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MicroReview

Staphylococcus and biofilms

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Summary

The genetic and molecular basis of biofilm formation in staphylococci is multifaceted. The ability to form a biofilm affords at least two properties: the adherence of cells to a surface and accumulation to form multilayered cell clusters. A trademark is the production of the slime substance PIA, a polysaccharide composed of β-1,6-linked *N*-acetylglucosamines with partly deacetylated residues, in which the cells are embedded and protected against the host's immune defence and antibiotic treatment. Mutations in the corresponding biosynthesis genes (*ica* operon) lead to a pleiotropic phenotype; the cells are biofilm and haemagglutination negative, less virulent and less adhesive on hydrophilic surfaces. *ica* expression is modulated by various environmental conditions, appears to be controlled by SigB and can be turned on and off by insertion sequence (IS) elements. A number of biofilm-negative mutants have been isolated in which polysaccharide intercellular adhesin (PIA) production appears to be unaffected. Two of the characterized mutants are affected in the major autolysin (*atlE*) and in D-alanine esterification of teichoic acids (*dltA*). Proteins have been identified that are also involved in biofilm formation, such as the accumulation-associated protein (AAP), the clumping factor A (ClfA), the staphylococcal surface protein (SSP1) and the biofilm-associated protein (Bap). Concepts for the prevention of obstinate polymer-associated infections include the search for new anti-infectives active in biofilms and new biocompatible materials that complicate biofilm formation and the development of vaccines.

Introduction

In numerous reports from the past two decades, it has been shown that especially biofilm-forming staphylococci, with *Staphylococcus epidermidis* as a leading species, cause a new infection that is best described as 'chronic polymer-associated infection'. The skin bacterium *S. epidermidis*, once considered harmless, is now known to be an opportunistic pathogen and very frequently infects catheter materials. Scanning electron micrographs from early studies show that *S. epidermidis* forms multiple cell layers on the polymer surface; the cells in these layers are enveloped and protected by an amorphous slimy material (Peters *et al*., 1981; Christensen *et al*., 1982; Marrie *et al*., 1982). The slime substance is not a true capsule, but is loosely bound to the staphylococcal cells. In most natural environments, a biofilm consists of a multispecies microbial community. In contrast, with biomaterial-associated infections, nearly 80% of the cells involved are *S. epidermidis*. This phenomenon can be explained by the easy access of this skin inhabitant to wounds and implants (von Eiff *et al*., 1999).

Biofilms are formed on nearly all kinds of catheters and on many other medical devices and implants (reviewed by Götz and Peters, 2000). Microscopy of biofilm formation *in vitro* suggests that two steps are involved: (i) the attachment of the bacterial cells to the polymer surface, which may occur very rapidly; and (ii) the growth-dependent accumulation to form multilayered cell clusters surrounded by a slimy matrix.

The slimy material is eye-catching when bacteria are examined by microscopy and, therefore, is believed to play a crucial role in polymer-associated infections. The chemical composition of slime has been investigated intensively. Earlier studies had the disadvantage that the slime material was not purified and was contaminated with medium-derived sugar compounds (e.g. from agar or yeast extract), which led to false results and artifacts (Drewry *et al*., 1990). The slime substance purified from *S. epidermidis* RP62A (ATCC 35984), galactose-rich capsular polysaccharide adhesin (PS/A), has, in contrast to teichoic acid, some protective activity when used as a vaccine (Kojima *et al*., 1990). Later, a slime-associated antigen (SAA) mainly composed of *N*-acetylglucosamine was isolated from the same strain (Baldassarri *et al*., 1996). The strain also excretes considerable amounts of teichoic acids (Hussain *et al*., 1993), which may contribute to the highly viscous appearance of the colonies and the strong biofilm formation of this strain. Up to 1996, reports

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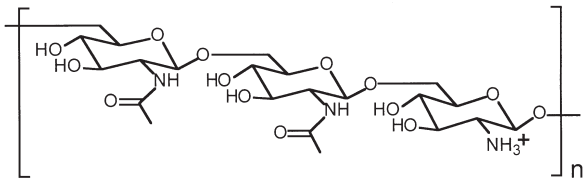

Fig. 1. Structure of polysaccharide intercellular adhesin (PIA). The polysaccharide is a linear homoglycan composed of β-1,6-linked *N*- acetylglucosamine residues; ≈ 20% of the residues are deacetylated and are thus positively charged. Adapted from Mack *et al.* (1996a).

on the chemical composition of 'slime' were conflicting; the most reliable work was carried out by Mack *et al.* (1996a), who purified a specific polysaccharide antigen of the biofilm-producing *S. epidermidis* strains 1457 and RP62A and were able to distinguish two polysaccharide fractions. Polysaccharide I (>80%) is a linear homoglycan of at least 130 residues of β-1,6-linked *N*-acetylglucosamine, 15–20% of which are deacetylated and therefore positively charged. Polysaccharide II (<20%) is structurally related to polysaccharide I, but has a lower content of non-*N*-acetylated D-glucosaminyl residues and contains phosphate and ester-linked succinate, rendering it anionic. The structure of this polysaccharide is unique (Fig. 1) and, according to its function in intercellular aggregation, it was referred to as polysaccharide intercellular adhesin (PIA). In retrospect, it appears that the above-mentioned SAA is most likely PIA.

Recently, a PIA-related substance has been described from *Staphylococcus aureus* MN8N. The *N*-acetylglucosamine residues of PNSG are completely succinylated, which led to its designation as poly *N*-succinyl β-1,6-glucosamine (PNSG) (McKenney *et al.*, 1999). It is now clear that the succinyl groups were an artifact (G. Pier, personal communication). Indeed, the existence of PNSG would contradict the findings that antibodies against PNSG and against PIA are completely cross-reactive and that only *N*-acetylglucosamine oligomers and no succinylated residues are found after *in vitro* synthesis with extracts of *Staphylococcus carnosus* clones expressing various *ica* genes (Gerke *et al.*, 1998).

Biological activity of PIA

In a collection of 179 *S. epidermidis* isolates, 51% produce PIA, and most of these strains form a biofilm (Mack *et al.*, 1996b). As the core polymer of PIA has a β-1,6-linked *N*-acetylglucosamine structure, we tested whether biofilm-positive strains could be distinguished from biofilm-negative strains using a phosphatase-labelled wheat germ agglutinin (WGA) as a specific probe for the $(GlcNAc\beta\text{-}1,4)_n$. This enzyme-linked lectinsorbent assay (ELLA) does not prove the presence of PIA, but is useful as an initial screening assay (Thomas *et al.*, 1997). Other detection methods, and genetic as well as biochemical techniques used to study biofilm formation in staphylococci, have been reviewed recently (Leriche *et al.*, 2000; Cramton *et al.*, 2001a). PIA-positive and PIA-negative strains can also be differentiated on Congo red agar (Heilmann and Götz, 1998); colonies of PIA-positive strains are black, whereas colonies of PIA-negative strains are red.

Haemagglutination of erythrocytes, a common property of *S. epidermidis* strains, may play a role in the pathogenesis of biomaterial-associated infections. PIA is somehow involved in haemagglutination; PIA-negative mutants (interruption or deletion of the *ica* genes) have a haemagglutination-negative phenotype (Fey *et al.*, 1999; Mack *et al.*, 1999). However, PIA alone does not mediate haemagglutination.

PIA also acts as an intercellular adhesin, and there are hints that PIA acts as an adhesin on glass and probably other hydrophilic surfaces (see below). The role of PIA in phagocytosis and host defence is currently being investigated.

PIA biosynthesis (*ica* genes)

The genes responsible for these various phenotypes were identified by isolating and analysing biofilm-negative transposon–insertion mutants of *S. epidermidis* O-47. Two classes of mutants were isolated. One is defective in attachment to polystyrene, and the other is defective in slime production and is unable to accumulate in multilayered cell clusters (Heilmann *et al.*, 1996a). For the first time, the two postulated steps in biofilm formation were genetically verified. The accumulation-defective mutants are unable to form a biofilm on both polystyrene and glass surfaces, display no intercellular aggregation and do not produce PIA. The transposon had integrated at various sites in an operon, which we named *ica* for intercellular adhesion (Heilmann *et al.*, 1996b). The operon is composed of the *icaR* (regulatory) gene and *icaADBC* (biosynthesis) genes. With PIA-specific antibodies and immunofluorescence studies, PIA was localized mainly on the cell surface. The *ica* genes cloned into the heterologous cloning host *S. carnosus*, which is apathogenic, lacks *ica*-related genes and is therefore unable to form a biofilm, produced interesting results: massive cell aggregation, formation of a biofilm on a glass surface and PIA production. Surprisingly, the *S. carnosus* clones hardly form a biofilm on polystyrene plates, which indicates that adherence of *S. carnosus* to polystyrene is weak, whereas adherence to glass is very pronounced. Although proof is lacking, we think that PIA itself mediates adherence to hydrophilic surfaces.

There are several pieces of evidence that *icaR* is a

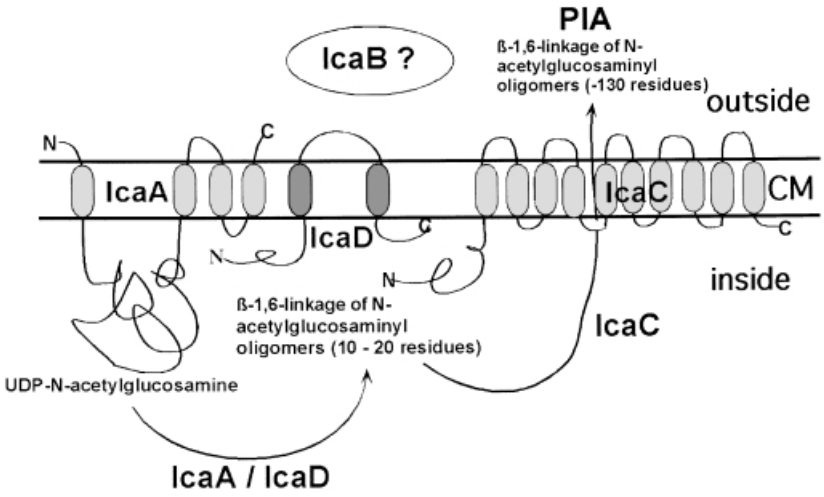

Fig. 2. Model of PIA biosynthesis. IcaA and IcaD synthesize oligomers derived from UDP-*N*-acetylglucosamine; synthesis comes to a halt when a length of 10–20 residues is reached. In the presence of IcaC, longer oligomers are formed that react with PIA-specific antiserum. Because of the sequence similarity with deacetylases, IcaB might catalyse the deacetylation reaction. CM, cytoplasmic membrane. Adapted from Heilmann *et al*. (1996a), Gerke *et al*. (1998) and O. Schweitzer (unpublished results).

repressor. An internal deletion in *icaR* in the chromosome of *S. epidermidis* O-47 augments PIA expression. By comparing *S. carnosus* PIA production in the presence or absence of *icaR*, again PIA production is lower in the presence of *icaR*. *icaA* promoter-directed reporter gene expression revealed that heterologously expressed *icaR* represses reporter gene expression (C. Gerke and F. Götz, unpublished). The exact IcaR-specific operator site has not yet been determined.

Functional analysis of the IcaABCD proteins

Our knowledge of the function of the IcaABCD proteins is only lowly. All encoding genes have been cloned individually or in combination in *S. carnosus*, and the *in vitro* biosynthesis of PIA has been analysed using the membrane fraction or cellular or extracellular extracts (Gerke *et al*., 1998). IcaA, C and D are located in the membrane fraction; IcaB is mainly present in the culture supernatant. IcaA contains four transmembrane helices and has *N*-acetylglucosaminyl-transferase activity with UDP-*N*-acetylglucosamine as substrate. Certain domains of the amino acid sequence show similarity to the chitinase (NodC) of rhizobia and the hyaluronan synthase (HasA) of *Streptococcus pyogenes*. IcaA alone has only low transferase activity; when *icaA* is co-expressed with *icaD*, the transferase activity increases ≈ 20-fold. IcaD might be a chaperone that directs the correct folding and membrane insertion of IcaA and, in addition, might act as a link between IcaA and IcaC. To date, no IcaD-like protein has been identified among the homologous β-glycosyl transferases; thus, IcaAD represents a novel enzyme combination (Gerke *et al*., 1998). *N*-Acetylglucosamine oligomers produced by IcaAD reach a maximal length of only 20 residues; only when the *icaAD* genes are co-expressed with *icaC* are longer oligomer chains synthesized that react with PIA-specific antiserum, which does not, however, react with short oligomeric *N*-acetylglucosamines (Fig. 2).

An undecaprenyl phosphate lipid carrier that is often involved in the biosynthesis of bacterial exopolysaccharides does not appear to participate in the activity of IcaAD – lipid-linked intermediates have never been detected *in vitro* or *in vivo*, and the antibiotics tunicamycin and bacitracin have no effect on the synthesis of IcaAD-dependent oligomers or PIA *in vitro* and *in vivo*.

In *in vitro* synthesis studies, no deacetylated residues have been detected by mass spectroscopic analysis. We therefore speculate that the primary product is a completely acetylated and therefore uncharged *N*-acetylglucosamine polymer. Deacetylation is apparently a second step that leads to the positively charged *N*-glucosamine residues. Although the PIA-specific deacetylase has not yet been identified, a candidate is the 33 kDa IcaB, which is secreted into the culture broth. IcaB shows sequence similarity to the *Rhizobium* NodB protein, which is involved in nodulation signal synthesis and is a chitooligosaccharide deacetylase (John *et al*., 1993).

The 42 kDa IcaC with its transmembrane helices is very likely an integral membrane protein. Inactivation of *icaA* or *icaC* in the *ica* gene-expressing plasmid pCN27 leads to complete loss of the intercellular adhesion phenotype of *S. carnosus*, which suggests that these two genes are necessary for PIA expression (Heilmann *et al*., 1996b). Internal frameshift and deletion mutants in either *icaB* or *icaD* led to a strong decrease in PIA expression (O. Schweitzer and F. Götz, unpublished). The function of IcaC is unknown, but we speculate that it might be involved in the translocation of the polysaccharide through the cytoplasmic membrane.

Adherence to polymers and extracelluar matrix

A transposon–insertion mutant of *S. epidermidis* strain

O-47 has been isolated that is biofilm negative even though the *ica* operon is intact and PIA is produced (Heilmann *et al*., 1996a). Although the mutant is unable to form a biofilm on polystyrene, biofilm formation on glass is entirely unaffected or even more pronounced, which led us to speculate and then demonstrate in binding assays that this mutant is defective in primary adhesion to polystyrene (Heilmann *et al*., 1997). The mutant is also significantly less hydrophobic than the wild type, but the most remarkable characteristic is the formation of huge cell clusters that resist even detergent treatment, which points to covalent interlinking of the cells. Both the altered surface hydrophobicity and the massive cell clumping suggest that cell surface compounds are changed. Indeed, in comparison with the wild type, the mutant lacks five cell surface-associated proteins with masses of 120, 60, 52, 45 and 38 kDa. This pleiotropic effect results from an 8 kb deletion, including a 4005 bp open reading frame (ORF) encoding the *S. epidermidis* major autolysin AtlE, which accompanied transposon insertion (Heilmann *et al*., 1997). AtlE is a homologue of *S. aureus* Atl, which has been identified by Oshida *et al*. (1995).

AtlE (1335 amino acids) is a multidomain protein composed of an N-terminal signal peptide, a propeptide (PP), an amidase domain (AM; 60 kDa), three highly cationic repeats (R1, R2, R3) and the glucosaminidase domain (GL; 52 kDa). Apart from the signal peptide processing, AtlE is also processed by an extracellular protease at two (or more) further positions: Val-303 and Thr-846. Differently sized degradation products are generated through partial processing, but all bind to the cell surface. Complementation studies have shown that production of only the 60 kDa AM is sufficient to restore the biofilm-positive phenotype and normal cell separation completely, which indicates that the amidase really acts as a major autolysin in *S. epidermidis*. The three cationic repeats might be involved in the attachment of the Atl proteins to the cell surface. Certain synthetic oligopeptides with sequences derived from the *S. aureus* Atl repeat-1 affect autolysis (Takano *et al*., 2000); therefore, the AtlE repeats might exert a similar function. AtlE binds strongly to vitronectin (Heilmann *et al*., 1997), a 70 kDa protein found in the extracellular matrix and in serum. Vitronectin is one of the few proteins that regulates both the complement and the coagulation systems; whether AtlE functions similarly is not known.

The staphylococcal major autolysins are multifunctional. Although *atl* and *atlE* mutants can survive, cell separation after division is severely affected, leading to covalently interlinked cell clusters. Atl and AtlE have binding motifs for the cell wall and vitronectin. We do not know whether the decrease in adherence of the *atlE* mutant to polystyrene results from the lack of hydrophobic interaction of the 60 kDa AM with the hydrophobic polymer or from formation of cell clusters that might be less resistant to shearing during the washing procedure.

Influence of the teichoic acid structure on biofilm formation

In a search for *S. aureus* mutants hypersensitive to positively charged antimicrobial peptides, mutants with an altered teichoic acid structure have been isolated (Peschel *et al*., 1999). The mutations are in the *dltA* operon, and the teichoic acids lack D-alanine. It has been postulated that the increased negative charge of the cell surface of the mutants leads to an increased scavenging of positively charged antimicrobial peptides, thus leading to the observed hypersensitivity. The *dltA* mutants are also more sensitive to vancomycin and antimicrobial host defence peptides, such as defensins, protegrins and platelet microbicidal proteins produced by mammalian skin, epithelia, phagocytes and platelets in response to staphylococcal infections (Peschel *et al*., 2000; Peschel and Vincent Collins, 2001).

Surprisingly, the *dltA* mutant is also biofilm negative even though PIA production appears to be unchanged. The mutant is severely affected in adherence to polystyrene or glass surfaces (Gross *et al*., 2001), the first step of biofilm formation. In this respect, the *dltA* mutant resembles the *atlE* mutant described above, with one exception: the *atlE* mutant is still able to form a biofilm on a glass surface. Biofilm formation of the *dltA* mutant can be completely restored by the addition of Mg^{2+} ions, which is in accordance with an earlier observation that low concentrations of Mg^{2+} (16 μM) significantly enhance the adhesion of all tested slime-positive *S. epidermidis* strains to plastic, whereas Ca^{2+} exerts little effect (Dunne and Burd, 1992). These findings corroborate the biological importance of the charge balance of the Gram-positive cell surface.

Other proteins that contribute to biofilm formation

Proteins other than the major autolysin AtlE contribute to biofilm formation in staphylococci, such as the accumulation-associated protein (AAP) and the biofilm-associated protein (Bap). Mitomycin mutagenesis of *S. epidermidis* RP62A led to the isolation of a biofilm-negative mutant, M7, which lacks a 140 kDa exoprotein (Schumacher-Perdreau *et al*., 1994). The 140 kDa antigen is predominantly found under sessile growth conditions, and not all biofilm-positive *S. epidermidis* strains produce this antigen. However, the positive strains produce significantly larger amounts of biofilm than those that lack the 140 kDa antigen. Therefore, the 140 kDa antigen is referred to as accumulation-associated protein (AAP) (Hussain *et al*., 1997).

An *S. aureus* transposon–insertion mutant has been isolated with a significant decrease in attachment to inert surfaces, intercellular adhesion and biofilm formation (Cucarella *et al*., 2001). The transposon is inserted in the *bap* gene, which encodes a novel cell wall-associated, 2276-amino-acid protein. The *bap* gene has been found in 5% of the *S. aureus* bovine mastitis isolates, but it was absent in the 75 clinical human *S. aureus* isolates analysed. The core region of Bap consists of 52% of the protein, with 13 successive nearly identical repeats, each containing 86 amino acids. All staphylococcal isolates harbouring *bap* are highly adherent and strong biofilm producers. In a mouse infection model, Bap is involved in pathogenesis, causing a persistent infection (Cucarella *et al*., 2001).

We have to be aware that biofilm formation does not only occur with foreign body infections. If one closely examines native tissues removed from patients with recurrent *S. aureus* infections (e.g. heart tissue, cartilage, chronic wounds), the cells are frequently organized in confluent colonies with a biofilm-like appearance. Therefore, not only should adherence to plastic polymers be considered, but also any bacterial factor that mediates adherence to components of the extracellular matrix of the host. *S. aureus* is especially capable of adhering to a large variety of matrix components to initiate colonization. This adherence is frequently mediated by protein adhesins of the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family, which in many cases are covalently anchored to the cell wall peptidoglycan (Foster and Höök, 1998). The fibronectin-binding protein A and B (FnBPA, FnBPB), the collagen-binding protein Cna and the fibrinogen-binding proteins, clumping factor A and B (ClfA and ClfB), belong to this family. ClfA and ClfB are prototypes of a recently identified multigene family of putative surface proteins characterized by a common domain composed of a unique Ser–Asp dipeptide repeat referred to as Sdr (SD repeat protein). There are other Sdr proteins (SdrC, SdrD and SdrE) whose function is unclear. Interestingly, it has been demonstrated that ClfA also mediates adherence to polyethylene or polyvinylchloride tubing, whereas FnBP has no effect (Vaudaux *et al*., 1995). This example shows that ClfA plays a dual role in wound and foreign body infection.

In general, *S. epidermidis* does not have that many adherence components for the extracellular matrix. It was therefore surprising when a ClfA-related fibrinogen-binding protein, Fbe (SdrG), was identified (Nilsson *et al*., 1998). Its contribution in biofilm has not yet been analysed.

Veenstra *et al*. (1996) identified a 280 kDa surface protein, SSP1, and its degradation product, SSP2. SSP1 is involved in the attachment of *S. epidermidis* 354 to polystyrene. Ultrastructural analysis suggests that this adhesin protrudes from the cell surface as a fimbria-like polymer. Unfortunately, the corresponding gene has not yet been identified, which would allow further analysis.

Regulation of slime production and biofilm formation

Environmental (stress) factors

In earlier literature, the term ‘slime’ was used to describe the mucous substance of colonies of staphylococcal strains, the slimy film coating on culture tube walls and the extracellular matrix encasing the catheter surface (Christensen *et al*., 1982). In retrospect, in most cases, the ‘slime’ was very likely PIA; unfortunately, the chemical composition of ‘slime’ was not or was incorrectly assessed. Only with the well-studied *S. epidermidis* strains RP62A and O-47 are we certain that PIA and ‘slime’ are the same. A further proof is that the expression of the *ica* genes in *S. carnosus* leads to PIA production and to the generation of colonies with a slimy mucous consistency (Heilmann and Götz, 1998).

The early observations on ‘slime’ regulation already indicated that slime (PIA) production was influenced by many parameters. In certain strains, such as *S. epidermidis* RP62A, slime production frequently varies (Christensen *et al*., 1987; Baddour *et al*., 1990), and Christensen *et al*. (1987) spoke of ‘phenotypic variation’. In recent years, many factors have been identified that influence PIA (‘slime’) or biofilm production (summarized in Table 1).

Variants of highly slime-producing *S. epidermidis* strains that produce markedly less slime (frequency 10^{-2}–10^{-3}) or no slime (frequency $>10^{-4}$) can easily be isolated. Oleic acid has an inducing effect on *S. aureus*, which could result from an ionic interaction of the positively charged PIA with the negatively charged oleic acid. Interestingly, the positive effect of oleic acid is more pronounced under oxygen-limited conditions (Campbell *et al*., 1986).

The effect of environmental factors on *ica* transcription in *S. epidermidis* has been studied directly by fusing the *ica* promoter on the chromosome with the *Escherichia coli* β-galactosidase gene *lacZ*. Subinhibitory concentrations of tetracycline and quinupristin–dalfopristin, as well as high temperature and osmolarity, increase the *ica* promoter activity, whereas penicillin, oxacillin, chloramphenicol, clindamycin, gentamicin, ofloxacin, vancomycin and teicoplanin have no effect (Rachid *et al*., 2000a). Subinhibitory concentrations of erythromycin induce *ica* expression weakly (2.5-fold). These results suggest that *ica* expression could be under the control of the alternative sigma factor SigB. Indeed, replacement of *sigB* on the

Table 1. Factors that influence biofilm formation.

Factor	Reference
Factors that influence PIA expression	
Glucose	Mack *et al*. (1992)
Glucosamine, *N*-acetylglucosamine	Gerke *et al*. (1998)
Oleic acid	Campbell *et al*. (1983)
Quinupristin–dalfopristin, tetracycline	Rachid *et al*. (2000a)
Urea (in *S. saprophyticus*)	Hjelm and Lundell-Etherden (1991)
Anaerobiosis	Cramton *et al*. (2001b)
Fe limitation	Deighton and Borland (1993); Elci *et al*., (1995); Evans *et al*. (1994)
High osmolarity, high temperature	Rachid *et al*. (2000b)
Ethanol (*S. epidermidis*)	Knobloch *et al*. (2001)
Global regulators	
SigB (*S. aureus*)	Rachid *et al*. (2000b)
RsbU (*S. epidermidis*)	Knobloch *et al*. (2001)
Agr (*S. aureus*)	Vuong *et al*. (2000)
Sar (*S. aureus*)	Pratten *et al*. (2001)

chromosome with the erythromycin cassette *ermB* leads to a complete loss of *ica* transcription and biofilm formation under non-osmotic and osmotic stress conditions. *sigB* complementation completely restores the wild-type phenotype (Rachid *et al*., 2000b). PIA expression also appears to be controlled by the *sigB* operon in *S. epidermidis*. Insertion of a transposon in the *rsbU* gene (the first gene of the *sigB* operon) of *S. epidermidis* leads to a biofilm-negative phenotype and almost undetectable PIA production, whereas primary attachment is uninfluenced (Knobloch *et al*., 2001). Interestingly, ethanol stress enables the mutants to form a biofilm, whereas salt stress does not.

Many virulence factors in *S. aureus* are controlled by the accessory gene regulator (Agr) and staphylococcal accessory regulator (Sar). This regulation may be affected by the environment in which the organisms are grown. A *sarA* mutant strain adheres to glass better than the *agrA* mutant or the wild-type strain. All three strains adhere better to fibronectin-coated glass than to uncoated glass. Thus, Agr and Sar have pleiotropic effects on the surface expression of molecules responsible for binding to different substrata (Pratten *et al*., 2001).

There also appears to be a correlation between the ability of *S. aureus* to adhere to polystyrene and the agr quorum-sensing system phenotype, as 78% of agr-negative, but only 6% of agr-positive strains tested form a biofilm. This has been confirmed with defined *agr* mutants and by inhibition of the agr system with quorum-sensing blockers. The observed effect is not the result of differential expression of the autolysin Atl or of the exopolysaccharide, PIA, but seems to be caused at least in part by the surfactant properties of δ-toxin (Vuong *et al*., 2000).

Anaerobiosis induces ica *expression*

Until recently, little was known about the ability of *S. aureus* to form a biofilm. In routine microtitre plate assays, many *S. aureus* isolates appear to be biofilm and PIA negative. We only became perplexed when it was discovered that the *ica* operon is present in nearly all *S. aureus* strains tested (Cramton *et al*., 1999). Therefore, the *ica* operon must be strictly regulated. Indeed, anoxic conditions lead to increased transcription of the *ica* operon and PIA production in *S. aureus* and *S. epidermidis*, although the regulation is less stringent in *S. epidermidis* (Cramton *et al*., 2001b). That anaerobiosis is an important stimulus for *ica* expression explains the biofilm-negative phenotype observed in earlier assays, which were carried out under oxic conditions.

Many clinical *S. aureus* isolates possess capsule polymers of predominantly polysaccharide of types 5 and 8 (CP5, CP8; Hochkeppel *et al*., 1987). It is therefore of interest whether PIA (slime) and capsule polymers are produced at the same time and under the same conditions. As it was thought that these microcapsules play a role in colonization of lung tissue of cystic fibrosis (CF) patients, it was surprising that CP5 and CP8 were only poorly expressed in the lung tissue and nasal polyps of two CF patients and in rats, but were well expressed when the pouch isolates were cultivated in the presence of oxygen (Herbert *et al*., 1997; McKenney *et al*., 1999). CO_2 (or rather anoxic conditions) is an environmental signal that downregulates the transcription of *cp5*; however, this regulation is strain dependent (Herbert *et al*., 2001). In the CP5-positive *S. aureus* strain Newman, adherence to endothelial cells is reduced, whereas in *cp5* mutants, adherence is increased (Pohlmann-Dietze *et al*., 2000). As CP5 expression is repressed under the anoxic conditions within the lung mucus, PIA might play a role in lung infection. McKenney *et al*. (1999) have shown that PIA (wrongly described as PNSG) is expressed in lungs infected with *S. aureus,* whereas CP8 is not expressed.

PIA polysaccharide and the capsule polysaccharides CP5 and CP8 are inversely controlled with respect to oxic and anoxic stimuli. As PIA is expressed in the lung environment, it is believed that PIA plays a crucial role in lung infection and any other kind of infection in which oxygen is, or becomes, limited. It would be interesting to determine how wild-type *S. aureus* and an *ica* mutant differ in virulence in a CF animal model.

*IS*256 *integrates into the* ica *operon*

An intact *ica* operon is more prevalent in clinical *S. epidermidis* isolates (septicaemic diseases, shunt-associated meningitis) than in skin isolates of non-hospitalized persons (Ziebuhr *et al*., 1997). Defined isolates of biofilm-negative variants occur at a frequency of $\approx 10^{-5}$ and, in 30% of these variants, the insertion sequence element IS*256* is integrated at specific hotspots within *icaA* or *icaC* (Ziebuhr *et al*., 1999; 2000a). Furthermore, the transposition of IS*256* into the *ica* operon is reversible. After repeated passages, the biofilm-forming phenotype can be restored. In these revertants, IS*256* is precisely excised, including the initially duplicated 8 bp target sites. These results elucidate, for the first time, a molecular mechanism for turning PIA expression on and off (Ziebuhr *et al*., 2000b). The recently sequenced genomes of *S. aureus* N315 and Mu50 contain, in addition to prophages and various transposons, 10 copies of IS*1181* and two of IS*431*, distributed over the genome (Kuroda *et al*., 2001). We have probably underestimated the contribution of IS elements and transposons to genetic flexibility and environmental adaptation in staphylococci.

Decreased virulence of biofilm-negative mutants in animal models

With the creation of defined isogenic staphylococcal mutants, it has been possible to compare wild type and mutants *in vivo*. Various animal models are currently used to investigate the significance of factors involved in staphylococcal biofilm formation. Some of the models have been reviewed recently (Yasuda *et al*., 1999; Rupp and Fey, 2001). Rupp *et al*. (1999a,b) have tested the relevance of PIA production in two different animal models – the rat central venous catheter (CVC)-associated infection model and a mouse model with a subcutaneously implanted intravenous catheter. The PIA-positive wild-type *S. epidermidis* 1457 causes CVC-associated infection (71% versus 14%) and subcutaneous abscesses significantly more frequently than the mutant strain. Furthermore, the wild-type strain adheres to the implanted catheter much better and is significantly less likely to be eradicated from the inoculation site by host defence than the mutant. *atlE* and *ica* mutants of *S. epidermidis* O-47 are significantly less virulent in a CVC infection model than the wild type (Rupp *et al*., 2001). Also, metastatic disease is less common in rats inoculated with *atlE* or *ica* mutants. These results confirm the importance of AtlE and PIA in the pathogenesis of *S. epidermidis* experimental CVC infection. In a rabbit endocarditis model, PS/A-negative strains develop significantly less endocarditis (Shiro *et al*., 1994).

In a comparison of *S. epidermidis* RP62A and its mitomycin-induced M7 mutant in a rabbit aortic valve endocarditis model, no differences were observed, indicating that AAP might not be an important virulence determinant *in vivo* (Perdreau-Remington *et al*., 1998). On the other hand, mutant M7 can be eradicated more easily *in vitro* and in animal models by amikacin, levofloxacin, rifampin or teicoplanin (Schwank *et al*., 1998).

Concepts for the prevention of polymer-associated infections

As soon as it was clear that biofilm-forming CNS play a major role in nosocomial bacteraemia and infection of prosthetic medical devices, many research groups investigated the potential of antibiotics in preventing biofilm formation or in curing staphylococcal foreign body infection (Götz and Peters, 2000). In general, staphylococcal cells embedded in a biofilm or in microcolonies are much more resistant to antibiotics than planktonic cells. Many patients with a chronic polymer-associated staphylococcal infection have been treated with various antibiotics, mostly without much success, and the replacement of the implant is usually unavoidable. In a new chronic osteomyelitis rat infection model, the influence of various antibiotics on biofilms of *S. aureus* has been investigated. Stainless steel implants precolonized for 12 h with a highly adherent *S. aureus* isolate were introduced into the rat tibiae and treated for 21 days with cefuroxime, vancomycin, tobramycin or ciprofloxacin. A reduction in bone colonization was achieved only with vancomycin or cefuroxime; however, cefuroxime was only able to sterilize one out of eight implants (Monzon *et al*., 2001). This recent study, like the many previous studies, emphasizes that, once a biofilm is formed, treatment with currently available antibiotics is difficult. In view of the failure of so many antibiotics to treat chronic staphylococcal infections, there is a great need for antimicrobials effective in biofilms. The question frequently raised is whether PIA (slime) is a diffusion barrier for antibiotics, which would explain the increased resistance of biofilm-imbedded cells. To answer this question, antibiotic (vancomycin and rifampin) penetration experiments have been carried out in a two-chamber system; the chambers were separated by a dialysis membrane covered on one side with an *S.*

epidermidis biofilm (Dunne *et al*., 1993). There was no indication that slime acts as a diffusion barrier. This finding is not quite unexpected, as even the multilayered staphylococcal cell wall provides no real barrier for antibiotics. It has also been discussed whether the different metabolic activities of biofilm and planktonic cells play a role in resistance; however, no real clues have been discovered. Thus, the reason for the increased antibiotic resistance of biofilm-imbedded staphylococci remains unknown (Mah and O'Toole, 2001).

Several immunization trials with PIA carried out by McKenney *et al*. (1999) indicate that PIA might be a useful antigen for vaccination to protect against *S. aureus* infection. Immunization against PIA (incorrectly regarded as PNSG) derived from *S. aureus* protected mice against kidney infections and death from strains that produce little PIA *in vitro*. Whether vaccination against *S. aureus* and *S. epidermidis* in the long run would be successful is questionable, especially as *S. aureus* produces numerous extracellular and surface-bound virulence factors, and *S. aureus* vaccination trials have a long tradition of failure – to date, no staphylococcal vaccine has found its way into practical application.

One of the prerequisites for chronic polymer-associated infections by staphylococci is adherence to the polymer surface; therefore, great efforts have been made to find materials that prevent adherence. In practice, however, there is no synthetic polymer or biocompatible metal onto which *S. epidermidis* does not bind under clinical conditions (Götz and Peters, 2000).

Jansen *et al*. (1989) started very early to investigate the influence of modification of polymeric materials on staphylococcal adherence and biofilm formation. Various antibiotics were incorporated or coupled to polymers, thus generating anti-infective catheters. Some of the materials exert a positive effect in the prevention of catheter-related infections. For example, a silicone ventricular catheter coated with a combination of rifampin and trimethoprim reduces colonization with *S. aureus in vitro* (Kohnen *et al*., 1998; Kockro *et al*., 2000).

The positive effect of silver-impregnated polymers, e.g. in the 'silver catheter', in reducing staphylococcal colonization or infection has been described (McLean *et al*., 1993; Dasgupta, 1994; Boswald *et al*., 1995; Jansen and Kohnen, 1995; Sampath *et al*., 1995; Gatter *et al*., 1998; Illingworth *et al*., 2000). Other materials tested were plasma-treated hydrogel-coated ionized fluoroplastics and heparinized hydrophilic polymers (Nagaoka and Kawakami, 1995; Appelgren *et al*., 1996; John *et al*., 1996; Biedlingmaier *et al*., 1998). Positive results have also been achieved with gelatine; for example, gelatine-impregnated polyester grafts show good *in vivo* resistance to coagulase-negative staphylococcal biofilm infection (Farooq *et al*., 1999).

Many *in vitro* results with new materials look very promising at the beginning, but the *in vivo* situation is frequently disillusioning. The main problem is that, as soon as the material is implanted, it comes into contact with blood, and the surface of the material is sooner or later covered with blood compounds to which staphylococci can adhere. *S. aureus* in particular has a broad arsenal of cell wall-associated compounds (murein, teichoic acids, various polysaccharides, binding proteins for tissue and plasma factors) that can interact with the one or other blood compound.

One must also be sceptical with respect to a long-range *in vivo* benefit of silver or other antimicrobial compounds in implant material. In a pilot *in vitro* study, *S. epidermidis* adhered even better to the surfaces of the silver-coated sewing cuff than to the uncoated cuff. These pilot *in vitro* results cast doubt on the anti-infective efficacy of silver-coated materials (Bechert *et al*., 1999; Cook *et al*., 2000). Furthermore, the use of silver ions or antibiotics in routine catheters will certainly lead to the selection of resistant strains. The search for alternative clinically applicable materials is not yet finished. Ideally, the material should allow binding of defence cells, but must resist the adherence of blood compounds and bacteria.

Conclusion and prospects

Biofilm formation in staphylococci is multifactorial, and the ability to form a biofilm makes the strains much better able to survive in the normally hostile environment of tissue and blood. Biofilm formation appears to be a bacterial survival strategy that is turned on when, for example, oxygen and Fe ions become limited, when sublethal concentrations of certain antibiotics are present or other stress situations emerge. The modulation of biofilm formation by various environmental conditions appears to be an advantage for successful infection. PIA (slime) production certainly plays a major role in generating a biofilm, and the corresponding *ica* genes are predominantly present in clinical isolates. On the other hand, PIA only leads to intercellular adhesion, which might also be mediated by proteins or non-proteinaceous cell wall structures. In this connection, it would be interesting to know more about the function of the biofilm cofactors, such as the accumulation-associated protein (AAP) and the biofilm-associated protein (Bap), or the D-alanine esterification of teichoic acid (DltA). Colonization of tissues or implants is frequently a first step in infection, and biofilm formation plays a crucial role. However, for the invasion of endothelial and epithelial cells, sepsis and spreading, it might be advantageous for some staphylococcal cells to be able to escape the biofilm network, to become biofilm negative and to move on to other tissues. On their way, they might regain a biofilm-positive phenotype and settle down on

heart valves, endocardium, periosteum, etc. One can imagine that the observed genetic variability in biofilm formation, which ranges from super- to non-biofilm formers, underlies a well-directed survival and spreading programme that we are only gradually learning to understand. This strategy makes it very difficult to master chronic and polymer-associated infections. If once a staphylococcal biofilm is formed the cells are nearly invulnerable, they are then shielded from the immune system and resist antibiotic treatment. Therefore, it is very important to understand the various aspects of biofilm formation. Only then shall we be able to develop more specific ways of overcoming staphylococcal resistance in chronic infections.

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