RESEARCH LETTER

Impact of oleic acid (*cis* -9-octadecenoic acid) on bacterial viability and biofilm production in *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is responsible for a broad variety of chronic infections. Most *S. aureus* clinical isolates show the capacity to adhere to abiotic surfaces and to develop biofilms. Because *S. aureus* growing in a biofilm is highly refractory to treatment, inhibition of biofilm formation represents a major therapeutic objective. We evaluated the effects of oleic acid on primary adhesion and biofilm production in eight genotypically different *S. aureus* strains as well as in the biofilm-negative *Staphylococcus carnosus* strain TM300. Oleic acid inhibited primary adhesion but increased biofilm production in every *S. aureus* strain tested. *Staphylococcus aureus* strain UAMS-1 was then selected as a model organism for studying the mechanisms triggered by oleic acid on the formation of a biofilm *in vitro*. Oleic acid inhibited the primary adhesion of UAMS-1 dose dependently with an IC_{50} around 0.016%. The adherent bacterial population decreased proportionally with increasing concentrations of oleic acid whereas an opposite effect was observed on the planktonic population. Overall, the total bacterial counts remained stable. Macroscopic detachments and clumps were visible from the adherent bacterial population. In the presence of oleic acid, the expression of *sigB*, a gene potentially involved in bacterial survival through an effect on fatty acid composition, was not induced. Our results suggest a natural protective effect of oleic acid against primary adhesion.

Introduction

Most pathogens involved in human infections are able to produce biofilms (Costerton *et al.*, 1999). The chemical nature of bacterial biofilms is either polysaccharidic or proteic. It constitutes a matrix enclosing bacteria following their attachment to a surface (Costerton *et al.*, 1978). Extracellular DNA may also be necessary for biofilm formation (Whitchurch *et al.*, 2002; Rice *et al.*, 2007). The adherent bacteria display gene patterns that differ profoundly from their planktonic counterparts (Sauer *et al.*, 2002; Beenken *et al.*, 2004). *Staphylococcus aureus* biofilms have been isolated and characterized on a plethora of different medical devices, causing major medical problems usually unsolved even after adapted and prolonged antibiotic treatments (Donlan & Costerton, 2002; Hall-Stoodley *et al.*, 2004). Biofilm-related infections usually require removal of the implanted material to avoid a persistent infection (Costerton *et al.*, 1999). Furthermore, *in vivo* detection of a biofilm is challenging and remains particularly difficult (Hall-Stoodley *et al.*, 2004; Trampuz *et al.*, 2007). Development of modified surfaces or screening for substances able to inhibit the formation of biofilm is an active field of research (Rodrigues *et al.*, 2006; Zhang *et al.*, 2006; Donelli *et al.*, 2007). Oleic acid has been reported to be the predominant bactericidal unsaturated fatty acid naturally present in staphylococcal abscesses and on the skin surface (Speert *et al.*, 1979; Dye & Kapral, 1981). The production of carotenoids in *S. aureus* has been found to confer resistance against oleic acid (Xiong & Kapral, 1992). Interestingly, production of staphyloxanthin, the major stationary-phase carotenoid (Marshall & Wilmoth, 1981),

is dependent on the activated form of the alternative sigma factor (*sigB*) (Katzif *et al.*, 2005), which in turn regulates the alkaline shock protein 23 (*asp23*) (Gertz *et al.*, 1999). In *S. aureus*, *sigB* is involved in the biogenesis of carotenoids (Kullik *et al.*, 1998; Giachino *et al.*, 2001; Katzif *et al.*, 2005). Oleic acid was reported previously to induce an *in vitro* 'slime production' under hypobaric oxygen conditions, as demonstrated in the case of a strain isolated from a sputum sample (Campbell *et al.*, 1986). Here, we investigated the effects of oleic acid on primary adhesion and biofilm production in eight unrelated strains of *S. aureus*. The viability of the planktonic or the adherent populations, as well as mRNA levels of *asp23* and *sigB* were evaluated under oleic acid stress in a model organism.

Materials and methods

Strains

Staphylococcus aureus strain UAMS-1 was isolated from a patient suffering from osteomyelitis and was shown previously to produce a biofilm in an experimental model of osteomyelitis (Smeltzer *et al.*, 1997) as well as *in vitro* (Beenken *et al.*, 2004). This strain and its *sigB* mutant have been described previously (Beenken *et al.*, 2004; Cassat *et al.*, 2006). The other strains used in this study are listed in Table 1. The identification of *S. aureus* Coch was based on Pastorex agglutination (Bio-Rad) and DNAse tests, and was further confirmed using a real-time PCR amplification procedure (Francois *et al.*, 2003).

Media and growth conditions

Staphylococcus aureus strains were grown in trypticase soy broth (TSB, Becton Dickinson, Le Pont de Claix, France) supplemented with 1% glucose. Oleic acid (free acid cell culture tested, *cis*-9-Octadecenoic acid, Sigma-Aldrich, Basel, Switzerland) was emulsified with TSBgluc (TSBglucOleic) by overnight agitation at 220 r.p.m. in a Lab-Shaker at 37 °C. Biofilm development was performed in TSBgluc (or TSBglucOleic emulsions) with 20 μL of overnight cultures mL^{-1} of medium. Bacterial colonies were counted on Mueller–Hinton agar plates (MHA, Bio-Rad, Marnes-La-Coquette, France).

Crystal violet biofilm assay

Biofilm staining assays were performed as described previously (O'toole & Kolter, 1998). Briefly, after bacterial growth, microtiter plates (Multiwell™ 6 well, Becton Dickinson) were washed twice with phosphate-buffered saline (PBS), fixed for 20 min at 80 °C and stained for 10 min with 1% (w/v) crystal violet solution freshly diluted twofold in 1% (v/v) ethanol/distilled water. Plates were then washed with water and photographed. The crystal violet was dissolved in dimethyl sulfoxide (DMSO) for 1 h before $OD_{600\,nm}$ measurements (Tu Quoc *et al.*, 2007). Half inhibitory concentration IC_{50} of biofilm inhibition was determined by curve fitting in Microcal™ Origin® (version 6.1, Northampton, MA).

Bacterial sample preparation and counting methods

UAMS-1 planktonic bacterial population was recovered from the supernatant, whereas the adherent bacterial population was detached from the surface with a scraper (Cell Scraper, BD Falcon™, Becton Dickinson) after addition of 0.5 M EDTA. Each sample was sonicated (Branson 2200, Geneva, Switzerland) and microscopically observed to exclude aggregation before bacterial measurements. $OD_{540\,nm}$ was used for indirect cell counting. Direct counting was performed in Neubauer chambers following the instructions of Marienfeld (http://www.marienfeld-superior.com/pgr06_info_e.htm). CFU determination was performed on MHA using a Countermat Flash colony counter (IUL, RB Scientific, Southampton, England).

Live/dead staining

A circular glass coverslip (diameter: 25 mm) was deposited on the microtiter plate before initiating bacterial culture. Live/dead staining was performed on the UAMS-1 planktonic population after centrifugation, allowing replacement

Table 1. Bacterial strains used in this study

Strain	Description	Sources/references
UAMS-1	Osteomyelitis isolate producing a proteinaceous and extracellular DNA-related biofilm	Gillaspy *et al.* (1995) Rice *et al.* (2007)
Newman	Sequenced strain	Baba *et al.* (2008)
N315	Sequenced strain	Kuroda *et al.* (2001)
Coch	PIA-positive strain isolated from a cochlear implant.	This laboratory
SA113	Restriction-deficient laboratory strain ATCC35556 producing an *ica*-dependent biofilm	Kristian *et al.* (2004)
S30	Pediatric isolate and strong PIA-dependent biofilm producer	Tu Quoc *et al.* (2007)
ISP479r	Laboratory strain derivative of RN8325 with restored *rsbU*. Strong biofilm producer	Pattee (1981), Toledo-Arana *et al.* (2005)
SA564	Recent human isolate	Somerville *et al.* (2002)
S. carnosus TM300	Biofilm-negative *icaADBC* and PIA-negative *S. carnosus* strain	Wagner *et al.* (1998)

of the supernatant by the staining mixture. The L7012 live/dead® BacLight™ bacterial viability kit (Invitrogen) was used to stain bacteria according to the manufacturer's protocol. Bacteria were then observed under an Axioskop 2 microscope using different filter combinations allowing detection of PI and SYTO 9. Pictures (resolution 2600 × 2600 pixels) were acquired with an Axiocam color camera (Zeiss).

Relative *sigB* and *asp23* mRNA quantification

Transcription of *S. aureus sigB* and *asp23* genes was monitored using quantitative real-time reverse-PCR (qPCR) from planktonic and adherent UAMS-1 (wt) and its Δ*sigB* mutant following the procedure described previously (Renzoni *et al.*, 2004; Garzoni *et al.*, 2007). qPCR was performed in a StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA) using the ThermoScript Reaction mixture (Invitrogen). The following oligonucleotides were designed using PRIMEREXPRESS software (Applied Biosystems). Forward 5′-TCGCGAACGA GAAATCATACAA, reverse 5′-ACCGATACGCTCACCTGT CTCT and the MGB probe 5′-TACGTTTATTGAGGGTTT-GAG (coupled with FAM and dark quencher) were used to quantify *sigB* gene expression. The transcript levels of *asp23* were determined as described previously (Renzoni *et al.*, 2004), except the forward primer 5′-GTTAAGCCACCTTT CATGTCTAAGATAC.

Results

Effect of oleic acid on *S. aureus* primary adhesion and biofilm formation

All tested strains were able to produce a biofilm in various amounts, except the nonbiofilm producer *Staphylococcus carnosus* strain TM300 that was used as a negative control (Fig. 1, column A). The presence of oleic acid during primary adhesion drastically affected biofilm production and resulted in the absence of crystal violet incorporation (Fig. 1, column B). The presence of oleic acid on an already formed biofilm showed a slight increase in crystal violet incorporation (Fig. 1, column C). These results were similar in the eight independent genetic backgrounds tested, whereas the absence of crystal violet incorporation was consistently observed for the biofilm-negative *S. carnosus* TM300.

Dose-dependent inhibition of primary adhesion in UAMS-1

Increasing oleic acid concentrations (ranging from 0.001% to 0.1%) led to a dose-dependent decrease of UAMS-1 biofilm detection. The curve fitted with a logistic equation revealed an IC50 of 0.016% (Fig. 2). The addition of 0.1% v/v oleic acid before primary adhesion and during bacterial growth precluded the formation of a detectable biofilm.

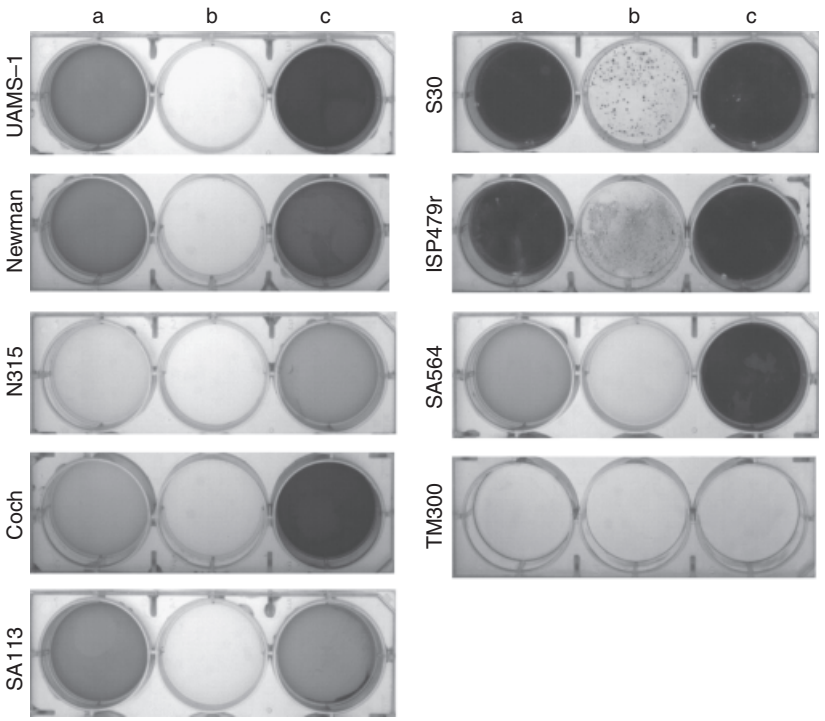

Fig. 1. Effect of oleic acid on primary adhesion and biofilm production in *Staphylococcus aureus*. Pictures of crystal violet staining assays used to quantify biofilms produced by eight genotypically different *S. aureus* strains as well as the biofilm-negative *Staphylococcus carnosus* strain TM300. Strains were incubated for 2 h on a first medium, which was replaced by a second bacterial-free medium for another 2 h. (a) Left wells show 4-h-old biofilms formed in TSBgluc without oleic acid. (b) Central wells show 4-h-old biofilms formed in TSBgluc in the presence of oleic acid. (c) Right wells show biofilm formation after primary adhesion for 2 h in TSBgluc, followed by a 2-h exposure to oleic acid. The oleic acid stress consisted of 0.1% v/v oleic acid emulsified in TSBgluc.

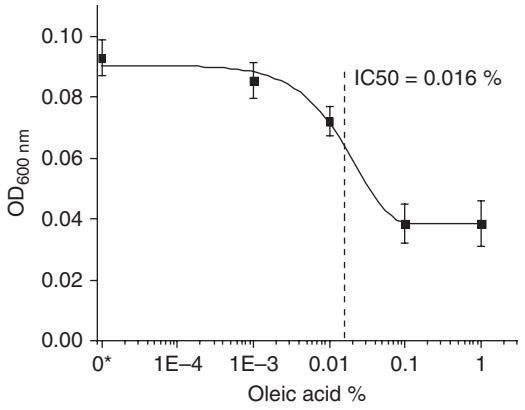

Fig. 2. Oleic acid inhibits primary adhesion of UAMS-1 dose-dependently. Spectrophotometric determination of crystal violet incorporated into a UAMS-1 biofilm after growth in the presence of different concentrations of oleic acid. Values are mean of three independent determination ± SEs. Exponential decay fit shows an IC50 value of 0.016% (dashed line). 0*; the 0% oleic acid concentration has been shifted artificially to 10^{-5}.

Effect of oleic acid on bacterial viability

The three different microbiological counting methods used in this study showed a dose-dependent decrease of the adherent population with increasing concentrations of oleic acid (Fig. 3a). An opposite effect was observed on planktonic cells but the total number of bacteria (i.e. adherent plus planktonic cells) remained stable across the different oleic acid concentrations and growth times tested in our study.

Live/dead fluorescence assay revealed that the proportion of living UAMS-1 cells remained stable under oleic acid conditions when grown as planktonic cells (Fig. 3b). In the planktonic population of the UAMS-1 *sigB* mutant, the proportion of living bacteria decreased from 83% to 14% in the presence of 0.1% oleic acid. In the adherent phenotype, both wild-type UAMS-1 and its *sigB* mutant revealed a marked decrease in viability in the presence of 0.1% oleic acid. Microscopic observations of adherent UAMS-1 revealed a viability of 81% in the absence of oleic acid and a drastic reduction to 19% in the presence of 0.1% oleic acid, whereas the *sigB* viability was 76% and 0%, respectively. Taken together, these results suggest a specific killing effect of oleic acid against all adherent bacteria but also against the UAMS-1 *sigB*-deficient mutant when grown in a planktonic phenotype.

Active SigB and *asp23* mRNA respective abundance under oleic acid stress conditions

UAMS-1 and its isogenic *sigB* mutant were plated on MHA to evaluate colony pigmentation. UAMS-1 clearly developed pigments visible through a yellowish coloration whereas *sigB* mutant colonies appeared white (not shown). Despite the presence of oleic acid, the relative transcript levels of *asp23* were similar in adherent and planktonic populations of UAMS-1 (not shown).

Discussion

Little is known about *S. aureus* biofilm formation in the presence of oleic acid, excluding an increased biofilm production reported under hypobaric oxygen conditions and in the presence of oleic acid (Campbell *et al.*, 1986). This lack of knowledge created the need for further test of oleic acid effects on UAMS-1 biofilm formation both under aerobic and under anaerobic conditions. In aerobic and anaerobic conditions, oleic acid stress led to the lack of detectable biofilm when added before primary bacterial adhesion. However, when oleic acid was added later, i.e. after primary adhesion, biofilm formation was found to have increased significantly under anaerobic conditions as compared with aerobic conditions. The previously described stimulating effect of oleic acid on *S. aureus* biofilm (Campbell *et al.*, 1986) could result from an ionic interaction of the positively charged polysaccharide intercellular adhesin (PIA) with the negatively charged oleic acid (Gotz, 2002) or from the specific response of an *S. aureus* strain to a peculiar chemical environment (Campbell *et al.*, 1986). Our *in vitro* studies show that oleic acid inhibited dose-dependently *S. aureus* UAMS-1 adhesion to polystyrene surfaces, resulting in a limited incorporation of crystal violet. However, once primary adhesion was developed, oleic acid stimulated biofilm formation. This effect was consistently observed across a panel of eight genotypically diverse *S. aureus* strains, ruling out a strain-specific effect.

Oleic acid is modifiable by a bacterial enzyme named fatty acid-modifying enzyme (FAME), ensuring bacterial survival in tissues (Mortensen *et al.*, 1992). *In vivo* FAME could esterify cholesterol with oleic acid, thus leading to a non-bactericidal product. There is no cholesterol in our assay and some strains of *S. aureus* did not produce FAME. Invasiveness defined by the authors as 'the ability either to survive within intraperitoneal abscesses or to multiply in the kidneys' was associated with FAME production in *S. aureus* strains P78, PG114, 18Z and TG, whereas strains 18Z-H, P78-22, 689 and 303 did not produce FAME; 5 other strains were not tested for FAME production (Mortensen *et al.*, 1992). The theoretical proteome of UAMS-1 (Hernandez *et al.*, in preparation) contains four putative esterases, but the FAME gene could not be identified.

Oleic acid has been reported previously as a killing agent for *S. aureus*, presumably through a mechanism involving bacterial lipids (Chamberlain *et al.*, 1991). Previous reports demonstrated that different staphylococci species are not capable of metabolizing oleic acid (James *et al.*, 2004) and

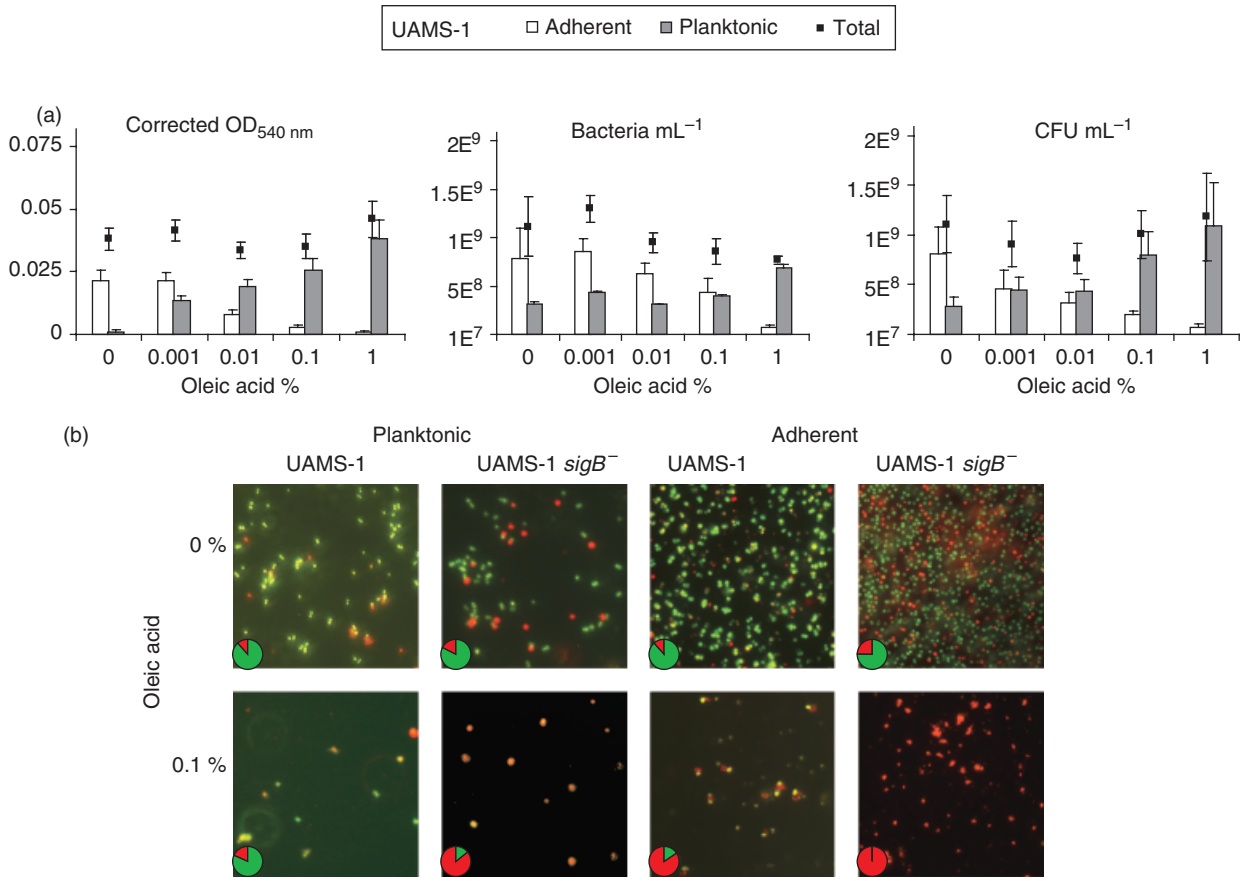

Fig. 3. Effect of oleic acid on planktonic and adherent bacterial cells. (a) Left, planktonic and adherent bacterial counts as measured by $OD_{540\,nm}$. Values are mean ± SEs of two independent determinations. Middle, direct counts of adherent and planktonic cells determined in a Neubauer chamber. Average values and SEs were calculated on six different squares for the dilutions 10e-3 and 10e-4. Right: CFUs on MHA Petri dishes were counted automatically using a Countermat Flash instrument. Average values and standard errors were calculated based on dilutions ranging from 10e-5 to 10e-7 on planktonic and adherent populations. The total bacterial count (i.e. adherent plus planktonic cells) is plotted as a square on each graph. b. Live/dead staining assay was performed on both adherent and planktonic UAMS-1 ± *sigB*. Pie charts represent the proportion of living bacteria in green and dead bacteria in red, as determined by counting bacteria on the live/dead staining images.

that oxidation compounds resulting from their oxidation are highly toxic for bacterial cells (Clarke *et al.*, 2007). This compound is present in abscesses and in exudates from cystic fibrosis patients at a concentration of *c.* 12 nmol mL^{-1} (Meyer *et al.*, 2000). This concentration appears to be lower than the IC_{50} determined in this study (*c.* 500 nmol mL^{-1}). Despite several *in vitro* studies that analyzed the effects of oleic acid on *S. aureus* (Chamberlain *et al.*, 1991; Xiong & Kapral, 1992; Xiong *et al.*, 1993), the potential of oleic acid as a bactericidal agent on *S. aureus* remains controversial. Owing to the plasticity of the *S. aureus* genome, which contributes to environmental adaptation, these observations are possibly strain dependent. Indeed, some reports suggest an adaptation of staphylococci to oleic acid (Speert *et al.*, 1979), whereas others report an *in vitro* bactericidal effect (Chamberlain *et al.*, 1991; Xiong & Kapral, 1992; Xiong *et al.*, 1993). The strains studied (18Z and 303) appeared to be most susceptible to oleic acid killing during the exponential phase of growth (Xiong *et al.*, 1993). The authors concluded that the *in vitro* killing property of oleic acid on *S. aureus* 18Z strain resulted from an increased membrane permeability and was indirectly correlated with the production of carotenoids (Chamberlain *et al.*, 1991). As carotenoids were shown to have a direct protective effect against oleic acid killing in *S. aureus* (Xiong & Kapral, 1992), this raises the question of a potential influence of oleic acid on *sigB* signaling.

In agreement with previous studies showing the effects of oleic acid on planktonic bacteria (Chamberlain *et al.*, 1991; Xiong & Kapral, 1992; Xiong *et al.*, 1993), our live/dead experiments performed with planktonic UAMS-1 and its Δ*sigB* mutant suggest a *sigB*-dependent resistance against oleic acid killing. However, the adherent bacterial population was affected by oleic acid independently of *sigB* expression as shown by the transcript levels of *asp*23 used to indirectly quantify the active form of SigB (Senn *et al.*,

2005). Thus, expression levels of *asp23* that are correlated to the abundance of protective compounds such as bacterial carotenoids were not induced by the presence of oleic acid during biofilm development in *S. aureus* strain UAMS-1.

During our biofilm development experiments in the presence of oleic acid, we observed the release of large bacterial clumps from already constituted biofilms. These clumps, containing mainly living bacteria, probably contributed to the overestimation of planktonic bacterial counts. The release of clumps was described previously during biofilm maturation (Stoodley *et al.*, 2001), probably creating free spaces that become parts of water channels contributing to the free circulation of metabolites in the biofilm (Stoodley *et al.*, 2002). The presence of free-floating bacterial cell clumps remains to be evaluated *in vivo* as it should be considered as a risk of systemic bacterial dissemination (Hall-Stoodley *et al.*, 2004).

In conclusion, identification or development of compounds able to inhibit biofilm formation or to initiate its release must be evaluated with the concern of bacterial viability. As suggested recently (Donelli, 2006), this issue appears to be crucial considering the release of viable clumps from biofilm-colonized surfaces and the risk of systemic bacterial dissemination. The different technical issues addressed in this study allowed testing potential inhibitors of surface-associated biofilm development and simultaneously evaluating detachment properties and impact on bacterial viability.

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