Staphylococcus epidermidis with the icaA⁻/icaD⁻/IS256⁻ genotype and protein or protein/extracellular-DNA biofilm is frequent in ocular infections

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In ocular infections (OIs) caused by Staphylococcus epidermidis, biofilms composed mainly of poly-N-acetylglucosamine (PNAG) have been widely studied, but PNAG-independent biofilms have not. Therefore, we searched for a relationship between the ica operon (involved in PNAG-biofilm) and the biochemical composition of biofilms in isolates from OI. Isolates from OI (n=62), from healthy conjunctiva (HC; n=45) and from healthy skin (HS; n=53), were used to detect icaA and icaD genes, and the insertion sequence 256 (IS256) using PCR. The compositions of the biofilms were determined by treatment with NaIO₄, proteinase K and DNase I. Multilocus sequence typing (MLST) was performed to characterize the isolates, and the expression of aap and embp genes was determined by real-time qPCR. A strong relationship between the icaA⁻/icaD⁻/IS256⁻ genotype and protein- or protein/extracellular DNA (eDNA)-biofilm composition was found in the isolates from OI (53.6%), whereas the icaA⁻/icaD⁻/IS256⁻ genotype and carbohydrate-biofilm was most prevalent in isolates from HC (25%) and HS (25%). Isolates with an icaA⁻/icaD⁻/IS256⁻ genotype and protein-biofilm phenotype were predominantly of the ST2 lineage, while carbohydrate-biofilm-producing strains were mainly of the ST9 lineage. The protein-biofilm-producing strains had higher expression levels of aap gene than carbohydrate-biofilm-producing strains; while embp gene did not have the same pattern of expression. These results suggest that S. epidermidis strains with icaA⁻/icaD⁻/IS256⁻ genotype and protein- or protein/eDNA-biofilms have a stronger ability to establish in the eye than S. epidermidis strains with icaA⁻/icaD⁻/IS256⁻ genotype and PNAG-biofilms.

INTRODUCTION

Staphylococcus epidermidis has been considered as an innocuous commensal micro-organism of the human skin and an important opportunistic pathogen. Staphylococcus aureus and S. epidermidis are the primary causative agents of nosocomial infections (National Nosocomial Infections Surveillance System, 2004). However, S. epidermidis is the most common source of ocular infections (OIs; Juárez-Verdayes et al., 2006) and has acquired mechanisms of adaptation to establish as an infectious micro-organism in the eye.

One of the most studied virulence factors in S. epidermidis is biofilm formation, which provides the basis for the bacterium’s resistance to many antibiotics and the immune response of the host (Costerton et al., 1999). Biofilms are formed by the initial adhesion of micro-organisms to abiotic or biotic surface, and their subsequent agglomeration to form a multicellular structure. Adherence to abiotic surfaces, such as catheters, depends on the hydrophobicity of the surface of bacterial cells (Vacheethasanee et al., 1998). Specific proteins involved in adhesion to abiotic...
surface in *S. epidermidis* include autolysin E (AtIE; Heilmann *et al.*, 1997) and biofilm-associated protein (Bap; Tormo *et al.*, 2005), which contribute to the hydrophobic character of bacterial cell surfaces, while the adherence to biotic surfaces, as occurring in extracellular matrix of the host, is conducted by the surface proteins called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), such as *S. epidermidis* SdrG that binds to fibrinogen (Arrecubieta *et al.*, 2007), and SdrF to collagen (Hartford *et al.*, 2001). Furthermore, it has been shown that AtIE (Heilmann *et al.*, 1997) and autolysin/adhesion (Aae; Heilmann *et al.*, 2003) proteins may bind to fibrinogen, fibronectin and vitronectin non-specifically. After the initial adhesion step, the development of biofilms through intercellular aggregation is mediated by many surface macromolecules, such as exopolysaccharides, proteins and teichoic acids; biofilms may even incorporate the macromolecules of lysed cells such as extracellular DNA (eDNA; Rice *et al.*, 2007).

Several *S. epidermidis* strains secrete poly-N-acetylglucosamine (PNAG), which surrounds the bacterium and enhances biofilm formation (Mack *et al.*, 1996). Therefore, in *S. epidermidis*, the production of PNAG is critical for biofilm formation (Heilmann *et al.*, 1996). The intercellular adhesion (*ica*) operon is formed by the *icaA*, *icaD*, *icaB* and *icaC* genes, which participate in the biosynthesis of PNAG (Heilmann *et al.*, 1996). The *IcaA* and *IcaD* proteins produce a chain from activated N-acetylglucosamine (GlucNac) monomers, which is exported by the *IcaC* protein (Gerke *et al.*, 1998). After exportation, the surface enzyme *IcaB* partially deacetylates the GlucNac residues (Vuong *et al.*, 2004). PNAG production depends on a variety of regulatory factors, and in *S. aureus* it has been demonstrated that expression of *icaADBC* can be turned off by transcriptional regulator of the teicoplanin-associated (TcaR) and intercellular adhesion regulatory (IcaR) proteins (Jefferson *et al.*, 2004). Besides, it has been shown that global regulator of stress response (Spx) protein induces the expression of *icaR* (Pamp *et al.*, 2006), and protein regulator of biofilm formation (Rfb) protein inhibits it (Cue *et al.*, 2009). Under anaerobic conditions, staphylococcal respiratory response regulator (SrAB) protein is synthesized and induces *icaADBC* expression (Ulrich *et al.*, 2007). Also, some *S. epidermidis* strains contain the insertion sequence 256 (IS256), which deactivates the *ica* operon (Ziebuhr *et al.*, 1999).

Recently, strains of *S. epidermidis* that do not require the *ica* genes to form biofilms have been reported (Kogan *et al.*, 2006), and strains with an *ica*-negative genotype that are still associated with biofilm infections have been isolated (Rohde *et al.*, 2007). These findings suggest that PNAG is not essential for biofilm formation in *S. epidermidis*. Moreover, two cell-surface-associated proteins have been implicated in the formation of the matrix of biofilms in *S. epidermidis*: accumulation-associated protein (Aap), a member of the family of Bap-like proteins, and extra-cellular matrix-binding protein (Embp). Full-length Aap is expressed as tufts of short fibrils (Banner *et al.*, 2007), and it has an external A-domain and an intermediate B-domain. The external A-domain of the Aap protein mediates adhesion to human corneocytes (Macintosh *et al.*, 2009), and the intermediate B-domain participates in biofilm formation (Rohde *et al.*, 2005). Full-length Aap is thought to be proteolytically processed to eliminate the external A-domain and to expose the intermediate B-domain because full-length Aap does not lead to biofilm formation. Furthermore, non-biofilm-producing strains treated with proteases in vitro have been observed to produce Aap-dependent biofilms (Rohde *et al.*, 2005). It has also been reported that the overexpression of a 460 kDa truncated isoform of Embp is necessary for biofilm formation and that Embp-mediated biofilm formation also protects *S. epidermidis* from phagocytosis via macrophages (Christner *et al.*, 2010).

Some *S. epidermidis* strains isolated from infections produce more biofilm than those isolated from healthy skin (Duggirala *et al.*, 2007; Suzuki *et al.*, 2005). Although the virulence capacity of *S. epidermidis* biofilms in the eye has been documented (Baillif *et al.*, 2008), to the best of our knowledge the biochemical compositions of biofilms in isolates from OIs have not been studied. Therefore, in this study the biochemical compositions of *S. epidermidis* biofilms from ocular infections (OI), healthy conjunctiva (HC) and healthy skin (HS) were analysed and compared.

**METHODS**

**Isolates.** Samples were obtained from ophthalmologist-diagnosed patients with conjunctivitis (*n=21*), corneal ulcers (*n=7*), endophthalmitis (*n=14*) or staphylococcal blepharitis (*n=20*). Corneal ulcer samples were obtained by scraping, and conjunctivitis and staphylococcal blepharitis samples were obtained by swabbing. The vitreous samples of patients with endophthalmitis were obtained mainly by vitrectomy. Swabbing samples were obtained from the skin of healthy individuals (HS; *n=53*), and the conjunctiva of healthy individuals (HC; *n=45*). All clinical samples were spread onto chocolate blood and mannitol salt agar plates, and incubated at 37°C for 12 h. For OI, where plates exhibited single colony morphology, these were regrown onto mannitol salt agar plates. *S. epidermidis* identification was performed by the Vitek Jr computerized system (BioMérieux), using the GPS-101 and V-1305 identification cards for Gram-positive bacteria. This study followed the tenets of the Declaration of Helsinki and was approved by the ethics review board of our institution. All patients agreed to participate in this study.

**PCR amplification of the* icaA* and *icaD*, and IS256.** DNA isolates were obtained using the method of Catalanotto *et al.* (2005). The partial amplifications of *icaA* and *icaD* genes, and IS256 were performed using primers described in Table 1. The PCRs were performed with 1 μl of DNA (100 ng), 1× buffer, 1 mM MgCl₂, 200 μM of each dNTPs, 1 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 0.2 μM of each specific primer. The optimal PCR conditions were 30 cycles of 30 s at 92°C, 40 s at 60°C and 30 s at 72°C. The PCR products were analysed on agarose gels.

**Determination of biofilm formation.** A semiquantitative determination of biofilm formation was performed in 96-well tissue
cells were harvested by scraping and washed with PBS 1×. Total RNA was extracted with TRIzol (Invitrogen), treated with DNase I (Invitrogen) and re-extracted. For the reverse transcriptase (RT) reaction, total RNA (3 μg) with 0.5 μg of oligo-hexamers (Invitrogen) was denatured at 70 °C for 10 min. Then, 1× single strand buffer, 0.5 mM DTT, 10 mM of each dNTP, and 200 U of MMLV RT (Invitrogen) were added. The RT reactions were performed at 42 °C for 1 h. Expression levels of RNA were analysed by RT-qPCR with the Fast SYBR Green qPCR kit (Applied Biosystems) according to the manufacturer’s instructions. Relative starting quantities of the RNAs for the genes of interest and the constitutive gene 16S rRNA (Table 1) were calculated by the standard curve method using genomic DNA of S. epidermidis as PCR template. Quantity of mRNA was normalized as PCR template. Quantity of mRNA was normalized

### RESULTS

#### Detection of the icaA and icaD genes, and IS256 in isolates of S. epidermidis

The icaA and icaD genes were homogeneously distributed among the isolates from HS, HC and OI, with an incidence from 15.1 to 26.7% (P>0.05 among different sources). The frequency of IS256 was similar to the rate of icaA and icaD genes (Table 2).

Next, the frequencies of the genotypes in each population were calculated, and eight different genotypes were exhibited (Table 3). The most common genotypes found in the three collections are: the genotype carrying the ica genes (icaA +/icaD +/IS256 + able to form PNAG-biofilms),

### Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5′→3′)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>icaA</td>
<td>Fw: TCTCTTGCAGGAGCAATCAGA Rv: AGGCACATAACATCCAGCA</td>
<td>Catalanotti et al. (2005)</td>
</tr>
<tr>
<td>icaD</td>
<td>Fw: ATGTCAGCCGCCAGCGACAG Rv: AGCTTTTTTCACATTTAATGCAA</td>
<td>Catalanotti et al. (2005)</td>
</tr>
<tr>
<td>IS256</td>
<td>Fw: TGAAGGCGAAGATGTAAAGC Rv: AGTGTAGGTTCCATAGGACAGC</td>
<td>Catalanotti et al. (2005)</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>Fw: AGGAGTCTCAGGACGTGGTTC Rv: CGGAGGCCGACCTGAGAG</td>
<td>This work (qPCR)</td>
</tr>
<tr>
<td>atlE</td>
<td>Fw: GCAGGGTCACACTTTACACA Rv: TCCACACAGATTACCTTCA</td>
<td>This work (qPCR)</td>
</tr>
<tr>
<td>aap</td>
<td>Fw: AGAAGAAGGTCGTCAG Rv: ATTCGATGATTGAAATC</td>
<td>This work (qPCR)</td>
</tr>
<tr>
<td>embp</td>
<td>Fw: GCTGATGAAGCCATAAAAATTAC Rv: AGAAATGCTTAGCATTC</td>
<td>This work (qPCR)</td>
</tr>
</tbody>
</table>

Fw: Forward; Rv: reverse.

### Table 2. Distribution of icaA and icaD genes, and IS256 in S. epidermidis isolates from different sources

<table>
<thead>
<tr>
<th>Source of isolation</th>
<th>icaA (%)</th>
<th>icaD (%)</th>
<th>IS256 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy skin, n=53</td>
<td>20.8</td>
<td>15.1</td>
<td>18.9</td>
</tr>
<tr>
<td>Healthy conjunctiva, n=45</td>
<td>15.5</td>
<td>26.7</td>
<td>13.3</td>
</tr>
<tr>
<td>Ocular infections, n=62</td>
<td>24.2</td>
<td>21.0</td>
<td>26.0</td>
</tr>
</tbody>
</table>

*Detection was by PCR according to Catalanotti et al. (2005). To compare data of percentages, a Fisher’s exact test was performed.
and the genotype that lost them (icaA−/icaD−/IS256− unable to form PNAG-biofilms). Regarding the icaA+/icaD+/IS256+ genotype, it was the least commonly found among the three collections of isolates (9.4% for HS, 15.6% for HC and 13% for OI, P=0.05 among themselves), in contrast with the icaA−/icaD−/IS256− genotype that was the most abundant (60.4% for HS, 64.5% for HC and 54.9% for OI, P=0.05 among themselves). Both genotypes are statistically different (P<0.05; Table 3). icaA−/icaD−/IS256− genotype might indicate two situations: (i) bacteria do not have the capacity to produce biofilms or (ii) bacteria produce PNAG-independent biofilms.

Relationship between biofilm production and genotypes

The production of biofilms by isolates in the three collections was examined and it was found that the largest number of isolates that produced biofilms occurred in the OI collection (45.2%). In comparison 17.7% of HC and 7.5% of HS isolates produced biofilms (P<0.003; Table 3).

Table 3 shows the relationships between biofilm production (phenotype) and the presence of the icaA and icaD genes and IS256 (genotypes). Biofilm-producing isolates from HS and HC had predominantly the icaA+/icaD+/IS256− genotype (3.7% for HS and 15.6% for HC) compared to the icaA−/icaD−/IS256− genotype (P<0.003); in contrast with non-biofilm-producing isolates, that had mainly the icaA−/icaD−/IS256− genotype (60.4% for HS and 64.5% for HC; P<0.003 compared to icaA+/icaD+/IS256− genotype). However, isolates from OIs showed a homogeneous distribution of genotypes between the two groups of biofilm-producing and non-biofilm-producing phenotypes, and the most frequent genotype was icaA−/icaD+/IS256− (27.4%) compared to icaA+/icaD+/IS256− genotype (4.8%; P<0.003; Table 3). Among the biofilm-producing isolates, the icaA+/icaD+/IS256− genotype, which was associated with carbohydrate-biofilms, was also the most strongly represented in the isolates from HS and HC, whereas the icaA−/icaD+/IS256− genotype was most prevalent in isolates from OIs (Table 3). These results suggest a relationship between the icaA+/icaD+/IS256− genotype and biofilm-producing isolates from OIs, unlike in the case of biofilm-producing isolates from HS and HC (in which the icaA+/icaD+/IS256− genotype was the most prevalent).

Table 3. Frequency of genotypes icaA, icaD, and IS256 in S. epidermidis isolates and their relationship with biofilm production

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Healthy skin, n=53 (%)</th>
<th>Healthy conjunctiva, n=45 (%)</th>
<th>Ocular infections, n=62 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (%)</td>
<td>B (+) (%)</td>
<td>B (−) (%)</td>
</tr>
<tr>
<td>icaA+/icaD+/IS256+</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>icaA+/icaD−/IS256−</td>
<td>5 (9.4)†</td>
<td>2 (3.7)†</td>
<td>3 (5.8)†</td>
</tr>
<tr>
<td>icaA−/icaD+/IS256−</td>
<td>3 (5.8)</td>
<td>2 (3.7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>icaA−/icaD−/IS256−</td>
<td>1 (1.9)</td>
<td>0 (0)</td>
<td>1 (1.9)</td>
</tr>
<tr>
<td>icaA+/icaD+/IS256−</td>
<td>4 (7.6)</td>
<td>1 (1.9)</td>
<td>3 (5.8)</td>
</tr>
<tr>
<td>icaA−/icaD−/IS256−</td>
<td>3 (5.8)</td>
<td>0 (0)</td>
<td>2 (3.7)</td>
</tr>
<tr>
<td>icaA+/icaD−/IS256−</td>
<td>7 (13.3)</td>
<td>1 (1.9)</td>
<td>6 (11.2)</td>
</tr>
<tr>
<td>icaA+−/icaD+/IS256−</td>
<td>32 (60.4)</td>
<td>0 (0)</td>
<td>32 (60.4)</td>
</tr>
</tbody>
</table>

*B. Biofilm producer and the results were analysed using a one-way ANOVA with a Tukey’s test.
†The icaA+/icaD+/IS256− vs icaA−/icaD−/IS256− genotypes were compared and a Fisher’s exact test was performed.

Relationship between biochemical compositions of biofilms and their genotypes

In case of the isolates from HS, the distribution of biochemical compositions of biofilms and their genotypes was as follows: one isolate formed a carbohydrate-biofilm, and its genotype corresponded to the expected icaA+/icaD+/IS256+; three isolates forming protein-biofilms, and in two of them showed genotypes missing one or both ica genes and IS256, however the third isolate interestingly carried the icaA+/icaD+/IS256+ genotype and biofilm-producing isolates from OIs, unlike in the case of biofilm-producing isolates from HS and HC (in which the icaA+/icaD+/IS256− genotype was the most prevalent).
with protein-eDNA-biofilm composition that were carrying icaA+/icaD−/IS256− genotype. Furthermore, two isolates with an ‘undetermined-biofilm’ presented icaA+/icaD+/IS256+ and icaA−/icaD−/IS256+ genotypes (Table 4).

There was an association between biochemical compositions of biofilms and genotypes, since it was found that 25% of the isolates from HS and HC carried icaA+/icaD+/IS256+ genotype and carbohydrate-biofilm composition, and not isolated with icaA+/icaD−/IS256− genotype and protein- or protein-eDNA-biofilm composition were observed (0%; P<0.05 by Fisher’s exact test). In contrast, 53.5% of the isolates from IOs carried icaA−/icaD−/IS256− genotype and protein- or protein-eDNA-biofilm composition; also, they were not isolated with icaA+/icaD+/IS256+ genotype and carbohydrate-biofilm composition (0%; P<0.05 by Fisher’s exact test). These results demonstrated a strong relationship between the icaA+/icaD+/IS256+ genotype and protein- or protein/eDNA-biofilm composition in the isolates from IO, whereas the icaA+/icaD+/IS256− genotype and carbohydrate-biofilm was the most prevalent in isolates from HC and HS.

In order to investigate whether there is a genetic relationship between the isolates, MLST analysis was performed. Twelve different ST lineages were found, and ST2 lineage was the most frequent among the isolates (50% for HS, 25% for HC and 46.5% for IO, P>0.05 among themselves) followed by ST9 lineage. When phenotypes/genotypes were associated with some ST lineage, carbohydrate-biofilm-producing strains were predominantly ST9 lineage (4/5; 80%), and none exhibited ST2 lineage. With regard to protein- or protein-eDNA-biofilm-producing strains, these were predominantly ST2 lineage (15/30; 50%) and very few displayed ST9 lineage (3/30; 10%, P<0.05). Furthermore, in isolates from HC and IO, there was greater variation of STs, in contrast to the isolates from HS (Table 4).

To determine if those strains with the genotype icaA+/icaD+/IS256− and protein-biofilm composition are capable of producing a carbohydrate-biofilm, these strains were grown in the presence of NaCl, or glucose, or ethanol (enhancers of biofilm production). It was found that strains grown with NaCl or ethanol, but not glucose, produced a change in the biochemical composition of their biofilms, producing protein- and carbohydrate-biofilms, in contrast to a carbohydrate-biofilm-producing strain treated under the same conditions, it did not change the composition of its biofilm (Table 5).

**Expression of icaD, atlE, aap and embp genes in protein-biofilm producer strains**

In order to relate atlE, aap and embp genes with protein-biofilm formation, the expression of these genes was determined. Comparing the expression levels of icaD between the protein-biofilm-producing isolates versus the
carbohydrate-biofilm-producing isolates, only carbohydrate-biofilm-producing strains expressed icaD gene (Fig. 1a, \( P < 0.05 \)); while for the atlE gene, there was a low expression level in carbohydrate-biofilm-producing isolates (Fig. 1b, \( P < 0.05 \)). Protein-biofilm-producing strains had higher expression levels of aap gene than carbohydrate-biofilm-producing strains (Fig. 1c, \( P < 0.05 \)); in contrast, there was no statistically significant difference in the expression of the embp gene between carbohydrate-biofilm-producing isolates and protein-biofilm-producing isolates (Fig. 1d).

**DISCUSSION**

Our findings have shown that a relationship exists between biofilm production and the icaA\(^+\)/icaD\(^-\)/IS\(^{256}\)\(^-\) genotype in isolates from HS and HC; conversely, the isolates from OIs displayed mainly the icaA\(^-\)/icaD\(^-\)/IS\(^{256}\)\(^-\) genotype. The non-biofilm-producing isolates from the three sources tended to lack the icaA and icaD genes, signifying a decreased ability to produce a biofilm (or, at least, a PNAG-biofilm). In the OI isolates, we observed a connection between the non-PNAG-biofilm-forming genotype (icaA\(^-\))

<table>
<thead>
<tr>
<th>Source of isolation</th>
<th>Genotype and ST</th>
<th>Biochemical composition in TSB*</th>
<th>Biochemical composition in TSB supplemented†</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS</td>
<td>icaA(^+)/icaD(^+)/IS(^{256})(^-) and ST9</td>
<td>Carbohydrate</td>
<td>Carbohydrate</td>
</tr>
<tr>
<td>HS</td>
<td>icaA(^+)/icaD(^+)/IS(^{256})(^-) and ST2</td>
<td>Protein</td>
<td>Carbohydrate and protein</td>
</tr>
<tr>
<td>OI</td>
<td>icaA(^-)/icaD(^-)/IS(^{256})(^-) and ST2</td>
<td>Protein</td>
<td>Protein</td>
</tr>
</tbody>
</table>

*The results were analysed using a one-way ANOVA with a Tukey’s test.
†NaCl 3% (w/v) or ethanol 4.5% (v/v) were added to the TSB medium (TSB supplemented).

**Table 5. Changes in the biochemical composition of the biofilm produced by addition of NaCl or ethanol**

![Fig. 1. Expression of icaD, atlE, aap and embp in protein-biofilm-producing strains. Measurements of mRNA expression levels for each gene were performed: icaD (a), atlE (b), aap (c), and embp (d), in three carbohydrate (CH)-biofilm-producing strains (icaA\(^+\)/icaD\(^+\)/IS\(^{256}\)\(^-\) genotype), and in three protein-biofilm-producing strains (icaA\(^-\)/icaD\(^-\)/IS\(^{256}\)\(^-\) genotype). SQ norm, start quantity normalized. Statistical differences between CH-biofilm and protein-biofilm were analysed by a Student’s t-test (*, \( P < 0.05 \)).
and/or icaD) and protein- or protein-eDNA-biofilm composition; to the best of our knowledge, this result has not previously been reported. However, this relationship was not observed in all the cases. We observed isolates that were lacking the icaA and icaD genes but continued to produce carbohydrate-biofilms, which could be explained by the formation of different carbohydrate-biofilms (distinct from the PNAG-biofilm). It has been observed that Streptococcus thermophilus strains produce exopolysaccharides of different monomer compositions and different molecular masses (Vaningelgem et al., 2004) and Pseudomonas aeruginosa can produce three distinct exopolysaccharides such as alginate, Pel and Psl (Ryder et al., 2007). Additionally, it was demonstrated that strains with icaA+/icaD+ /IS256 genotype and protein-biofilm composition were able to change the biochemical composition of their biofilm (from protein-biofilm to carbohydrate-protein-biofilm) when they were grown in NaCl or ethanol. This result indicates that the ica operon is functional and inducible by NaCl or ethanol. It has been demonstrated that NaCl increases biofilm production by activation of ica operon expression and ethanol induces the expression of icaR (Conlon et al., 2002).

A high genetic variability has been observed in S. epidermidis, so it is considered that this species has an open pan-genome (Conlan et al., 2012). This fact was also found in our strains, since it was observed that among 40 biofilm-producing strains, 12 STs were observed, with ST2 lineage the most frequent. It has been proposed that the high incidence of ST2 lineage may be caused by the presence of IS256 and ica genes, since all ST2 isolates contain these two factors, which have been linked to the invasiveness of S. epidermidis (Kozitskaya et al., 2004). Moreover, most of the ST2 isolates exhibit an ability to form biofilms in vitro (Li et al., 2009). It has been observed that biofilm-forming strains and ica-positive strains occur at a higher frequency than non-biofilm-producing strains and ica-negative strains in the ST2 lineage (Li et al., 2009). To our knowledge, no studies exist that associate the ST lineage with the composition of the biofilm. In this study, 50% of strains with ST2 lineage have a biofilm composed of protein- or protein-eDNA, and 80% of the ST9 lineage strains have a carbohydrate-biofilm. This suggests that the composition of the biofilm produced by S. epidermidis may be associated with the particular lineages.

The fact that protein-biofilms predominated in isolates from OIs suggests that this type of biofilm may function to promote virulence in the eye. Few studies comparing the strength of protein-biofilms with the PNAG-biofilms have been performed. However, carbohydrate-biofilms have been documented as being more resistant and rigid than protein-biofilms (Hussain et al., 1997). Other researchers have also isolated strains that form protein-biofilms from infections (Kogan et al., 2006; Hussain et al., 1997), suggesting that protein-biofilms might have the same functions as carbohydrate-biofilms. Furthermore, an in vitro assay demonstrated that protein-biofilms protect against phagocytosis via neutrophils (Rohde et al., 2005). These data suggest that protein-biofilms may have the same functions as PNAG-biofilms.

In S. epidermidis, two cell-surface-associated proteins have been implicated in the formation of the matrix of biofilms: Aap and Embp. One study reported that 27% of clinic isolates from prosthetic joint infections after total hip arthroplasty were S. epidermidis strains producing Aap-mediated biofilms (Rohde et al., 2007). Our results suggest that Aap is involved in the formation of protein-biofilm in our strains; the expression levels of aap were higher in protein-biofilm-producing strains than in carbohydrate-biofilm-producing strains. Previously it has been shown that aap expression is constitutive among various strains (Patel et al., 2012). With respect to the Embp protein, there was a similar expression level in both phenotypes, which suggests that it might not be involved in protein-biofilm formation. However, more studies are required to elucidate its participation.

Biofilms containing eDNA have been detected in various bacteria isolated from wastewater environments (Frolund et al., 1996). DNA is a major structural component of S. aureus biofilms, whereas it is a minor component of S. epidermidis biofilms (Izano et al., 2008). Our results show that eDNA-biofilms occur more frequently in OI isolates than in HC and HS isolates. A previous study demonstrated that eDNA-biofilms were present in 100% of clinical S. epidermidis isolated from implant infections (Ravaïoli et al., 2011).

With regard to the isolates with an ‘undetermined-biofilm’ composition that were observed, we can envisage two possibilities: the biofilms were formed by other biomolecules, or carbohydrates and proteins were subjected to further modifications (acetylation, methylation, etc.), and were therefore resistant to the treatments used in our assay. NaIO4 oxidizes the diol groups of carbohydrates to aldehydes, breaking the –C–C bonds, so we think that the isolates with undetermined-biofilm composition may have modifications such as amination or acetylation in the hydroxyl groups of their carbohydrates, since they were NaIO4 resistant. In the future, it would be interesting to test this hypothesis. This assertion can be supported by the findings of exopolysaccharide-biofilm carrying modifications with organic and inorganic substituents (Conrad et al., 2003).

In this work, biofilm-producing isolates from OIs exhibited a variety of genotypes and biochemical biofilm compositions in comparison with HC and HS isolates, suggesting that different morphotypes of biofilms may occur and perform similar protective functions. Further studies in vivo are required to corroborate the functions of different types of biofilm in S. epidermidis isolates from OIs.

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