

Quantitative analysis of adhesion and biofilm formation on hydrophilic and hydrophobic surfaces of clinical isolates of *Staphylococcus epidermidis*

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Abstract

Staphylococcus epidermidis is now well established as a major nosocomial pathogen associated with infections of indwelling medical devices. The major virulence factor of these organisms is their ability to adhere to devices and form biofilms. However, it has not been established that adherence and biofilm formation are closely linked phenotypes for clinical isolates. In this study, the initial adhesion to different materials (acrylic and glass) of 9 clinical isolates of *S. epidermidis*, along with biofilm-positive and biofilm-negative control strains, was assayed using physico-chemical interactions to analyze the basis for bacterial adherence to the substratum. X-ray photo electron spectroscopy (XPS) analysis of the cell surface elemental composition was also performed in an attempt to find a relationship between chemical composition and adhesion capabilities. Biofilm formation on the two surfaces was evaluated by dry weight measurements. Human erythrocytes were used to evaluate the ability of *S. epidermidis* strains to cause hemagglutination, an indicator of the production of a poly-*N*-acetyl glucosamine cell surface polysaccharide also involved in biofilm formation. The clinical isolates exhibited different cell wall physico-chemical properties, resulting in differing abilities to adhere to surfaces. Adhesion to hydrophobic substrata for all strains occurred to a greater extent than that to hydrophilic surfaces. Bacterial cell hydrophobicity seemed to have little or no influence on adhesion. X-ray photoelectron spectroscopy analysis showed a high ratio of oxygen/carbon for all strains, which is a common characteristic of *S. epidermidis* species. No relevant relationship was found between XPS data and adhesion values. All strains forming biofilms were able to agglutinate erythrocytes. However, no direct relationship was found between the amount of biofilm formed and the initial adhesion extent. These results indicate that high levels of initial adherence do not necessarily lead to thick biofilm formation. These two aspects of the pathogenesis of medical device related-infection may need to be evaluated independently to ascertain the contribution of each to the virulence of *S. epidermidis* causing device-related infections.

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1. Introduction

Staphylococcus epidermidis normally colonizes the human skin and mucous membranes and represents a major component of the normal bacterial flora of this habitat. In

predisposed hosts, usually with an indwelling medical device, *S. epidermidis* has become a significant nosocomial pathogen [31,34,39,40]. The major virulence factor associated with this organism's ability to cause infections is dependent on adherence to medical devices and formation of a biofilm [41].

Microbial adhesion to surfaces has been shown to be a complex process, involving physico-chemical, protein and polysaccharide factors [2,6,7,9,13,16,21,25,26,30]. From

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an overall physico-chemical point of view, microbial adhesion can be mediated by non-specific interactions, with long-range characteristics, including Lifshitz–van der Waals forces, electrostatic forces, acid–base interactions, and Brownian motion forces [3,37]. As soon as microorganisms reach a surface, they will be attracted or repelled by it, depending on the sum of the different non-specific interactions [17]. In biological systems, hydrophobic interactions are usually the strongest of all long-range non-covalent forces [5], and adhesion to surfaces is often mediated by these types of interactions [35]. It has been demonstrated that hydrophobicity plays an important role in a wide range of microbial infections [15]. Microbial hydrophobicity is defined by the energy of attraction between apolar or slightly polar cells immersed in an aqueous phase [38], and can be assessed by several methods, although, according to Doyle [15], the best method for determining bacterial hydrophobicity is by contact angle measurements.

After initial adhesion, mature biofilm formation is often associated with the production of specific molecules by the microorganisms that mediate cell-to-cell adhesion. In *S. epidermidis* a polymer of *N*-acetyl glucosamine, initially defined biologically as the polysaccharide intercellular adhesin (PIA) and chemically as Poly-*N*-acetyl-glucosamine (PNAG), have been identified as the molecules responsible for biofilm formation [10,22,29]. The PNAG molecule is synthesized from genes contained in the *ica* locus [12,29]. PNAG/PIA is also involved in the agglutination of erythrocytes, which is a common property of *S. epidermidis* strains [27,32]. This characteristic can be used to identify the presence of PNAG/PIA in a *S. epidermidis* strain by hemagglutination assays.

Expression of PNAG/PIA, adhesion to synthetic surfaces and biofilm formation are virulence factors of *S. epidermidis* clinical strains. As different clinical isolates of *S. epidermidis* are expected to exhibit different phenotypic behaviors [34,40] it is further expected that they might have different capabilities to adhere and to form biofilms on synthetic surfaces.

In this study the ability of 11 strains of *S. epidermidis* to adhere to acrylic (hydrophobic surface) and glass (hydrophilic surface) was determined, as well as the importance of physico-chemical interactions, namely, hydrophobicity, in the adhesion process. The ability to form a mature biofilm, in the same surfaces, was also evaluated, in an attempt to determine if there is a relationship between these two phenomena in clinical isolates of *S. epidermidis*.

2. Materials and methods

2.1. Bacterial strains

The *S. epidermidis* clinical strains used in this work were: IE75, IE186, IE214 (isolated from cases of infective en-

docarditis), M129, M187 (isolated from cases of dialysis-associated peritonitis), and FJ6, JI6, LE7 and PE9 (isolated from blood). In addition, we used strain 9142, a known producer of PIA/PNAG, and strain 9142-M10, which has a transposon inserted into the *ica* locus and does not produce PIA/PNAG. These two strains were provided by D. Mack, Hamburg, Germany.

2.2. Media and growth conditions

Tryptic soy broth (TSB) and tryptic soy agar (TSA) plates were prepared according to the manufacturer's instructions. All strains were grown for 24 (\pm 2) h at 37 °C in a shaker rotating at 130 rpm in 15 ml of TSB using bacteria grown on TSA plates not older than 2 days as inocula. Then, 50 μ l of each cell suspension was transferred to 30 ml of fresh TSB, which was incubated for 18 (\pm 2) h at 37 °C at 130 rpm. After being harvested by centrifugation (for 5 min at 10 500 *g* and 4 °C), cells were washed twice and resuspended in saline (0.9% NaCl prepared in distilled water) at a concentration of approximately 1×10^9 cells/ml, determined by the optical density at 640 nm. These cell suspensions were used in the subsequent adhesion and surface characterization assays.

2.3. Substrate preparation

Glass was obtained by slicing microscope slides into 2×2 cm squares. Acrylic was also cut into 2×2 cm squares. These substrates were immersed in a 0.2% solution of a commercial detergent overnight, after which they were transferred to a new solution of 0.2% of the commercial detergent and washed at 40 °C with strong agitation for 5 min. The squares were then well rinsed with distilled water, and finally each individual square was well rinsed with ultra-pure water and dried at 60 °C, overnight.

2.4. Physico-chemical characterization of surfaces

2.4.1. Bacterial hydrophobicity assay

The method used for measuring contact angles on bacterial lawns has been described by Busscher et al. [8]. Briefly, a suspension of *S. epidermidis* cells in saline solution was deposited onto a 0.45 μ m cellulose filter by first washing the filter with 10 ml of distilled water for wetting, and then 20 ml of the cell suspension was added, obtaining a thick lawn of cells after filtration. The lawn of cells was then air-dried for at least 3.5 h, until the so-call "dried-plateau" was obtained. The hydrophobicity parameters were obtained using the sessile-drop contact angle technique, using an automated contact angle device [8]. Contact angles were standardized using as reference liquids: water, formamide and α -bromonaphtalene. All experiments were done in duplicates, with 4 repeats.

2.4.2. Cell preparation for XPS analysis of the bacteria surface

X-ray photo electron spectroscopy (XPS) analysis of the elemental composition of the bacterial cells was performed as described by van der Mei et al. [36]. Briefly, fresh cells in TSB (18 ± 2 H) were harvested and centrifuged for 5 min at $10\,500\text{ g}$, 4°C . Cells were then resuspended in physiological serum at a density of approximately 1×10^9 cells/ml, and 20 ml of that suspension was filtered through a cellulose filter previously wetted with 10 ml of distilled water. After filtering, the cellulose filter covered with bacteria was sliced into small 1 cm^2 squares and quickly frozen in liquid nitrogen. Frozen filters were stored at -80°C for 1–2 h, followed by 24 h of lyophilization (Christ Alpha 2-4, B. Braun, Germany). The XPS analysis was performed using an ESCALAB 200A apparatus, with a VG5250 software and data analysis. The spectrometer used monochromatized $\text{Mg}(K\alpha)$ X-ray radiation (15000 eV). The constant pass energy of the analyzer was 20 eV and it was calibrated with reference to $\text{Ag}3d_{5/2}$ (368.27 eV). The pressure during analysis was under 1×10^{-6} Pa. The spectra were recorded following the sequence C1s, O1s, N1s, P2p. The elemental composition was defined as the ratio between oxygen and carbon (O/C), nitrogen and carbon (N/C), or phosphorous and carbon (P/C).

2.4.3. Substratum surface hydrophobicity

Cleaned and dried substratum surfaces were used for determining the hydrophobicity parameters of the surface. The hydrophobicity parameters were obtained using the sessile-drop contact angle technique, using an automated contact angle measurement apparatus [8]. Contact angles were standardized using as reference liquids: water, formamide and α -bromonaphthalene. All experiments were done in triplicate, with 4 repeats.

2.5. Adhesion assays

2.5.1. Initial adhesion to substrates

Initial adhesion was performed as described previously [11]. Briefly, squares of acrylic or glass were placed in 6 well tissue-culture plates containing 5 ml of a suspension of 1×10^9 cells/ml in saline solution. Initial adhesion to each substratum was allowed to occur for 2 h at 37°C , in a shaker rotating at 120 rpm. Negative controls were obtained by placing acrylic or glass squares in a saline solution without bacterial cells. The squares were then gently transferred to 100 ml glass beakers containing distilled water, and were allowed to rest there for approximately 10 s. Afterwards, a new transfer was made to a different 100 ml glass beaker with distilled water, followed by a third transfer 10 s later. These washing steps were carefully performed in order to remove only the cells that were suspended in the liquid interface formed along the surface, and to minimize cell detachment from the surface. The substrate squares with ad-

hered cells were dried at 37°C . All experiments were done in triplicate, with 4 repeats.

2.5.2. Image analysis

Before image observation and analysis of adhered cells, the substrate squares were stained with a 0.2% safranin solution, for better image contrast. Direct bacterial counts were done using a phase contrast microscope coupled to a 3 CCD video camera that acquires images with 820×560 pixels resolution and at a magnification of $400\times$. With this magnification 1 cm^2 is equivalent to 1.823×10^4 captured images (as determined by a Neubauer chamber). For each surface analyzed, 20 pictures were taken, covering the entire surface. Each image was then analyzed using automated enumeration software, for determining the number of adhering cells.

2.6. Biofilm assays

2.6.1. Biofilm formation on surfaces

Biofilm formation was performed as described previously [10]. Briefly, clean squares of each type of substrate (glass or acrylic) immersed in distilled water were autoclaved for 15 min at 121°C . Each sterilized square was placed in a well of a 6 well tissue-culture plate containing 5 ml of TSB supplemented with 0.25% of glucose. Then 50 μl of a cell suspension of 1×10^9 cells/ml prepared in a 0.9% NaCl solution was added. Microbial growth was allowed to occur for 72 h at 37°C while rotating at 120 rpm. Every 12 h the TSB medium containing suspended cells was removed and fresh TSB + 0.25% glucose was added. Negative controls were obtained by placing sterilized squares in TSB + 0.25% glucose without adding bacterial cells.

2.6.2. Biofilm dry-weight determination

Biofilm dry-weight determinations were performed as described by An and Friedman [1]. Briefly, after 72 h of growth, the squares were removed from the wells of the tissue culture plates and dried at 80°C , for 24 h. The squares were weighed and placed again at 80°C for 2 more h, and weighed once more to check the stability of the dry weight. Then, the biofilms were scraped from the squares and the dried cell mass weighed. The substrate squares were then further cleaned with 0.2% of the commercial detergent solution. Cleaned surfaces were dried overnight at 80°C and then weighed. Biofilm dry-weights were obtained as the differences between the 2 measurements. All experiments were done in triplicate, with 3 repeats.

2.7. Hemagglutination assays

2.7.1. Erythrocytes

Human blood collected with heparin was used to retrieve erythrocytes, by adding 5 ml of blood to 45 ml of saline solution which was then centrifuged twice at 2500 g for 10 min. Next, 100 μl of the pellet was added to 10 ml of a saline so-

lution, obtaining a 1% erythrocyte solution to be used in the hemagglutination assays.

2.7.2. Hemagglutination assays

The hemagglutination assay was performed as described elsewhere [27] with some modifications. Briefly, *S. epidermidis* cells from an overnight culture in TSB were grown in fresh TSB supplemented with 0.25% glucose for 18 h. Cells were then resuspended in saline, and adjusted to a concentration of approximately 3×10^9 cells/ml. Five twofold dilutions of each cell suspension were made (100 μ l) in 96 well (U-shaped) microtiter plates. Then 100 μ l of the 1% erythrocyte solution was added to each well. To ensure thorough mixing of the bacteria and erythrocytes, the total volume of each well was pipetted in and out with a micropipette. Incubation was at room temperature for 2 h, and hemagglutination titers were evaluated macroscopically. Erythrocytes that appeared to be negative for macroscopic hemagglutination were also evaluated microscopically. All experiments were done in duplicate with 3 repeats.

2.8. Statistical analysis

All the assays were compared using one-way analysis of variance (ANOVA) by applying Levene's test of homogeneity of variances and the Tukey multiple comparisons test, and also the paired sample *t*-test, using SPSS software (Statistical Package for the Social Sciences). Student's *t*-test was applied to all experimental data for rejection of some experimental values. All tests were performed with a confidence level of 95%.

3. Results

3.1. Surface physico-chemical analysis

Bacterial cell surface physico-chemical characteristics are presented in Table 1. Water contact angles formed by bacterial lawns can be used as a qualitative indication of cell surface hydrophobicity, with lower values indicating a more hydrophilic surface. The values obtained for all strains assayed were quite similar, ranging from contact angles of 19.9° (strain 9142-M10) to 33.7° (strain M187), indicating that these different bacterial strains have comparable levels of cell surface hydrophobicity. All clinical *S. epidermidis* strains examined here could be considered hydrophilic. From Table 1 it can also be observed that all strains had surfaces that were predominantly electron donors (higher values of γ_S^-), with a low electron acceptor parameter (γ_S^+). The exception was strain IE214 that had the greatest electron acceptor parameter. Interestingly, under the growth conditions used in this study this strain grew as highly aggregated cells, forming a flocculent suspension in the growth medium.

Table 1

Water contact angle (in degrees) of *S. epidermidis* strains, and cell surface hydrophobicity parameters (in mJ/m²)

Strain	Water contact angle (±SD)	γ_S^{LW}	γ_S^+	γ_S^-	ΔG_{iwi}^{TOT}
9142	26.1 (1.6)	29.07	4.32	43.79	17.59
9142-M10	19.9 (1.5)	28.82	4.66	47.09	19.97
IE75	26.8 (2.8)	26.63	5.06	43.83	17.10
IE186	19.6 (2.0)	27.53	5.12	47.37	19.75
IE214	18.7 (0.9)	19.51	8.96	48.44	15.58
M129	28.5 (2.6)	28.67	4.73	41.16	14.76
M187	33.7 (1.6)	27.63	4.93	37.49	11.45
FJ6	30.1 (3.8)	30.15	3.73	41.42	15.94
JI6	28.8 (5.1)	28.43	4.86	40.84	14.36
LE7	25.1 (3.8)	31.21	3.45	45.04	19.52
PE9	29.7 (1.2)	30.29	3.72	41.61	16.08

SD means standard deviation; γ_S^{LW} represents the apolar Lifshitz–van der Waals surface free energy component; γ_S^+ represents the electron acceptor surface free energy component; γ_S^- represents the electron donor surface free energy component; ΔG_{iwi}^{TOT} represents the degree of hydrophobicity.

Table 2

Water contact angle (in degrees) of the substratum, and surface hydrophobicity parameters (in mJ/m²)

Surface	Water contact angle (±SD)	γ_S^{LW}	γ_S^+	γ_S^-	ΔG_{iwi}^{TOT}
Glass	23.0 (2.2)	34.53	2.72	45.46	19.98
Acrylic	85.5 (2.2)	38.06	0	7.59	−50.82

SD means standard deviation; γ_S^{LW} represents the apolar Lifshitz–van der Waals surface free energy component; γ_S^+ represents the electron acceptor surface free energy component; γ_S^- represents the electron donor surface free energy component; ΔG_{iwi}^{TOT} represents the degree of hydrophobicity.

Table 3

Bacterial surface elemental composition of cells grown in TSB during 18H at 37 °C

Strain	N/C	O/C	P/C
9142	0.203	0.479	0.042
9142-M10	0.189	0.530	0.046
IE75	0.195	0.408	0.038
IE186	0.193	0.463	0.041
IE214	0.197	0.420	0.028
M129	0.187	0.502	0.050
M187	0.200	0.408	0.030
FJ6	0.171	0.539	0.054
JI6	0.173	0.570	0.055
LE7	0.202	0.513	0.048
PE9	0.192	0.473	0.041

Substrate surface physico-chemical characteristics are presented in Table 2. Water contact angles of the 2 materials used clearly differed, with glass being hydrophilic, as expected (water contact angle of 23.0° and $\Delta G_{iwi}^{TOT} > 0$) and acrylic being hydrophobic (water contact angle of 85.5° and $\Delta G_{iwi}^{TOT} < 0$).

The elemental composition of the surface of the bacterial strains, determined by XPS, is given in Table 3. All strains used in this study exhibited high O/C values, ranging from

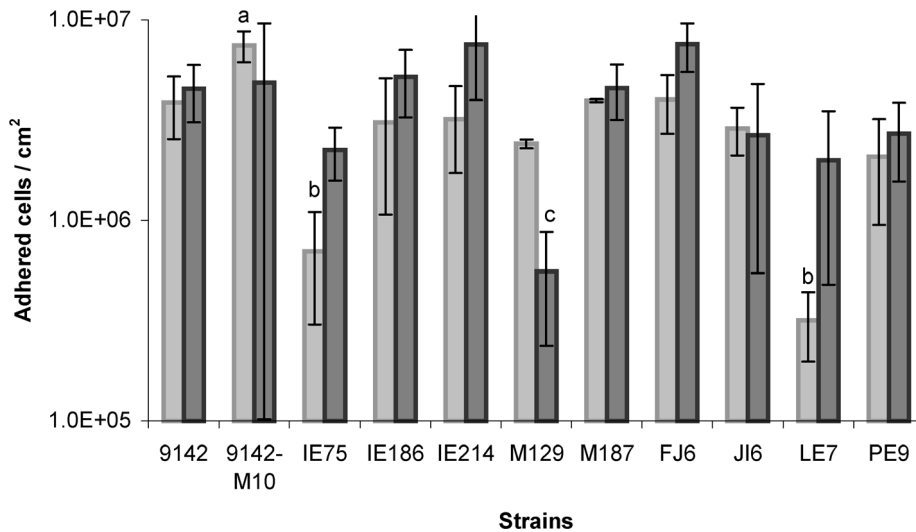


Fig. 1. Differences in adhesion of *S. epidermidis* strains to glass (■) and acrylic (■). (a) Strain with high extent of adhesion to glass ($P < 0.05$); (b) strain with low extent of adhesion to glass ($P < 0.05$); (c) strain with low extent of adhesion to acrylic ($P < 0.05$).

0.408 (strain M187) to 0.570 (strain JI6). On the other hand, P/C values were low, ranging from only 0.028 (strain IE214) to 0.055 (strain JI6).

3.2. Initial binding to substratum

Initial binding of bacteria to both substrate surfaces is presented in Fig. 1. A wide range in the number of bound cells was obtained, ranging from 3.19×10^5 cells/cm² (strain LE7 on glass) to 7.55×10^6 cells/cm² (strain FJ6 on acrylic). For 8 of 11 strains the number of bacterial cells initially bound to glass was lower when compared to acrylic ($P < 0.05$, paired-*t*-test). Strains 9142-M10, M129 and JI6 were the exceptions, with the latter not showing a significant difference in binding to the two substrates ($P < 0.05$, paired-*t*-test).

Strain 9142-M10 had the highest initial binding to glass (7.4×10^6 cells/cm²), whereas strains LE7 and IE75 had low initial binding to glass (3.2×10^5 and 7.0×10^5 cells/cm² respectively), making these phenotypes markedly different from the remaining strains ($P < 0.05$, ANOVA and Tukey's multiple comparison test). When binding to acrylic, strain M129 can be considered a low binding strain (5.6×10^5 cells/cm²), being markedly different from most of the remaining strains ($P < 0.05$, ANOVA and Tukey's multiple comparison test).

3.3. Hemagglutination assays

Most strains were able to agglutinate human erythrocytes (Table 4); however as can be seen by the titers, the overall degree of hemagglutination activities was relatively weak. Bacterial dilutions from a standardized initial amount that causes hemagglutination ranged from 1:1 (strain LE7) to 1:16 (strain IE214). Strains 9142-M10 and IE75 did not cause any agglutination of human erythrocytes. For those

Table 4

Hemagglutination macroscopic titers and microscopic confirmation of *S. epidermidis* strains

Strain	Hemagglutination titers	Microscopic observation
9142	1:2	–
9142-M10	No hemagglutination	No hemagglutination
IE75	No hemagglutination	Hemagglutination observed
IE186	1:4	–
IE214	1:16	–
M129	1:2	–
M187	1:4	–
FJ6	1:2	–
JI6	1:8	–
LE7	1:1	–
PE9	1:8	–

strains, microscopic observations of the suspension containing bacterial cells and erythrocytes were performed. With this observation, strain IE75 microagglutinated the erythrocytes, whereas the *ica*-interrupted strain 9142-M10 did not induce any hemagglutination.

3.4. Biofilm formation

The amount of biofilm formed on both the glass and acrylic substrate surfaces is presented in Fig. 2. Most of the strains were able to form biofilms on both materials tested, except for the *ica*-interrupted strain 9142-M10, which was used as a negative control for biofilm formation. It was observed that only a small amount of biofilm was formed by strain IE75 on both materials. The amount of biofilm produced by almost all of the other strains on acrylic surfaces was greater than on glass. The only exception was strain PE9. Therefore, a hydrophobic surface appears to promote biofilm formation by most clinical isolates of *S. epidermidis*.

Strains 9142, IE186, JI6 and PE9 showed significantly different abilities from the remaining strains to form biofilms

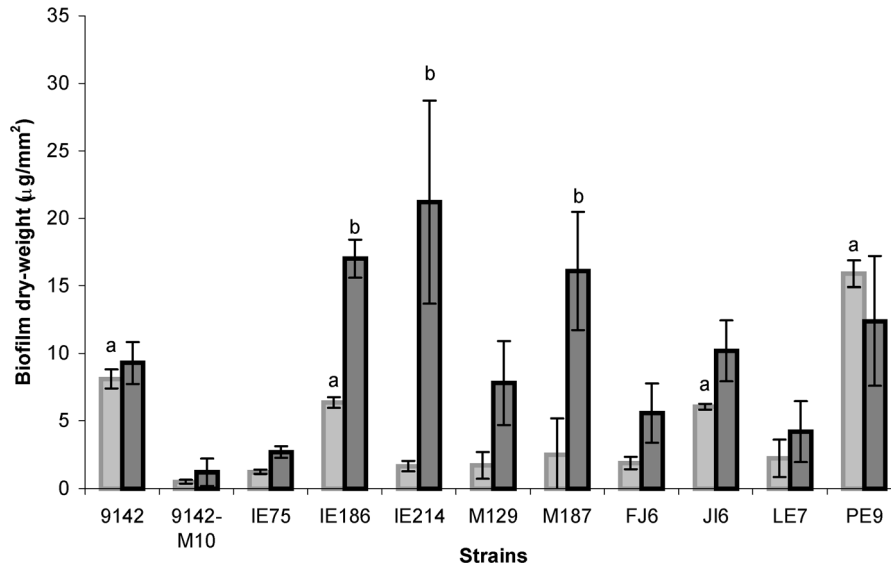


Fig. 2. Biofilm formation of *S. epidermidis* strains on glass (■) and acrylic (■). (a) High biofilm producers on glass ($P < 0.05$); (b) high biofilm producers on acrylic ($P < 0.05$).

on glass (8.2, 6.4, 6.0 and 15.0 $\mu\text{g}/\text{mm}^2$, respectively), being considered high biofilms producers on glass ($P < 0.05$, ANOVA and Tukey's multiple comparison test). On acrylic, strains IE186, IE214 and M187 were the highest biofilm producers (16.9, 20.9 and 16.1 $\mu\text{g}/\text{mm}^2$, respectively), being markedly different from most of the remaining strains ($P < 0.05$, ANOVA and Tukey's multiple comparison test). Since only one strain, IE186, was a high biofilm producer on both surfaces it appeared that different clinical isolates have differing abilities to form biofilms on hydrophobic versus hydrophilic surfaces.

4. Discussion

S. epidermidis is now recognized to be one of the most common causes of serious nosocomial infections [39], and this is related to the organism's ability to adhere to indwelling medical devices and form biofilms on them [41]. Bacterial adhesion is thought to be the first of two steps of biofilm formation [10,41], the second stage being accumulation of the bacterial cells in the biofilm mass. Biofilms play an important role in implant infections such as catheters [40], voice prostheses [7] and also bone-cement implants, intraocular artificial lens and cranioplastic implants [14].

In this study, we first evaluated the adherence of 9 clinical *S. epidermidis* strains along with an isogenic pair of strains with one lacking an intact *ica* locus to 2 different substrata using a static adhesion assay. This methodology of assessing adherence has been controversial due to the use of washing steps necessary to remove non-adherent and loosely adherent cells [4]. It has been demonstrated that the passage of an air-liquid interface through a lawn of adherent bacteria can detach some of the cells [19], although this effect is attenuated in the presence of a more hydrophilic substrate or

with more rapid washing of adherent cells [20]. In a previous study [13], several different washing procedures were assayed and the effect of each was evaluated as reported by Suárez et al. [19]. The results obtained suggested that when using hydrophilic glass no effect is observed from the different washing procedures. Conversely, for the hydrophobic acrylic substrate, an effect was sometimes observed, but was attenuated by rapid washing to minimize the time of exposure of the adherent cells to the air-liquid interface.

As expected, the clinical isolates exhibited differing abilities to adhere to the substrate surfaces and form biofilms. A quantitative value of hydrophobicity, expressed in International System (SI) units, can be obtained through the van Oss approach [37,38]. According to this theory hydrophobicity is defined as the free energy of interaction between two entities (i) when immersed in water (w)— ΔG_{iwi} . If $\Delta G_{iwi} < 0$, there is a preferential interaction between entities (i) rather than between an entity (i) and water, and the substance (i) is considered hydrophobic. By the same reasoning, if $\Delta G_{iwi} > 0$ the substance (i) is hydrophilic. For all strains used in this study, the highest level of initial binding occurred when the hydrophobic acrylic ($\Delta G_{iwi}^{\text{TOT}} = -50.82 \text{ mJ}/\text{m}^2$) was used as a substratum, whereas a lower level of initial binding was generally obtained with the hydrophilic glass ($\Delta G_{iwi}^{\text{TOT}} = 19.98 \text{ mJ}/\text{m}^2$). Clearly, substratum hydrophobicity greatly influences the initial binding of *S. epidermidis*, a result implicated in other studies but never fully examined with a range of clinical isolates of this organism.

However, no relationship was found between the bacterial strain's surface hydrophobicity and the extent of initial binding to either a hydrophilic or hydrophobic substrate. For instance, strain FJ6 exhibited one of the lowest hydrophilic surfaces (water contact angle of 30.1°) but was also one of the strains with high initial binding to the substrata. Con-

versely, strain IE214 had the highest hydrophilic surface (water contact angle of 18.7°) and was also one of the strains with high initial binding to the substrata. These findings are similar to those of other authors [7,13,30].

Strain IE214 had the greatest electron acceptor parameter (Table 1). Interestingly, under the growth conditions used in this study, this strain grew as highly aggregated cells, forming a flocculent suspension in the growth medium. This can be explained by both higher electron acceptor and donor parameters, meaning that this strain can establish a great number of acid–base interactions between one cell and another [37].

Many prior studies suggested that hydrophobic interactions contribute to the initial binding of pathogens to tissues, leading to colonization, invasion or tissue destruction [15]. A microorganism may adhere to a substratum via the hydrophobic effect if the associating sites possess sufficiently high densities of apolar areas [15]. Compared to glass, acrylic has such high densities of apolar areas, as can be seen by γ_S^- and γ_S^+ values presented in Table 2, and the bacteria bound better to this substrate than to glass. Thus, *S. epidermidis* microorganisms may be more prone to binding to hydrophobic surfaces such as acrylic compared to glass.

It is generally accepted that the outermost cell surface plays a crucial role in bacterial binding to surfaces, as it interacts directly with the substratum surface [6,36]. In an attempt to correlate adhesion results with surface elemental composition, XPS analysis of the cell surface was performed. The ratios O/C, N/C and P/C of the various strains studied were in the same range of values reported by Van der Mei et al. [36]. In their study, a cluster analysis of 210 microbial strains, including 33 staphylococci, revealed that O/C values for staphylococci are very high, ranging from 0.355 to 0.638 [36]. It has been suggested that high O/C may be an indicator of capsular polysaccharide material on the outer membrane [36]. No relation was found between the extent of adhesion and XPS data. Nevertheless, N/C values can be correlated with cell surface hydrophobicity—less hydrophobic cells exhibited lower N/C.

In order to determine whether the initial adhesion event was a determinant of the subsequent amount of biofilm formed, biofilm dry weights were obtained on the same substrates used in the initial binding experiments. Several prior studies evaluated biofilm formation on the bottom of U-shaped polystyrene microtiter plates [10,22,23,27,29], but such studies did not consider the influence of different substrates on biofilm formation [29]. Furthermore, by weighing the dried biofilm mass, a direct quantitative comparison of biomass formation by different strains could be made. In the present study, no direct relationship between the ability of strains to initially bind and subsequently form biofilms was found. For instance, strains IE75, M129 and JI6 had comparable initial binding levels; however, while the last 2 formed relatively thick biofilms, IE75 hardly formed more than a monolayer. This result indicates that biofilm formation is not

dependent on the extent of initial adherence of bacteria to the substrate. In a study with 54 clinical isolates, a qualitative analysis of biofilm formation was performed and similar conclusions were withdrawn [18]. However, the study conducted here provides more detailed information because it is based on a quantitative analysis of biofilm formation and thus it makes it possible to obtain a better evaluation of the relationship between initial binding and biofilm formation. Heilmann et al. demonstrated in 1996, that biofilm formation and initial adhesion were two distinct phenomena, using one mutant *S. epidermidis* strain [23]. However, a recent study by Maira-Litran et al. [28] demonstrated that the original conclusions from Heilmann et al. [22] reporting adherence and biofilm formation on polystyrene plates, was dependent on the manufacturer of the plates, since different results obtained with the exact same strains and using the exact same methodology were obtained with plates made in the US compared with those made in Germany.

Biofilm formation is more likely to be dependent on cell-to-cell adhesion rather than on the amount of cells initially attached to the surface. As no relationship between cell surface properties and biofilm formation was found, cell-to-cell adhesion is probably established by specific interactions and not influenced by the physico-chemical interactions of the bacteria with the attachment substrate. Likely, adherence is a complex phenomenon involving a variety of surface factors on the bacterium. One such factor, that has been proposed is the *S. epidermidis* autolysin encoded by the *atlE* gene [24]. However, while an *atlE* mutant was deficient in attachment to polystyrene and this was restored in a complemented strain, it was never definitively shown in this study that it was the *atlE* protein itself, and not a secondary effect of the *atlE* mutation, such as disruption in PNAG/PIA production, that caused the defect in adherence.

PNAG/PIA can also agglutinate erythrocytes [32] and a direct relationship was found between the ability of a strain of *S. epidermidis* to agglutinate erythrocytes and to form a biofilm. For 9 of the 11 strains, a linear relation was derived describing the relationship between biofilm formation (B) and hemagglutination titers (T): $B = 1.0807 \times T + 3.6025$, $r = 0.9054$. For instance, strain IE214 formed the greatest amount of biofilm ($21.2 \mu\text{g}/\text{mm}^2$ on acrylic) and exhibited the highest hemagglutination titer (1:16). Conversely, strain IE75 formed small amounts of biofilm on both surfaces (1.2 on glass and $2.7 \mu\text{g}/\text{mm}^2$ on acrylic) and no macroscopic hemagglutination was observed, for this strain. However, via microscopic examination, it was possible to verify that strain IE75 could bind to human erythrocytes and cause some degree of agglutination. As expected, strain 9142-M10, the biofilm-negative control strain, did not agglutinate human erythrocytes because this strain cannot express PNAG/PIA [27]. Probably the clinical isolate IE75 expressed small amounts of PNAG/PIA, giving rise to both low biofilm formation and small hemagglutination capabilities, but the amount of PNAG/PIA produced by this strain was not formally quantified. Taking together hemagglutination data

that reflect the level of expression of PNAG/PIA, although it may not be entirely quantitative, it is possible to conclude that PNAG/PIA is a clear determinant of biofilm formation, but not for initial adhesion of *S. epidermidis* under the conditions studied. However, using shorter-term adherence assays to catheters, it has been found that PNAG/PIA contributes to adherence in this setting [30], emphasizing the effect of test conditions on the outcomes obtained.

Overall, it also appears from our studies that there is a wide range of variation in adherence and biofilm formation among clinical *S. epidermidis* strains. For instance, the number of adherent cells of clinical strain LE7 was 23 times lower than that of *ica*-interrupted strain 9142-M10 (on glass) and the number of adherent cells of clinical strain M129 was 13 times lower than that of clinical strain FJ6 (on acrylic). Strain IE214 formed a biofilm almost 8 times greater than that of strain IE75 and 5 times greater than that of strain LE7 (on acrylic). Thus, initial adhesion and biofilm formation on inert surfaces, which are considered to be manifestations of one of the major virulence factors of *S. epidermidis*, are strain-dependent and not a consistent phenotypic characteristic of the species or of clinical isolates. This means that when evaluating either bacterial adherence or biofilm formation, it is important to take into account that the relative levels of these phenotypes do not reflect the potential of the organism to cause a clinically significant infection. Obviously, host factors associated with susceptibility to infection make a major contribution to the outcome of an infectious process, and this cannot be determined by measuring bacterial phenotypes. Therefore, it might be important to consider that properties of PNAG/PIA unrelated to bacterial adherence and biofilm formation, such as providing resistance of the bacteria to host immune effectors [33,42], may be important in the outcome of an *S. epidermidis* infection.

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