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**CHARACTERIZING THE *IN VITRO* BIOFILM PHENOTYPE OF *STAPHYLOCOCCUS*
EPIDERMIDIS ISOLATES FROM CENTRAL VENOUS CATHETERS**

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27

28 **Abbreviations¹**

29

¹ **Abbreviations:**

CVC: central venous catheter, CRBI: catheter-related bloodstream infection, MTP: microtiter plate, PIA: polysaccharide intercellular adhesion, ICU: intensive care unit

ABSTRACT

Central venous catheter (CVC)-related infections are commonly caused by *Staphylococcus epidermidis* that is able to form a biofilm on the catheter surface. Many studies involving biofilm formation by *Staphylococcus* have been published each adopting an own *in vitro* model. Since the capacity to form a biofilm depends on multiple environmental factors, direct comparison of results obtained in different studies remains challenging. This study characterized the phenotype (strong versus weak biofilm-producers) of *S. epidermidis* from CVCs in four different *in vitro* biofilm models, covering differences in material type (glass versus polymer) and nutrient presentation (static versus continuous flow). A good correlation in phenotype was obtained between glass and polymeric surfaces independent of nutrient flow, with 85% correspondence under static growth conditions and 80% under dynamic conditions. A 80% correspondence between static and dynamic conditions on polymeric surfaces could be demonstrated as well. Incubation time had a significant influence on the biofilm phenotype with only 55% correspondence between the dynamic models at different incubation times (48h versus 17h). Screening for the presence of biofilm-related genes only revealed that *ica A* was correlated with biofilm formation under static but not under dynamic conditions. In conclusion, this study highlights that a high level of standardization is necessary to interpret and compare results of different *in vitro* biofilm models.

1. INTRODUCTION

Staphylococcus species are a common cause of device-related infections due to their biofilm-forming properties, especially in immunocompromised individuals (1). Biofilms are generally described as structured microbial communities attached to a surface and encapsulated in a matrix of self-produced extracellular polymeric substances. Not all bacteria in a biofilm express the same genes or show the same growth rate due to nutrient and oxygen gradients from the surface to the deeper layers of the biofilm (2, 3). Bacteria living in a biofilm community are also phenotypically different from their free-living planktonic counterparts and have been shown to express different genes compared to planktonic bacteria (4). The biofilm matrix protects the microbes within the slime layer against both conventional antimicrobials and the host defence mechanisms, which often impede their treatment resulting in chronic infections (4, 5).

Biofilm formation on medical devices is a particular problem in intensive care units (ICU) where catheters are frequently used to monitor critically ill patients. Coagulase-negative *Staphylococci*, particularly *S. epidermidis*, are the main colonizers of central venous catheters (CVCs) (1) and hence an important cause of catheter-related bloodstream infections (CRBIs) (6, 7). CRBIs are associated with increased morbidity, mortality and healthcare costs (7-9) and biofilm formation is believed to be one of the major bacterial virulence factors with a key role in disease pathogenesis (10).

Appropriate laboratory models are essential to expand our knowledge on bacterial biofilms and different *in vitro* models are currently available, including static and dynamic models (reviewed by Coenye et al., 2010) (11). In static models, biofilms are grown in the absence of any fluid flow, while in dynamic models growth medium is added to the reactor and waste products are removed by a constant fluid flow. Many of these laboratory models are used to evaluate phenotypic biofilm characteristics of *Staphylococcus* clinical isolates. Microtiter (MTP)-based static models are most

frequently used (12-18). Other studies adopt dynamic models that may be more relevant to study catheter-related infections because of the impact of shear forces on biofilm formation (19, 20). Since the experimental conditions used for the determination of the biofilm phenotype of *Staphylococcus* isolates vary significantly between studies, results are often difficult to compare (21-24).

Biofilms develop in a number of successive stages, starting with the attachment of bacteria to a surface. CVCs become rapidly coated with human serum proteins such as fibronectin, collagen, fibrinogen and vitronectin. Adhesion of *S. epidermidis* to these serum proteins is mediated by 'microbial surface components recognizing adhesive matrix molecules' (MSCRAMMs) (10, 16, 25). *S. epidermidis* contains several MSCRAMMs including the fibrinogen binding protein (Fbe) (26) and the fibronectin binding protein (Embp) (27). The bifunctional adhesin/autolysin Atle not only binds vitronectin but is also responsible for the release of eDNA, which is a matrix component (28-30). Following surface attachment, matrix production is initiated followed by biofilm expansion. The polysaccharide intercellular adhesion (PIA), synthesized by the gene products of the *ica* operon, is an important component of the *S. epidermidis* matrix. Besides PIA and eDNA, the matrix also consists of proteins like the biofilm accumulation protein homologue Bhp (Bap in *S. aureus*) (31) and the accumulation associated protein Aap (16, 32). The *ica* operon is present in a high percentage of clinical isolates; however, some PIA-negative *Staphylococci* are able to form a biofilm as well. The latter suggests that some isolates possess other mechanisms for biofilm formation. Therefore, it is hypothesized that proteinaceous factors like Embp, Aap and Bhp can replace PIA during biofilm formation both in PIA-positive and -negative strains (33, 34).

Using four different *in vitro* models, we determined the biofilm phenotype of *S. epidermidis* isolates cultured from CVCs obtained from an intensive care unit (ICU)

102 setting and of *Staphylococcus* lab strains. Two static models, a standard 96-well
103 microtiterplate model and a model using Lab-Tek™, and two dynamic models, a
104 catheter model and the commercial BioFlux™, were included. Under identical cultivation
105 conditions, we evaluated how the physical environment may affect biofilm formation.
106 Both the influence of static versus dynamic flow conditions and polymeric versus glass
107 surfaces on the biofilm-forming process were investigated. To correlate the biofilm
108 phenotype with genotypic characteristics, the impact of the presence of biofilm-related
109 genes *ica A*, *fbe*, *bhp*, *atlE*, *embp* and *aap* on biofilm phenotype was scrutinized.

110

111 2. MATERIAL AND METHODS

112 2.1 Bacterial strains and culture conditions

113 Clinical *Staphylococcus* strains were isolated from CVCs from different patients of the
114 ICU of the Antwerp University Hospital (Clinical Trial Registration: 12/12/112). All CVCs
115 had been removed as part of the routine procedure to remove foreign material when the
116 patient experienced clinical and laboratory signs of infection. At the bedside, the last 2
117 cm of the tip of the catheter was separated from the bulk of the catheter by cutting with
118 sterile scissors and transported into a sterile recipient. The catheter tip was then placed
119 in Brain Heart Infusion broth (BHI, BD, Belgium) and vortexed to remove the bacteria
120 from the catheter. Next, 100 µl was spread on a blood agar (Oxoid™, UK) and agars
121 were incubated up to 48h. Initially, isolates were identified using standard
122 microbiological techniques including Gram-staining, catalase and coagulase tests and
123 their identity was confirmed using MALDI-TOF (Bruker Daltonics, Germany). If
124 Staphylococcal species were cultured in the routine lab, the catheter tip was further
125 used for this study. In order to include only isolates underscoring the pathogenicity of
126 biofilms (the susceptibility of inert catheter material to microbial colonization and
127 dissemination through the circulation from and to the biofilm) those isolates only

detected identically both on the catheter and in the blood cultures of the same patient were incorporated in this study (n=40). To assess the degree of biofilm formation, 8 reference strains were included. *S. aureus* ATCC 6538, *S. aureus* ATCC 29213, *S. aureus* ATCC 25923, *S. aureus* NCTC 8325, *S. epidermidis* 1457, and *S. epidermidis* RP62A are known biofilm-forming strains while *S. aureus* ATCC 5374 and *S. epidermidis* ATCC 12228 are consistently negative for biofilm formation (35-40). Bacteria were subcultured in tryptic soy broth (TSB; Lab M, UK) at 37°C and stored in aliquots of 1 ml at -80°C in 10% glycerol.

2.2 Biofilm assays

Cryostabilates were slowly thawed and plated on Colombia blood agar (Oxoid, Hampshire, England) supplemented with 5% horse blood. Next, agar plates were aerobically incubated overnight at 37°C and one colony was added to TSB and adjusted to a concentration of 10^6 CFU/ml.

2.2.1 Static biofilm assays

In the static assays, biofilms were cultivated in polystyrene microtiter plates (MTPs, Greiner Bio-One, USA) or in Lab-Tek™ chamber slides with a glass surface. The start inoculum was added to the wells and incubated for 48h. Every 24h, the growth medium was changed and non-inoculated TSB was included as a blank. The total biofilm biomass was quantified using crystal violet as described previously by Toté *et al.*, 2009 (41). In brief, after discarding the growth medium, the wells were rinsed twice with PBS and the remaining biofilm was fixed with methanol (Merck Millipore, Germany). Next, a 0.005% crystal violet solution (Merck Millipore, Germany) was added to each well and the excess stain was removed by a washing step under running tap water. Plates were air dried and the bound crystal violet was resolubilised in 33% glacial acetic acid

154 (Sigma-aldrich, USA). After 15 minutes, the optical density (OD) was measured at 570
155 nm (Labsystems Multiskan MCC/340 microplate reader, Fisher Scientific, USA). Clinical
156 isolates were evaluated in three independent tests (MTP 10 replicates/test and Lab-
157 Tek™ 4 replicates/test).

158

159 2.2.2. Dynamic biofilm assays

160 Biofilms were cultivated using two dynamic models with a different abiotic surface. The
161 BioFlux™ (Fluxion) (42) is equipped with a glass capillary, while the catheter model is
162 made of polyurethane. To grow biofilms in the BioFlux™ microfluidic system, the
163 bacterial suspension was pushed into the flow channel from the output wells as
164 described in Vanhommerig *et al.*, 2014 (36) and statically incubated for 30 min to allow
165 the bacteria to adhere. Thereafter, a flow was initiated and the growth medium was
166 pumped through the flow cell at 0.5 dyne/cm² (~ 0.06 ml/h). To avoid clogging problems,
167 biofilms were cultivated for 17h, stained with SYTO9 (life technologies™, Belgium).
168 Syto9 (12.5 µM) was flown through the channels for 10 min and stained biofilms were
169 visualized using fluorescence microscopy (Axio observer Z1, Carl Zeiss) (Figure 1).
170 Biomass was quantified as the surface coverage of the microfluidic channel.

171

172

173 **Figure 1: Fluorescence microscopy image of a bioflux™ biofilm.** The clinical strain *S. epidermidis*
174 1307 1356 was stained with 12.5 µM Syto9 after 17h incubation.

175

176 In the second model, biofilms were grown in polyurethane tubings (3 French, Instech
177 Solomon, USA). The catheters were locked with the bacterial suspension during 30 min
178 to allow the bacteria to attach. Next, a TSB flow was generated and medium was
179 pumped through the catheter with a flow rate of 1 ml/h. Biofilms were cultivated at 37°C

for 17 or 48h and stained with crystal violet to quantify the total biomass. In the dynamic assays, clinical isolates were characterized in at least two independent tests (1 replicate/test).

2.3 Detection of biofilms-associated genes by PCR

Bacterial cultures were grown overnight in TSB, pelleted by centrifugation and incubated with 20 mg/ml lysozyme in 20 mM Tris-HCl (pH 8.0); 2 mM EDTA and 1.2% Triton for 30 min at 37°C. DNA was extracted using the QIAmp DNA mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

Specific primers were obtained with the primer blast tool (43) based on sequences retrieved from the Nucleotide sequence database of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/nucleotide>). Synthesis of the primers was performed by Integrated DNA Technologies (Leuven, Belgium) and the primer sequences are presented in Table 1.

Table 1: Primers used in this study. Primers were obtained with the primer blast tool (43) or based on reference (17)* (FP: forward primer, RP: reverse primer; bp: base pairs, T_m : annealing temperature).

Gene	Primers (5'→3')	T_m (°C)	Amplicon (bp)	Accession number
<i>ica A</i>	FP: ATT GAT GAC GAT GCG CCT TTT RP: GCC CAT CGA ACC CTT TGT TT	60	389	DQ149646.1
<i>aap</i> *	FP: AAA CGG TGG TAT CTT ACG TGA A RP: CAA TGT TGC ACC ATC TAA ATC AGC	58	466	AJ249487.1
<i>bhp</i>	FP: TGC TTG CTG CTA CTG AGG AC RP: CGT TGG TGT GAT AGA GCC GA	60	533	AY028618.1
<i>atle</i> *	FP: CAA CTG CTC AAC CGA GAA CA RP: TTT GTA GAT GTT GTG CCC CA	58	682	U71377.1

<i>fbe</i>	FP: AGT TCA GGT CAA GGA CAA GGT RP: GAA TGT TTA AGC GTC GGC GT	59	278	Y17116.1
<i>embp</i>	FP: CGA CTT GCA ACT GAG AAG AGC RP: ACG TTG ATT GAG TGA GCT GAG A	59	142	AY101364.1

197

198 Amplification of the genes was performed by PCR in a total reaction volume of 20 µl
199 containing Go Taq® Green mastermix (Promega, USA), water for molecular biology
200 (Sigma-Aldrich, Bornem, Belgium), primers (10 µM) and bacterial DNA. The PCR was
201 conducted using an initial denaturation step at 95°C for 5 min, 40 amplification cycles
202 each consisting of a denaturation step at 95°C for 30 s, annealing for 45 s and
203 elongation at 72°C for 30 s, along with a final extension step at 72°C for 5 min in a
204 UnoCycler (VWR, USA). The presence and size of the PCR products were confirmed by
205 gelelectrophoresis on 1.5% (m/v) agarose (Electran) gels in Tris Borate EDTA buffer
206 (Applichem Panreas) at 120 V for 45 min.

207

208 **2.4 Statistical analysis**

209 Statistical analysis was performed with the GraphPad Prism 6 software using Spearman
210 rank correlation and Fisher's exact test.

211

212 **3. RESULTS**

213 **3.1 Discriminating cut-off between strong and weak biofilm-producing strains**

214 To discriminate between weak and strong biofilm-forming strains, eight reference strains
215 were cultivated in the four biofilm models (Figure 2). Two separate populations could be
216 observed in the static MTP models and in both dynamic models. None of the
217 *S. epidermidis* reference strains were strong biofilm-producers in all models. As
218 *S. aureus* ATCC6538 was the only reference strain with a strong biofilm-forming
219 capacity in all the models after 17h and 48h, half of the OD of this strain was used as a

cut-off to distinguish between a strong and weak biofilm phenotype. For the MTP-based model, this threshold was $OD_{570nm} \geq 0.36$, for the Lab-Tek™ $OD_{570nm} \geq 0.33$ and for the dynamic catheter model $OD_{570nm} \geq 0.48$. To determine the cut-off value for the BioFlux™, the surface coverage of all strains were compared in a dot plot revealing a large gap between 10% and 18% surface coverage. All strains below 18% surface coverage were considered weak biofilm-forming strains and all strains above that value were classified as strong biofilm-formers.

Figure 2: Definition of cut-off value to discriminate between strong and weak biofilm-producing strains. Eight reference strains were tested in the four in vitro models and a dot plot was created. Based on the OD-values or the % area for the BioFlux™ assay, two populations of weak and strong biofilm-formers could be observed for both dynamic models and the static microtiterplate (MTP) model.

3.2 Biofilm phenotype in clinical isolates

Forty *Staphylococcus* strains isolated from CVCs were evaluated, including 27 *S. epidermidis*, 6 *S. capitis*, 5 *S. aureus*, 1 *S. warneri* and 1 *S. hominis* strain. Their biofilm-forming capacity was checked in the static MTP model since this method is considered

as a standard procedure in biofilm research (11). Thirteen isolates demonstrated a strong biofilm-forming capacity (10 *S. epidermidis* strains and 3 *S. capitis* strains) while all the *S. aureus* strains were weak biofilm-producers. Since *S. epidermidis* was the most abundant species, all strong (n=10) and 10 weak *S. epidermidis* biofilm-producing strains were retained for the subsequent experiments comparing the biofilm-forming characteristics in the four *in vitro* biofilm models.

To determine the influence of polymeric and glass surfaces on biofilm formation, *S. epidermidis* isolates were cultured on both surfaces under static (MTP versus Lab-Tek™) and dynamic (catheter versus BioFlux™) growth conditions. In the static models, biofilms were grown for 48h and a positive correlation could be demonstrated for biofilm formation in MTPs and Lab-Tek™, with 85% (17/20) of strains demonstrating the same phenotype on respectively polystyrene and glass (p<0.0001) (Table 2).

Table 2: Influence of a polymeric and glass surface on *in vitro* biofilm formation by 20 *S. epidermidis* isolates. 2x2 correlation matrix comparing static (MTP and Lab-Tek™) and dynamic biofilm models (catheter model and BioFlux™) with a polymeric and glass surface respectively. In all models biofilms were grown during 48h except for the BioFlux™ model where biofilms were cultivated during 17h (+ = strong biofilm-producers; - = weak biofilm-producers).

Static	Lab-Tek™ +	Lab-Tek™ -	Dynamic	BioFlux™ +	BioFlux™ -
MTP +	45%	5%	Catheter +	10%	40%
MTP -	10%	40%	Catheter -	5%	45%

In the dynamic models, flow rate was kept constant and biofilms were grown for 48h in the catheter model and only for 17h in the BioFlux™ since longer periods were practically not possible (clogging of the microfluidic channels due to extensive growth of biofilm-positive strains,...). Hence, all biofilms were grown for 17h in the BioFlux™ and for 48h in the other models unless otherwise mentioned. Only 55% (11/20) of the

isolates exhibited a similar biofilm phenotype on both polyurethane catheters and BioFlux™ glass surfaces (Table 2). Of the remaining 45% (9/20) showing a different biofilm phenotype, 40% (8/20) were strong biofilm-producers in the catheter model while only one strain formed a strong biofilm in the BioFlux™. A logic explanation for this poor correlation could be the difference in incubation time between both dynamic models. To investigate this, all strains showing a positive biofilm phenotype in the catheter model (10/20) were also cultivated for 17h whereby 70% (7/10) showed negative. All isolates with a negative phenotype after 48h (10/20) were obviously negative after 17h as well. A correlation of 80% (16/20) could be observed with 75% (15/20) of the isolates being weak biofilm-producing strains in both models after 17h (Table 3). These results indicate that the surface material has no significant effect on biofilm formation under flow conditions, in contrast to the incubation time.

Table 3: Influence of a polymeric and glass surface on *in vitro* biofilm formation by 20 *S. epidermidis* isolates in dynamic models. 2x2 correlation matrix comparing the catheter model and BioFlux™ when biofilms were grown during 17h (+ = strong biofilm-producers; - = weak biofilm-producers).

17h incubation	BioFlux™ +	BioFlux™ -
Catheter+	5%	10%
Catheter -	10%	75%

To evaluate the effect of static or dynamic incubation on biofilm formation, *S. epidermidis* clinical isolates were cultured on polymeric (MTP versus catheter) or on glass (Lab-Tek™ versus BioFlux™) surfaces. On polymeric surfaces, 80% (16/20) displayed a similar phenotype independent of the flow condition, suggesting that biofilm formation of *S. epidermidis* is not much affected by fluid flow (Table 4). On glass surfaces, half of the isolates exhibited a similar phenotype. Also in this case, the

different incubation time between biofilms grown in Lab-Tek™ and the BioFlux™ could explain this poor correlation.

Table 4: Influence of static and dynamic growth conditions on biofilm formation by 20 *S. epidermidis* isolates. 2x2 correlation matrix comparing static (MTP plate and Lab-Tek™) and dynamic (catheter model and BioFlux™) models with a polymeric or glass surface respectively. In all models biofilms were grown during 48h except for the BioFlux™ model where biofilms were grown during 17h (+ = strong biofilm-producers; - = weak-biofilm-producers).

Polymeric	Catheter +	Catheter -	Glass	BioFlux™ +	BioFlux™ -
MTP +	40%	10%	Lab-Tek™ +	10%	45%
MTP -	10%	40%	Lab-Tek™ -	5%	40%

When comparing the results of all four *in vitro* models, only 40% (8/20) of the isolates exhibited the same phenotype, with 10% (2/20) strong and 30% (6/20) weak biofilm-forming strains (Table S1). Exclusion of the BioFlux™ results increased this figure to 70% (14/20). Of the eight reference strains, only four demonstrated the same phenotype in all four models, increasing to five (three strong and two weak biofilm-formers) upon exclusion of the BioFlux™ system (Table S1). For three strains the ability to form a biofilm varied with the model used.

3.3 Detection of biofilm associated genes

To correlate the biofilm phenotype of all *S. epidermidis* isolates with genotypic variations, the presence of the biofilm-related genes *ica A*, *atlE*, *embp*, *fbe*, *aap* and *bhp* was investigated. Since *atlE*, *embp* and *fbe* were present in all isolates, no correlation with biofilm formation could be found. The results for *ica A*, *aap* and *bhp* are summarized in Table 5.

311

312 **Table 5: Presence of biofilm-related genes in 20 *S. epidermidis* clinical isolates.** Correlation with
 313 biofilm phenotype in both static and dynamic models with a polymeric (MTP and catheter model) or glass
 314 (Lab-Tek™ and BioFlux™ model) surface. In all models biofilms were grown during 48h except for the
 315 BioFlux™ model where biofilms were grown during 17h.

Static	<i>ica A</i> +	<i>ica A</i> -	<i>aap</i> +	<i>aap</i> -	<i>bhp</i> +	<i>bhp</i> -
MTP +	37%	0%	22%	15%	4%	33%
MTP -	22%	41%	56%	7%	48%	15%
Lab-Tek™ +	55%	0%	40%	15%	15%	40%
Lab-Tek™ -	25%	20%	35%	10%	20%	25%
Dynamic						
Catheter +	45%	5%	30%	20%	5%	45%
Catheter -	30%	20%	45%	5%	30%	20%
BioFlux™ +	11%	0%	0%	11%	0%	11%
BioFlux™ -	48%	41%	78%	11%	52%	37%

316

317 Fifty-nine % of all *S. epidermidis* isolates were *ica A* positive (Table S1). In both static
 318 models (MTP and Lab-Tek™), a correlation between the presence of *ica A* and biofilm
 319 formation could be demonstrated (MTP, $p = 0.001$ and Lab-Tek™, $p = 0.026$). In the
 320 dynamic models (catheter and BioFlux™), no association could be detected between
 321 *ica A* and biofilm formation ($p > 0.05$). Regardless of the model, all strong biofilm-
 322 producing strains were *ica A* positive whereas all *ica A* negative strains were weak
 323 biofilm-formers except one. This isolate demonstrated a strong biofilm phenotype in the
 324 Lab-Tek™ model, although its OD-value (0.34 ± 0.07) barely exceeded the threshold
 325 (0.33). On the other hand, not all *ica A* positive isolates were strong biofilm-forming
 326 strains, suggesting that *ica A* plays an important role in the biofilm-forming process but
 327 that its presence alone is not sufficient.

328 Seventy-eight % of the isolates were positive for *aap* although no association could be
 329 demonstrated in both static and the dynamic catheter model ($p > 0.05$). In the BioFlux™,
 330 the presence of *aap* was negatively correlated with biofilm formation ($p = 0.007$).

331 The *bhp* gene was present in 52% of the isolates. Its presence was negatively
332 associated with biofilm development since the gene was absent in 90% of the isolates
333 with a biofilm positive phenotype in at least two models. In MTP, a significant correlation
334 could be demonstrated between the absence of *bhp* and biofilm formation ($p=0.001$),
335 while no association was found for the other models ($p>0.05$).

336

337 4. DISCUSSION

338 *S. epidermidis* is an important pathogen causing nosocomial infections due to its ability
339 to adhere to indwelling medical devices such as CVCs and to produce biofilms. As
340 biofilm formation is considered a major virulence factor, it is particularly useful to
341 characterize the biofilm phenotype of clinical isolates. To evaluate this phenotype,
342 appropriate laboratory models are required. Currently, several biofilm models have been
343 described and each study is using its own laboratory model. Therefore, it is often
344 impossible to compare results obtained in different studies as biofilm formation may be
345 influenced by many factors, (21). This study specifically evaluated the biofilm-forming
346 ability of *S. epidermidis* strains isolated from CVCs in different *in vitro* models, as well
347 as the presence of some genes that are assumed to play a role in adherence and
348 biofilm formation (10).

349

350 The biofilm phenotype was first evaluated in a static MTP model. Of all *S. epidermidis*
351 isolates, 37% were strong biofilm-formers. These data are in accordance with previous
352 studies using the MTP model where 21 to 69% of *S. epidermidis* isolates originating
353 from different infection sites showed a biofilm-positive phenotype (14, 16, 44). Since the
354 surface material might play an important role in bacterial adherence (22, 45, 46), the
355 effect of polymeric and glass surfaces on the biofilm phenotype of *S. epidermidis*
356 isolates was investigated. Under static conditions, a good correlation could be

demonstrated between polystyrene (MTP) and glass (Lab-Tek™), with only three out of twenty *S. epidermidis* isolates showing a divergent phenotype. Under dynamic growth conditions, biofilms were grown for 48h in the catheter model and for 17h in the BioFlux™. A 55% correlation was found between biofilm formation on polyurethane catheters and BioFlux™ glass surfaces. This lower correspondance is most likely due to the difference in incubation time. For practical reasons, it was not possible to grow biofilms longer than 17h in the BioFlux™ mainly because of clogging of the microfluidic channels by biofilm-positive strains. A second reason may be that penetration of SYTO9 in thick biofilms in the BioFlux™ (including some reference strains) becomes hampered, making quantification impossible. For all these reasons, we could state that the BioFlux™ should be used for short term experiments and is a good model to investigate the early stages of biofilm formation. When biofilms were grown for 17h in both the catheter and BioFlux™, a fairly good correlation could be found with 80% of the isolates showing the same biofilm phenotype. The majority (75%) were weak biofilm-producing strains. When biofilms were grown for 48h in the catheter model, 50% showed a strong biofilm phenotype compared with only 15% after 17h, indicating the occurrence of false-negatives after this short incubation time. Related to incubation time, these data indicate that the biofilm phenotype of strong biofilm-producers is not affected by the surface material both under static or dynamic cultivation conditions. The biofilm phenotype of the weak biofilm-producing strains is probably not affected. Previous studies demonstrated a good correlation between biofilm formation in glass tubes and MTPs (47, 48). Another study observed that initial adhesion to different surface materials is affected by the surface material although it was not related with the final amount of produced biofilm biomass (45). This suggests that the ability to form biofilms probably depends more on bacterium-bacterium interactions than on the physicochemical characteristics of the surface.

383

384 Other factors that may contribute to the different biofilm phenotypes obtained in the
385 catheter model and the BioFlux™ are the flow conditions. Growth medium is pumped
386 through the catheters at a flow rate of 1 ml/h while the flow rate in the BioFlux™ was
387 approximately 0.06 ml/h. The flow that can be generated in the BioFlux™ is typically
388 limited by the amount of volume that can be added to the input well. It is, however, very
389 difficult to compare the flow conditions in both models as the diameters of the channels
390 and catheters are different, leading to different shear forces which in turn can influence
391 biofilm development.

392

393 Next, the influence of fluid flow on the biofilm phenotype of the *S. epidermidis* isolates
394 was investigated, since fluid flow and shear forces can have a profound impact on
395 biofilm production (20). When comparing the static MTP model and the dynamic
396 catheter model both having a polymeric surface, the majority of the isolates (80%)
397 displayed the same biofilm phenotype. In strains containing the *ica* operon, fluid flow
398 can promote biofilm formation by increasing the production of polysaccharide
399 intercellular adhesin (PIA). PIA is one of the most important molecules in
400 *Staphylococcus* biofilm formation, although *ica*-independent biofilms were also reported
401 (16, 33). In this study, strains without *ica A* generally did not form biofilms although
402 some of the isolates positive for *ica A* demonstrated a weak biofilm phenotype
403 regardless of static or dynamic growth conditions, suggesting that other factors do play
404 a role in biofilm formation. For the models with a glass surface, (Lab-Tek™ and
405 BioFlux™), only half of the isolates showed the same phenotype in both models, while
406 forty-five% demonstrated a strong biofilm-producing phenotype in the Lab-Tek™ only.
407 In the latter case, the 48h versus 17h incubation time could be an explanation for this
408 poor correlation.

409

410 When comparing the biofilm-forming capacity of the *S. epidermidis* isolates using all
411 models, 40% exhibited the same phenotype. Exclusion of the BioFlux™ results
412 increased this figure to 70%. For the remaining 30%, the biofilm phenotype was
413 dependent on the model. These results indicate that there is a relatively good
414 correlation between all models except for the BioFlux™. The different incubation time,
415 17h in the BioFlux™ versus 48h in all other models could be an explanation for its poor
416 correspondence to the other models. Besides differences in surface or fluid flow, other
417 environmental factors and bacterial growth medium composition may have a profound
418 impact on biofilm development. For example addition of glucose, NaCl or ethanol can
419 promote biofilm production (15, 21, 24, 40, 49). Given the strong impact of incubation
420 time and external conditions, standardized laboratory models are indispensable to
421 characterize and compare the biofilm-forming capacity of clinical isolates. Standardized
422 laboratory models are defined as models that are approved by standard setting-
423 organizations like ASTM International and that are validated in large inter-laboratory
424 studies. Currently, ASTM has approved five biofilm methods including three biofilm
425 models (the rotating disk reactor, the drip flow biofilm reactor and the CDC biofilm
426 reactor) and two assays for the evaluation of antimicrobial agents against
427 *Pseudomonas aeruginosa* biofilms (50-54). Hence, for large-scale biofilm studies
428 involving different strains all three ASTM approved models are not suitable in contrast to
429 the four models used in this study.

430

431 The differences in biofilm formation between the various laboratory models can be
432 applied to the reference strains as well, where only 5 out of 8 strains demonstrated the
433 same phenotype in all models except the BioFlux™. This may have implications as the
434 selection criteria to discriminate between biofilm- and non-biofilm-producing strains are

often based on the phenotype of a reference strain (21). In order to obtain a reliable cut-off value, 8 reference strains were incorporated in this study, including *S. epidermidis* RP62A. Although this strain is considered as a biofilm-positive strain in literature, little or no biofilm formation could be observed in this study in any of the models (55). A possible explanation could be the use of different growth media like BHI instead of TSB (55) or the addition of glucose to the medium (40, 56). On the other hand, *S. epidermidis* 1457, another well-known biofilm-positive strain (40), was able to form a biofilm in all models except the BioFlux™ whereas *S. epidermidis* ATCC 12228, a biofilm-negative strain lacking the *ica* operon (39), demonstrated a weak biofilm-producing phenotype in all models. *S. aureus* ATCC6538 was the only reference strain with a strong biofilm-forming capacity in both dynamic models after 17h and both static models after 48h incubation. As such, only this strain could definitely be considered as a good positive reference.

To correlate biofilm phenotype with genotypic variation, the presence of several biofilm-related genes, including *ica A*, *aap*, *bhp*, *fbe*, *embp* and *atlE*, was investigated. All clinical isolates were positive for *Fbe*, *embp* and *atlE*, which is in accordance with Rhode *et al.*, and could therefore not be used as a marker to discriminate between biofilm- and non-biofilm-producing strains (16, 17). In line with previous results, 59% of the isolates were positive for *ica A* of which two thirds were able to form a biofilm *in vitro* (14, 16, 18, 44, 55, 57). None of the *ica A* negative isolates were capable of producing a biofilm in any of the models, except for one strain. This strain was classified as a strong biofilm-producing strain in the Lab-Tek™ model although its OD-value (0.34 ± 0.07) barely exceeded the threshold (0.33). These results suggest that *ica A* plays indeed an important role in the biofilm formation process of these set of isolates, but that its

460 presence alone is not sufficient since not all of the *ica A* positive isolates were able to
461 produce a biofilm.

462
463 The accumulation associated protein (Aap) is a proteinaceous factor which plays a role
464 in PIA-independent biofilm formation (58). In this study, *aap* was present in 78% of the
465 isolates but no correlation with biofilm formation could be demonstrated except in the
466 BioFluxTM. In this model, the presence of *aap* was negatively correlated with biofilm
467 formation which is probably due to the occurrence of false-negatives after 17h of
468 incubation. Another protein involved in PIA-independent biofilm formation is the biofilm-
469 associated protein (Bhp) analogue. In a study by Tormo et al., *bhp* was present in all *ica*
470 *A* negative biofilm-positive *Staphylococcus* isolates (31). In this study, no *ica A* negative
471 *Staphylococcus* strains were observed as biofilm-positive and the presence of *bhp* was
472 negatively correlated with biofilm formation since most biofilm-producing isolates did not
473 contain *bhp*.

474
475 In summary, a good correlation between glass and polymeric surfaces and between
476 static and dynamic growth conditions was obtained, although biofilm formation was still
477 model-dependent for 30% of the isolates illustrating the profound impact of
478 environmental factors on the phenotypic characteristics of bacterial strains and
479 demonstrating that certain strains may be more susceptible to external changes than
480 others. To obtain a reliable classification of the biofilm-forming capacity of clinical
481 isolates and to identify genetic factors contributing to biofilm formation, it appears useful
482 to integrate different laboratory models. Ultimately, the *in vitro* phenotype of clinical
483 isolates should be compared with the *in situ* phenotype in the patient to validate any
484 standardized clinically relevant laboratory model. Finally, this study demonstrated that
485 the BioFluxTM is only suited to study the early phases of biofilm formation.

486

487

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488

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