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Staphylococcus epidermidis biofilm quantification: Effect of different solvents and dyes

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ABSTRACT

Staphylococcus epidermidis biofilm formed in the presence of the solvents DMSO, ethanol or methanol was quantified using safranin or crystal violet staining protocols. We found that biofilm quantification was the most accurate when safranin protocol was applied. Moreover, both DMSO and ethanol stimulated biofilm formation.

1. Introduction

Staphylococcus epidermidis is known to produce biofilm on indwelling medical devices causing chronic infections. Hence, approaches to inhibit biofilm formation or to disperse formed biofilm have been proposed (Kostakioti et al., 2013). Many of the tested anti-biofilm compounds, however, are not water-soluble and must be dissolved with the help of solvents for in vitro tests. Dimethyl sulfoxide (DMSO) is commonly the solvent of choice because it is able to dissolve polar and apolar compounds. This solvent has been used when testing candidate compounds to inhibit biofilm formation by *Pseudomonas aeruginosa* (Bijtenhoorn et al., 2011), *Escherichia coli* (Begde et al., 2012), *S. aureus* (Fallarero et al., 2013) and *S. epidermidis* (Panmanee et al., 2013). DMSO has been used at concentrations ranging from 0.1% (to dissolve thiazolidione derivatives; Huang et al., 2012) to 5% (to dissolve tea tree oil; Karpanen et al., 2008) in tests against *S. epidermidis* biofilm. Panmanee et al. (2013) reported that 0.2% DMSO did not affect *S. epidermidis* biofilm analysis when applying crystal violet staining. Using this same dye, Lim et al. (2012) showed that *E. coli* biofilm formation was stimulated (increase of 140%) in response to an exposure to 4% DMSO. Ethanol is also applied as solvent in bacterial biofilm tests because of its ability to dissolve polar and apolar substances (Vestby et al., 2014). Chaieb et al. (2007) indicated that 2% ethanol induced *S. epidermidis* biofilm formation, and this stimulation was strain dependent, where increase in biofilm ranged from 10 to 75%. Only in few cases, was methanol used as a solvent (Reck et al., 2011), and no reports showing the interference of this solvent on bacterial biofilm formation are available. A simple assay is often used to quantify bacterial biofilm formation (Christensen et al., 1985) applying specific protocols for the dyes safranin and crystal violet. It is remarkable that most biofilm reports are based on crystal violet staining (Kaplan et al., 2004; Croes et al., 2009; Waldrop et al., 2014). Ayed et al. (2010) reported *S. epidermidis* ability to degrade crystal violet after 12 h incubation with this dye. However, it is unknown if crystal violet can be decolorized by *S. epidermidis* after a short-term incubation. Safranin staining is also used to quantify bacterial biofilm, but to a lesser extent (McKenney et al., 1998; Melchior et al., 2006). In the present study, we used a biofilm formation assay to compare the effect of different solvents (DMSO, ethanol and methanol) on *S. epidermidis* biofilm formation. Moreover, both crystal violet and safranin staining protocols were tested. To estimate the direct effect of *S. epidermidis* on both dyes, a direct decolorization test was performed.

S. epidermidis (ATCC 35984) was cultured in tryptic soy broth + 0.25% glucose (pH 7.0) (TSB^+) under aerobic conditions at 37 °C for 24 h before exposure to DMSO (≥99.5%), absolute ethanol or methanol (≥99.8%) (Merck KGaA, Darmstadt, Germany). Each solvent was separately diluted in TSB^+ to a work concentration of 2% before use. Safranin and crystal violet (Sigma-Aldrich, St. Louis, MO) were individually diluted with de-ionized water to a working concentration of 0.1%. For the exposure test, 100 μl of bacterial suspension (10^6 CFU/ml) was transferred to a U-bottomed 96-well microtiter polystyrene plate (Costar, Corning, NY, USA) containing different concentrations of DMSO, ethanol or methanol (0.0078–2%). Hence, the final tested concentrations of DMSO, ethanol or methanol were 0.0039–1%. There were six replicate wells per treatment group and wells with sterile TSB^+ alone served as

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blanks. The plates were incubated on a microplate shaker (Heidolph titramax 100) at 600 rpm, 37 °C for 24 h. Biofilm formation was quantified by safranin or crystal violet staining protocols. Regarding safranin, the supernatant from all wells was discarded and the biofilms adhering to the bottom of the wells were washed with de-ionized water three times, and then incubated with 0.1 M HCl for 1 h at room temperature (RT). After which, HCl was replaced by safranin (0.1% in water) and incubation was performed for 45 min at RT. Non-bound safranin was removed by rinsing the stained biofilm three times with de-ionized water, and thereafter incubation was performed in 125 µl 0.2 M N_aOH at 57 °C for 1 h. At the end of incubation, 100 µl of solution from each well was pipetted to a new flat-bottom 96-well microtiter polystyrene plate. The absorbance of each sample was determined at a wavelength of 540 nm using a microplate reader (3550-UV, Bio-Rad, Hercules, CA). Crystal violet staining was carried out as O'Toole (2011) reported before, with slight modifications. In brief, the adhering biofilms were washed with de-ionized water three times, and incubated with crystal violet (0.1% in water) for 15 min (RT). Non-bound crystal violet was removed by rinsing the biofilm three times with de-ionized water and dried out for 2 h (RT). Thereafter incubation was performed in 125 µl acetic acid (30% in water) (RT) for 15 min. At the end of incubation absorbance was measured at a wavelength of 540 nm. As described above, protocols used for safranin and crystal violet staining present some notable differences, e.g. time of incubation with dye (45 min for safranin vs. 15 min for crystal violet), elution (0.2 M NaOH for 1 h at 57 °C for safranin vs. 30% acetic acid for 15 min at RT for crystal violet) and presence (safranin) or absence (crystal violet) of an inactivation step before staining. Therefore, to determine if the variation in approaches used for the two methods were responsible for the discrepancy in sensitivities between the two dyes, we switched safranin and crystal violet staining protocols. Each test was repeated four times. Finally, to elucidate the possible decolorization effect of *S. epidermidis* (biofilm and planktonic) on crystal violet during short-term exposure, we performed a decolorization test as described by Parshetti et al. (2011). For biofilm, 200 µl of bacterial suspension (10^6 CFU/ml) was cultured in a 96-well plate, under aerobic conditions at 37 °C for 24 h. The supernatant from all wells was discarded and the biofilms adhering to the bottom of the wells were washed with de-ionized water three times, and then incubated with safranin (0.01% in water) or crystal violet (0.01% in water), both for 15 or 45 min (RT). Incubation was performed, either or not with previous inactivation with 0.1 M HCl (safranin) or 100% ethanol (crystal violet). We used 100% ethanol of inactivation (Christensen et al., 1985) because HCl was not suitable for crystal violet; neither was ethanol suitable for safranin. At the end of incubation, 100 µl of dye solution from each well was pipetted to a new flat-bottom 96-well microtiter polystyrene plate. Absorbance was measured at a wavelength of 540 nm. Stock dye solutions of 0.01% safranin or crystal violet served as control. For planktonic bacteria, one colony of *S. epidermidis* bacteria was cultured in 10 ml TSB^+ under aerobic conditions at 37 °C for 24 h. Supernatant (1 ml) was taken into a new Eppendorf tube and incubated with safranin and crystal violet at a final concentration of 0.01%. Incubation, with both dyes, was performed for 15 and 45 min (RT); TSB^+ alone and with dyes served as control. At the end of incubation, bacterial cells were separated by centrifugation at 5000 rpm for 20 min. 100 µl of dye supernatant from each tube was pipetted carefully to a new flat-bottom 96-well microtiter polystyrene plate. Absorbance was measured at a wavelength of 540 nm. Each test was repeated three times. Biofilm formation data were analyzed using a Kruskal–Wallis test followed by post hoc Dunn's multiple comparisons. Degradation data was evaluated using one-way ANOVA followed by Tukey multiple comparisons post hoc test. Differences were considered significant at P values <0.05. For all statistical analyses, GraphPad Prism version 6.04 was used.

S. epidermidis biofilm formation was stimulated ($P < 0.05$) when bacteria was exposed to 0.0078–1% DMSO (increase of 13.28 and 42.2%, respectively) by safranin staining and to 0.125–1% DMSO (increase in 12.87–24.35%, respectively) by crystal violet staining (Fig. 1, panel A). Likewise, based on both safranin and crystal violet staining, *S. epidermidis* exposure to 0.0625–1% ethanol boosted ($P < 0.05$) biofilm formation by 20.22–60.68% for safranin and 12.92–30.21% for crystal violet staining (Fig. 1, panel B). Lim et al. (2012) reported that much higher concentrations of DMSO (4%) and ethanol (2%) were necessary to stimulate biofilm formation (increase of 140% and 10%) by *E. coli*. According to these authors, *E. coli* is capable of adapting to DMSO and ethanol exposure by increasing their biofilm matrix via amyloid production, which is involved in bacterial attachment (Otzena and Nielsen, 2008). In the present study, using the same staining procedure, 0.125% DMSO was sufficient to stimulate *S. epidermidis* biofilm formation in 21.7%, and quantification of biofilm was more sensitive by safranin staining when compared to crystal violet. Although this increase in biofilm might not be biologically relevant, there is still a concern that choosing inaccurate solvents and staining techniques may provide misleading results. There was no significant change of *S. epidermidis* biofilm formation in any tested concentrations of methanol when compared with control (Fig. 1, panel C). Detailed information on the percentages of biofilm stimulation is given in Supplementary Table 1. As reported

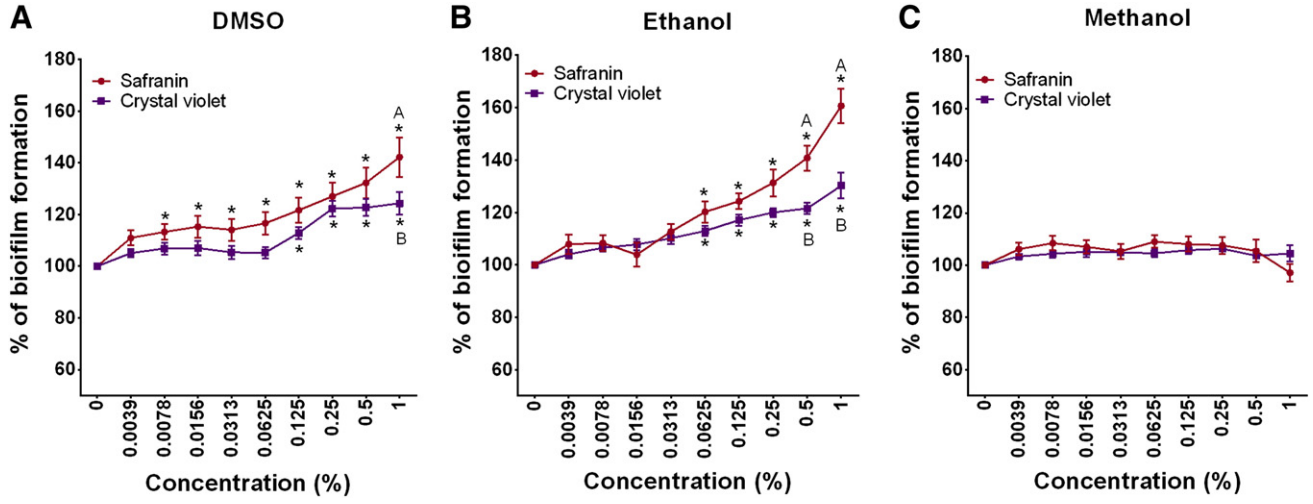

Fig. 1. Mean percentage (±SEM) of *S. epidermidis* (ATCC35984) biofilm formation after exposure to different concentrations (0.0039–1%) of DMSO (A), ethanol (B) and methanol (C). * indicates significant ($P < 0.05$) difference between treatments and control (0%).