Biofilm characteristics of *Staphylococcus epidermidis* isolates associated with device-related meningitis

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Staphylococcus epidermidis biofilm causes device-related meningitis in neurosurgical patients. This study assessed the contribution of polysaccharide and protein to the development of a strong biofilm-positive phenotype in four *S. epidermidis* isolates associated with probable device-related meningitis, under varying environmental conditions. RT-PCR analysis of the intercellular adhesion operon (*icaADBC*) and assessment of polysaccharide intercellular adhesin (PIA) production indicated a correlation between increased *icaA* transcription and PIA production in *ica*⁺ isolates grown in medium with 4 % ethanol and 4 % NaCl. Treatment of biofilm with sodium metaperiodate caused dispersion of adhered cells ($P < 0.0001$), indicating involvement of PIA. Transcriptional levels of protein factors revealed that *atlE* transcription levels were similar in all isolates, whilst *aap* levels were variable, with induction being seen in two isolates following growth in the presence of alcohol or salt. Transcription of *agr* did not influence protein expression and *RNAIII* transcription varied among the strains. Although *aap* transcription was induced, the treatment of biofilm with proteinase K did not always disperse the biofilm. Our data suggest that, among the three *ica*⁺ *S. epidermidis* isolates clinically associated with meningitis that were studied, PIA contributed to the strong biofilm-positive phenotype, whereas protein factors appeared to have a secondary role.

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INTRODUCTION

Successful treatment and prevention of hydrocephalus, a common neurosurgical condition, often involves the insertion of an external ventricular drain (EVD) or a shunt. However, this can be complicated by infections such as meningitis, often caused by coagulase-negative staphylococci (CoNS), with *Staphylococcus epidermidis* being isolated most frequently (Diaz-Mitoma *et al.*, 1987). Mounting evidence suggests that such infections are a result of biofilm formation on the device (Diaz-Mitoma *et al.*, 1987; Tojo *et al.*, 1988; Kockro *et al.*, 2000).

Staphylococcal biofilm formation involves both host and bacterial factors. Early investigations have shown that production of polysaccharide is a primary mechanism employed by *S. epidermidis* (Peters *et al.*, 1987; Tojo *et al.*,

1988; Christensen *et al.*, 1990; Mack *et al.*, 1992). The polysaccharide is a polymeric homoglycan consisting of repeating units of β -1,6-*N*-acetylglucosamine (Mack *et al.*, 1996; McKenney *et al.*, 1998; Maira-Litran *et al.*, 2002; Sadvskaya *et al.*, 2005), termed polysaccharide intercellular adhesin (PIA). Synthesis of PIA is linked to the biosynthetic enzyme-coding genes located in the intercellular adhesion operon (*icaADBC*) (Heilmann *et al.*, 1996), and biofilm production involving PIA is known to be *ica*-dependent and is considered the primary mechanism employed by staphylococci (Stevens *et al.*, 2008).

Other *ica*-independent mechanisms of biofilm development in staphylococci have been identified and protein factors such as the major cell-wall autolysin (AtlE) have been found to promote the initial adhesion of *S. epidermidis* to surfaces (Heilmann *et al.*, 1997). More recently, it was found that *atlE* is influenced by the accessory gene regulator (*agr*) two-component system (Batzilla *et al.*, 2006). Furthermore, Hussain *et al.* (1997)

Abbreviations: CoNS, coagulase-negative staphylococci; CSF, cerebrospinal fluid; EVD, external ventricular drain; PIA, polysaccharide intercellular adhesin.

identified a mutant strain of *S. epidermidis* that displayed a lack of ability to accumulate on glass and polystyrene surfaces, associated with the absence of a 140 kDa surface protein (Hussain *et al.*, 1997), the accumulation associated protein (Aap). Aap requires proteolytic cleavage for activation, and it is the B domain that is involved in the accumulation of cells (Rohde *et al.*, 2005).

Aap has an LPXTG motif, and, similar to other proteins such as the biofilm-associated protein (Bap) in *Staphylococcus aureus* and the Bap homologue (Bhp) in *S. epidermidis*, is important in the staphylococcal *ica*-independent mechanism of biofilm formation (Cucarella *et al.*, 2001, 2004; Rohde *et al.*, 2005; Tormo *et al.*, 2005).

We have demonstrated previously that carriage of both *ica* and *aap* in *S. epidermidis* meningitis-causing isolates is associated with higher levels of biofilm production and significant infection (Stevens *et al.*, 2008). Here, we have described further strongly biofilm-positive *S. epidermidis* isolates that were associated with EVD-related infections and identified *ica*-dependent and protein-dependent biofilm mechanisms.

METHODS

Bacterial strains and isolates. The well-characterized biofilm-forming laboratory strain CSF41498 (Conlon *et al.*, 2002a, b) is a clinical cerebrospinal fluid (CSF) isolate obtained from the microbiology diagnostic laboratory at Beaumont Hospital, Dublin, which contains the National Neurosurgical Unit and in which approximately 10–15 patients each year have CoNS isolated from their CSF deemed to be of clinical significance. The biofilm-positive reference strain RP62A (ATCC 35984) was used as a control in haemagglutination assays. The four *S. epidermidis* isolates examined were collected from CSF specimens from neurosurgical patients at Beaumont Hospital. These isolates, associated with EVD-related meningitis, have been described in an earlier study (Stevens *et al.*, 2008). Briefly, 62 CoNS isolates from the CSF of 46 patients were assessed for biofilm production by two phenotypic methods: the presence of *ica* and *aap* by PCR, and clonal variability by PFGE. Among the isolates associated with meningitis, 71.4% produced biofilm and *ica*⁺/*aap*⁺ isolates produced more biofilm than *ica*⁺/*aap*[−] isolates (Stevens *et al.*, 2008).

Haemagglutination assay: indirect indicator of PIA production. Indirect analysis of PIA activity was determined according to the method outlined by Rupp & Archer (1992). The assay was performed in a 96-well round-bottomed microtitre plate (Nunc) as follows: 100 µl of a 1:10 bacterial suspension was placed in the first test well and then serially diluted twofold (1:10–1:1280), so that each test well contained 50 µl. To each well, 50 µl of a 1% suspension of sheep erythrocytes (Sigma) was added. Plates were sealed with Cellophane and shaken gently to ensure even mixing of the bacterial suspension and erythrocytes. Plates were incubated at room temperature for 2 h. Haemagglutination was scored according to the criteria outlined by Rupp & Archer (1992).

RNA purification and analysis. RNA purification and RT-PCR were performed as described previously (Conlon *et al.*, 2002a, b). The constitutively expressed *gyrB* gene was used as an internal standard in this set of experiments using the primers described by Goerke *et al.* (2000). Additional primers used in this study are outlined in Table 1.

Table 1. *S. epidermidis* RT-PCR target genes, primers and primer sequences

Target gene	RT-PCR primers	Primer sequence (5'→3')
<i>gyrB</i>	<i>gyrB</i> 1	TTATGGTGTCTGGACAGATACA
	<i>gyrB</i> 2	CACCGTGAAGACCGCCAGATA
<i>icaA</i>	KCA1	AACAAGTTGAAGGCATCTCC
	KCA2	GATGCTTGTTTGATTCCCT
<i>icaR</i>	KCR1	GGTAAAGTCCGTCAATGGAA
	KCR2	CGCAATAACCTTATTTTCCG
<i>RNAIII</i>	RNAIIIF	TGAAGTTATGATGGCAGCAGAT
	RNAIIIR	GTTGGGATGGCTCAACAACT
<i>atlE</i>	ALT1	AAAGAAACGGCATCTAAT
	ALT2	TTCTTCAATTCTTGGTGCTT
<i>aap</i>	AAP1	AAAATTCAGTGTGGTACGG
	AAP2	AGAAGACATCTGTGGTGCTT

RT was performed at 55 °C for 30 min, terminated at 95 °C and followed by 24 cycles of PCR for *gyrB*, *icaA*, *icaR* or *atlE* transcript amplification, and 16 and 21 cycles for amplification of *RNAIII* and *aap*, respectively.

Biofilm assay and treatments. Semi-quantitative determinations of biofilm formation in 96-well tissue culture plates (Nunc) were performed based on the method of Christensen *et al.* (1985) and as described by Ziebuhr *et al.* (1997).

Bacteria were grown at 37 °C in brain–heart infusion (BHI) broth or under conditions known to induce biofilm formation: BHI supplemented with 4% (v/v) ethanol or 4% (w/v) NaCl. Plates were incubated for 24 h. Following incubation, treatments of adhered cells were performed according to methods described previously (Mack *et al.*, 1996; Rohde *et al.*, 2005; O'Neill *et al.*, 2007). Following this, test wells were treated with vehicle or with either sodium metaperiodate buffer (10 mM sodium metaperiodate, 50 mM sodium acetate) or proteinase K [100 µg ml^{−1} in 20 mM Tris/HCl (pH 7.5), 100 mM NaCl]. Plates were incubated at 37 °C for 2 h and then washed vigorously three times with dH₂O, dried for 45 min at 65 °C and stained with a 0.4% (w/v) crystal violet solution. After staining, the plates were again washed three times with sterile H₂O. The A₄₉₂ of the adhered, stained cells was measured using a Multiskan plate reader (Flow Laboratories). The criteria outlined by Christensen *et al.* (1985) were used to determine whether isolates were non-adherent and biofilm-negative (A₄₉₂ ≤ 0.12) or strongly biofilm-positive (A₄₉₂ > 0.24). Assays were performed in triplicate and the mean biofilm absorbance value is shown.

PIA immunoblot assay. PIA immunoblot assays were performed according to methods described previously (Cramton *et al.*, 1999; O'Neill *et al.*, 2007) with some modifications. Overnight cultures (5 ml containing ~5 × 10⁹ cells) were harvested by centrifugation and the pellet was resuspended in 0.5 mM EDTA, boiled for 5 min and centrifuged again. An aliquot of the supernatant was then treated with proteinase K (200 µg ml^{−1}; Sigma) at 65 °C for 1 h and then boiled for 5 min to inactivate the enzyme. Samples were diluted in Tris-buffered saline [TBS: 20 mM Tris/HCl (pH 7.4), 0.9% (w/v) NaCl] containing bromophenol blue (0.01%, v/v; Sigma).

An Immobilon-P nitrocellulose membrane (Millipore) was pre-treated with methanol for 15 s, washed with dH₂O for 2 min and soaked in TBS for 5 min. Samples were diluted as needed and transferred to the prepared membrane using a vacuum blotter. The membrane was allowed to dry, washed, rehydrated and blocked with

blocking buffer [TBS plus 1 % (w/v) BSA (Sigma)] for 1 h, treated with primary antibody solution comprising anti-PIA antibody (a gift from G. B. Pier, Harvard) diluted 1:5000 in TBS-T [TBS plus 0.001 % (v/v) Tween; Sigma] with 0.1 % (w/v) BSA for 1 h, and then with secondary antibody (horseradish peroxidase-labelled anti-rabbit antibody diluted 1:5000 in TBS-T plus 0.1 % skimmed milk) for 1 h. The blots were developed using ECL detection reagents (GE Healthcare) and detected using a Bio-Rad Fluor-S Max CCD camera system.

Congo red agar: indirect indicator of polysaccharide production. Congo red agar was used to examine indirectly the potential of *S. epidermidis* isolates to produce polysaccharide or slime (Ziebuhr *et al.*, 1997; Arciola *et al.*, 2003; Stevens *et al.*, 2008). Colony phenotypes were identified according to criteria described previously (Handke *et al.*, 2004).

Statistical analysis. Biofilm assays were performed in triplicate and mean biofilm absorbance values $\pm$ SD were calculated using Microsoft Office Excel 2003. One-tailed Student's *t*-tests were calculated using GraphPad Prism 4. Biofilm production was deemed significantly different when $P \leq 0.05$.

RESULTS AND DISCUSSION

Regulation of *ica* and PIA production

Initially, haemagglutination assays were performed as an indirect measure of PIA production (Table 2). Strain BM3, a known *ica*[−] strain, failed to agglutinate sheep erythrocytes. Strains BM6, B11 and BM13 showed similar patterns of haemagglutination to the biofilm-positive reference strain RP62A. All caused haemagglutination following growth in BHI broth. Growth in 4 % ethanol or 4 % NaCl led to a higher degree of agglutination. CSF41498 was also used as a control. This biofilm-positive laboratory strain of *S. epidermidis* showed enhanced haemagglutination following growth in ethanol but not in salt.

Table 2. Haemagglutination of erythrocytes by the four meningitis-causing *S. epidermidis* isolates following growth under various environmental conditions

0, No haemagglutination; +, loose pellet with surrounding halo of haemagglutination; ++, diffuse haemagglutination with some poorly organized sedimentation of erythrocytes; +++, diffuse haemagglutination. The dilution is the most dilute bacterial cell suspension that caused the highest degree of haemagglutination (range 1:10–1:1280).

Isolate	Degree of haemagglutination (dilution)		
	BHI	BHI + 4 % ethanol	BHI + 4 % NaCl
BM3	0	0	0
BM6	++ (1:10)	+++ (1:20)	+++ (1:10)
BM11	++ (1:10)	+++ (1:40)	+++ (1:10)
BM13	+++ (1:20)	+++ (1:40)	+++ (1:80)
CSF41498	++ (1:20)	+++ (1:40)	++ (1:10)
RP62A	+++ (1:10)	+++ (1:40)	+++ (1:80)

Next, we assessed *icaA* and *icaR* gene transcription (Fig. 1). Of the four clinical isolates, three were *ica*⁺. Strain BM3, which had failed to cause haemagglutination suggesting a lack of PIA production, was confirmed as *ica*[−] by RT-PCR (Fig. 1b). For all three *ica*⁺ strains, growth in 4 % ethanol decreased *icaR* expression and induced a concomitant increase in *icaA* (Fig. 1c–e). NaCl had a similar effect on *ica* gene expression in strains BM11 and BM13. Immunoblot analysis confirmed these transcriptional observations at the level of PIA production.

The best-understood pathway of biofilm production in *S. epidermidis* is that involving the production of PIA by the *ica*-operon encoded biosynthetic enzymes (IcaADBC) (Mack *et al.*, 1996; Maira-Litran *et al.*, 2002). An early study reported that CoNS, isolated from paediatric shunt infections, were capable of producing a visible mucoid extracellular matrix *in vitro* (Bayston & Penny, 1972). However, more recent studies have demonstrated the existence of *ica*-independent and protein-dependent pathways (Rohde *et al.*, 2005; Kogan *et al.*, 2006). The variation in biofilm-forming capabilities of *S. epidermidis* is likely to be great, given the possible combinations of interactions between the organism itself and the surrounding environment. Recently, in *S. aureus* clinical isolates it has been shown that different mechanisms of biofilm formation are used depending on the susceptibility of the isolate to methicillin (O'Neill *et al.*, 2007). In methicillin-resistant *S. aureus* isolates, a novel mechanism of protein-mediated biofilm formation seems to exist, which in part is promoted by the surface-expressed, LPXTG-containing, fibronectin-binding proteins FnBPA and FnBPB (O'Neill *et al.*, 2008). In comparison with *S. aureus*, *S. epidermidis* expresses fewer cell-surface proteins to participate in biofilm formation.

Examination of *icaA* and *icaR* transcription and subsequent PIA production revealed that all of our *ica*⁺ isolates showed similar patterns under each environmental condition: high levels of PIA production, detected by immunoblot assays and haemagglutination assays, were seen following decreased *icaR* transcription and increased *icaA* transcription when the medium was supplemented with 4 % ethanol or 4 % NaCl. The *icaA* and *icaR* transcriptional patterns were in accordance with the earlier findings of Conlon *et al.* (2002a, b), and the production of PIA indicated that *ica*-dependent formation is likely to be important.

AtlE and Aap

Biofilm formation correlates with carriage of *ica* in *S. epidermidis* clinical isolates associated with device-related infections (Fitzpatrick *et al.*, 2005). We have shown previously that carriage of the *ica* operon, either alone or in combination with *aap*, is common in the majority of meningitis-causing isolates and that the *ica*⁺ *aap*⁺ genotype is associated with significantly more biofilm production than in isolates positive for the *ica* locus alone

Fig. 1. Correlation between *ica* operon transcription and PIA production in neurosurgical *S. epidermidis* isolates under three different environmental growth conditions. Neurosurgical *S. epidermidis* isolates (b–e) were grown in BHI, or in BHI supplemented with 4% ethanol or 4% NaCl. The *ica*⁺ and biofilm-positive laboratory strain CSF41498 (a) and the previously characterized *ica*⁺ and biofilm-positive isolate BM3 (b) acted as positive and internal negative controls, respectively. Transcription of the constitutively expressed *gyrB* (control), as well as that of *icaA* and *icaR*, was measured by RT-PCR using RNA isolated from cultures grown at 37 °C to an OD₆₀₀ of 2.0 (upper panels). PIA production (lower panels) was detected in undiluted cell extracts of overnight cultures using PIA-specific antibody.

(Stevens *et al.*, 2008). In addition to PIA production, protein factors are likely to contribute strongly to the development of mature biofilms *in vivo* in some isolates. Therefore, we assessed the expression levels of *aap* and *atlE*.

A previous proteomic analysis revealed that mutation of the *agr* two-component system in *S. epidermidis* was associated with decreased levels of extracellular autolytic proteins such as AtlE and also influenced the accumulation of extracellular proteases (Batzilla *et al.*, 2006). For the four isolates studied here, *atlE* transcript levels did not vary significantly under different growth conditions (Fig. 2). High levels of *RNAIII* transcription were generally associated with high levels of *atlE* transcription. However, although growth in ethanol led to variable decreases in *RNAIII* transcription in all four isolates, no change in *atlE* expression was measured. Growth in 4% NaCl completely repressed expression of *RNAIII* in strain BM13 but was not associated with decreased *atlE* transcription (Fig. 2e). These data suggested that the Agr system does not control AtlE at the transcriptional level. Autolysins such as AtlE play an important role in cell-wall processing and

recycling, and it is interesting to speculate that the major role they have in the process of biofilm formation may be to orientate and expose cell-surface adhesins correctly to maximize and ensure effective interaction with either host factors or device surfaces.

In CSF41498, no *aap* transcription was detected (Fig. 2a). Induction of *aap* gene expression was evident in isolates BM3 and BM13 when the medium was supplemented with 4% ethanol and 4% NaCl (Fig. 2a, e). In isolates BM6 and BM11, *aap* transcription did not vary greatly under the different environmental conditions. The *agr* system may also influence the role of Aap in the accumulative phase of biofilm formation indirectly by controlling extracellular protease production (Rohde *et al.*, 2005; Lindsay & von Holy, 2006). It is known that Aap requires proteolytic cleavage for activity (Rohde *et al.*, 2005). The positive effect of *agr* on the accumulation of extracellular proteases, and the subsequent activation of Aap, may be a crucial factor in allowing *S. epidermidis* to adapt to changes in environmental conditions and to determine biofilm persistence and spread (Rohde *et al.*, 2005).

Fig. 2. Transcription levels of protein factors important in biofilm formation and the possible influence of *agr*. Neurosurgical *S. epidermidis* isolates (b–e) were grown in BHI, or in BHI supplemented with 4 % ethanol or 4 % NaCl. The *atlE*⁺*aap*⁺ biofilm-positive laboratory strain CSF41498 (a) acted as the control strain. Transcription of the constitutively expressed *gyrB* (control), as well as that of *atlE*, *aap* and *RNAIII*, was measured by RT-PCR using RNA isolated from cultures grown at 37 °C to an OD₆₀₀ of 2.0.

It could be that, although important biofilm factors such as Aap are being expressed, they are not being activated by proteolytic cleavage in some strains and therefore not always contributing to biofilm formation when there is a completely functional *ica* operon present. This is supported, in part, by the lack of *RNAIII* transcription in isolate BM13, which may be controlling extracellular protease production downstream and indirectly controlling the involvement of Aap in the biofilm formation process, which could possibly vary among strains. The lack of *aap* transcription in the control strain CSF41498 indicated that Aap is not always required for strong biofilm formation when *ica* is fully active, and may serve as a back-up mechanism should *ica*-dependent biofilm formation cease (Rohde *et al.*, 2005; Hennig *et al.*, 2007).

Alternatively, Aap may serve to enhance localization of PIA at the cell surface, thus allowing rapid accumulation of cells in some strains where PIA production is not as efficient. Recently, Banner *et al.* (2007) demonstrated and correlated the presence of localized tufts on the surface of *S. epidermidis* strain NCTC 11047 with the presence of Aap. It is possible that these fimbrial tufts, along with Aap, enhance biofilm production by binding PIA, increasing intercellular adhesion and thus allowing the rapid

accumulation of cells. This is supported by the presence of a conserved pentaglycine domain in Aap, which has been reported to have affinity for *N*-acetylglucosamine, the major component of PIA (Hussain *et al.*, 1997; Bateman *et al.*, 2005). In addition, it has been shown that sequence repeats known as G5 domains from Aap are zinc-dependent adhesion modules analogous to mammalian cadherin domains, leading to speculation that this leads to extensive contact between cells, a form of 'zinc zipper' (Conrady *et al.*, 2008).

RT-PCR analysis of *atlE* transcription indicated this was consistently high under each environmental growth condition, with only slight variations being seen. Unfortunately, no apparent correlation was obvious with *atlE* levels and *agr* transcription. Interestingly, it appears that growth in the presence of alcohol and under salt stress caused some induction of *aap* transcription in two isolates (BM3 and BM13), being most evident in isolate BM13.

Contribution of PIA and protein to *S. epidermidis* biofilm formation *in vitro*

Taken together, these data suggest that *ica* gene expression may be important in biofilm production by strains BM6,

BM11 and BM13 but not strain BM3. Furthermore, as all strains expressed *atlE* and *aap*, it also suggests a role for protein-mediated biofilm production. Therefore, in order

Fig. 3. Identifying the mechanism of biofilm production: PIA versus protein. Biofilm assays were performed in a 96-well microtitre plate. Neurosurgical *S. epidermidis* isolates were cultured at 37 °C for 24 h in BHI (a), or under conditions known to induce biofilm formation, namely BHI supplemented with 4 % ethanol (b) or 4 % NaCl (c). Test wells were either left untreated or were treated with sodium metaperiodate (10 mM) or proteinase K (100 µg ml⁻¹) to examine the dispersion of PIA-mediated and protein-mediated biofilm, respectively. Dispersion of biofilm by sodium metaperiodate or by proteinase K was considered significant when $P \leq 0.05$ (* vs untreated for sodium metaperiodate and # vs untreated for proteinase K). Isolates were deemed to be strongly biofilm-positive when the biofilm A_{492} was >0.24 (indicated by the dotted line).

to determine the mechanism of biofilm formation of each of the clinical isolates, we used sodium metaperiodate and proteinase K to disperse adhered biofilm (Fig. 3).

Treatment with sodium metaperiodate should disperse polysaccharide- or PIA-mediated biofilm. Under standard growth conditions (BHI, 37 °C for 24 h), all four isolates examined produced a strong biofilm (absorbance >0.24) as shown in Fig. 3(a).

Dispersion of adhered biofilm, produced under standard growth conditions (Fig. 3a) or following growth in the presence of 4 % ethanol (Fig. 3b), using sodium metaperiodate caused a significant decrease in biofilm formation for strains BM6, BM11 and BM13. As expected, it had no effect on biofilm formed by the *ica*⁻ isolate BM3. In this instance, proteinase K treatment was sufficient to significantly disperse the biofilm matrix.

Similarly, NaCl-induced biofilm production was dispersed by sodium metaperiodate in strain BM6, BM11 and BM13 and once again by proteinase K in strain BM3. Interestingly, proteinase K also dispersed biofilm produced by strain BM11, suggesting a role for protein in biofilm production by this strain.

Protein-mediated biofilm formation is the most likely mechanism of biofilm formation in isolate BM3, as it lacks the *ica* operon and the necessary biosynthetic enzymes to produce PIA, but protein also seemed to contribute to biofilm formation in isolate BM11. Biofilm phenotypic variation among staphylococci has been reported previously and shown to involve the insertion sequence IS256 (Ziebuhr *et al.*, 1997). The importance of protein factors such as Aap was highlighted by Hennig *et al.* (2007), who found that a *S. epidermidis* *icaC*::IS256 insertion mutant spontaneously switched to protein-mediated biofilm production with higher levels of *aap* transcription. Only isolate BM11 was positive for IS256 by PCR (data not shown), and this isolate was the sole isolate where protein appeared to contribute significantly to a strongly biofilm-positive phenotype.

It is clear that genes other than *icaADBC* and *aap* can be important to the biofilm phenotype. Both staphylococcal

surface proteins SSP-1 and SSP-2 have been implicated as being important factors for efficient primary attachment at the initial stages of the process (Veenstra *et al.*, 1996). Further investigations into the role of *S. epidermidis* cell-surface adhesins, as well as examining the global regulatory systems that control their expression, are likely to lend more insight into the contribution that protein factors have to the biofilm-forming process. In addition to PIA and protein, extracellular DNA has also been shown to be important in stabilizing the structure (Izano *et al.*, 2008).

Our findings indicate that both polysaccharide and protein are important in the development and maintenance of a mature biofilm in CSF isolates deemed to be clinically significant and *ica*⁺/*aap*⁺, but this appears to be complex and variable. We did not include *ica*⁺/*aap*⁻ isolates, as these did not form biofilm or produce PIA and were predominantly clonal, and the number of isolates studied was relatively small. However, even among these well-characterized *ica*⁺/*aap*⁺ isolates, there was some variation in the contribution of PIA and protein to the biofilm matrix that formed under different *in vitro* environmental growth conditions. Also, examination of the *agr* two-component system indicated the influence that this may have on protein factors, and biofilm formation is likely to vary among strains. Understanding the role and interaction of all potential biofilm factors with each other is necessary to identify new targets and possibly to develop new therapeutic approaches for treating device-related, clinically significant infections involving *S. epidermidis*.

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