MINIREVIEW

ica and beyond: biofilm mechanisms and regulation in Staphylococcus epidermidis and Staphylococcus aureus

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Abstract
Recent progress in elucidating the role of the icaADBC-encoded polysaccharide intercellular adhesin (PIA) or polymeric N-acetyl-glucosamine (PNAG) in staphylococcal biofilm development has in turn contributed significantly to our understanding of the pathogenesis of device-related infections. Nevertheless, our understanding of how the ica locus and PIA/PNAG biosynthesis are regulated is far from complete and many questions remain. Moreover, beyond ica, evidence is now emerging for the existence of ica-independent biofilm mechanisms in both Staphylococcus aureus and Staphylococcus epidermidis. Teichoic acids, which are a major carbohydrate component of the S. epidermidis biofilm matrix and the major cell wall autolysin, play an important role in the primary attachment phase of biofilm development, whereas the cell surface biofilm-associated protein and accumulation-associated protein are capable of mediating intercellular accumulation. These findings raise the exciting prospect that other surface proteins, which typically function as antigenic determinants or in binding to extracellular matrix proteins, may also act as biofilm adhesins. Given the impressive array of surface proteins expressed by S. aureus and S. epidermidis, future research into their potential role in biofilm development either independent of PIA/PNAG or in cooperation with PIA/PNAG will be of particular interest.

Introduction
Bacterial biofilms are now known to play an important role in a range of chronic infections such as native valve endocarditis, otitis media, cystic fibrosis pneumonia and infections associated with implanted biomaterials (Costerton et al., 1999). The capacity of both Staphylococcus epidermidis and Staphylococcus aureus to form biofilm is an important virulence factor in the development of device-related infections. Device-related infections, in turn, represent a serious clinical problem, given that the majority of hospital patients undergo procedures for the insertion of foreign devices, from catheters to artificial heart valves, etc. Moreover, patients susceptible to device-related infections are often colonized with hospital-acquired, multiple antibiotic-resistant organisms and may be further compromised by serious underlying disease or trauma. Significantly, the majority of biofilm-mediated device-related infections are caused by either S. epidermidis or S. aureus (O’Gara & Humphreys, 2001).

Biomaterials are rapidly coated with a conditioning film composed primarily of host-derived extracellular matrix proteins following insertion into a patient. Some of these host proteins can act as receptors for bacterial attachment and can thus clearly play an important role in the early stages of biofilm development. Staphylococcal binding to extracellular matrix proteins is mediated by microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Foster & Hook, 1998). Following initial attachment to an implanted device surface, biofilm formation involves a cellular accumulation process.

ica-mediated biofilm development
Although multiple bacterial and external factors influence attachment and accumulation, production of an extracellular polysaccharide adhesin, termed polysaccharide intercellular adhesin (PIA) or polymeric N-acetyl-glucosamine (PNAG) by ica operon-encoded enzymes (Mack et al., 1996; Maira-Litran et al., 2002), is currently the best-
understood biofilm mechanism in staphylococci (Fig. 1). Initially identified in *S. epidermidis* using transposon mutagenesis to isolate *S. epidermidis* mutants defective in biofilm formation (Mack *et al.*, 1994; Heilmann *et al.*, 1996a; Heilmann & Gotz, 1998), the *ica* locus was subsequently implicated in *S. aureus* biofilm formation (Cramton *et al.*, 1999, 2001). In *S. epidermidis*, the *ica* gene cluster appears to have an important role in the pathogenesis of device-related infections. For instance, Ziebuhr *et al.* (1999) reported that c. 85% of *S. epidermidis* blood culture isolates contain the *ica* genes compared with 6% of saprophytic isolates, while a number of other studies have indicated that the *ica* locus may be a significant marker discriminating between significant and contaminating isolates (Frebourg *et al.*, 2000; Galdbart *et al.*, 2000; Fitzpatrick *et al.*, 2002; Rohde *et al.*, 2004; Li *et al.*, 2005). Recent *in vitro* studies have provided evidence that PIA/PNAG is also required for immune evasion and virulence expression of the *1999; Fluckiger* glucosamine in an isogenic molecule (Vuong *et al.*, 2004). The surface-attached protein IcaB is then respon-

The divergently transcribed *icaR* gene, which is located upstream of the *icaABCD* operon in both *S. epidermidis* and *S. aureus*, appears to be a member of the *tetR* family of transcriptional regulators. It has recently been reported that the *icaR* gene encodes a transcriptional repressor with a central role in the environmental regulation of *ica* operon expression in *S. epidermidis* (Conlon *et al.*, 2002a). Modification of the bacterial environment by the addition of NaCl or ethanol to the growth medium can activate the *ica* operon via separate regulatory pathways in an *icaR*-depen-

The global stress response regulator σ^B^ and RsbU (a positive regulator of σ^B^) control biofilm development in *S. epidermidis* (Knobloch *et al.*, 2001) but not *S. aureus* (Valle *et al.*, 2003). σ^B^ controls *ica* operon expression in *S. epidermidis* by repressing transcription of *icaR* via an unidentified regulatory intermediate (Knobloch *et al.*, 2004). Interestingly, biofilm induction by NaCl is impaired footprint analysis, Jefferson *et al.* (2004) demonstrated that purified IcaR protein from *S. aureus* bound the *ica* operon promoter region close to the *icaA* start codon. Using DNA affinity chromatography to isolate proteins that bound to the *ica* promoter region, Jefferson *et al.* (2004) also identified TcaR as a weak repressor of *ica* operon transcription. TcaR is a member of the MarR family of transcriptional regulators and controls expression of the teicoplanin-associated locus. However, deletion of *tcaR* alone in *S. aureus* was not associated with any changes in PNAG or biofilm production, whereas deletion of both *icaR* and *icaR* was found to have a synergistic effect on these phenotypes (Jefferson *et al.*, 2004).

In addition to *IcaR* and *TcaR*, the insertion sequence element IS256 can also play a negative role in the control of *S. epidermidis* biofilm development. Ziebuhr *et al.* (1999) first demonstrated that reversible integration of the IS256 element into the *ica* locus results in biofilm-positive to biofilm-negative phenotypic switching. It was subsequently demonstrated that IS256 insertions in the *rsbU* gene of the sigB operon or the *sraA* locus, which both result in diminished *ica* operon expression, were also associated with phenotypic switching (Conlon *et al.*, 2004; Handke *et al.*, 2004). Clearly, IS256 mutations in loci encoding global regulators will affect multiple traits in staphylococci, suggesting that this genetic element may play a broader role in facilitating rapid phenotypic responses to specific external environmental signals.

An important component in bacterial biofilms is the capacity to communicate using quorum-sensing systems. The most important and best-characterized quorum-sensing system in staphylococci is the accessory gene regulator (agr) system, which does not seem to affect *ica* expression and the production of PIA/PNAG (discussed below). A second quorum-sensing system, the LuxS system has recently emerged as another negative regulator of biofilm formation in *S. epidermidis* (Xu *et al.*, 2006). Mutation of the *luxS* gene, which is required for synthesis of the AI-2 autoinducer molecule, positively impacted on *ica* operon expression, PIA/PNAG production and biofilm development. Significantly, the *luxS* mutant also exhibited enhanced virulence in a rat model of biofilm-associated infection (Xu *et al.*, 2006). Interestingly, Doherty *et al.* (2006) concluded that unlike *S. epidermidis*, mutation of *luxS* in *S. aureus* was not associated with altered biofilm-forming capacity.
Biofilm mechanisms in staphylococci

in S. epidermidis rsbU mutants, whereas ethanol-mediated biofilm activation is unaffected (Knobloch et al., 2001; Conlon et al., 2004), further suggesting the existence of two regulatory pathways controlling biofilm expression. Consistent with this ethanol-mediated icaR repression is $\sigma^B$-independent (Knobloch et al., 2004). The differential effect of $\sigma^B$ on ica operon expression in S. epidermidis and S. aureus explains, at least in part, differences in the regulation of biofilm development in these two species. Interestingly, $\sigma^B$ also appears to be important for the long-term stability of S. epidermidis biofilms (Jager et al., 2005). Using ethanol to induce biofilm formation in $\sigma^B$ mutants, it was observed that, over time, biofilms formed by sigB mutants were more rapidly dispersed than wild-type biofilms. Although this further highlights the importance of $\sigma^B$ for both biofilm development and stability, it is interesting to note that biofilm dispersal may also be beneficial for the pathogen in terms of dissemination. In addition, although $\sigma^B$ does not appear to be required for biofilm development in S. aureus, it would be interesting to examine the stability of biofilms formed by S. aureus sigB mutants.

In S. aureus, the Spa protein, which interacts directly with the z subunit of RNA polymerase to regulate transcription initiation, also contributes to stress resistance and biofilm regulation. Specifically, Spa appears to activate icaR transcription, leading to decreased ica operon expression (Frees et al., 2004; Pamp et al., 2006). Interestingly, Spa is normally degraded by the ClpXP protease. Consistent with this, biofilm formation was impaired in a clpX mutant (Frees et al., 2004). However, the absence of ClpP was associated with enhanced biofilm formation (Frees et al., 2004), leading the authors to speculate that although ClpP may positively contribute to PIA/PNAG production, it may also play a negative role in an ica-independent biofilm mechanism (Pamp et al., 2006).

Two studies published in 2003 reported that the sarA locus was required for ica operon transcription, PIA/PNAG production and biofilm formation in S. aureus (Beenken et al., 2003; Valle et al., 2003). SarA is a major global regulator of a diverse range of virulence determinants in S. aureus (Cheung & Zhang, 2002), with gene chip analysis revealing that this protein can affect the transcription of at least 120 genes in S. aureus (Dunman et al., 2001). Given that SarA also regulates agr (Cheung & Projan, 1994), the impact of agr mutations on biofilm development was also examined. These agr mutants had similar biofilm-forming capacity compared with the wild-type strains, indicating that SarA affects biofilm formation by an agr-independent pathway (Beenken et al., 2003; Valle et al., 2003). Because $\sigma^B$ regulates sarA expression in S. aureus and ica operon transcription is decreased in S. epidermidis $\sigma^B$ mutants, Valle et al. (2003) also examined the role of $\sigma^B$ in the biofilm-negative phenotype of the S. aureus sarA mutants. Mutants lacking $\sigma^B$ alone and sarA – $\sigma^B$ double mutants were constructed. Interestingly, these $\sigma^B$ mutants were able to form a biofilm (Valle et al., 2003), which contradicted the earlier findings of Rachid et al. (2000b), who reported that $\sigma^B$ positively regulates biofilm formation in strain M12. This may suggest that $\sigma^B$ regulates S. aureus biofilm in a strain-dependent manner.

The SarA protein of S. epidermidis is 84% identical to SarA of S. aureus despite significant divergence at the nucleotide level outside the coding region. The three S. aureus sarA promoters P1, P2 and P3 are present in S. epidermidis but the organization and spacing between these promoters are different from S. aureus (Fluckiger et al., 1998), suggesting that SarA regulation and activity may differ significantly between the two species. As described above and elsewhere, sarA mutations in S. epidermidis are also associated with a biofilm-negative phenotype (Conlon et al., 2004; Tormo et al., 2005b). Purified SarA protein from S. aureus binds to the ica operon promoter of both S. epidermidis and S. aureus with high affinity (Tormo et al., 2005b) and positively regulates ica operon expression via an IcaR-independent mechanism (Conlon et al., 2004; Tormo et al., 2005b). A Tn917 insertion in the S. epidermidis purR gene, whose homologue regulates purine biosynthesis in other gram-positive organisms, negatively impacts ica expression and biofilm development (Mack et al., 2007). However, the absence of identifiable PurR recognition sequences upstream of the icaA or icaR genes or an obvious correlation between purine biosynthesis and the biofilm phenotype suggests that PurR plays an indirect role in ica regulation (Mack et al., 2007). Nevertheless, although connections between biosynthetic pathways and PIA/PNAG production may not be immediately obvious, external stresses that increase PIA/PNAG production and activate biofilm formation also repress central metabolism (Vuong et al., 2005). Furthermore, Vuong et al. (2005) reported that the growth of S. epidermidis in the presence of a subinhibitory concentration of the TCA cycle inhibitor fluorocitrate dramatically increased PIA/PNAG production, perhaps leading the authors to make the interesting suggestion that alterations in the intracellular levels of biosynthetic intermediates, ATP or the redox status of the cell in response to external signals may influence PIA/PNAG biosynthesis (Vuong et al., 2005).

Interestingly, a recent screen of a Mu transposition insertion library in the S. aureus paediatric isolate S30 revealed that the majority of insertion mutations outside the icaADBC locus resulting in a biofilm-impaired phenotype were associated with diminished PIA/PNAG production (Tu Quoc et al., 2007). Clearly, additional regulators of ica operon expression and PIA/PNAG production remain to be described.
**ica**-independent biofilm mechanisms

Despite the undeniably important role of the **ica**ADBC locus and the regulatory pathways controlling **PIA/PNAG** production in staphylococcal biofilm development, recent studies have begun to highlight the existence of **PIA/PNAG**-independent biofilm mechanisms in both **S. aureus** and **S. epidermidis** (Beenken et al., 2004; Fitzpatrick et al., 2005; Rohde et al., 2005, 2007; Kogan et al., 2006). This is perhaps not surprising in **S. aureus**, given the diverse array of cell surface adhesins used by this pathogen in binding to the host extracellular matrix (Fig. 1). Nevertheless, it is interesting to note that the **ica** locus is more likely to be associated with clinically significant **S. epidermidis** isolates than carriage isolates (Ziebuhr et al., 1999; Fitzpatrick et al., 2002), whereas in **S. aureus** the vast majority of isolates appear to carry this gene cluster (Knobloch et al., 2002; Fitzpatrick et al., 2006). It therefore seems reasonable to assume that the role of the **ica** locus in the **S. aureus** biofilm phenotype is complex and likely to be both strain- and environment-dependent. Moreover, it also seems likely that the role of the **ica** locus in **S. aureus** and **S. epidermidis** biofilm development may vary significantly. However, as observed recently in **S. aureus**, a number of reports have described **biofilm**-positive **S. epidermidis** strains that lack the **ica** operon (Ziebuhr et al., 1999; Fitzpatrick et al., 2002, 2005; Frank et al., 2004; Rohde et al., 2004; Kogan et al., 2006).

The first well-characterized **ica**ADBC-independent biofilm mechanism was described by the research groups of J. Penades and I. Lasa, who demonstrated that the biofilm-associated protein (Bap) was essential for both initial adherence and intercellular accumulation during biofilm development by the **S. aureus** bovine mastitis isolate V329 (Cucarella et al., 2001). Mutation of the **ica** locus in this isolate had no impact on the biofilm phenotype (Cucarella et al., 2004). However, SarA acts as a transcriptional activator of the **bap** gene and therefore a positive regulator of Bap-mediated biofilm development (Trotonda et al., 2005). A Bap homologue protein, which can also mediate **ica**-independent biofilm, has been identified in some **S. epidermidis** and other coagulase-negative staphylococcal species (Tormo et al., 2005a). Although the **bap** gene is present in only 5% of bovine isolates and appears to be absent in human clinical isolates of **S. aureus** (Cucarella et al., 2001), the Bap protein has now emerged as a member of a group of over 100 surface proteins with conserved structural and functional characteristics from diverse bacterial species, which play an important role in biofilm development [reviewed by Lasa & Penades (2006)].

The research group of M. Smeltzer subsequently demonstrated that deletion of the **ica** locus in a biofilm-positive **S. aureus** human clinical isolate UAMS-1 had no impact on biofilm development (Beenken et al., 2004). In contrast, mutation of the sarA gene in **S. aureus** UAMS-1 resulted in impaired biofilm forming capacity. We recently reported that **ica**ADBC-independent biofilm formation was possible in four clinical methicillin-resistant **S. aureus** (MRSA) isolates (Fitzpatrick et al., 2005). Interestingly, by examining the environmental regulation of biofilm development and using phage transduction to construct **ica** deletion mutants, evidence for different mechanisms of biofilm in MRSA and methicillin-sensitive **S. aureus** (MSSA) isolates was obtained (Fitzpatrick et al., 2006). Specifically, NaCl was significantly more likely to induce biofilm formation in MSSA isolates than MRSA isolates. NaCl is a known activator of **ica** operon transcription (Rachid et al., 2000a; Fitzpatrick et al., 2005) and, consistent with this, biofilm development in MSSA isolates is apparently **ica**ADBC-dependent and involves the production of **PIA/PNAG**. Biofilm development in MRSA isolates is primarily glucose-induced, is **ica**-independent and apparently involves a protein adhesin (Fitzpatrick et al., 2005; O’Neill et al., 2007). These data reveal a previously unknown level of complexity in terms of both biofilm mechanisms and regulation in **S. aureus**. Nevertheless, consistent with the findings of Beenken et al. (2004) and Trotonda et al. (2005), we recently demonstrated that mutation of the sarA locus abolished biofilm development in eight MRSA and seven MSSA strains (O’Neill et al., 2007). Thus, given that considerable progress has already been made in understanding the role of SarA in controlling **ica**-mediated biofilm (Valle et al., 2003; Tormo et al., 2005b), these data strongly suggest that SarA may act as a master regulator controlling both **ica**-dependent and **ica**-independent biofilm mechanisms.

In contrast to the role of SarA, mutation of the global regulator **agr** has a neutral effect on biofilm development (Beenken et al., 2003) or can lead to increased biofilm formation (Vuong et al., 2000; Toledo-Arana et al., 2005). Moreover, under different growth conditions, the influence of **agr** on biofilm formation can vary from positive to neutral to negative, perhaps reflecting the responsiveness of the **Agr** system to external environmental cues (Yarwood et al., 2004). We recently observed that mutation of the **agr** locus in 21 **S. aureus** isolates had a variable (either neutral or positive) effect on biofilm development (O’Neill et al., 2007). In **S. aureus**, Vuong et al. (2000) observed that expression of the major cell wall autolysin **atlE**, which has previously been implicated in the early stages of **S. epidermidis** adherence by directly interacting with hydrophobic surfaces (such as medical devices) (Heilmann et al., 1997) or **PIA/PNAG**, was not affected by **agr** mutation. This led them to hypothesize that **Agr** may influence biofilm development, at least in part, because of the surfactant properties of the **agr**-encoded **δ**-toxin, which might prevent hydrophobic interactions between the cell and material surfaces. Consistent with this possibility, when **δ**-toxin was added to...
the medium of an *S. aureus* *agr* mutant strain, biofilm-forming capacity decreased (Vuong *et al*., 2000). Similar to *S. aureus*, mutation of the *agr* system in *S. epidermidis* was also associated with increased biofilm formation, at least in part through the δ-toxin, and independent of changes in PIA/PNAG production (Vuong *et al*., 2003). However, unlike *S. aureus*, Agr also regulates AtlE production in *S. epidermidis*. The role of the *agr* system in the *Staphylococcus* biofilm phenotype has been covered in depth in a recent review (Kong *et al*., 2006).

Like Atl, the *S. epidermidis* surface proteins SSP-1 and SSP-2 have also been implicated in the primary attachment phase of biofilm development (Veenstra *et al*., 1996). In contrast, the accumulation-associated protein (Aap) has been implicated in biofilm accumulation in *S. epidermidis*. Aap was originally identified by Hussain *et al*. (1997), who used mitomycin mutagenesis to generate a mutant that lacked a 140 kDa protein required for biofilm accumulation (Hussain *et al*., 1997). Interestingly, the observation that anti-Aap antibodies could inhibit PIA/PNAG-dependent biofilm suggested that Aap might somehow anchor PIA/PNAG to the cell surface (Hussain *et al*., 1997). Consistent with this possibility, a novel repeat domain termed the G5 domain (after conserved glycine residues) implicated in
N-acetylglucosamine binding has also been identified in AAP and a number of other Staphylococcus surface proteins (Bateman et al., 2005). However, Rohde et al. (2005) recently demonstrated that proteolytic cleavage of Aap by staphylococcal or host proteases, which converts the protein into an adhesin, is sufficient to mediate biofilm in a PIA/PNAG-independent mechanism. The Aap homologue in S. aureus, designated SasG, is known to mediate binding to the surface of nasal epithelial cells (Roche et al., 2003) but its potential role in biofilm has not yet been investigated.

These data naturally highlight the importance of cell surface molecules in mediating adherence and biofilm development. Teichoic acids, which are now known to be surface molecules in mediating adherence and biofilm potential role in biofilm has not yet been investigated. (Bateman et al., 2005) D-alanine incorporation into teichoic acids, was biofilm-AAP and a number of other (Bateman et al., 2005).

The intensification of interest in the Staphylococcus biofilm phenotype has yielded significant insights into the mechanisms and regulation of this important virulence determinant. Considerable progress has been made in profiling the role of the icaADBC-encoded exopolysaccharide PIA/PNAG in biofilm development and the pathogenesis of device-related infections. Nevertheless, our understanding of how the ica locus and PIA/PNAG biosynthesis is regulated is far from complete and many questions remain, such as the identity of the regulatory intermediates controlled by σB and ethanol, which repress icaR transcription.

Beyond ica, recent developments have now revealed that surface proteins such as Aap and Bap can mediate PIA/PNAG-independent intercellular accumulation during...
biofilm development by ica-deficient strains of S. aureus and S. epidermidis. These findings open up the intriguing prospect that other Staphylococcus surface proteins may also be involved in biofilm development, a possibility that is perhaps predictable given the numbers of surface proteins in staphylococci; S. aureus is predicted to encode 28 surface proteins (Roche et al., 2003; Gill et al., 2005), while 18 have been identified in S. epidermidis (Bowden et al., 2005; Gill et al., 2005). Among the 28 surface proteins identified in S. aureus, 21 are predicted to contain LPXTG motifs required for surface display. Anchoring of LPXTG proteins to the Gram-positive cell wall is catalyzed by an extracellular transpeptidase, Sortase, which is required for virulence (Mazmanian et al., 2000, 2002). Staphylococcus epidermidis possesses 11 LPXTG-motif containing surface proteins and apart from Aap, their role in biofilm development is largely unknown. The remaining seven non-LPXTG surface proteins in S. aureus and S. epidermidis include Ail and AtlE, respectively. The role of surface proteins as biofilm adhesins (as opposed to their role as MSCRAMMs) needs to be further investigated in order to advance our understanding of the pathogenesis of device-related infections. Of particular importance in this context is the observation that almost all S. aureus clinical isolates possess and express the ica operon under various growth conditions, but many are incapable of biofilm development under the same conditions (Fitzpatrick et al., 2005, 2006). These findings represent something of a paradox; if the ica operon is conserved and expressed in most S. aureus clinical strains, then why does it not always mediate biofilm development? Thus, progress in understanding the role of surface proteins as biofilm adhesins may help in answering this question. Alternatively, under appropriate conditions, proteins and other surface molecules may cooperate with PIA/PNAG to mediate intercellular adhesion. Such a scenario would also explain why the ica locus is maintained, expressed and regulated in many Staphylococcus clinical isolates capable of ica-independent biofilm formation.

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References


