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Farnesol induces cell detachment from established *S. epidermidis* biofilms

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Abstract

Antibiotic resistance is a serious problem in *S. epidermidis* infections since many clinical isolates of this organism are resistant up to eight different antibiotics.The increased resistance to conventional antibiotic therapy has lead to the search for new antimicrobial therapeutic agents. Farnesol, an essential oil found in many plants, has been shown to be active against *S. epidermidis*. Using a typecontrol strain we recently described that while farnesol was not efficient at killing biofilm bacteria, a strong reduction on biofilm biomass was detected, and we hypothesize that farnesol could, somehow, induce biofilm detachment. In this report, to test our hypothesis we used 36 representative clinical strains of *S. epidermidis* from different geographic locations and characterized them in terms of genetic variability by MLST and SSCmec. Strains were tested for biofilm formation and the presence of *ica*, *bhp* and *aap* genes was determined. Stronger biofilms had always the presence of ica operon but often co-harbored *bhp* and *aap* genes. Farnesol was then used in biofilm forming strains and biofilm detachment was detected in half of the strains tested. Furthermore, we also showed that farnesol inability to kill biofilm bacteria was not the result of the biofilm structure but was related to high cell density. Our results demonstrate, for the first time, that the biomass reduction previously found by us, and many other groups, is the result not of cell killing but instead is the result of biofilm detachment.

Keywords: *S. epidermidis*, biofilm detachment, clinical strains

Introduction

Staphylococcus epidermidis is a Gram-positive bacterium that normally colonizes the human skin and mucous membranes. Previously regarded as an innocuous commensal microorganism it is now seen as an important opportunistic pathogen, becoming,in the past few decades, the most frequent causative agent of nosocomial infections. This is mainly due to the increasing use of medical devices, allowing for biofilm formation in such surfaces [1]. *S. epidermidis* biofilm-related infections normally begin with the introduction of bacteria from the skin of the patient or health care personnel during device insertion. While these infections rarely lead to mortality, they are associated with increased patient morbidity [1].An important aspect of the biofilm-related infections is their economic burden on the public health system at an annual cost of over 2 billion Dollar in the US alone [2].

Microbial biofilms are communities of bacteria that live adhered to a surface and are surrounded by an extracellular polymeric matrix, in an increased antibiotic resistance and tolerance to the immune system[3]. Multiple mechanisms have been proposed for the high resistance of bacterial biofilms to antibiotics, including 1) the low diffusivity of antibiotics through the matrix, 2) the inactivation of the antibiotics by matrix components, 3) the presence of a sub-population of bacteria, known as persisters, that are unaffected by antibiotics, or 4) the heterogeneous nature of the biofilm composition and tridimensional structure [4].These mechanisms only partially explain the increased resistance and probably this phenotype is the result of more than one specific mechanism.

Increased bacterial resistance toward antibiotics has led to the search for new antimicrobial therapeutic agents such as essential oils from plants. Farnesol is a naturally-occurring sesquiterpene that was originally isolated from essential oils found in many plants [5]. Farnesol has also been found to be produced by *Candida spp,* being involved in quorum sensing [6]. Of high importance is the fact that farnesol has been shown to have antimicrobial potential against several bacteria, including *S. epidermidis*[7].However, the mechanism of action of farnesol is not yet fully understood, but it seems to be related to cell membrane integrity [8].

We recently described a bacterial strain where farnesol had no detectable antibiotic effect but strongly reduced biofilm biomass. We hypothesized that farnesol could be inducing biofilm detachment[9]. In this manuscript we tested this hypothesis and assayed the effect of farnesol in biofilm-forming clinical isolates of *S. epidermidis,* representing a wide diversity in terms of genetic background, geographic and clinical origins.

Materials and Methods

Bacterial strains

Two well known biofilm-forming strains were selected to be used as control strains, based on our previous work:*S. epidermidis* 9142 biofilm biomass is reduced by farnesol while *S. epidermidis* 1457 biofilms shows no reduction[7].Furthermore, we also used 25 clinical strains previously characterized by several different molecular typing techniques, namely staphylococcal chromosome cassette *mec* (SCC*mec*) and multilocus sequence typing (MLST), were selected from a total of 217 nosocomial isolates collected between 1996 and 2001 in 17 different countries, from disease (107 isolates) and from carriage (87 isolates). Isolates were selected in order to include the highest diversity as possible in terms of genetic backgrounds, geographic and clinical origins. Finally a subset of 9 clinical strains isolated from indwelling devices in Boston, MA, USA, were also included. All strains are listed in the results section.

Cell density and cell metabolism effect on farnesol antimicrobial activity

Tryptic soy broth (TSB, Oxford, UK, 1 mL) was inoculated with one single colony of each *S. epidermidis* control strain, in a 10 mL sterile test tube and incubated at 37°C in a shaker at 120 rpm for 24 ± 2 h. Each bacterial suspension was gently sonicated on ice, at 8Wfor 10 s, with the sonicator tip placed at the air/liquid interface (Ultrasonic Processor, Cole-Parmer, USA). This treatment did not reduce cell cultivability. To test farnesol on stationary-phase planktonic cells, serial 10-fold dilutions were performed in 2 mL of fresh TSB supplemented with 300 µM of 96% pure trans-trans-farnesol from Sigma ((*E,E*)-3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol, *trans,trans*-3,7,11-trimethyl-2,6,10-dodecatrien-1-ol), a concentration previously optimized, and needed to affect *S. epidermidis* biofilms [7]. A control was used where no farnesol was added. Each cell concentration was incubated for 4 hat 120 rpm at 37 ºC. Colony forming units (CFU) were determined by the standard plating method, using Tryptic Soy Agar (TSA) plates. To test farnesol on exponential-phase planktonic cells, serial 10-fold dilutions of inocula were performed in 2 mL of fresh TSB without farnesol. Each cell concentration was pre-incubated for 8 h at 120 rpm at 37 ºC before 300 µM farnesol was added to the medium, except to the control. After adding farnesol, cells were allowed to incubate for additional 4 h in the same conditions, before determining CFUs. These experiments where repeated three to five times with duplicates.

Biofilm quantification

Biofilms were quantified using 3 different approaches. TSB was inoculated with one single colony of each *S. epidermidis* control strain, in a 10 mL sterile tube, and incubated at 37°C in a shaker at 120 rpm for 24 ± 2 h. Then a 1:200 dilution was performed in fresh TSB supplemented with 1% (w/v) of glucose (TSBG), and incubated at 37°C in a shaker at 120 rpm in 96 well culture plates (Orange Scientific, Braine-l'Alleud, Belgium) for 24 h. Biofilms were then washed twice with 0.9% NaCl and fresh TSBG was added with or without 300 µM of farnesol and allowed to incubate in the same conditions for further

24 h. For biofilm biomass determination, the standard crystal violet staining method was used as described elsewhere [10]. To determine cell viability, biofilms were washed twice with 0.9% NaCl and resuspended in 0.9% NaCl followed by gentle sonication as described above. CFUs were determined by the standard plating method, using TSA plates. To determine the percentage of growth of bacteria inside the biofilm after 48 h, we quantified the relative population density of biofilms and the respective suspension formed during the last 24 h of growth, on each 96 well, as described before [11]. These experiments were repeated three to five times with 8 replicates.

Molecular characterization of the clinical strains

All strains were characterized by multilocus sequence typing (MLST)following the scheme proposed by Thomas *et al.*[12]. The MLST data wereanalysed using the goeBURST algorithm (http://goeBURST.phylowiz.net).This analysis was performed on March 21st, 2012. Isolates were considered as belonging to the same clonal complex (CC) if sharing six out of seven loci.The SCC*mec* type was determined by the combination of the class of *mec* complex and the type of *ccr* complex. SCC*mec* was considered non-typable when either *mec* complex or *ccr* complex, or both, were non-typable by the methods used or when the isolate carried more than one *ccr* type. SCC*mec* was considered to be new if a new combination of *mec* complex and *ccr* complex was found. The presence of the genes associated to biofilm formation, namely *icaA*, *aap* and *bhp* was detected by PCR, using DyNAzyme II PCR mix (Finnzymes, Vantaa, Finland), in the following thermal conditions: initial denaturation at 94ºC for 5 min followed by 35 cycles of 94ºC at 30 s, 54ºC at 30 s and 72ºC at 45 s. A final extension step was performed for 10 min at 72 ºC. Negative results were re-checked with a second set of primers. Oligonucleotide primers were designed using the Primer3 software, having *S. epidermidis* RP62A genome as template: *icaA* (Fw - tgcactcaatgagggaatca, Rv- tcaggcactaacatccagca; amplicon size of 417), *aap* (Fw - gctctcataacgccacttgc, Rv - ggacagccacctggtacaac; amplicon size of 617), bhp (Fw - tggactcgtagcttcgtcct, Rv - tctgcagatacccagacaacc; amplicon size of 213).For biofilm biomass determination, the standard crystal violet staining method was used [10].

Statistical analysis

All the assays were compared using the paired sample t-test, using SPSS. All tests were performed with a confidence level of 95%.

Results

Effect of farnesol on established biofilms

To better understand the effect of farnesol on biofilm physiology, we characterized three different parameters on the biofilm formation ability of *S. epidermidis* exposed to farnesol: 1) biofilms were quantified regarding biomass accumulation, 2) viable cell concentration and also by the 3) percentage of bacteria that grew in the biofilm mode.*S. epidermidis* 9142 (farnesol susceptible) and *S. epidermidis* 1457 (farnesol tolerant) biofilms were formed and exposed to 300 μM of farnesol. Biofilms were initially grown for 24 h, after which the medium was removed and replaced by fresh TSBG or TSBG supplemented with 300 μM of farnesol. As can be seen in Figure 1 only strain 9142 was significantly affected by farnesol (paired samples t-test, $p<0.05$). While there was a significant reduction in the biofilm biomass (paired samples t-test, $p<0.05$), the total number of viable cells was not affected. Interestingly, in the presence of farnesol, there was a significant (paired-samples t-test, $p<0.05$) increase of 44.1% in the number of cells living outside the *S. epidermidis* 9142 biofilm.

The effect of cell density and metabolic activity on farnesol activity against planktonic cells

To further understand the action of farnesol on planktonic cells, populations of bacteria at different cell concentrations and in different growth phases were exposed to 300 μM of farnesol for 4 h, after which viable bacteria were determined. The killing rate was defined as the logarithmic difference between

viable bacteria after the 4 h treatment and the viable bacteria before the treatment with farnesol. The data shown in Table 1 indicate that farnesol had no effect on stationary-phase populations equal or above 10^8 CFU/mL. However, in diluted cell populations, approximately 90% of bacteria were killed during the 4 h test. In log phase, at a cell density of 10^9 and 10^8 cells/mL (resulting from the initial inocula of 10^7 and 10^6 CFU/mL) farnesol showed a bacteriostatic effect, reducing cell growth, but not killing bacteria. Similarly to the stationary-phase planktonic populations, at cell densities bellow 10^7 CFU/mL, farnesol killed around 90% of bacteria.

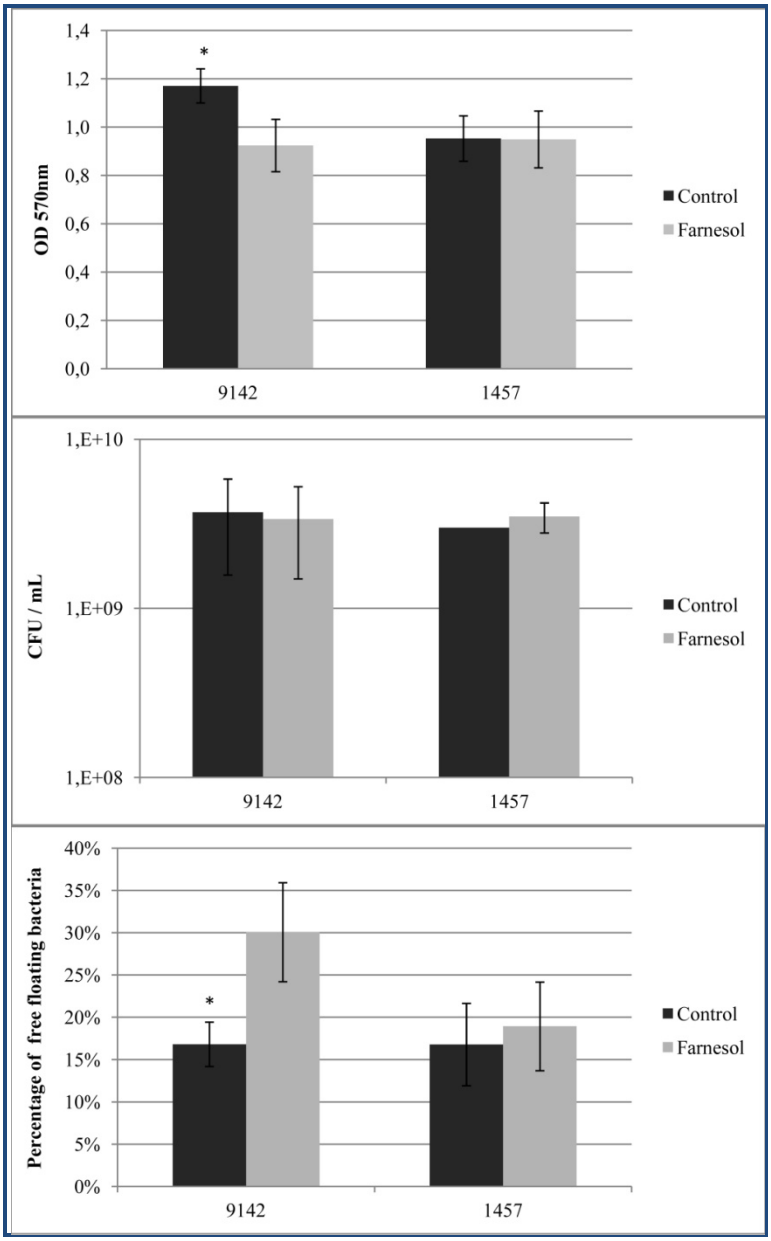

Figure 1 | Effect of farnesol on *S. epidermidis* biofilms. Bacteria were allowed to form biofilms for 24 h, after which fresh medium was added with or without 300 μM of farnesol and allowed to grow for further 24 h. Top: crystal violet staining; middle: CFUs determination; bottom: percentage of planktonic cells living outside the biofilms.* significant difference, paired samples t-student, $p<0.05$.

Table 1 | Effect of farnesol exposure on planktonic cells in stationary or exponential phase at different concentrations, expressed as the logarithmic variation of cell number during the 4 h exposure time to farnesol. Stationary planktonic cells were grown for 24 h in TSB, and then diluted down in fresh TSB and allowed to grow for 4 h in the presence of farnesol. Log phase cells were grown for 24 h, and then diluted in fresh TSB after which they were grown for another 8 h before adding farnesol. After farnesol exposure, cells were allowed to grow for more 4 h. *significant bactericidal effect or **significant bacteriostatic effect (paired samples t-student, $p<0.05$).

	Stationary phase		Log phase	
Inoculum cell density	**control**	**farnesol**	**control**	**farnesol**
10^9 / mL	0.27 ± 0.13	0.14 ± 0.13	N/A	N/A
10^8 / mL	0.24 ± 0.26	0.02 ± 0.30	N/A	N/A
10^7 / mL	0.13 ± 0.26*	-1.12 ± 0.41*	0.85 ± 0.10**	0.03 ± 0.07**
10^6/ mL	0.75 ± 0.56*	-1.42 ± 0.76*	1.56 ± 0.27**	0.29 ± 0.27**
10^5 / mL	0.93 ± 0.13*	-1.01 ± 0.47*	1.50 ± 0.06*	-1.07 ± 0.07*
10^4 / mL	0.63 ± 0.38*	-1.50 ± 0.39*	1.16 ± 0.08*	-0.61 ± 0.09*

N/A – not applied

Farnesol induced cell release from biofilms of clinical strains

The data indicate that farnesol induces strain-dependent cell detachment from established biofilm. To understand whether the reported different bacterial responses to farnesol were associated with different strain genetic backgrounds, we test the effect of farnesol in a collection of well characterized nosocomial *S. epidermidis* clinical isolates. Some strains were previously characterized regarding genetic typing [13] while others were only analysed for biofilm formation ability [14]. For this study all strains were screened by MLST, SSCmec, the presence of biofilm formation associated genes (*icaA*, *aap*, *bhp*) and the ability to form biofilms (Table 2).Of the 36 strains used in this study, 20 were biofilm positive (55.6%) and *icaA* was detected in 19 strains (52.7% incidence), *aap* in 20 strains (55.6% incidence) and *bhp* in 13 strains (36.1 % incidence). Farnesol-induced cell detachment was detected in 10 of the 20 biofilm-forming strains (50% incidence). No correlation was found between the effect of farnesol and a specific genetic background.

Discussion

S. epidermidis currently ranks the first among the causative agents of biofilm-related nosocomial infections[1]. Due to the high antibiotic resistance found in these microorganisms, the search for new antimicrobial agents has increased in recent years[15]. Farnesol is one of the compounds that has been tested for antimicrobial potential in several bacterial species. On an early report, we demonstrated that while a concentration as low as 30 µM of farnesol was able to kill actively growing planktonic cells, only concentrations as high as 300 µM of farnesol was able to reduce established biofilm biomass [7]. The observed decrease in biofilm thickness could be a result of bacterial death or bacterial dispersion from the biofilm. To address this issue we analysed 3 complementary parameters of biofilm physiology and demonstrated that while biofilm biomass could be reduced, resulting on an increased concentration of bacteria living as planktonic cells, total number of cultivable bacteria was not affected.

While the exact mechanism of action of farnesol is yet unkown, Inoue *et al*. demonstrated that farnesol antimicrobial mechanism has been linked to cell membrane integrity [8]. Furthermore, we have also shown that farnesol has a somewhat similar effect as vancomycin [9], which is known to be less effective in non-growing cells and high density populations, such as biofilms. To better understand the inability of farnesol to kill biofilm bacteria, the effect of cell metabolism and cell density was addressed. As many

Table 2 | Phenotypic and molecular characterization of the *S. epidermidis* strains used, and farnesol-mediated cell release.

Strain	SCC*mec* typing	ST	CC	Biofilm genes	Biofilm formation	Farnesol induced cell release
1457	MSSE	86	2	*ica, aap, bhp*	+++	-
9142	II	10	2	*ica, aap, bhp*	+++	+
COB17	33	33	33	*n/d*	-	n/a
CV45	IV	79	2	*ica, bap*	-	n/a
DEN110	IV	68	66	*aap*	-	n/a
DEN116	I	42	2	*n/d*	-	n/a
DEN120	A/C	40	2	*n/d*	-	n/a
DEN185	IV	21	2	*aap, bhp*	++	+
DEN19	IV	1	2	*aap*	-	n/a
DEN69	V	56	S56	*ica*	+++	+
DEN94	IV	49	247	*n/d*	-	n/a
FJ6	MSSE	10	2	*ica, aap bhp*	+	-
GRE26	IV	11	11	*n/d*	-	n/a
HUR51	B/3	47	33	*n/d*	-	n/a
ICE102	IV	52	2	*aap, bhp*	++	-
ICE192	IV	5	2	*aap, bhp*	-	n/a
ICE20	IV	89	2	*ica, aap*	++	-
ICE21	I	36	2	*ica, aap*	++	+
ICE24	IV	38	2	*aap*	-	n/a
ICE5	IV	23	2	*ica, bhp*	++	+
ICE9	III	6	2	*ica, bhp*	+++	-
IE186	IV	367*	S367	*ica, aap*	+++	-
IE214	NT	10*	2	*ica, aap*	++	+
IE75	IV	1	2	*ica, bhp*	+	+
ITL34	IV	66	66	*n/d*	-	n/a
JI6	III	366*	2	*ica, aap, bhp*	+++	+
LE7	III	9	2	*ica, aap*	+	-
M129	III	366	2	*ica, aap*	++	-
M187	IV	367	S367	*ica, bhp*	++	-
MCO150	IV	46	2	*aap, bhp*	-	n/a
MEX37	II	71	11	*n/d*	-	n/a
MEX60	NT/2	61	2	*Ica*	++	+
PE9	II	10*	2	*ica, aap bhp*	+++	-
PLN64	NT/2	64	247	*n/d*	-	n/a
TAW113	MS	85	2	*aap*	++	+
URU23	IV	86	2	*ica, aap*	+++	-

*inferred due to similarity of PFGE macrorestriction pattern. n/a – non applied; n/d – none detected)

other antibiotics that target cell-wall synthesis, such as vancomycin, the cell-density and cell physiology have been widely described in the literature [16]. Similar to vancomycin, our results confirm that farnesol is also cell-density dependent with reduced reducing bactericidal activity on cell densities above 10E8 CFU/mL. Interestingly, contrary to vancomycin, farnesol seemed not to be cell-physiology dependent, since the same effect was found on stationary or log-phase grown cells.

In order to analyze whether a genetic background could be associated with the reported strain to strain variable responses to farnesol, we determined farnesol induced biofilm detachment using a collection of well characterized nosocomial *S. epidermidis* isolates. Interestingly all the strongest biofilm-forming strains presented at least the *icaA* gene. A similar result was described by Rodhe *et al.* in a collection of 51 clinical isolates of *S. epidermidis*[17]. Despite no correlation between the effect of farnesol and a specific genetic background, farnesol was able to detach cells from the biofilm of nine strains tested belonging to clonal complex 2 (CC2), which is the main clonal lineage in hospitals worldwide. The results suggest that other phenomena independent of the genetic background, such as the overall metabolism or the type of biofilm formed may influence the outcome of farnesol in clinical *S. epidermidis* isolates. Nevertheless it was observed activity against *S. epidermidis* of highly clinical and epidemiological relevance which clearly substantiate its use in the future.

Our previous findings regarding the role of farnesol as an adjuvant in antimicrobial chemotherapy [18] can now be better explained. We hypothesized that despite the inability of farnesol to kill the bacteria inside biofilms, by inducing, via a yet unknown mechanism, cell detachment from biofilms, the suspended bacteria will be potentially more susceptible to antibiotic attack, taking into consideration that no diffusion barrier would be present in such cases. While we did not address this issue, it would be interesting to determine the role of farnesol over longer periods of time, in order to access its effect of mature biofilms. Furthermore, to better understand the molecular mechanisms underlying farnesol mode of action, it would be important to test different concentrations of farnesol, including sub-MIC concentrations.

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