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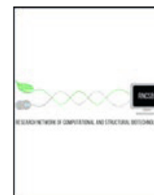

Review

The staphylococcal exopolysaccharide PIA – Biosynthesis and role in biofilm formation, colonization, and infection

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A B S T R A C T

Exopolysaccharide is a key part of the extracellular matrix that contributes to important mechanisms of bacterial pathogenicity, most notably biofilm formation and immune evasion. In the human pathogens *Staphylococcus aureus* and *S. epidermidis*, as well as in many other staphylococcal species, the only exopolysaccharide is polysaccharide intercellular adhesin (PIA), a cationic, partially deacetylated homopolymer of N-acetylglucosamine, whose biosynthetic machinery is encoded in the *ica* locus. PIA production is strongly dependent on environmental conditions and controlled by many regulatory systems. PIA contributes significantly to staphylococcal biofilm formation and immune evasion mechanisms, such as resistance to antimicrobial peptides and ingestion and killing by phagocytes, and presence of the *ica* genes is associated with infectivity. Due to its role in pathogenesis, PIA has raised considerable interest as a potential vaccine component or target.
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Abbreviations: CoNS, coagulase-negative staphylococci; PIA, polysaccharide intercellular adhesin; PNAG, poly-N-acetylglucosamine; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*.

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1. Introduction

The genus *Staphylococcus* comprises more than 40 species [1], of which at least 10 are found in the human skin microbiome [2]. Several species are among the most frequently isolated bacteria from the human skin and mucous membranes [3]. While many coagulase-negative staphylococci (CoNS), such as *S. epidermidis*, are skin colonizers in virtually all humans [3], the coagulase-positive species *S. aureus* persistently colonizes only ~20%, and intermittently about another 30% of the human population [4]. Many staphylococci are opportunistic pathogens with the ability to cause numerous infections. CoNS and *S. aureus* are both involved in subacute and chronic infections, particularly device-associated infections [5], while *S. aureus* can also cause severe lung, blood, and bone infections [6]. The success of the staphylococci in human colonization and infection is due to a plethora of factors. Similar to many other pathogenic bacteria [7], staphylococci produce extracellular polysaccharide (EPS), which has multiple functions in pathogenesis, including biofilm formation and immune evasion. The term EPS is used to differentiate from other bacterial polysaccharides, such as internal polysaccharides that have storage function, and capsular polysaccharides, which are also external but more closely related to the surface and usually covalently surface-linked [8].

Unlike some other bacteria, such as *Pseudomonas aeruginosa*, which have several types of EPS [9], staphylococci only produce one dominant EPS molecule [7]. This EPS has been named polysaccharide intercellular adhesin (PIA) based on function [10], or poly-β-1-6-N-acetylglucosamine (PNAG) based on its chemical nature [11]. We will use the original term PIA in this review. The genes necessary for PIA biosynthesis are encoded in the *ica* (intercellular adhesion) locus [12]. Like all Gram-positive bacteria, staphylococci also produce teichoic acids, which are polymers of sugars and alcohol phosphates and which – similar to EPS - have been implicated in colonization and biofilm formation [13]. However, due to their ubiquitous presence and covalent surface linkage, teichoic acids are generally not considered EPS.

PIA is also found in many other, phylogenetically diverse bacteria. In *Escherichia coli,* PIA is called PGA and the *ica* locus, *pgaABCD* [14]. In *Yersinia pestis*, *Pseudomonas fluorescens*, *Bordetella bronchiseptica*, *B. pertussis*, and *B. parapertussis,* homologues of *pga* were discovered, named *hmsHFRS* for *Y. pestis* and *bpsABCD* for *Bordetella* [15–19]. PIA homologues were shown to be directedly related to biofilm formation in these species [15,16,18,19]. Similar findings were obtained in *Acinetobacter baumannii* [20], *Actinobacillus actinomycetemcomitans* and *A. pleuropneumoniae* [21,22], *Burkholderia ambifaria, B. cenocepacia, B. cepacia*, *B. multivorans* and *B. vietnamiensis* [23], *K. pneumoniae* [24,25] and *Bacillus subtilis* [26].

The wide distribution of PIA and its frequently established importance in infection has resulted in considerable interest in this molecule in recent years. While there are many reviews on biofilm formation, there is no comprehensive review on this specific key biofilm molecule. Here, we present a review of PIA in staphylococci, including its structure, biosynthesis and regulation, role in biofilm formation, colonization, and infection, and finish with a discussion of the potential of PIA-targeting therapeutics.

2. Distribution and genetic encoding of PIA in staphylococci

The production of PIA is mediated by the *ica* locus, which consists of a regulatory gene, *icaR*, and the biosynthetic operon *icaADBC* [12]. PIA and the *ica* locus were first described in *S. epidermidis* [12,27] but then also found in *S. aureus* and other staphylococcal species with significant conservation [28–30]. Presence and expression of the *icaADBC* operon can vary significantly among the many staphylococcal species in which *ica* genes have been detected. For example, while most *S. aureus* strains have the *ica* genes [31], only some appear to rely on PIA expression for biofilm formation in vitro and in vivo [32–34]. In *S. epidermidis*, which has been in the focus of PIA research, recent findings indicate that presence of the *ica* genes is linked to a specific genetic cluster. Namely, *ica* genes are present in the *S. epidermidis* A/B cluster at ~37%, as opposed to only 4% in cluster B [35,36]. Furthermore, the *ica* genes are virtually the only genes of *S. epidermidis* whose presence has been found to be significantly higher in isolates from device infection, or device-associated blood infection, versus colonization isolates [37–42]. However, this association has been doubted [43,44]. So far, other staphylococcal species have rarely been investigated for a correlation of *ica* gene presence, PIA production, and source from infection. In the species where this was analyzed, presence of the *ica* genes generally was highly strain-specific and associations with infection were similar to those found for *S. epidermidis* [45–49]. Altogether, it has been difficult to attribute roles in infection and colonization to the *ica* genes and their biosynthetic product solely based on epidemiological data, which is why functional research on this EPS molecule has focused on investigation of deletion strains and in some cases, purified PIA.

3. Structure of PIA

PIA was discovered when what was previously called slime underwent in-depth chemical analysis. In 1996, PIA from *S. epidermidis* was identified to be a linear, positively charged, partially (~15–20%) deacetylated polymer of β-1–6-N-acetylglucosamine [27], whose expression was highly correlated with biofilm formation [50]. Beside N-acetylation, around 10% of N-acetylglucosamine residues of PIA have been reported to be O-succinylated in *S. epidermidis* and *S. aureus* [27,51,52]. Before the chemical description of PIA by Mack et al. [27], preliminary studies had identified slime-associated staphylococcal polysaccharides that were given different names (SAA or PS/A) [53,54]. Mack et al. described PIA to contain about 130 residues of N-acetylglucosamine (NAG) with some degree of deacetylation corresponding to an estimated molecular weight of ~30 kDa [27]. Subsequently, McKenney et al. identified PS/A from *S. epidermidis* and *S. aureus* as a >250 kDa molecule with considerable N-succinylation (65–100%) that is synthesized from the same locus as PIA (*ica*), ultimately calling it poly-N-succinyl-β-(1–6)-glucosamine (PNSG) [55,56]. Later, the same group reported PS/A to have a size of 21 kDa, 100 kDa and 460 kDa but no degree of N-succinylation, henceforth calling the molecule PNAG [poly-N-acetyl-β-(1–6)-glucosamine] [11]. It was confirmed by detailed NMR analyses that N-succinylation was indeed an analytical artifact in a study that referred to *S. aureus* exopolysaccharide as SAE, a PIA-related molecule of high molecular weight (>300 kDa) having about 45–60% N-acetylation and 10% O-succinylation [51]. Notably, when using the same strain and growth condition as well as a similar purification strategy as used by Maira-Litran et al. [11], Sadovskaya et al. showed that PIA, PS/A, SAA and SAE are all of the same chemical entity [52]. Furthermore, all these molecules were shown to be synthesized by the *ica* locus [55]. Therefore, variable reports on the size and slightly different characteristics of PIA are likely due to differences in the degree of polymerization as well as variation in experimental approaches used in different studies (Fig. 1).

There are reports on a similar glucosamine-containing EPS molecule in *S. epidermidis* of only 20 kDa, whose biosynthesis is not mediated by the *ica* locus [57]. This 20-kDa partially sulfated acidic polysaccharide was claimed to be both a major slime component and a distinct antigen with potential to induce specific

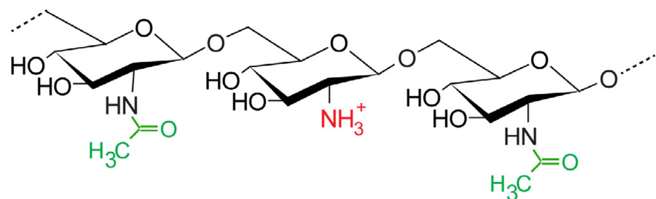

Fig. 1. Structure of PIA. PIA is a homopolymer of N-acetylglucosamine (GlcNAc) residues with β-1–6 linkage. About 15 to 20% of the GlcNAc residues are de-acetylated. In the figure, acetyl groups are in green and the free amino group that results from IcaB-catalyzed deacetylation, which is positively charged at neutral or basic pH, is in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and protective antibody in *S. epidermidis* [58,59]. Later this molecule was reported to be a partially sulfated polymer of N-acetylglucosamine and glucose, expressed exclusively in *S. epidermidis* but not in other CoNS species [60]. It was stressed not to be synthesized from the *ica* locus [60], but in the absence of a defined biosynthetic locus, these reports on a second EPS molecule in staphylococci that is different from PIA have to be regarded as preliminary and in need of verification.

4. PIA biosynthesis

The *icaADBC* locus contains four different genes, *icaA*, *icaD, icaB* and *icaC*, which are arranged in an operon. The *ica* operon was first described in *S. epidermidis* in 1996 and reported to comprise three genes that are co-transcribed from one promoter [12]. Later, it was found that the locus also contains a small fourth gene, *icaD,* which is located between *icaA* and *icaB* [61]. Expression of all four genes is required for the synthesis of fully functional PIA [61] (Fig. 2).

The major PIA-synthesizing enzyme is encoded by *icaA* [61]. IcaA is an N-acetylglucosaminyltransferase that synthesizes PIA oligomers from UDP-N-acetylglucosamine. However, the transferase activity of IcaA is low and only reaches high efficacy in the presence of IcaD. IcaA and IcaD are located in the plasma membrane [61]. IcaA is a 412 amino-acid polypeptide having four predicted transmembrane domains, while IcaD is much smaller, having only 101 amino acids with two potential transmembrane domains [61]. IcaAD was shown to produce PIA with a maximal length of only 20 residues, while further elongation of PIA required assistance of IcaC [61]. Together with the predicted transmembrane structure of IcaC, these findings led to the assumption that IcaC exports the growing PIA chain and possibly forms a complex with IcaA and IcaD [61]. However, it has been proposed - based on comparison of *ica* homologues in different bacteria but without experimental evidence - that IcaAD may also export PIA, while IcaC may be responsible for modifications of PIA, such as O-succinylation, that appear to be limited to staphylococci [62]. IcaB, a 259 amino-acid polypeptide with a potential signal sequence, is a cell surface-attached enzyme that has PIA deacetylase activity [63]. Via deacetylation, IcaB introduces a positive net charge into PIA, which makes the polymer attach stably to the bacterial surface and which is crucial for PIA-mediated phenotypes [63]. IcaB enzymatic activity is metal-dependent and preferentially targets the second or third sugar residues from the reducing terminal of pentamer or hexamer PIA [64] (Fig. 2).

5. Regulation of PIA biosynthesis

PIA can be produced in large amounts in a presumably highly energy-consuming process [27]. This requires tight regulation of *ica* expression. PIA production and *ica* expression have been found to be dependent on environmental conditions, such as anaerobiosis, salt, glucose and alcohol concentration, and antibiotics [65–68]. Over the years, a large number of regulatory genes and proteins have been found to regulate *ica* expression, which likely underlies the strongly differential expression of PIA in different staphylococcal strains [69]. It is believed that *ica* expression is more variant and dependent on environmental conditions in *S. aureus* than in *S. epidermidis* [70].

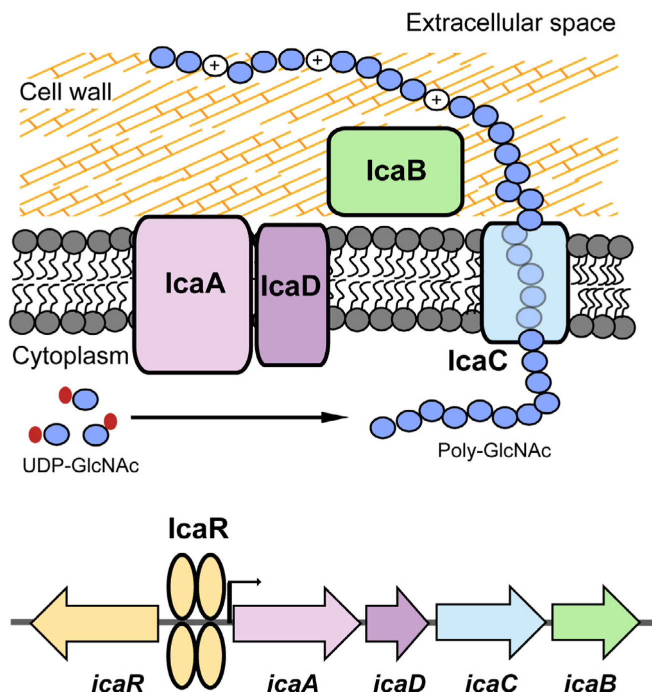

Fig. 2. Genetic encoding and biosynthesis of PIA. PIA is synthesized by the products of the *icaADBC* operon. The *icaADBC* operon is under control of the product of the *icaR* gene, which is encoded upstream. IcaR, which is itself subject to control by manifold regulators and environmental conditions, binds in two dimers to the *icaADBC* promoter region, repressing *icaADBC* transcription. IcaA and IcaD, two membrane proteins, synthesize a growing poly-GlcNAc chain from activated precursor GlcNAc units. This chain is likely exported by the membrane protein IcaC, although IcaC has also, alternatively, been speculated to be involved in PIA O-succinylation. IcaB is an enzyme that is attached to the bacterial outer surface and introduces positive charges in the otherwise neutral PIA molecule by de-acetylation of some GlcNAc residues. The cationic character is vital for surface attachment and functionality of PIA.

The *ica* locus contains a dedicated regulator, IcaR, which is encoded upstream of the *icaADBC* operon [66]. IcaR, whose crystal structure has been obtained, is a member of the TetR family of transcriptional regulators [71]. It binds to a specific DNA region upstream of *icaA* resulting in strong suppression of *icaADBC* transcription [66,72] (Fig. 2). Deletion of *icaR* leads to PIA overproduction [66]. Aminoglycoside antibiotics can interfere with the binding of IcaR to DNA, thus resulting in the induction of biofilm formation [71]. Some but not all of the environmental influences on PIA production as well as the impact of global regulators discussed below are mediated by IcaR. Interestingly, the 3′ untranslated region (UTR) interferes with the Shine-Dalgarno sequence of the *icaR* transcript, producing a substrate for RNAse III, thereby reducing *icaR* translation [73].

TcaR, a MarR-type transcriptional of the *icaR* transcript regulator, provides IcaR-independent regulation of *icaADBC* [74]. TcaR negatively regulates *icaADBC*, however to a much smaller extent than IcaR [72]. In *S. epidermidis,* TcaR can become the primary *ica* repressor in the absence of IcaR [74]. Interestingly, while IcaR binds to only one specific site upstream of *icaA*, TcaR can bind to multiple sites, including the binding site of IcaR as a competitor of IcaR as well as to the *icaR* promoter region as a repressor [74].

SarA is the most extensively studied regulator among the staphylococcal accessory regulator (Sar) family. This protein is relatively small, containing 124 amino acids with a winged-helix DNA binding domain [75]. SarA strongly activates the *icaA* promoter via high binding affinity [76–78]. In *S. aureus*, mutations in *sarA* decrease but do not stop the production of PIA [76], while in *S. epidermidis*, deletion of *sarA* can result in complete abortion of PIA production [77,79]. Interestingly, in *S. aureus*, SarA induces not only the transcription of *icaADBC* but also its suppressor *icaR*, suggesting binary control to prevent the overproduction of PIA [78]. On the other hand, in *S. epidermidis*, SarA regulation of PIA production is IcaR-independent [77]. SarA represents a global regulator with manifold influences on staphylococcal physiology, many of which are mediated via its impact on the Agr quorum-sensing system [80], another major regulator of staphylococcal gene expression [81]. Agr itself does not impact *ica* transcription but similar to SarA impacts many unrelated biofilm factors such as proteases and phenol-soluble modulins (PSMs) [81,82]. Overall, the impact of *sarA* and *agr* deletion on staphylococcal biofilm formation is negative and mostly PIA-independent, because both regulators strongly upregulate protease and PSMs, which are biofilm detachment factors [83]. Other members of the Sar protein family that regulate *ica* in *S. epidermidis* comprise SarX and SarZ. SarX binds to the *icaADBC* promoter, upregulating transcription, while SarZ also upregulates *ica* transcription in an unknown fashion [84,85].

Sigma B is an alternative sigma factor that regulates a number of virulence and virulence-associated genes in response to environmental stimuli. It has been reported to be important for *S. aureus* and *S. epidermidis* biofilm formation. While initial studies reported that sigmaB increases *ica* transcription in a potentially IcaR-dependent way [78,86], this has been controversial at least for *S. aureus* [76], and recent research suggests that the impact of sigma B on PIA production in *S. aureus* is due to altered proteolytic turnover of PIA biosynthesis proteins [87].

In addition to the regulators discussed in detail above, a number of other regulator factors/ systems have been shown to affect PIA synthesis, including Rbf [88], LuxS [89], Spx [90], SrrAB [91], Ygs [92], GdpS [93], and CcpA [94]. SrrAB, for example, appears to be important for the increase of PIA production under anerobic conditions [91]. Moreover, recent findings also add non-protein factors to the list of PIA regulators. IcaZ, a non-coding 400-nucleotide RNA, which is encoded downstream of *icaR*, was found to inhibit *icaR* mRNA translation, leading to increased PIA production [95]. IcaZ is found inclusively in *ica*-positive *S. epidermidis* but no other staphylococcal species [95]. Additionally, a regulatory RNA named RsaE binds in its processed form to the 5′UTR of the *icaR* mRNA, also increasing PIA production [96].

Finally, another distinctly different way to regulate PIA synthesis that was found in *S. epidermidis* is the reversible insertion of IS256 into either *icaA, icaC, rsbU* or *sarA*, which causes a “phase variation” phenotype of abolished or decreased PIA production [97–100]. Similar to *ica*, IS256 is associated with infection origin of *S. epidermidis* isolates [101,102], suggesting that this type of PIA regulation is important for pathogenesis.

6. Role of PIA in biofilm formation

Biofilm is a consortium of microbial cells that aggregate with each other and to a surface via a self-synthesized slimy extracellular matrix (ECM). This matrix is chemically heterogenous, comprising extracellular DNA (eDNA), lipids, EPS, and proteins that frequently form amyloid fibers [103]. The types and ratio of each component depend on the bacterial species and environmental conditions. In many staphylococci, particularly *S. epidermidis*, the EPS PIA is the major component of the biofilm matrix [12,104] (Fig. 3). Biofilm formation develops in at least three main stages: (i) attachment of microbial cells to a surface, followed by (ii) production of the ECM and maturation of the biofilm, and finally (iii) detachment of microbial cells or clusters [105,106].

Attachment to an abiotic surface, such as that of an indwelling medical device, is governed by the physicochemical properties of the surface and the bacterial envelope and is reversible [107]. Studies mostly performed in *S. epidermidis* have attributed key roles to charge and surface hydrophobicity in staphylococcal attachment to abiotic surfaces [108–110]. However, in vivo, surface attachment is mediated predominantly via specific adhesion molecules, such as those of the MSCRAMM family, which cover the abiotic surface of an indwelling medical device soon after insertion [111].

Despite its positive charge, PIA appears to contribute to surface hydrophobicity of *S. epidermidis* [112] and may thus mediate initial adherence to some extent. However, the adherence properties often attributed to PIA in the literature [113] likely rather reflect its contribution to the beginning second, accumulation stage of biofilm formation. By representing a major component of the extracellular matrix, PIA fixes staphylococcal cells in the fibrous net it produces and thereby builds up biofilm mass [114], which

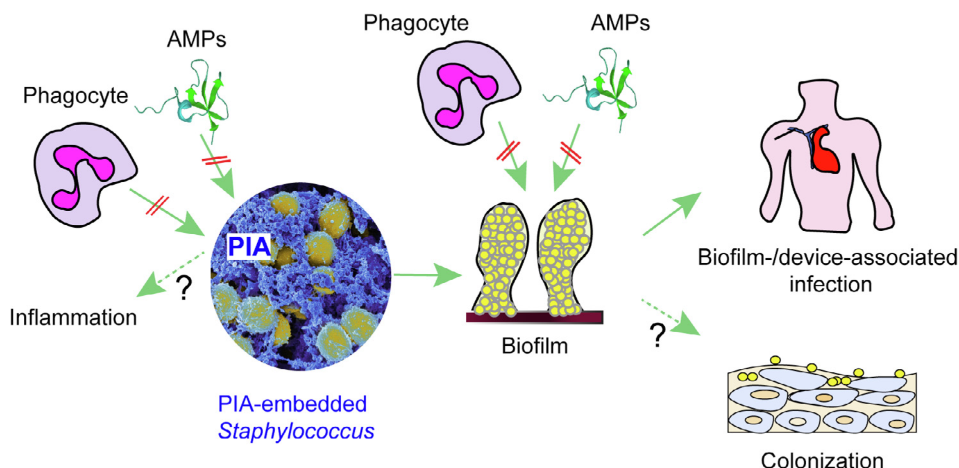

Fig. 3. Functions of PIA. PIA embeds staphylococcal cells in a dense extracellular matrix network. This network protects the cells from attacks by mechanisms of innate host defense (AMPs, phagocytes). Furthermore, some reports have suggested direct pro-inflammatory functions of PIA. However, most of the biological functions of PIA are mediated by its contribution to biofilm formation. This includes most notably device- and other biofilm-associated infections. Biofilm formation also further contributes to the protection from AMPs and phagocytosis. Finally, PIA may contribute to epithelial colonization under specific conditions.

leads to increased resistance of the biofilm to mechanical force. Accordingly, PIA is crucial for biofilm formation under high-shear flow conditions like those found inside catheters [40,115–119], but it becomes less important under low-shear conditions like those in subcutaneously implanted tissue [120], ocular infections [121], or platelet concentrate [122]. Single-cell force spectroscopy data demonstrated multivalent electrostatic interaction of the cationic PIA polymer with the negatively charged wall teichoic acids on staphylococcal cells, confirming on the molecular level that the cationic character previously shown to be crucial for PIA function [63] has an important role in the attachment of PIA to the cell surface and PIA-mediated intercellular adhesion [123]. There is no evidence for a covalent linkage of PIA to the cell surface.

In the final stage of biofilm formation, staphylococcal cells are detached from the biofilm. Detachment can happen via mechanic force in a process often called sloughing, by enzymatic digestion via proteases or nucleases, or via detergents [124]. The role of the PSM detergent-like molecules in this process has been demonstrated in vivo in *S. epidermidis* and *S. aureus* and is independent of whether the biofilm is PIA-dependent or -independent [125–127], while enzymatic digestion depends on the chemical nature of the biofilm [128–130]. Notably, PIA-degrading enzymes have not been found in staphylococci. The only known enzyme to degrade PIA is dispersin B, which is found in a periodontal disease-causing pathogen, *Actinobacillus actinomycetemcomitans* [131]. This enzyme hydrolyzes the 1,4-β-glycosidic linkage of PIA, causing detachment and dispersion of cells from biofilms [131], effectively inhibiting PIA-dependent staphylococcal biofilm formation and immune evasion capacity [114,132,133]. It is often used to determine PIA dependence of staphylococcal biofilm formation [134]. Whether an open reading frame that has similarity to the dispersin B gene and is found in *S. lugdunensis* close to the *ica* locus codes for a PIA-hydrolase remains to be shown [135].

For a long time, PIA has been deemed crucial for staphylococcal biofilms, but beginning in the early 2000s, there have been reports on PIA-independent staphylococcal biofilm formation, in which isolates from biofilm infection were shown to be *ica*-negative and form in-vitro biofilms [33,136,137]. However, strains using PIA-independent biofilm formation seem to form weaker and less stable biofilm than those whose biofilms are based on PIA [33,138]. Furthermore, PIA production results in dense, rough colonies as opposed to smooth colonies formed by PIA-negative biofilm-forming *S. epidermidis* [114]. It has also been reported that *ica*-negative and -positive clinical staphylococcal isolates show enhanced biofilm production when induced by heparin [139], staphylococcal or host proteases [134], trypsin [140] or by special conditions like those found within platelet concentrates [141–143]. Furthermore, PIA-dependent biofilm is frequently found in methicillin-sensitive (MSSA), while PIA-independent biofilm is prevalent in methicillin-resistant *S. aureus* (MRSA) [32,136,144]. There have been attempts to link this difference to the *mecA* gene that is responsible for methicillin resistance [145]. However, how the *mecA* gene is mechanistically involved in the difference of PIA usage for biofilm formation in MSSA and MRSA remains largely undetermined. Lastly, *S. epidermidis* appears to have the ability to switch to a protein-dependent biofilm upon disruption of the *ica* gene locus by IS256 [98].

7. Contribution to host colonization

Research on staphylococci has traditionally been focused on infection, while their commensal lifestyle has received only minor attention. This is now changing due to the increased interest in microbial communities and the human skin microbiome. The abundant skin commensal *S. epidermidis* has recently been shown to occur in two major genetic clusters (A/C and B), of which virtually only the A/C cluster isolates contain *ica* genes (37% versus 4% in cluster B) [35,36]. A/C cluster isolates also exhibit *ica*-unrelated phenotypes, such as protease production and matrix protein binding, making them potentially more pathogenic, while B cluster isolates seem to have evolved to adapt to conditions found in sebaceous glands and hair follicles [35]. Furthermore, an earlier study performed in human volunteers showed that presence of the *ica* genes appeared to be disadvantageous for survival on the skin due to a high fitness cost [146]. Together with the many reports that associate presence of *ica* with infection, these results suggest that PIA production may only be of advantage on the skin under certain conditions and that presence of *ica* genes together with other genetic features makes specific *S. epidermidis* isolates more prone to infect the host. How PIA affects skin colonization in other staphylococci has not yet been investigated.

8. PIA in infection

Animal studies that analyzed the contribution of PIA to different types of infection have yielded conflicting results. Most frequently these studies investigated *S. epidermidis* device-related infection. Before the discovery of PIA, some studies reported an impact of slime production on the pathogenesis of *S. epidermidis* device-related infection [147] and an association with origin from infection, for example from nosocomial bacteremia [148], while others did not [148,149]. In addition to the assumption that clumping/slime production increases the success of infections on indwelling medical devices, recent research also has suggested that this phenotype increases the chances of staphylococcal dissemination through the bloodstream [150].

After the discovery of the *ica* genes, isogenic deletion mutants were used to directly investigate the impact of PIA on infection. Most of those studies used the *ica*-negative M10 transposon mutant of *S. epidermidis* strain 1457 [10]. The first studies were performed in the Rupp laboratory and consistently showed a significant impact of the *ica* genes on catheter-related infection in mice and rats [117,151,152]. Later, it was shown that introduction of the *ica* genes alone is sufficient to render a commensal *S. epidermidis* strain invasive [153], and several further studies showed similarly reduced infectivity of isogenic *ica*-negative *S. epidermidis* as compared to the parental strain in device-related infection [154], independently of the used biomaterial [155]. Moreover, the importance of PIA deacetylation for device-related infection in mice, as investigated using an *icaB* isogenic deletion mutant, further confirmed PIA's importance for pathogenesis [63]. Additionally, *ica*-positive *S. aureus* or *S. epidermidis* showed better in-vivo survival than their corresponding *ica* mutants in wild-type/mutant mouse co-infection models [70]. Finally, a significant impact of the *ica* genes on *S. epidermidis* infection was confirmed in a *C. elegans* infection model, where *ica* genes were required for lethal infection produced by feeding challenge [156]. Together, these results add to those already mentioned above showing increased prevalence of *ica* genes in infective *S. epidermidis* isolates to substantiate a role of *ica* in *S. epidermidis* device-related infection [37–42] (Fig. 3).

However, other researchers found no impact of the *ica* genes on virulence in device-related infection models. Chokr et al. reported a lack of impact of *ica* on infection in a guinea pig tissue cage model for *S. epidermidis* and Francois et al. for both *S. epidermidis* and *S. aureus* in the same model [120,157]. Kristian et al. reported a similar outcome when using a mouse tissue cage model and *S. aureus* strain SA113 [158]. Furthermore, in a *C. elegans* infection model no correlation of PIA-production and virulence was found comparing 30 *S. epidermidis* isolates from infective endocarditis [159]. The

most likely reason for the conflicting reports as for the impact of *ica* on virulence is a differential relative effect of PIA as compared to other staphylococcal virulence factors and dependence on strains and models used. Interestingly, at least two of the three strains that were used by Francois et al. and Kristian et al. [120,158] are Agr-dysfunctional (*S. epidermidis* O47, *S. aureus* SA113) [82,160], suggesting that the resulting complete absence of PSM production [161] and concomitant increased compact biofilm [125–127] abrogates a measurable impact of PIA on biofilm expansion.

In-vivo investigations on the impact of *ica* on infection in staphylococcal species other than *S. epidermidis* are generally rather scarce. PIA is produced by *S. aureus* in vivo and significantly impacts *S. aureus* systemic infection in mice [162] despite variability and strain-dependence of in-vitro production [70,163]. As for device-related infection, the abovementioned studies that did not find a role for *S. aureus ica* are the only studies that have been performed [120,158]. Interestingly, loss of PIA in an *S. aureus* strain that overproduces PIA due to a mutation in the *icaADBC* promoter [164] produces a fitness gain by a compensatory mutation that was also detected in clinical isolates; however, this was only determined in vitro [165]. Later, the same mutation that leads to PIA overproduction and an associated immunoprotective "mucoid" phenotype was detected in *S. aureus* isolates from cystic fibrosis patients, in which also similar compensatory mutations with a non-mucoid phenotype occurred over time [166]. Altogether, these findings suggest that PIA production, while likely important for device-associated infection and associated with a high fitness cost in *S. epidermidis*, is subject to dynamic alterations in production, especially in vivo and in *S. aureus*.

During co-infection with other organisms, PIA may play a role to increase overall virulence. This was shown for coinfection with *Candida albicans*, a pathogenic fungus that often occurs together with staphylococci in catheter-related infection, wound infection, cystic fibrosis, periodontitis and denture stomatitis [167]. In mixed in-vitro biofilms of *S. epidermidis* strain RP62A and *C. albicans*, slime, which is mainly composed of PIA in that strain [52], protected *C. albicans* from fluconazole penetration [168]. EPS produced by *S. epidermidis* also increased the overall virulence of a mixed *S. epidermidis* and *C. albicans* challenge in *C. elegans*, resulting in reduced survival of the infected worms [169]. In *S. aureus*, MSSA and MRSA grew synergistically with *C. albicans* within biofilms [170], and *C. albicans* increased *S. aureus* resistance to vancomycin [171], suggesting mutual benefit.

9. PIA and the host immune system

There are multiple studies that have investigated the role of PIA in the interaction with the immune system (Fig. 3). In cell culture assays, the *ica*-negative mutant M10 was more susceptible to antimicrobial peptides (human beta-defensin 3, LL-37 and dermcidin) and to non-opsonic phagocytosis and killing by human polymorphonuclear leucocytes (PMNs) than the parental strain *S. epidermidis* 1457 [104]. PIA-mediated resistance to opsonic PMN killing was shown in another study by Kristian et al., which also demonstrated diminished immunoglobulin and complement (C3b) deposition on the surface of *ica*-positive *S. epidermidis* biofilms in a device-related infection model in addition to increased local infection, bacterial burden, and larger edema [154]. Furthermore, PIA-producing *S. epidermidis* biofilm was shown to lead to less pronounced granulocyte activation and cytokine release than the reduced biofilm produced by its isogenic *ica* mutant [172]. Finally, PIA was shown to decrease susceptibility to phagocytosis by macrophages [114] and restoration of PIA production in the M10 mutant resulted in reduced NF-kB activation and diminished IL-1β production in macrophages [114]. In *S. aureus,* depletion of PIA resulted in increased IL-12 production in murine dendritic cells [173], decreased blood CFU in intravenously challenged mice and increased complement-mediated phagocytic killing [162]. Together, these results indicate that most of the effects of PIA production on immune evasion are mediated by its impact on biofilm formation, which shelters the cells from recognition by phagocytes and from killing by antimicrobial peptides. Vuong et al. [104] investigated single cells after biofilm disruption and found similar immune evasion effects, suggesting that cellular "coating" with PIA provides immune evasion properties also in the absence of a biofilm, which thus may play a role also in non-biofilm-related, acute infection.

On the other hand, several ex-vivo and in-vivo studies reported increased inflammatory reactions to PIA-positive versus isogenic PIA-negative bacteria. Fredheim et al. showed increased complement activation ex vivo [172], and Ferreirinha et al. increased neutrophil recruitment in vivo by PIA-positive strains. Al-Ishaq et al. reported association of C5a concentration with PIA mode of biofilm formation in clinical samples [174]. These effects are likely due to higher bacterial survival and more pronounced infection that PIA producers cause via their above-mentioned immune evasion effects. Additionally, when assessing altered pro-inflammatory effects of PIA-negative mutants, one should keep in mind that such effects may be due to differential release of the strongly pro-inflammatory PSMs, or lipopeptides, whose release is PSM-dependent [175], in a biofilm setting [125,126], rather than direct effects of PIA itself.

Finally, based on investigation using purified PIA, it has been suggested that the PIA molecule is itself pro-inflammatory [172,174,176]. For example, studies using incubation of purified PIA with human astrocytes implicated that PIA can induce IL-6, IL-8, and MCP-1 expression via TLR-2 [176]. However, the purification of PIA is difficult, making it hard to rule out effects by contaminating strongly pro-inflammatory molecules, which is why further verification of the pro-inflammatory capacities of the PIA molecule is certainly warranted.

10. PIA and antimicrobial resistance

Biofilm formation is widely known to decrease susceptibility to antibiotics and other antimicrobial agents [177]. The underlying mechanisms comprise a reduced metabolic state, persister formation, and decreased penetration through the biofilm extracellular matrix, among others [178]. As for staphylococci, oxacillin, cefotaxime and vancomycin reportedly penetrate poorly through *S. aureus* and *S. epidermidis* biofilms [179], while some other antibiotics, such as amikacin and ciprofloxacin, were unaffected by staphylococcal biofilm formation [179]. While it has also been reported that rifampin and vancomycin have at least some capacity to penetrate through the biofilm matrix [180–182], their antibacterial efficacy was shown to depend on biofilm age or infection duration [183], or concentration and conditions [184,185], respectively.

As PIA is part of the extracellular matrix, it is reasonable to assume that it mostly affects antibiotics whose penetration through the biofilm matrix is impaired. However, due to its essentiality for biofilm formation in many isolates, PIA may also theoretically impact the activity of antibiotics that easily penetrate through the matrix. In correlative studies, *ica*-positive *S. epidermidis* and *S. aureus* strains showed increased resistance as compared to *ica*-negative strains to a variety of antibiotics, such as oxacillin, gentamicin, ciprofloxacin, levofloxacin, co-trimoxazole, erythromycin, vancomycin, and the cell-wall degrading enzyme lysostaphin [186–189]. It is also noteworthy that subinhibitory concentrations of some antibiotics can increase transcription of

the *ica* locus; yet the underlying mechanisms are not understood. This was found for tetracycline and quinupristin-dalfopristin, and to some extent erythromycin, while most antibiotics tested did not show such an effect [65]. Finally, PIA may enhance horizontal gene transfer via its impact on biofilm formation, inasmuch as plasmid transfer by conjugation in *S. aureus* was observed to be 10,000 times higher in biofilm than in planktonic states, which can be explained by increased cell-to-cell contact in biofilms [190].

11. PIA as an immunotherapeutic target

With surface location representing a key prerequisite of a vaccine target, PIA, as an important surface-located biofilm component, was early considered as a potential vaccine candidate. However, the immunogenicity of polysaccharides is generally low [191]; and presence of capsule or EPS generally represents an immune evasion mechanism by which the bacteria minimize opsonization [192]. Nevertheless, anti-PIA antisera may overcome such limitation if they are highly reactive. In the 1990s, immunization of rabbits with PIA [capsular polysaccharide/adhesin (PS/A)] was shown to reduce disease severity in rabbit models of catheter-related *S. epidermidis* bacteremia and endocarditis [193,194]. ELISA and immunoelectron microscopy data also clearly indicated adsorption of anti-PIA antibodies by various PIA-positive staphylococcal strains [55]. A PIA-based vaccine was then developed and showed protective effects in mice against kidney infection and death caused by *S. aureus* strains Reynolds and MN8 [56], which interestingly produced undetectable levels of PIA in vitro [56]. Furthermore, when PIA was conjugated with diphtheria toxoid (DT), the vaccinated mice or rabbits produced significant anti-PIA antibody titers [195]. The obtained anti-PIA antibodies opsonized and induced killing of various staphylococcal strains and their transfusion cleared *S. aureus* from mouse blood [195]. Importantly, the conjugated deacetylated PIA (85% deacetylation)/DT was markedly more effective as a vaccine than native PIA (15% deacetylation)/DT [195]. The stronger potential of deacetylated PIA (>75% deacetylation, dPIA) compared to native PIA in inducing protective antibodies was again shown in a later study, in which dPIA was conjugated with tetanus toxoid (TT) for immunization [196], eliciting anti-PIA antibodies in mice and rabbits, mediating opsonic killing of various *S. aureus* strains and *E. coli*, and protecting the animals from skin abscess caused by *S. aureus* and peritonitis caused by *E. coli* [196]. The stronger potential of dPIA compared to native PIA in protecting challenged animals is likely due to the increase of surface attachment of PIA following deacetylation [63,197] and may explain why natural antibody against native PIA is unable to trigger protective effects despite being common in human and animals [198–200]. In another study, a PIA vaccine in form of a bacterin preparation resulted in high production of anti-PIA antibodies and significant protection against *S. aureus* infection and mastitis in sheep [201]. Furthermore, PIA was expressed in *E. coli* in outer membrane vesicles (OMVs) together with staphylococcal IcaB and the produced PIA-decorated OMVs were highly immunogenic and protected mice from infection not only by *S. aureus* but also the PIA-positive *Francisella tularensis* subsp. *holarctica* [202].

PIA has also been combined with other molecules in vaccines. For example, covalent conjugation of dPIA to clumping factor A (ClfA), but not a mixture of the two unconjugated molecules, was highly immunogenic in mice, rabbits, goats and rhesus monkeys [203]. Transfusion of goat antisera to dPIA-ClfA vaccine to mice significantly reduced blood CFU of different *S. aureus* strains [203]. When glycerol teichoic acid (Gly-TA) and PIA were used to immunize mice, both anti-Gly-TA and anti-PIA antibodies were obtained and the anti-Gly-TA/-PIA sera were able to inhibit biofilm formation of *S. epidermidis* and *S. aureus* in vitro significantly better than anti-Gly-TA or anti-PIA sera alone [204]. Combination of PIA and recombinant SesC protein as a conjugated vaccine induced the production of opsonic antibodies, suppressed biofilm production and protected mice from intravenous challenge with *S. epidermidis* [205].

Despite multiple promising results in animal models, the clinical potential of a PIA vaccine remains somewhat questionable because of the limited prevalence of *ica* in several clinically important staphylococci, such as *S. epidermidis*, and the varying expression of PIA. However, due to the fact that there is no effective *S. aureus* vaccine despite numerous attempts, it may be worth to further invest in PIA-based vaccine development [206], particularly as PIA immunization may be valuable for infections also by *Streptococcus pneumoniae* [207], *Rhodococcus equi* [208], and multiple other species [209]. Finally, immunization against PIA only affects pathogens but leaves microbial diversity virtually unaffected [210].

12. Summary and outlook

Despite increasing reports in the last 20 years on PIA-independent biofilm formation, PIA is still recognized as a major biofilm component particularly in *S. epidermidis*, many other CoNS, and in MSSA. It contributes to immune evasion via its biofilm-forming ability and possibly even independently of it, and affects several directly and indirectly biofilm-related infection types.

Important open questions comprise PIA's role in colonization and how this is related to the association of *ica* gene presence with specific clades. As for PIA's role in infection, a thorough investigation of its contribution especially to the many different types of *S. aureus* infection, relative to the contribution of other virulence factors, is warranted. Furthermore, it should be analyzed what the precise function of IcaC is and whether and how the PIA molecule has direct pro-inflammatory effects. Finally, given the problems with obtaining a working *S. aureus* vaccine, PIA should not be given up on as a vaccine component or target.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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