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Note

Revisiting an agar-based plate method: What the static biofilm method can offer for biofilm research

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ABSTRACT

The development of biofilms in static plates was monitored. Glass coupons were placed on agar covered with filter paper, which was inoculated with suspended bacteria. The viable cell density, biofilms matrix and biomass were quantified. The method is excellent for adhesion and material studies, due to its simplicity and flexibility.

The unique properties of bacterial biofilms call for the development of reliable and specific research methods, different to the ones optimized for planktonic bacteria (Cos et al., 2010). Accordingly, various biofilm culturing methods have been developed, in which model biofilms grow under various fluid shear conditions in specific reactors. The choice of the biofilm reactor is based upon the research question under investigation (Buckingham-Meyer et al., 2007; Coenye and Nelis, 2010).

Biofilm formation in liquid cultures has been studied in detail, and it is thought to involve several distinctive phases. At the initial phase, planktonic bacteria are irreversibly attached to the surface. The attachment is followed by aggregation and proliferation of the cells. The production and accumulation of extracellular polymeric substances then result in the formation of a mature biofilm, where the cells are organized into a three-dimensional community surrounded by the biofilm matrix. At the final phase, cells return into a planktonic stage and detach from the biofilm (Dunne, 2002; Kiedrowski and Horswill, 2011; Otto, 2008).

Although bacterial biofilms may also form on surfaces that are not immersed in liquid, the solid-state growth methods are not as common as the well-studied liquid cultures. Among the reported solid-state methods are the colony biofilm model (Anderl et al., 2000) and the static biofilm method (Charaf et al., 1999). In the colony biofilm model, polycarbonate membrane filters are inoculated and regularly transferred onto fresh agar medium, while in the static biofilm method the biofilm is left to develop on a single filter-covered agar plate for the whole incubation period (Fig. 1). The static biofilm method has been shown to be useful in antimicrobial efficacy testing against *Pseudomonas aeruginosa* PAO1, ATCC 15442 and *Staphylococcus aureus* ATCC 6538 biofilms (Buckingham-Meyer et al., 2007; Charaf et al., 1999). However, the formation of the biofilms on static method plates has not been thoroughly characterized, nor its potential as a research choice fully explored.

Consequently, in this contribution we set off to gain a deeper understanding of the development of static method biofilms. For this purpose, the kinetics of biofilm development was studied for the Gram-positive *S. aureus* ATCC 25923, a model biofilm-forming organism. Furthermore, the results were compared with *S. aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 35984, and the Gram-negative *Escherichia coli* XL-1 Blue and *P. aeruginosa* ATCC 15442. In all cases, biofilms were grown exactly according to the previously described method (Buckingham-Meyer et al., 2007). Briefly, sterile borosilicate glass coupons (diameter 1.27 cm, height 0.4 cm, BioSurface Technologies Corporation) were placed on an agar plate covered with an inoculated filter paper (Whatman Qualitative Grade 2, 70-mm diameter, GE Healthcare). The purpose of the filter paper is to provide a barrier between the coupons and the agar surface that allows for diffusion of nutrients while

Abbreviations: CFU, colony forming unit; CV, crystal violet; TSB, tryptone soy broth; WGA, wheat germ agglutinin.

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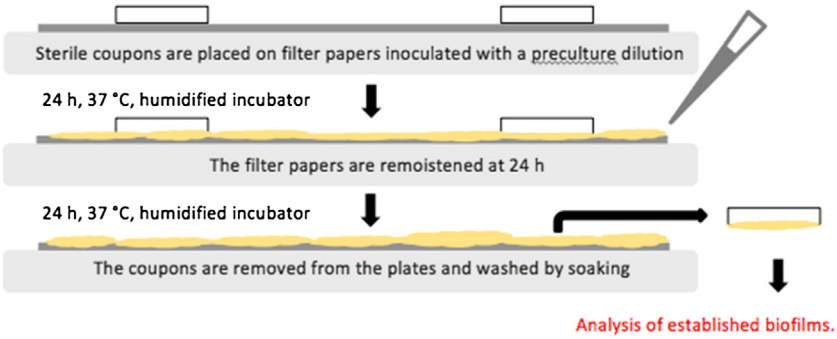

Fig. 1. An overall scheme of the main steps of the static biofilm method. The light gray boxes represent TSA or R2A (*P. aeruginosa*) plates, the gray lines above the plates represent inoculated filter papers, and the coupons on the filter papers are drawn as empty boxes. The biofilms are drawn in yellow. Only the original 48-h incubation period is shown here. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

preventing the agar from sticking to the coupon surface. The inoculum consisted of 1.5 mL of a 1:10 dilution of an 18–24 h old preculture in either tryptone soy broth (TSB, Sigma-Aldrich) or in a 100-fold diluted TSB (only for *P. aeruginosa* ATCC 15442). After 24 h of incubation in a humidified incubator at 37 °C, the filters were remoistened with 1.5 mL of 10-fold or 1000-fold (*P. aeruginosa* ATCC 15442) diluted TSB (Fig. 1, Buckingham-Meyer et al., 2007).

In this contribution, longer incubation periods for growing the biofilm were studied (1, 2, 4, 24, 32 or 48 h), and the coupons were analyzed by either viable cell counts or crystal violet (CV) staining of the total biomass. In the case of *S. aureus* ATCC 25923 the coupons were additionally analyzed by staining with a wheat germ agglutinin (WGA) Alexa Fluor 488 conjugate (Molecular Probes, Thermo Fisher Scientific). At the end of the selected incubation period, each coupon was rinsed by dipping it in TSB, and subsequently analyzed according to a specific protocol. Differences in the protocols were only owed to practical reasons, as the work with three of the strains (*S. aureus* ATCC 25923, *S. epidermidis* ATCC 35984 and *E. coli* XL-1 Blue) was performed in Finland while the other strains (*S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 15442) were studied in the United States.

For viable cell counting, the biofilms (except those formed by *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 15442) were removed and dispersed by a sonication-based method, simpler than the one originally reported (Buckingham-Meyer et al., 2007; Charaf et al., 1999), which was based on scraping biofilm off the surface. Coupons were fully immersed in 1 mL of 0.5% (wt./vol.) Tween 20 (Sigma-Aldrich) in TSB, followed by quick vigorous mixing and 5 min of sonication in a water bath sonicator (Sonorex Digitec, Bandelin) at 25 °C, 35 kHz. In the case of *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 15442 biofilms, a previously published protocol was followed (ASTM International, 2013). Briefly: each coupon was fully immersed in a conical vial containing 10 mL of buffered water (KH_2PO_4 0.3 mM, MgCl $6H_2O$ 2.0 mM; pH 7.2). The vial was vortexed (30 s) and sonicated (30 s, 45 kHz, 25 °C) in 2 cycles followed by one more vortexing step for 30 s. In all cases, dilution series of the resulting suspensions were prepared and plated for the determination of the colony forming units (CFUs). All plates were incubated at 37 °C for 24 h.

Alternatively, the washed coupons with intact biofilms were analyzed by staining with CV or the WGA–Alexa fluor conjugate. For all biofilms except those formed by *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 15442, the following modifications were made to a previously described CV protocol (Sandberg et al., 2008). The coupons with the intact biofilm side up were transferred to a 12-well plate, and gently covered with 70 μL of 2.3% (wt./vol.) CV solution (Sigma-Aldrich) for 5 min. This volume was enough to cover the surface of the glass coupon where the biofilm had formed. Washing was done three times with 3 mL of Milli-Q water, and the bound dye was dissolved in 1.5 mL of 96% (vol/vol) ethanol (1 h). In the case of the intact, washed *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 15442 biofilms, the staining was performed for 15 min in 5 mL of 0.5% (wt./vol.) CV (Acros) in Milli-Q water, followed by washing three times in 30 mL of sterile buffered water. The dye was dissolved in 5 mL of 95% (vol/vol) ethanol (10 min).

For WGA staining of the *S. aureus* ATCC 25923 biofilms, a previously described protocol (Skogman et al., 2012) was followed with minor modifications: the coupons were washed three times in a 12-well plate with 3 mL of PBS, and the dye was dissolved in 1 mL of 33% acetic acid.

The results revealed that for *S. aureus* ATCC 25923 there were no significant changes in the viable counts or in the amount of the attached biomass during the first 2 h in a humidified incubator. As expected, the initial attachment phase was followed by proliferation of the cells, characterized by a steady increase in the viable counts. Also, a prominent accumulation phase was observed as a time-shift in the biomass accumulation: the amount of the biomass attached to the coupons, measured by CV staining, continued to increase after 24 h, when the number of the CFUs had already reached a maximum

Table 1
The results of the viable counts, CV staining and WGA staining of *S. aureus* ATCC 25923 coupons incubated in a humidified vs. non-humidified incubator. In the latter case, the volume of the inoculum was increased from 1.5 mL to 2.25 mL. The WGA stain was quantified at $\lambda_{excitation} = 495$ nm and $\lambda_{emission} = 520$ nm. The average value of two biological replicates is presented with the standard deviation in parentheses. Samples obtained in each time point were compared in humidified vs. non-humidified conditions with an unpaired comparison t-test with Welch's correction ($p < 0.05$) (GraphPad Prism program, San Diego, USA).

Time (h)	Log (CFU/cm^2)		CV staining (A_{595})		WGA staining (fluorescence)	
	Humidified	No humidification	Humidified	No humidification	Humidified	No humidification
1	5.55 (±0.24)	5.84 (±0.06)	0.14 (±0.00)	0.14 (±0.01)	0.23 (±0.05)	0.44 (±0.08)
2	5.77 (±0.03)	5.94 (±0.02)	0.14 (±0.01)	0.14 (±0.00)	0.46 (±0.25)	0.68 (±0.25)
4	7.36 (±0.03)	7.38 (±0.22)	0.20 (±0.04)	0.17 (±0.00)	1.89 (±0.25)	2.49 (±0.88)
24	8.23 (±0.62)	7.61 (±0.18)	0.58 (±0.30)	0.94 (±0.27)	9.68 (±1.31)	9.17 (±2.24)
32	8.08 (±0.33)	7.75 (±0.09)	0.85 (±0.09)	1.16 (±0.35)	N.a.	N.a.
48	8.01 (±0.25)	7.49 (±0.30)	1.31 (±0.54)	1.74 (±0.48)	12.03 (±4.28)	11.83 (±1.77)

N.a. = not analyzed.