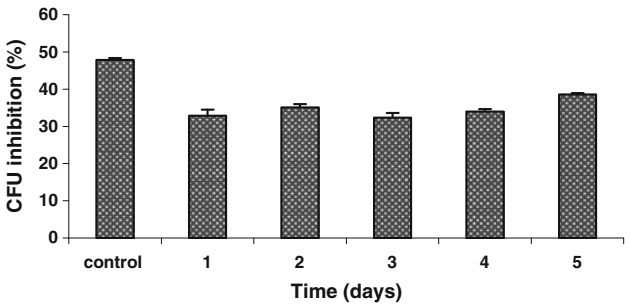

Fig. 4 Percentage of CFU inhibition by farnesol against planktonic *S. epidermidis* cells (strain 1457). Control corresponds to cells not exposed to sub-inhibitory concentrations of farnesol. *Error bars* represent standard deviation

The results of CFU determination evidenced an acquisition of tolerance to farnesol by the cells after being exposed to farnesol at a concentration of 30 μM. However, over time, no resistance to farnesol was observed.

Discussion

Nowadays, *S. epidermidis* ranks first amongst the causative agents of nosocomial infections and represents the most common cause of infections on indwelling medical devices [15]. Simultaneously, the resistance to antibiotics has become an important problem in *S. epidermidis* infections. In this context, the interest in studying the antimicrobial activity of potential alternatives to antibiotics has increased in recent years. In a previous study, the effect of farnesol against biofilm cells of six clinical isolates of *S. epidermidis* strains was assessed after 24 h of exposure. The strains assayed were 1457, 9142, IE186, IE75, IE214 and LE7 [8], and no significant difference was observed in the effect of farnesol on biofilm cells of the different strains

(some results not shown). However, as determined by Sousa et al. [17], *S. epidermidis* 1457 strain is the one, which has the highest level of extracellular polysaccharides content and, consequently, the strain that formed thicker biofilms. Taking this into consideration, we selected this strain as the most extreme example to evaluate the role of farnesol in biofilm structure and matrix composition.

First, the polysaccharide and protein biofilm matrix content, as well as the total biomass of biofilm were quantified. The results of this study showed an increase in proteins and polysaccharides per gram dry weight of biofilm after treatment with farnesol at 300 μM. This may be derived from the bursting of the cells and consequent release of cellular content. This fact can also be due to an overexpression of some *S. epidermidis* virulence genes responsible for the production of PNAG/PIA and other exopolymers, which can be a protective mechanism triggered by cells under stress. On the other hand, there is a slight decrease in the amount of polysaccharides and proteins in the extracellular matrix after exposure to 30 μM of farnesol. This sub-inhibitory concentration has no effect either on cell metabolic activity and consequently in cell replication or in the total biofilm biomass (Fig. 1b) ($P > 0.05$) [7]. Thus, although this concentration was not significantly inhibitory against *S. epidermidis* biofilm, it can possibly decrease the matrix development and therefore the biofilm formation over time.

Many *S. epidermidis* strains produce exopolymers, namely poly-gama-glutanic acid and a PNAG homopolymer, also named PIA, which surrounds and connects *S. epidermidis* cells inside biofilms [15]. In general, *S. epidermidis* exopolymers protect the cells from antibody recognition and consequently protect the bacterium from important mechanisms of innate host defence. Relative to PNAG/PIA, in addition to its role as part of the extracellular biofilm matrix, it has been found to protect *S. epidermidis* from neutrophil killing, complement deposition, immunoglobulins and antimicrobial peptides (AMPs) [4, 15]. Moreover, our immune system may have evolved to react less strongly to prevalent colonising bacteria, hampering *S. epidermidis* biofilm eradication.

The presence of PNAG/PIA in *S. epidermidis* biofilm was detected by WGA binding [3]. WGA is a carbohydrate-binding protein of approx. 36 kDa, which selectively recognizes sialic acid and *N*-acetylglucosaminyl sugar residues which are predominantly found in biofilm matrix and namely in PNAG/PIA molecules. Farnesol at 300 μM promoted a modification in biofilm structure and a decrease in biofilm thickness. The decrease in biofilm thickness could either be a result of biofilm bacteria death or biofilm bacteria dispersion. To clarify this point, biofilms were stained with live/dead, and as indicated by these results the observed biofilm reduction was in fact mainly due to cell death (Fig. 2b). As we have previously shown *S. epidermidis* planktonic cells after 12-h exposure to 300 μM farnesol have an average reduction of about 4 log. Therefore, any cells released from 24-h-treated biofilms are most likely killed when assuming a planktonic lifestyle.

To test the potential antimicrobial action of farnesol against *S. epidermidis*, it is crucial to determine the cells ability to acquire tolerance/resistance. For this purpose, *S. epidermidis* cells were exposed to sub-inhibitory and inhibitory concentrations of farnesol.

Staphylococcus epidermidis 1457 seems to be capable of rapid adaptation after an initial contact with farnesol but this effect was not a progressive mechanism. Therefore, it can be concluded that this is a reversible mechanism and therefore, a case of tolerance and not resistance. Moreover, resistance is translated by an increased survival of individuals which are immune to the effects of the antibacterial agent, whose offsprings then inherit the resistance creating a new population of resistant bacteria. The results of this study demonstrated that over cell generations there is no decrease in the susceptibility of farnesol.

In conclusion, the results obtained by the quantification of extracellular polymers and by WGA fluorescent detection of PNAG/PIA support the hypothesis that farnesol causes disruption of the cytoplasmic membrane and consequently release of cellular content. In addition to cell death, farnesol seems to destroy the biofilm structure reducing its biomass. This general promotion of biofilm weakness may be a potential help for the human immune system to eradicate the focus of *S. epidermidis* infection.

Acknowledgment Fernanda Gomes and Pilar Teixeira fully acknowledge the financial support of Fundação para a Ciência e Tecnologia (FCT) through the grants SFRH/BD/32126/2006 and SFRH/BPD/26803/2006, respectively.

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