BIOFILM FORMATION BY STAPHYLOCOCCI AND STREPTOCOCCI: STRUCTURAL, FUNCTIONAL AND REGULATORY ASPECTS AND IMPLICATIONS FOR PATHOGENESIS

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BIOFILM FORMATION BY STAPHYLOCOCCI AND STREPTOCOCCI: STRUCTURAL, FUNCTIONAL AND REGULATORY ASPECTS AND IMPLICATIONS FOR PATHOGENESIS

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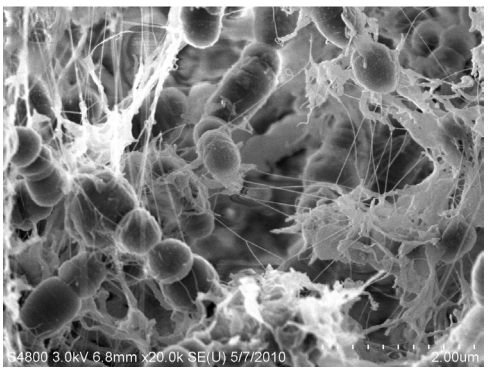

Scanning electron micrograph image of Streptococcus mutans biofilm on an apatitic surface

Members of the genus Staphylococcus and Streptococcus are the causative agnets of many human and animal diseases. Over the past decade the complete sequencing of many staphylococcal and streptococcal genomes has promoted a significant advance in our knowledge of these important pathogens. The pathogenicity of these bacteria is due to the expression of a large variety of virulence factors. Such determinants, which are cell wall-associated and secreted proteins, include adhesins that confer to the pathogen the ability to attach to extracellular matrix/plasma and host cell surfaces, proteins that contribute to host cell invasion and intracellular survival and soluble factors that decrease phagocytosis and modulate the immune response. Furthermore, these Gram-positive cocci in many natural environments (heart valve, lung, oral cavity, throat) and infections on implanted devices live in matrix-encased groups known as biofilms. Biofilms are specialized bacterial communities with high order organization analogous to that of a tissue in multicellular organism that adhere to abiotic or biological substrata and produce an exopolymeric matrix composed of polysaccarides, proteins, DNA or combination thereof. Bacteria within a biofilm persist in adverse conditions, show resistance to killing by antibiotics and to host immune defences and are difficult to eradicate and treat clinically. Therefore, understanding the mechanisms of biofilm development will allow us to effectively combat staphylococcal/streptococcal biofilm-based infections.

This Research Topic will focus on the molecular components involved in biofilm formation by staphylococci and streptococci, the role they play in the development, maturation and dispersal of biofilm and on the regulatory aspects of such complex processes. The implication for the pathogenesis of infective diseases and potential therapeutic strategies against biofilm-based infections will be also discussed. The articles will highlight both the recent advances and future challenges inherent in this rapidly evolving area.

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EDITORIAL
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Biofilm formation by staphylococci and streptococci: structural, functional, and regulatory aspects and implications for pathogenesis

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Keywords: Staphylococcus, Streptococcus, biofilm, structure, regulation, pathogenesis

Members of the genus *Staphylococcus* and *Streptococcus* are commensals and opportunistic pathogens that cause a variety of infections in human and animals. Biofilm formation is an important survival strategy adopted by these Gram-positive cocci. Biofilms are surface-associated, specialized multicellular bacterial communities embedded in a self-produced matrix of polysaccharides, proteins, DNA or combinations thereof. Biofilm growth allows bacteria to persist *in vivo* by resisting host immune defenses and antibiotics meaning that these infections are difficult to eradicate and treat clinically. Biofilms form in many natural niches occupied by staphylococci and streptococci (such as the nasopharynx, heart valves, lungs, and oral cavity) and are important during the infection of implanted medical devices.

Biofilms develop in multiple stages to form highly ordered multicellular communities (O'Toole et al., 2000; Otto, 2013). Initially the bacteria must adhere to a surface or host tissue (primary attachment phase) before they proliferate to form multicellular aggregates (accumulation phase). During the maturation stage, channels and mushroom-shaped structures are created to allow nutrients to penetrate the deeper layers of the biofilm (O'Toole et al., 2000). Finally, during the dispersal stage, bacteria detach from the biofilm and disseminate to new sites (Otto, 2013).

In this series of articles, the authors provide an overview of the molecular components involved in biofilm formation by staphylococci and streptococci. Particular emphasis is placed on the mechanisms of development and dispersal of biofilm and the regulation of biofilm formation. Novel therapeutic strategies for the prevention and eradication of medical biofilms are also discussed.

Arciola et al. (2015) describe our evolving understanding of the enzymes involved in the biosynthesis of polysaccharide intracellular adhesion (PIA), a polymer that mediates staphylococcal biofilm accumulation. They discuss the genetic control of factors involved in biofilm formation and disruption. McCarthy et al. (2015) explore the different mechanisms used by methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) to form biofilm and the impact of methicillin resistance on PIA production and virulence *in vivo*. Speziale et al. (2014) describe proteins of staphylococci that contribute to primary attachment and biofilm accumulation. The current understanding of the mechanistic basis of cell wall anchored surface protein-dependent biofilm accumulation is described. Büttner et al. (2015) capture recent advances in our understanding of the contribution of *Staphylococcus epidermidis* multifunctional surface proteins to the primary attachment and accumulation phases of biofilm formation. They also illustrate the integration of biofilm-promoting factors into regulatory networks and emphasize how these factors contribute to the adaptation of *S. epidermidis* to changing environmental conditions. The review by Le et al. (2014) presents evidence of the critical activity of phenol soluble modulins (PSMs) as mediators of biofilm structuring and dispersal, stressing their role

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in the formation of biofilm channels and dissemination of clusters of biofilm to distal organs *in vivo*. In keeping with the theme of dispersal, Lister and Horswill (2014) report the current understanding of enzymatic dispersal mechanisms (proteases, nucleases, and dispersin B) and the newly described broad-spectrum effects of D-amino acids and synthetic cationic peptides.

Gilley and Orihuela (2014) describe the reasons why biofilm-forming *Streptococcus pneumoniae* colonizing the nasopharynx of humans are not invasive. The bacteria in the biofilm have a decreased rate of metabolism, produce less capsular polysaccharide and less pneumolysin than their planktonic counterparts. The authors suggest that this may explain long-term, asymptomatic nasopharyngeal colonization by *S. pneumoniae* in humans. Chao et al. (2015) describe the increased propensity of *S. pneumoniae* in a biofilm to exchange genetic material. Mechanisms involved in dispersal during colonization and disease are also considered.

Fiedler et al. (2015) review the clinical relevance of biofilm formation by the human pathogen *Streptococcus pyogenes*, in particular during oto-nasopharyngeal and skin infection. Moreover, they report the involvement of capsule carbohydrates, pili, surface proteins, and secreted enzymes and the regulatory aspects of the biofilm lifestyle of *S. pyogenes*. Rosini and Margarit (2015) discuss the role of pili and other surface components and the influence of environmental conditions on biofilm production by *Streptococcus agalactiae*. Klein et al. (2015) present evidence of how *Streptococcus mutans* assembles a cariogenic biofilm in the mouth and how extracellular polysaccharide provides mechanical stability and facilitates the creation of highly acidic microenvironments inside the matrix. Extracellular DNA enhances the local synthesis of extracellular polysaccharide, increases the adherence of *S. mutans* to saliva-coated apatitic surfaces and increases the cohesive properties of biofilm.

In conclusion, these reviews summarize one of the most vital areas of research in the pathogenesis of staphylococcal and streptococcal infections. The limitations of *in vitro* techniques, and the urgent need to advance our understanding of biofilm formation using *in vivo* models, are considered. The knowledge generated from this research has the potential to lead to novel approaches to diagnosing and treating biofilm-related infections, and it lays the groundwork for deeper investigation into the basic biology of these diseases.

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REVIEW ARTICLE
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Polysaccharide intercellular adhesin in biofilm: structural and regulatory aspects

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Staphylococcus aureus and *Staphylococcus epidermidis* are the leading etiologic agents of implant-related infections. Biofilm formation is the main pathogenetic mechanism leading to the chronicity and irreducibility of infections. The extracellular polymeric substances of staphylococcal biofilms are the polysaccharide intercellular adhesin (PIA), extracellular-DNA, proteins, and amyloid fibrils. PIA is a poly-β(1-6)-*N*-acetylglucosamine (PNAG), partially deacetylated, positively charged, whose synthesis is mediated by the *icaADBC* locus. DNA sequences homologous to *ica* locus are present in many coagulase-negative staphylococcal species, among which *S. lugdunensis*, however, produces a biofilm prevalently consisting of proteins. The product of *icaA* is an *N*-acetylglucosaminyltransferase that synthetizes PIA oligomers from UDP-*N*-acetylglucosamine. The product of *icaD* gives optimal efficiency to IcaA. The product of *icaC* is involved in the externalization of the nascent polysaccharide. The product of *icaB* is an *N*-deacetylase responsible for the partial deacetylation of PIA. The expression of *ica* locus is affected by environmental conditions. In *S. aureus* and *S. epidermidis ica*-independent alternative mechanisms of biofilm production have been described. *S. epidermidis* and *S. aureus* undergo to a phase variation for the biofilm production that has been ascribed, in turn, to the transposition of an insertion sequence in the icaC gene or to the expansion/contraction of a tandem repeat naturally harbored within *icaC*. A role is played by the *quorum sensing* system, which negatively regulates biofilm formation, favoring the dispersal phase that disseminates bacteria to new infection sites. Interfering with the *QS* system is a much debated strategy to combat biofilm-related infections. In the search of vaccines against staphylococcal infections deacetylated PNAG retained on the surface of *S. aureus* favors opsonophagocytosis and is a potential candidate for immune-protection.

Keywords: *Staphylococcus*, biofilm, ica locus, Polysaccharide intercellular adhesin (PIA), poly-β(1-6)-*N*-acetylglucosamine (PNAG), anti-PIA vaccine

INTRODUCTION

The recognition of "slime" as a mucilaginous material elaborated by certain microrganisms such as molds and bacteria represents a very early discovery in the history of microbiology. Impressively, the first papers describing the ability of bacterial species to form slime date back to the beginning of the 20th century. The description of specific extracellular polymeric components that structurally contribute to slime composition started with the second half of the century, when Wilkinson (1958) and Catlin and Cunningham (1958) began to report the existence of extracellular polysaccharides and deoxyribonucleic acids.

The medical importance of bacterial biofilm was for the first time enlighten by Bill Costerton, recognized as the "Father of Biofilm," who, in 1978 established an extraordinarily new microbiological paradigm, the "biofilm theory." In an article, published in Scientific American, he asserted that bacteria stick on available surfaces in glycocalyx-enclosed biofilms and that the sessile bacterial population becomes predominant particularly in medical ecosystems (Costerton et al., 1978). Costerton's observations shifted the medical research from the attention to microbial cell-wall structures, which are the interface of planktonic bacteria with the environment, to the biofilm, which is the interface of sessile bacteria with their environment (Costerton, 1989). The introduction of the *biofilm theory* opened two lines of research: the study of biochemistry and genetics of biofilms and their formation and, on the other side, the improvement of the medical diagnosis and treatment of biofilm-centered infections.

The profound influence that the Costerton's insight exerted on the bio-molecular knowledge of bacterial adhesion and on the need of appropriate medical methods for diagnosis and treatment of biofilm-related infections is analyzed in a review written by one of us together with the Director of the Costerton's Institute. The review was thought in the sad circumstance of the passing away of Bill, remembered as a charming *Maestro* for a large number of colleagues and students (Ehrlich and Arciola, 2012).

The early experimental works on slime producing bacteria were just a prelude to the more elaborated concept of biofilm (Mack et al., 1975; Costerton et al., 1978), where the sessile life-cycle phase was progressively associated to the social behavior of communicating and mutually interacting bacterial cells (Stoodley et al., 2002), forming communities encased within a protective extracellular matrix (Costerton et al., 1987) derived from the elaboration of an exocellular slime (the glycocalix) (Gristina and Costerton, 1984).

Over the years, progressive light was cast on the importance of glycocalix elaboration and biofilm formation not only as a fundamental mechanism of adhesion and colonization of biomaterial surfaces (Jacques et al., 1987), but also as a critical virulence mechanism enabling bacteria to escape the host immune-response and resist medical antibiotic chemotherapies (Gilbert et al., 1997; Costerton et al., 1999). The possibility for bacteria with a planktonic phenotype, otherwise susceptible to the host defenses and medical treatments, to switch to a sessile form of life more adapt to survive to the aggressive environment of host tissues, assumed a specific meaning in the pathogenesis of clinical infections, especially those associated to implant materials.

The great difficulty to eradicate microbial infections generated by biofilm-forming bacteria in presence of implant materials has received increasing attention over the last decades. Indeed, once established on a biomaterial surface, biofilm-forming strains are capable to resist and survive common antibiotic regimens formulated against and active on planktonic bacteria.

Soon after the launch of the *biofilm theory*, *Staphylococcus epidermidis* was early discovered to be one of the principal actors of biomaterial-associated infections and, certainly, its ability to colonize implant surfaces and produce resistant biofilms was recognized as a key factor in determining its success.

With the studies on slime/biofilm production by clinical strains, the biofilm was progressively demonstrated to be an important mechanism in bacterial adherence and pathogenesis of infections associated to biomaterials surfaces (Christensen et al., 1982). The production of biofilm was searched both by phenotypic methods, such as the microtiter plate (MtP) test (Christensen et al., 1985), the Congo red agar (CRA) plate test (Freeman et al., 1989) and its optimization (Arciola et al., 2002b), and by molecular detection of the *ica* locus, that had been identified as the genetic basis of biofilm production in *S. epidermidis* (Heilmann et al., 1996). The presence of *ica* locus in *S. epidermidis*, detected together with the positivity of phenotypic evidence of biofilm production, was then proposed as a marker of virulence of *S. epidermidis* strains responsible for implant-associated infections (Arciola et al., 2002a,b). PIA, besides described in *S. epidermidis* strains (Mack et al., 1996), was also described in *S. aureus* and the role of *ica* locus recognized also in this species (Cramton et al., 1999). The *ica* locus was then found widespread present in biofilm producing *S. aureus* strains responsible for catheter and implant infections (Ammendolia et al., 1999; Montanaro et al., 1999; Arciola et al., 2001).

The great attention that staphylococcal biofilms have received in recent years is justified by the large prevalence of *S. aureus* and *S. epidermidis*, but also of other emerging coagulase-negative staphylococci, as etiological agents of implant-infections. For instance, in orthopedics epidemiological studies have shown that staphylococci are the primary cause of implant-infections, causing nearly 80% of all prosthetic infections (Arciola et al., 2005, 2014; Montanaro et al., 2011).

Bacteria in biofilms can resist antibiotics at concentrations up to 1000 times higher than those active on the same bacteria in the planktonic state (Ceri et al., 1999). Antibiotic substances targeting the biofilm phenotype are therefore urgently needed. In search for the most efficacious antibiotic treatments active on biofilms, it has become common to assay antibiotic substances not only on planktonic bacteria to extrapolate minimal inhibitory concentrations (M.I.C.), but also screen them on sessile bacteria to achieve relevant information on the minimum biofilm eradication concentration (M.B.E.C.) (Ceri et al., 1999; Parra-Ruiz et al., 2010).

Apart from the efforts to identify the most active combinations of conventional antibiotics to eradicate bacterial biofilms, increased attention is being paid to identify new antibacterial molecules specifically targeting the biofilm state and therefore defined anti-biofilm substances. The great interest to counteract biomaterial-associated infections caused by biofilm-producing bacterial species has therefore seen the proliferation of studies screening different classes of compounds ranging from herb extracts, natural antimicrobial peptides, molecules of microbial origin to synthetic molecules. The intent has become not just the treatment but also the prevention of biofilm formation. This important goal can be achieved by the better knowledge of the molecular mechanisms of biofilm production and control and by the development of anti-biofilm biomaterial surfaces (Arciola et al., 2012; Campoccia et al., 2013). In this context, the knowledge of biofilm pathophysiology plays a fundamental role, as different strategies can be developed to contrast bacterial colonization and biofilm formation. They all rely on a deep understanding of the mechanisms implicated in biofilm production and of the fine mechanisms that rule the expression of the biofilm phenotype and govern biofilm formation. Two main anti-biofilm strategies are presently under consideration. One is based on biofilm disaggregating agents, such as enzymes that attack the PNAG (Dispersin B) or the extracellular DNA (DNase I) or the biofilm proteins (proteinase k) (Arciola, 2009; Kaplan, 2009; Arciola et al., 2012).

The other strategy is based on the modulation of the effector molecules, an approach called *quorum quenching* (QQ), which consists in interfering with the intercellular bacterial communications, with the aim at artificially inducing bacteria to assume a planktonic rather than sessile phenotype. In other words, the latter target consists in fooling the *quorum sensing* (QS) system. Based on signal molecules often referred to as *pheromones* or *autoinducers*, the QS system enables bacteria to sense their own density in the *milieu* and modify their phenotype accordingly. This involves ruling the expression of distinct traits for the specific cellular phase of growth and/or *milieu* colonization.

While some of the principal components of the QS system in *S. aureus* and *S. epidermidis* have been unveiled, much still remains to be elucidated as significant functional differences exist between the two bacterial species and, even, among different

strain types, which often happen to compete in the environment just using different allelic forms of the signal molecules. These different alleles of signal molecules often exert the function of QS interference in the competition among different types of strains.

A brief description of some fundamental aspects of staphylococcal biofilm pathophysiology concerning the ultrastructural composition of the extracellular biofilm matrix, and the genetic mechanisms governing biofilm formation will be depicted in the next paragraphs.

CHEMISTRY OF PIA AND ALTERNATIVE FORMS OF BIOFILM

The investigation of the chemical nature of the extracellular biofilm matrix began rather early around the years '50 s. However, in spite of many efforts made to elucidate its composition, the difficult purification of bacterial extracellular matrix and the multiplicity of analytical techniques adopted, often generating artifacts, initially led to divergent results. Thus, depending on their different experimental approach, different authors described different main exopolysaccharide components: the capsular polysaccharide-adhesin (PS/A) (Muller et al., 1993), the polysaccharide intercellular adhesin (PIA) (Mack et al., 1996), poly-β(1-6)-*N*-acetylglucosamine (PNAG) (Maira-Litrán et al., 2002), and *S. aureus* exopolysaccharide (SAE) (Joyce et al., 2003). In 2005, was finally demonstrated that PIA from *S. epidermidis* was structurally identical to the poly-β(1-6)-*N*-acetylglucosamine from the PS/A-overproducing strain *S. aureus* MN8m (Sadovskaya et al., 2005), definitively solving a dilemma lasted for more than a decade on the real chemical formula of the main exopolysaccharide component of the biofilms of most *S. aureus* and *S. epidermidis* clinical strains.

Currently, poly-β(1-6)-*N*-acetylglucosamine, alternatively named with the synonymous terms PIA or PNAG, has therefore been identified as the main exopolysaccharide component of staphylococcal biofilm matrix. Interestingly, the same exocellular polysaccharide has recently been identified even in numerous other gram-negative bacterial species members of the *Proteobacteria* family, including *Escherichia coli*, *Yersinia pestis*, *Pseudomonas fluorescens*, *Bordetella* spp., *Xenorhabdus nematophila*, *Aggregatibacter actinomycetemcomitans*, and *Actinobacillus pleuropneumoniae* (Ganeshnarayan et al., 2009), suggesting a convergent evolution in phylogenetically diverse bacteria.

Although PIA certainly represents a main mechanism of biofilm formation in *S. aureus* and *S. epidermidis*, numerous advancements in the study of biofilm have shown the existence, especially for *S. aureus*, of alternative forms of biofilm that are PIA-independent. The observation that a minor proportion of *S. aureus* strains can form biofilm even in the absence of the *ica* locus and that certain strains carrying such locus continue anyway to produce biofilm even after deletion of the locus suggested the existence of *ica*-independent pathways (O'Gara, 2007). The assumption of the possibility of alternative mechanisms to produce biofilms was long debated despites some very early studies had already suggested the complexity of biofilm architecture and the inclusion of extracellular polymeric substances different than PIA, such as e-DNA and teichoic acids, in the biofilm matrix.

A number of proteins localized in the extracellular matrix of biofilms have been identified that can generate PIA-independent biofilms. The biofilm-associated protein (Bap), a 2276-amino acid surface protein, is one of these proteins enabling biofilm production even in the absence of production of the exopolysaccharide component (Cucarella et al., 2004; Tormo et al., 2005). The Bap protein seems to play a role prevalently in human staphylococcal infections caused by coagulase-negative staphylococcal (CoNS) species. The *bap* gene encoding for Bap has been identified in *S. epidermidis*, *S. chromogenes*, *S. xylosus* and a few other CoNS species, where it is often carried in a putative composite transposon (Cucarella et al., 2004; Tormo et al., 2005). However, as far as *S. aureus* is concerned, the role of Bap seems less relevant in isolates from human infections (Vautor et al., 2008; Tang et al., 2013) and, up to now, exclusively strains of veterinary origin (e.g., bovine mastitis) have been found to harbor the *bap* gene (Valle et al., 2012). However, the functions of Bap are still far from being totally understood and are not limited to biofilm formation. Indeed, it has been found that Bap not only promotes the adhesion, but also prevents the entry of *S. aureus* into epithelial cells (Valle et al., 2012). Bap involvement in the pathogenesis of infections is therefore more complex as this protein influences the extent of bacterial internalization into host cells and consequently tissue invasiveness.

In the production of biofilm more clinically significant is the role of another protein factor, namely the accumulation associated protein (Aap). Among biofilm-forming *S. epidermidis* isolates from total hip or total knee infected arthroplasties, up to 27% of the strains were found to be endowed with this surface protein and negative to the *ica* locus (Rohde et al., 2007).

Differently, surface proteins such as SasG (Geoghegan et al., 2010), SasC (Schroeder et al., 2009), Protein A (Merino et al., 2009), and two fibronectin-binding proteins, namely FnBPA and FnBPB (O'Neill et al., 2008), have been documented to contribute to biofilm formation in *S. aureus*. Interestingly, *S. aureus* clinical strains are all generally endowed with the *ica* locus. These alternative mechanisms of biofilm formation can probably concur and could be switched on in different phases of the pathogenesis of infections, adapting the characteristics of the biofilm extracellular matrix in response to external stimuli (Houston et al., 2011), in order to colonize and establish the infection in host tissues, while evading the immune response and the effects of antibiotic treatments.

The protein components of staphylococcal biofilms are discussed in depth in a recent review that illustrates how a multitude of proteins intervene at different stages of the biofilm formation, with certain proteins contributing to the biofilm accumulation and others mediating the primary attachment to the surfaces (Speziale et al., 2014).

THE SYNTHESIS OF PIA

PIA, as described above, is now well-established to consist of poly-β(1-6)-*N*-acetylglucosamine, a linear glucosaminylglycan that plays a fundamental function in mediating intercellular adhesion of bacterial cells. In addition to bacterial aggregation, this exopolysaccharide has important structural functions in the biofilm matrix architecture, and is implicated in bacterial

adhesion to biomaterial surfaces as well as evasion from host immune-response (Vuong et al., 2004). PIA synthesis is mediated by the *icaADBC* locus, first discovered and investigated by Heilmann et al. (1996). The *icaADBC* locus was initially described in *S. epidermidis*, but few years later its presence was confirmed even in many other species of the *Staphylococcus* genus, *S. aureus* included (Cramton et al., 1999). The *ica* locus is however part of the "accessory genes" genome and not of the so-called "core" genome, meaning that it is not found in all bacterial strains. Its presence is exclusively observed as part of the virulon of exopolysaccharide-based biofilm-forming staphylococcal strains. In early studies, the *ica* locus was reported in a large proportion of staphylococcal strains isolated from implant related-infections (catheter-associated infections) (Arciola et al., 2001). Important differences in the prevalence of *ica*-positive strains had previously been observed also comparing clinical isolates (blood cultures) with saprophytic strains (85% vs. 6%) (Ziebuhr et al., 1997).

Further evidence of the important role of PIA as a virulence factor came also from experimental studies in rat models of intravascular catheter-associated infections with isogenic mutant strains deficient in PIA production (Rupp et al., 1999, 2001).

The *icaADBC* locus consists of four genes. The first two genes of this gene cluster, respectively *icaA* and *icaD*, exert a primary role in the exopolysaccharide synthesis. The former gene encodes for a transmembrane enzyme with *N*-acetylglucosaminyl transferase activity, necessary for the synthesis of the poly-*N*-acetylglucosamine polymer. However, the enzymic activity of the product of *icaA* becomes significant and olygomers longer than 20 residues are synthesized only when coexpressed with the product of the *icaD* gene (Gerke et al., 1998). Conversely, the product of the *icaC* gene appears to translocate the poly-*N*-acetylglucosamine polymer to the bacterial cell surface, while the *icaB* product operates the deacetylation of the molecule (Vuong et al., 2004). Deacetylation of poly-*N*-acetylglucosamine polymer is relevant for the structural development of exopolysaccharide-based biofilm, enabling the fixation of the polymer to the outer bacterial surface. The negative regulator termed intercellular adhesin locus regulator (*icaR*) gene governs the expression of the *ica* locus under the influence of SarA and the stress sigma σ^B (Cerca et al., 2008).

While DNA-sequences homologous to the *ica* locus have been identified also in many staphylococcal species other than *S. aureus* and *S. epidermidis*, up to now they have not been reported for two staphylococcal species, respectively *S. haemolyticus* and *S. saprophyticus*. Species such as *S. lugdunensis*, although endowed with the *ica* locus, appear to produce a biofilm whose matrix prevalently consists of protein material (Frank and Patel, 2007). By CLSM and specific staining with fluorescent dyes we have observed that the biofilm of *S. lugdunensis* is composed of both PIA, which is stained with FITC-WGA, and proteins, stained with Sypro®-Ruby, specific for the protein component (**Figure 1**).

PHASE VARIATION IN STAPHYLOCOCCI FOR THE BIOFILM FORMATION

An interesting question is the phase variation of staphylococci, which consists in a process of switching on/off for the expression/silencing of the *ica* locus, leading to a quantitative variation of biofilm production. In *S. epidermidis*, the phase variation for biofilm formation is thought to be a mechanism of persistence and relapse. The insertion/excision of the insertion sequence IS*256* in the gene *icaA* or in *icaB*, or, more frequently, in *icaC* as a possible mechanism of the phase variation was hypothesized by Ziebuhr et al. (1999). These Authors observed the phase variation in two *S. epidermidis* reference strains that had been subjected to repeated *in vitro* subcultures. In a different way, the natural occurrence of the IS*256* insertion element either in *ica* locus or in the genomic DNA of clinical strains of *S. epidermidis* - just as they had been isolated, that is without artificial manipulations to induce phase variations - was searched by Arciola et al. (2004). The search for *ica* genes was carried out in 120 *S. epidermidis* isolates from prosthesis-associated infections and in 4 *S. epidermidis* reference strains and was compared with the bacterial phenotypes (ability/inability to produce biofilm). Moreover, two biofilm-negative RP62A-derived acriflavin mutants (D9 and HAM892) were analyzed. The four genes of the *ica* locus appeared, in all cases of that collection, strictly linked each other, so that they were either all present or all absent, nor were detected gene deletions within the *ica* locus. IS*256* was present in eight out of the 69 *ica*-negative strains and in 34 out of the 51 *ica*-positive strains. However, when IS*256* was found in the bacterial genomic DNA, it was never found within the *ica* locus, this observation suggesting that the insertion/excision of IS256 is not a natural occurring mechanism for off/on switching the biofilm production. In the same study, for the first time, two RP62A-derived acriflavin mutants, D9 and HAM892, unable to produce biofilm, were shown to harbor within their *icaC* genes, at the base 3319, a 1300-bp insertion corresponding to IS*256*. Although the insertion was found within *icaC*, as in the study of Ziebuhr et al. (1999), it was traced in a position rather different from that described by Ziebuhr et al. (1999). The different point of insertion was ascribed by the Authors to the different mutagenesis conditions, namely repeated subcultures in the experiments reported by Ziebuhr et al. (1999) and, instead, the chemical mutagenesis by acriflavin in the study of Arciola et al. (2004). However, interestingly, in none of the 120 *S. epidermidis* clinical isolates from prosthesis associated infections the IS256 insertion element was found within the *ica* operon, neither in some strains that, although *ica*-positive, were biofilm *non*-producers. Thus, the insertion/excision of IS*256* in *ica* operon does not appear as a natural occurring mechanism for switching off/on the biofilm production, but rather as the consequence of either a chemical mutagenesis or of manipulative mutations. Afterwards, in the same 2004 year, only some months later, the phase variation of biofilm formation by an insertion sequence was described also in *Staphylococcus aureus* by Kiem et al. (2004). A biofilm-negative phase-variant *S. aureus* mutant was detected from six strains subjected to repeated subcultures. Again, the *ica*C gene of the phase-variant strain was found to be inactivated by the insertion sequence IS*256*.

More recently, a mechanism of phase variation of the poly-N-acetylglucosamine expression in *Staphylococcus aureus* has been described that does not involve the insertion/excision of IS*256* but the expansion or contraction of a simple tetranucleotide tandem repeat housed within *icaC*. Inactivation of IcaC by the expansion

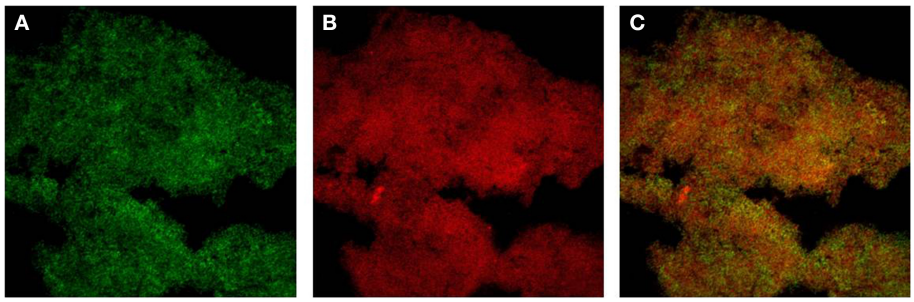

FIGURE 1 | Double staining with FITC-Wheat Germ Agglutinin (FITC-WGA, for exopolysaccharide detection) and SYPRO Ruby (FilmTracer™ SYPRO® Ruby Biofilm Matrix Stain, for protein detection) was carried out as described in Ravaioli et al. (2012). (A) Green channel image showing PNAG stained with FITC-WGA. **(B)** Red channel image showing the proteic component stained with SYPRO® Ruby. **(C)** Merged image of the two channels.

or contraction of this tetranucleotide tandem repeat results in a PIA/PNAG-negative phenotype (Brooks and Jefferson, 2014). And indeed, the expansion (or contraction) of a 4-nt tandem "ttta" repeat shifts the reading frame of *icaC* and leads to a premature stop codon, truncating the IcaC protein at 303 amino acids, 47 amino acids shorter than the full-length protein. The Authors suggest that, under certain conditions, the loss of PIA/PNAG production may be advantageous during infection. Inactivation of *icaC* is therefore a mechanism of phase variation for PIA/PNAG expression and *icaADB* may contribute to the bacterial fitness, by a mechanism still unknown and involving the absence of an intact *icaC* gene and of PIA/PNAG production. All these findings indicate that the mutation of i*caC* is the preferred "off switch" for PIA/PNAG production.

GENETIC CONTROL OF BIOFILM METABOLISM

The numerous studies on the genetic control of biofilm production in staphylococci have led to consider the expression of the biofilm forming phenotype very complex. This complexity is in part because there is a multiplicity of factors contributing to the biofilm extracellular matrix, these varying with the bacterial species but also, within the same species, with the strain type. In addition, biofilm production derives from a complicated equilibrium of production of extracellular polymeric substances, including amyloid fibrils and polymerized phenol soluble modulins (PSMs), and their catabolism determined by expression of enzymes such proteases, nucleases and soluble, still non-polymerized, PSM peptides (Schwartz et al., 2012).

A fine control of sessile and planktonic phenotypes is highly required to explicate a well coordinate and efficacious action during the invasive phase. The expression of biofilm is therefore governed by mechanisms of collective coordination. These mechanisms enable not only the sensing of environmental stimuli, but also the density of bacterial cells belonging to the same group and sharing the same pheromone system.

Diverse studies have documented as *ica*-positive *S. epidermidis* easily express their biofilm-forming phenotype under *in vitro* conditions. Differently, in *S. aureus*, whose strains are generally all endowed with *ica* locus, the biofilm production is not always fully expressed *in vitro* and often requires a modified atmosphere (anaerobiosis) or supplementation of the growth broth with nutrients in order to be fully manifested (Arciola et al., 2001; Cramton et al., 2001; Stepanović et al., 2003, 2007).

Vice versa, *S. aureus* strains manifestly express increased biofilm production *in vivo* (Ammendolia et al., 1999; O'Gara, 2007). *In vitro* stress conditions induced by iron limitation, starvation, thermal stress or subinhibitory concentrations of ethanol, salt and some antibiotics have also been found to increase the amount of biofilm produced.

Therefore, a series of stimuli from the environment and from bacterial density are sensed by bacterial cells and influence the expression of biofilm-forming phenotype. As far as the response to environmental stimuli is concerned, important regulators of staphylococcal biofilm production are represented by the two-component signal transduction systems (TCSs) and σ^B. TCSs mediate a diverse range of adaptive responses in response to environmental stresses. First identified in *S. aureus* (Fournier and Hooper, 2000), the presence of ArlRS TCS was later confirmed also in *S. epidermidis* (Zhu et al., 2010). Recent data show that ArlRS plays an important role in the regulation of *S. epidermidis* biofilm formation, and acts in an *ica*-dependent manner distinct from the role of ArlRS in *S. aureus* biofilm formation, which was found to be *ica*-independent (Wu et al., 2012). In addition to influencing biofilm formation, ArlRS is also involved in the modulation of bacterial autolysis and, consequently, of e-DNA release contributing to the biofilm extracellular matrix.

In *S. aureus* another two-component regulatory system is the *lytSR* operon that affects murein hydrolase activity and autolysis (Brunskill et al., 1996). The LytS sensor component, interacting with its cognate response regulator LytR, activates the transcription of genes under its control. The target of the system is the *lrg/cid* operon, which has been shown to be a regulator in the control of cell death and lysis during biofilm development (Rice et al., 2003; Rice and Bayles, 2008). The *cidA* gene encodes a putative holin protein that is an effector of murein hydrolase activity and cell lysis, while *lrgA* encodes a putative antiholin that is an inhibitor of these processes (Rice et al., 2007), Thus, the biological function of the *cid* and *lrg* operons is to provide a source of extracellular genomic DNA (eDNA) for interweaving and strengthening with this molecule the scaffold of the matrix of biofilm.

The complex intervening factor influencing biofilm production and, in particular PIA synthesis, are depicted in **Figure 2**.

It has been earlier anticipated that biofilm is also influenced by bacterial density. Intercellular communication in staphylococci is enabled by the so-called *quorum sensing* (QS) systems.

The role of QS system in controlling staphylococcal biofilm dispersal and structuring is presented in details in another review of this series (Le et al., 2014) and will be treated here only summarily.

In staphylococci, the system that regulates the expression of virulence factors in response to cell density is the accessory gene regulator (*agr*) system (Recsei et al., 1986; Novick et al., 1993). The importance of the *agr* in biofilm formation in both *S. aureus* and *S. epidermidis* was first shown by the group of Michael Otto (Otto, 2001), who highlighted that, while *agr* leads to increase expression of toxins, serine protease, DNase, fibrinolysin, enterotoxin B, and toxic shock syndrome toxin-1, it decreases the expression of colonization factors and biofilm formation. Since biofilm is one of the most important virulence factors in chronic bacterial infections, the use of *agr*-inhibiting substances for anti-staphylococcal treatment has been proposed, but it may have severe drawbacks, as it might turn an acute into a chronic infection by promoting the expression of colonization factors (Otto, 2001).

Among the peptides strictly controlled by the *agr* locus, a family of short staphylococcal peptides, Phenol-Soluble Modulins (PSMs), characterized by amphipathic a-helical structure conferring surfactant-like properties, have been shown to be key effector molecules in biofilm structuring, dispersal and dissemination, by a mechanism consisting in the disruption of non-covalent interactions between biofilm matrix macromolecules (Otto, 2013). These peptides have been demonstrated to be relevant in the pathogenesis of *S. aureus* and *S. epidermidis* biofilm-associated infection, under both *in vitro* and *in vivo* models (Le et al., 2014).

THE DEBATED PROMISE OF QUORUM QUENCHING (QQ)

In the introduction, quorum quenching (QQ), alternatively known as quorum interference, has been mentioned as one of the latest strategies to counteract staphylococcal infections. In nature, QQ is a strategy pursued by antagonistic bacterial strains competing for the environment (Geisinger et al., 2009). QQ can be achieved by alternative tactics, these including quorum sensing disruption through inhibition of signal molecule biosynthesis, signal molecule inactivation and blockade of signal transduction (Kiran et al., 2008; Rampioni et al., 2014). A weakness of the QQ approach resides in the fact that not only QS systems markedly differ between Gram-positive and Gram-negative bacteria, but also their functions are different in the same *genus* and sometimes even in diverse strain types of the same species (Geisinger

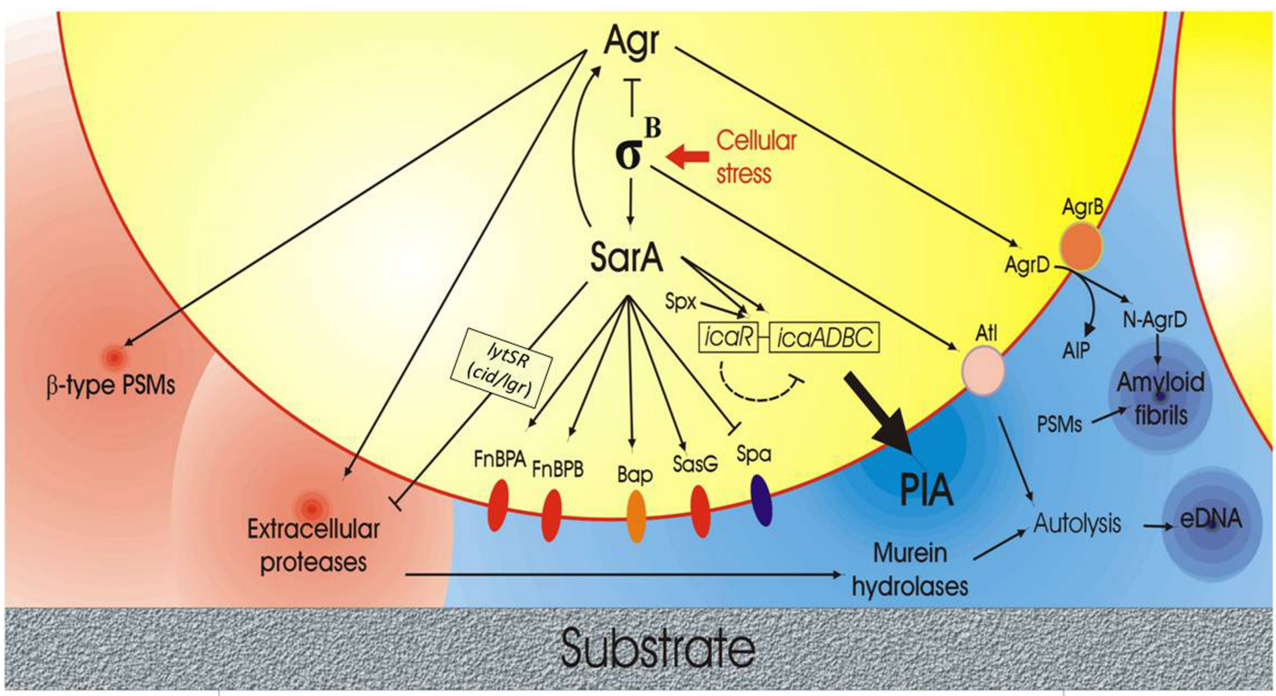

FIGURE 2 | Scheme of the complex network of interactions governing biofilm formation and disruption in *S. aureus* based on current scientific evidences. The right side of the figure illustrates the anabolic phase of biofilm with the production of some fundamental extracellular polymeric substances (EPS) such as PIA, extracellular-DNA (eDNA) and amyloid fibrils. The *lytSR* operon with its target genes *lrg/cid,* which affects murein hydrolase activity, is also represented. The center of the figure reports the protein membrane components implicated in biofilm formation, these including the FnBPs adhesins, the Biofilm associated protein (Bap), SasG and Spa. Conversely, in the left side of the figure, the molecules playing a role in biofilm catabolism and extracellular biofilm matrix disruption, such as phenol-soluble modulins (PSMs) and extracellular proteins, are reported. Agr QS system, σ^B factor and SarA appear to act as central regulators, orchestrating the bacterial behavior in response to stress factors, cellular densities and cell cycle phases.

et al., 2012). This means that it may result hard to find universal key molecules able to exert their desired action on all pathogens and that what can inhibit biofilm-formation in a species could stimulate it in another.

However, while inhibiting *agr* would be counterproductive and the use of QS inhibitors is debatable for combatting biofilm-associated infections by staphylococci, *vice versa*, QS interference is expected to become a powerful strategy to control virulence and antibiotic tolerance of Gram-negative bacteria.

To date, methods that can be used to disrupt quorum sensing include: (1) antagonizing signal binding to LuxR-family receptor, (2) inhibiting signal production, (3) degrading signals, (4) trapping signals, and (5) suppressing synthase and receptor activities, stabilities or productions (Hirakawa and Tomita, 2013).

Overall QQ appears an important strategy for applications in medicine (but also veterinary and agriculture), opening new horizons for preventive/therapeutic measures alternative or adjuvant to conventional antibiotics (Costerton et al., 2007).

PIA IN IMMUNOTHERAPEUTIC STRATEGIES TO COMBAT STAPHYLOCOCCAL INFECTIONS

The poly-*N*-acetyl-β-(1,6)-glucosamine, PNAG, also designed as PIA, is a high-profile candidate for a vaccine that could possibly provide protection against both coagulase-positive and coagulase-negative staphylococci. As outlined above, the native form of PIA/PNAG is partially de-*N*-acetylated (dPNAG). It has been observed that only antibodies to the deacetylated epitopes of PNAG are able to give protection, favoring opsonization of *S. aureus* and killing by human neutrophils (Kelly-Quintos et al., 2005, 2006).

Antibodies that bind to PNAG with either low (<15%) or high (>90%) levels of acetate were shown to have a superior opsonic and protective activity than antibodies that bind to PNAG with only high levels (>70%) of acetate (Cerca et al., 2007). Both in *S. epidermidis* and in *S. aureus*, IcaB is a deacetylase that causes partial deacetylation of PNAG and thus ensures a better surface retention of PNAG and optimal biofilm formation.

By studying the acetylation of PNAG by *icaB* negative *S. aureus* mutants, Cerca et al. (2007) observed that less PNAG was associated to the bacterial surface, and this strain was highly susceptible to antibody-independent killing by neutrophils. A *S. aureus* mutant with over-expression of *icaB* producing primarily surface-associated PNAG, was more susceptible to opsonophagocytosis with antibody to deacetylated PNAG. The higher retention of deacetylated PNAG on the surface of *S. aureus* provides a molecular mechanism explaining the superior opsonic and protective activity of antibody to dPNAG (Cerca et al., 2007).

While there is a large consensus on the need of a vaccine protecting people from staphylococcal infections, there is much less clarity about the choice of efficacious candidates for a component vaccine. Projan et al., in discussing the possible targets of an anti-*Staphylococcus* vaccine, list almost eight diseases and about thirty possible molecular targets (Projan et al., 2006).

The choice of an appropriate antigen for immunotherapy should attentively consider the issue of the immune evasion. In fact, PIA/PNAG, in addition to its role in intercellular adhesion and biofilm formation, has been pointed out to play a role just in the immune evasion by bacteria. Evidence suggests that antibodies against PIA/PNAG often recognize the secreted PIA/PNAG rather than its surface-associated form, this behavior resulting in an ineffective immune response (Cerca et al., 2007). An effective immune response against surface-associated PIA/PNAG has been evoked by a conjugate vaccine, composed of the *Staphylococcus aureus* PNAG and Clumping Factor A, which has been proved successful in eradicating infection (Maira-Litrán et al., 2012). Thus, PIA/PNAG protects the bacteria from immune defenses but, otherwise, it could actually be the target of an effective immune response.

Although this field is rich of interests and researches, and progress have been made, further experimental, epidemiological, pre-clinical and clinical studies are required before an efficacious vaccine could be achieved.

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Structural basis of *Staphylococcus epidermidis* biofilm formation: mechanisms and molecular interactions

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Staphylococcus epidermidis is a usually harmless commensal bacterium highly abundant on the human skin. Under defined predisposing conditions, most importantly implantation of a medical device, *S. epidermidis*, however, can switch from a colonizing to an invasive life style. The emergence of *S. epidermidis* as an opportunistic pathogen is closely linked to the biofilm forming capability of the species. During the past decades, tremendous advance regarding our understanding of molecular mechanisms contributing to surface colonization has been made, and detailed information is available for several factors active during the primary attachment, accumulative or dispersal phase of biofilm formation. A picture evolved in which distinct factors, though appearing to be redundantly organized, take over specific and exclusive functions during biofilm development. In this review, these mechanisms are described in molecular detail, with a highlight on recent insights into multi-functional *S. epidermidis* cell surface proteins contributing to surface adherence and intercellular adhesion. The integration of distinct biofilm-promoting factors into regulatory networks is summarized, with an emphasis on mechanism that could allow *S. epidermidis* to flexibly adapt to changing environmental conditions present during colonizing or invasive life-styles.

Keywords: *Staphylococcus epidermidis* biofilm formation, regulation, PIA, Aap, Embp, AtlE, primary attachment, biofilm accumulation

MEDICAL RELEVANCE OF *STAPHYLOCOCCUS EPIDERMIDIS*

Staphylococcus epidermidis, member of the group of coagulase-negative staphylococci, belongs to the commensal skin flora of every human individual (Kloos, 1980, 1997; Kloos and Schleifer, 1986). In its natural niche, the species is of significant importance for maintenance of local homoeostasis—a role that is so far understood only superficially (Grice and Segre, 2011). Only recently light was shed onto the potential importance of *S. epidermidis* to interfere with colonization with *Staphylococcus aureus* via expression of a serine-type protease termed Esp, thereby interfering with colonization mechanisms of *S. aureus*, e.g., biofilm formation (Iwase et al., 2010; Sugimoto et al., 2013). Given the tremendous abundance of *S. epidermidis* on the skin, it is not surprising that *S. epidermidis* is the most common cause of contamination in clinical specimens, and it is a challenge for medical microbiologist to reliably identify true invasive isolates (Mack et al., 2006). Improved abilities to discriminate between clinically relevant and contaminating *S. epidermidis* isolates is of utmost importance, as the species is today one of the most important bacteria related to hospital acquired infections. If the invasive behavior of *S. epidermidis* follows a clear pathogenic strategy that extends its colonizing abilities (Mack et al., 2009), or merely can be regarded as an accident during which mechanisms usually contributing to commensalism turn into virulence features (Otto, 2009), is still a matter of debate. Nevertheless, without doubt *S. epidermidis* is a true opportunistic pathogen that only causes disease in patients with predisposing factors. These include individual patient characteristics (i.e., premature birth, congenital immune defects) or concomitant medical conditions—like HIV infection, immunosuppression after bone marrow or solid organ transplantation and chemotherapy related neutropenia (Goldmann and Pier, 1993; Rupp and Archer, 1994). Most significantly, *S. epidermidis* is the leading organism isolated from foreign material related infections (FMRI) (Darouiche, 2004; Geipel and Herrmann, 2005) such as infected prosthetic joints, central venous catheters (CVC), cerebrospinal fluid shunts, intracardiac devices, artificial heart valves, and vascular grafts (Mack et al., 2006; Rogers et al., 2009). Use of implanted medical devices increases in number, and certainly, this will further propel the importance of *S. epidermidis* as an important human pathogen.

S. epidermidis is responsible for the vast majority of nosocomial catheter related blood stream infections (CRBSI) in the United States (Wisplinghoff et al., 2003; Hidron et al., 2008) and also in German intensive care units (ICUs) (Geffers and Gastmeier, 2011). Evaluation of a multicenter international data collection calculated a risk of 6.8 CRBSI per 1000 central line-days in ICUs (Rosenthal et al., 2014). Results from the Surveillance and Control of Pathogens of Epidemiological Importance (SCOPE) indicate that coagulase-negative staphylococci are the most frequent cause of nosocomial blood stream infections (Wisplinghoff et al., 2003). Species discrimination identified eighty percent

of CoNS from these infections as *S. epidermidis* (Jukes et al., 2010).

CoNS rank as third most common infective agent in native (NVIE) and first in prosthetic valve infective endocarditis (PVIE) clearly demonstrating the importance of CoNS in these clinical entities (Murdoch et al., 2009). Among CoNS isolates, about eighty percent were identified as *S. epidermidis* (Chu et al., 2004, 2009). Evaluation of the results of the International Collaboration on Endocarditis database showed a significantly higher rate of complicated clinical courses of PVIE due to CoNS with respect to heart failure compared to *S. aureus* or viridans streptococci (Lalani et al., 2006).

S. epidermidis is a significant cause of infections of prosthetic joint implants. The lack of non-invasive curative treatment options for joint implant infections often necessitates surgical intervention including replacement surgery. In the UK, CoNS and *S. epidermidis* were isolated in 36% of total hip and 49% of total knee arthroplasty infections (Phillips et al., 2006; Nickinson et al., 2010). In another study of infected total hip and knee arthroplasties about 77% of the isolated CoNS were confirmed *S. epidermidis* (Rohde et al., 2007).

First evidence suggesting a pathogenetic link between foreign-material implantation and *S. epidermidis* infection came from early electron microscopic analysis of explanted central venous catheters. Here, bacteria were found to colonize artificial material in large agglomerations, embedded into an amorphous material (Peters et al., 1982). While this specific mode of growth was first referred to as "slime," today it is termed biofilm formation (Götz, 2002). In fact, there is significant evidence connecting the biofilm mode of growth to the general persistent course of *S. epidermidis* foreign-material infections (Scherr et al., 2014a), and to the regular failure to eradicate infections by antimicrobial therapies (Lewis, 2005; Mack et al., 2009; Otto, 2009; Rohde et al., 2010). The latter aspect has been subject to extensive review recently (Lewis, 2010). Although *S. epidermidis* infections are regarded as prototypic biofilm infections (Costerton et al., 1999; Otto, 2009), it must be noted that it is by far not clear that biofilms observed under *in vitro* conditions indeed correlate with the biofilm growth evident *in vivo*. While this is most probably the case in central venous catheter (Peters et al., 1981) or cerebrospinal fluid shunt (Kockro et al., 2000) infections, there is some doubt that this model can be easily transferred to infections occurring at the interface of an implant and the surrounding tissues, e.g., prosthetic joint infections (Broekhuizen et al., 2008; Zaat et al., 2010). Clearly, much needs to be learned with regard to the exact spatial organization of *S. epidermidis* in implant infections, e.g., by making use of *ex vivo* or *in vivo* imaging approaches.

STRUCTURAL FACTORS CONTRIBUTING TO *S. EPIDERMIDIS* BIOFILM FORMATION

Traditionally, the process of biofilm formation is divided into at least three steps. During the phase of primary attachment, bacteria adhere to the surface to be colonized, while during the accumulative phase, bacteria initiate the establishment of a three dimensional, multi-cellular and multi-layered architecture in which, intriguingly, most bacteria do not have direct contact to the surface (Mack et al., 2009; Otto, 2009; Rohde et al., 2010). *S. epidermidis*, then, is able to disassemble the biofilm structure again, and liberated cells are believed to allow *S. epidermidis* to colonize additional body sites. The process of biofilm formation needs a wide range of functional activities, ranging from molecules mediating binding to native or conditioned (i.e., host extracellular matrix covered) surfaces, over glue-like factors fostering cell-cell aggregation, to activities that break down matrix components (Otto, 2009). The tremendous diversity of specific functional requirements during biofilm formation is on the bacterial side significantly mirrored by the expression of a plethora of different, highly specialized factors characterized by very distinct profiles of biological functions. Thus, related to their specialized functions during *S. epidermidis* biofilm morphogenesis, specific factors are assigned to groups representing mechanisms being active either during primary attachment or during biofilm accumulation, respectively.

FACTORS INVOLVED IN PRIMARY ATTACHMENT

Tight binding of bacteria to foreign-materials is a pivotal step toward establishment of a device-associated infection. Not unexpectedly, factors specifically involved in mediating bacterial–surface interactions were identified and further characterized. Some genetic evidence suggests that bacterial binding to unmodified polystyrene is fostered by the *S. epidermidis* autolysin AtlE (Heilmann et al., 1997). AtlE is a 115 kDa protein, which belongs to a group of bacterial peptidoglycan (PGN)-hydrolases playing a pivotal role in the degradation of the bacterial cell wall (Biswas et al., 2006). The protein consists of an N-terminal signal peptide, a propeptide, a catalytic domain with *N*-acetylmuramyl-L-alanine amidase activity, three repeats (R1–3), and a C-terminal catalytic domain with *N*-acetylglucosaminidase activity (Schlag et al., 2010; Zoll et al., 2010). In addition to its general role in cell wall turnover, AtlE also is of importance for binding to unmodified polystyrene, as demonstrated by the defect of an *atlE*::Tn*917* transposon mutant of *S. epidermidis* O-47 that lost its ability to adhere to plastic surfaces (Heilmann et al., 1997). Intriguingly, the importance of distinct domains for recruitment of AtlE to the bacterial cell wall (i.e., internal repeats) and enzymatic activities have been identified (Zoll et al., 2010). High resolution structural information is available, however so far it remains unclear, which exact AtlE domains are relevant for the primary attachment process and stable bacterial surface binding. In fact, at present it appears possible that expression and functional activation of AtlE induces significant changes in cell surface hydrophobicity, and thus, the AtlE effect on primary attachment might be secondary (Otto, 2014). In addition, a significant role of AtlE in eDNA mediated *S. epidermidis* biofilm formation is apparent (Qin et al., 2007; Christner et al., 2012).

While interactions between *S. epidermidis* and unmodified artificial surfaces most likely does not involve specific receptor-ligand interactions, it is well-known that *S. epidermidis*, similar to *S. aureus*, expresses cell surface proteins that mediate specific interactions with host extracellular matrix (ECM) components (Patti et al., 1994). Proteins with ECM-binding activity are believed to be of significant importance for the initiation of a device infection, since foreign materials become, as soon as they are inserted into the body, covered by ECM material (e.g.,

FN, fibronectin; Fg, fibrinogen; Vn, vitronectin; Cn, collagen) (Arrecubieta et al., 2006; Mack et al., 2009). In fact, *S. epidermidis* can use AtlE to adhere to surface organized Vn, while the lipase GehD is involved into interactions with collagen (Bowden et al., 2002). In addition to these proteins, for which their enzymatic activities might be of primary importance for *S. epidermidis* physiology, *S. epidermidis* also expresses proteins with a primary and dedicated function in bacterial-ECM interactions. These proteins belong to the group of serine-aspartate repeat (Sdr) proteins (McCrea et al., 2000), a widely investigated protein family of microbial surface components recognizing adhesive matrix molecules (MSCRAMM) (Josefsson et al., 1998; Foster et al., 2014). In *S. epidermidis*, three Sdr proteins referred to as SdrF, SdrG, and SdrH have been identified (Josefsson et al., 1998). SdrG, a LPXTG-motif containing protein covalently attached to the bacterial cell surface, is crucial for *S. epidermidis* adherence to fibrinogen-coated surfaces. It is therefore also being referred to as fibrinogen binding protein of *S. epidermidis* (Fbe) (Nilsson et al., 1998; Pei et al., 1999; Hartford et al., 2001). The gene encoding Fbe/SdrG is common in clinical *S. epidermidis* isolates (Nilsson et al., 1998; Rohde et al., 2004, 2007). Fbe/SdrG protein contains five distinct regions: an N-terminal export motif sequence, an A region that contains the Fg binding activity, a B region of so far unknown function, and the R region containing serine-aspartate repeat sequences. Fbe/SdrG specifically binds to a peptide sequence of 14 amino acids found in the N-terminus of the β-chain of Fg (Ponnuraj et al., 2003), and structural analysis of the interaction revealed a unique "dock, lock, and latch" mechanism ensuring a particularly strong interaction (Bowden et al., 2008; Herman et al., 2014). SdrF, sharing overall organizational similarity with SdrG, has been shown to mediate *S. epidermidis* binding to collagen I (Arrecubieta et al., 2007). In contrast to Fg-binding properties of Fbe/SdrG, the collagen binding epitopes of SdrF are located within the B repeat region (Arrecubieta et al., 2007). So far no specific functionality has been attributed to the N-terminal A domain of SdrF. A single B domain repeat of SdrF was sufficient to interact with collagen I, and apparently, this binding occurs via interactions with the α1 and α2 chains of type I collagen (Arrecubieta et al., 2007). Using a *Lactococcus lactis* heterologous expression system and a murine infection model evidence was generated that SdrF may contribute to cardiac assist device driveline infections (Arrecubieta et al., 2009). SdrF also mediates binding to unmodified Dacron surfaces covering drivelines. In contrast, *L. lactis* expressing GehD bound only weakly to driveline surfaces (Arrecubieta et al., 2009). Anti-SdrF inhibited *S. epidermidis* 9491 binding in the *in vivo* model only by roughly 50%, indicating that additional *S. epidermidis* collagen binding factors may be involved (Arrecubieta et al., 2009).

Extensive work has addressed the role of extracellular DNA (eDNA) in *S. epidermidis* and *S. aureus* biofilm formation. Data confirm that eDNA is a structural component of the biofilm matrix in both species, although evidence anticipates that eDNA has, at least partially, different functions in both species. Several independent studies have demonstrated that eDNA is released through increased cell lysis (Allesen-Holm et al., 2006; Rice et al., 2007; Christner et al., 2012). In *S. epidermidis* autolysis is determined to a large extent by the activity of the major autolysin AtlE (Biswas et al., 2006). A role for eDNA in *S. epidermidis* 1457 during primary attachment was deduced from observations showing that addition of DNase I abrogated bacterial attachment to glass surfaces. These findings were confirmed in additional, genetically independent *S. epidermidis* backgrounds (i.e., RP62A) (Qin et al., 2007). In extent to its effect on primary attachment, eDNA functions as an intercellular adhesin contributing to the stabilization of biofilms (Whitchurch et al., 2002). Based on the finding that DNase I has biofilm disintegrating activity when added within the first 6 h of biofilm accumulation currently it is believed that eDNA mediated intercellular adhesion is critical especially during the early accumulative phase (Qin et al., 2007). A role of eDNA in earlier stages of staphylococcal biofilm formation has recently been underpinned by observations showing that during *S. aureus* surface colonization under flow conditions eDNA, while not having impact on primary attachment, is critical during the transition from attachment to accumulation (Moormeier et al., 2014). It should again be stressed that functional differences of eDNA during *S. epidermidis* and *S. aureus* biofilm formation are apparent (especially with respect to the function during accumulation) (Izano et al., 2008; Christner et al., 2012), and observations in one species cannot easily be extrapolated to the other. This is especially true for the role of eDNA as a target during biofilm detachment events. By *saeRS* regulated expression of nuclease Nuc *S. aureus* can remodel the biofilm ultrastructure and control the release of bacteria from established biofilms (Mann et al., 2009; Olson et al., 2013). The lack of nuclease activity questions if this biofilm-escape mechanism is, in addition to *agr*-mediated biofilm dispersal (Vuong et al., 2003; Wang et al., 2011a), of relevance in *S. epidermidis*.

MECHANISMS OF *S. EPIDERMIDIS* BIOFILM ACCUMULATION

The hallmark of the accumulative phase is expression of intercellular adhesive properties, ultimately leading to cell aggregation and subsequent development of a multicellular, multilayered biofilm architecture (Costerton et al., 1995). Parallel to the discovery of factors with dedicated functions during primary attachment, the nature of intercellular adhesins, functioning as the "biofilm glue," was partially unraveled. Based on the early electron microscopic studies, showing *S. epidermidis* cells embedded in an amorphous extracellular matrix (Peters et al., 1981), focus was set onto the biochemical analysis of biofilm matrix components. These efforts ultimately resulted in the discovery of the polysaccharide intercellular adhesin (PIA), which is at present the most extensively studied intercellular adhesin (Mack et al., 1994b).

Structural analysis of PIA and comparison of PIA isolated from *S. epidermidis* and *S. aureus* has been recently reviewed (Mack et al., 2013). The structure of PIA was first described for biofilm-forming *S. epidermidis* 1457 and RP62A. PIA was extracted from the cells by sonication after the strains had been cultured in trypticase soy broth, which revealed the existence of both a major polysaccharide I (>80%), and a minor polysaccharide II (<20%), which are structurally closely related and could be separated due to differing ionic properties (Mack et al., 1996). Chemical analyses and NMR spectroscopy have demonstrated

that polysaccharide I is a linear homoglycan of β-1,6-linked 2-amino-2-deoxy-D-glucopyranosyl residues. Approximately 80–85% of them are *N*-acetylated; the rest are non-*N*-acetylated and carry a positive charge. Polysaccharide II of PIA has a lower proportion of de-N-acetylated 2-amino-2-deoxy-D-glucopyranosyl residues and is modified by ester-linked succinate residues rendering it anionic (Mack et al., 1996). Despite a high apparent molecular weight indicated by elution in the void volume of Sephadex G200 (Mack et al., 1994a, 1996) or Sephacryl S300 columns (C. Fischer and D. Mack, unpublished results), the ratio of reducing terminal sugar residues to total sugar residues was shown by methylation analysis to be 1:130, implying an average M_r of 30,000 for PIA polysaccharide chains (Mack et al., 1996). This implies aggregation of PIA polysaccharide chains in solution. PIA was shown to function also as the hemagglutinin of *S. epidermidis* (Rupp and Archer, 1992; Fey et al., 1999; Mack et al., 1999). Production of a functionally active PIA molecule requires expression of all four *icaADBC* genes (Gerke et al., 1998). The process has been the subject of detailed study in recombinant strains of *S. carnosus* which expressed different combinations of the *icaADBC* genes and with UDP-GlcNAc as a sugar donor (Gerke et al., 1998). IcaA belongs to the glycosyltransferase 2 family. It is an integral membrane protein with 412 aa and four predicted transmembrane domains (Heilmann et al., 1996; Gerke et al., 1998; Gill et al., 2005), and directs the synthesis of β-1,6-linked GlcNAc oligosaccharides of up to 20 GlcNAc units. IcaD is required for full activity of IcaA *in vitro*. It is a 101 aa integral membrane protein with two potential membrane spanning domains: it may be a chaperone directing folding and membrane insertion of IcaA and may act as a link between IcaA and IcaC (Gerke et al., 1998). Also essential for the synthesis of fully functioning PIA is IcaC, a 355 aa integral membrane protein with 10 predicted transmembrane domains, which may be involved in externalization and elongation of the growing polysaccharide (Gerke et al., 1998). IcaB is a member of the polysaccharide deacetylase family, including, for example, chitin deacetylases or the chitooligosaccharide deacetylase NodB of *Rhizobium melioti*. In its mature form it is a 259 aa secreted protein with a predicted signal sequence, responsible for the de-*N*-acetylation of PIA, and crucial for PIA activity in biofilm formation and for virulence in *S. epidermidis* (Vuong et al., 2004a). In Δ*icaB*-mutants, where the *icaB* gene has been deleted, PIA is poorly retained on the cell surface as it does not contain non-*N*-acetylated GlcNAc (Vuong et al., 2004a).

Early observations made by biochemical analysis of biofilm matrix extracts not only showed the presence of(poly-)saccharides but pointed toward the additional presence of proteins and nucleic acids (Hussain et al., 1991). Specific proteins have been identified and characterized. Apart from biofilm associated protein Bap (Tormo et al., 2005a), that is only rarely found in invasive *S. epidermidis* from human infections (Tormo et al., 2005a; Rohde et al., 2007; Piessens et al., 2012), SesC has been proposed to play a role in biofilm formation (Shahrooei et al., 2009). SesC is an LPXTG motif-containing 68 kDa surface protein of *S. epidermidis* distantly related to clumping factor A of *S. aureus* and is expressed more strongly in biofilm-associated as compared to planktonic *S. epidermidis* 1457 and 10b cells *in vitro* and *in vivo* (Shahrooei et al., 2009; Lam et al., 2014). Rabbit anti-SesC inhibited biofilm formation of a number of *S. epidermidis* isolates *in vitro*, which may be related to changes in primary attachment to fibrinogen-coated surfaces in the presence of anti-SesC. All of 105 *S. epidermidis* isolates recovered from nose swabs or infections were in possession of the *sesC* (Shahrooei et al., 2009). Passive and active immunization using SesC as a target protein was shown to decrease *S. epidermidis* biofilm formation in an *in vivo* model of central venous catheter infections (Shahrooei et al., 2012). A specific role of SesC as an intercellular adhesin in biofilm accumulation remains to be demonstrated.

MULTIFUNCTIONAL PROTEIN FACTORS IN *S. EPIDERMIDIS* BIOFILM FORMATION

Although having partially additional enzymatic functions (e.g., AltE, GehD), it is a common feature of many factors contributing to *S. epidermidis* surface colonization that they carry out functions either during the primary attachment or accumulative phase of biofilm formation (Rohde et al., 2006; Mack et al., 2009; Otto, 2009). With the increasing interest in protein factors contributing to staphylococcal biofilm accumulation, it became apparent that, at least in *S. aureus*, many factors (e.g., FnBPA, ClfA) must be regarded as multifunctional proteins not having an exclusive role in either primary attachment or accumulation (Foster et al., 2014). This concept of multifunctional proteins with important roles during several phases of biofilm formation and surface colonization is now also evolving in *S. epidermidis*, with the accumulation associated protein (Aap) and the extracellular matrix binding protein (Embp) being the most prominent factors.

Embp and its ortholog in *S. aureus* designated Ebh were almost simultaneously identified during studies aiming at identifying *S. epidermidis* or *S. aureus* protein factors with Fn binding activities (Clarke et al., 2002; Williams et al., 2002). By using a phage display approach a phage was isolated, which exhibited fibronectin binding activity and contained a DNA fragment from a 30,500 bp open reading frame (ORF) coding for a 10,203 aa protein that was referred to as extracellular matrix binding protein Embp (Williams et al., 2002) (**Figure 1**). Using bioinformatics the architecture of Embp was predicted to consist of an N-terminal YSIRK-motif containing export signal (aa 58–84), followed by an unordered region of approximately 2500 amino acids (aa 85–2586). The overall architecture of Embp mainly is characterized by 21 repetitive "Found In Various Architectures" (FIVAR) repeats (aa 2587–4500) and 38 alternating "G-related Albumine-binding" (GA) motifs and FIVAR repeats—termed the FIVAR-GA repeats—(aa 4501–9443) that span roughly 7000 amino acids in the central proportion of the Embp protein. Finally the C-terminus consists of four domains of unknown function (DUF1542) (aa 9444–9841), followed by a potential transmembrane motive (aa 10,070–10,088) (Christner et al., 2010) (**Figure 1**). Although the *S. aureus* homolog Ebh displays at least functional homologies with respect to fibronectin binding activity (Clarke et al., 2002) some functional predictions found in Ebh, e.g., an N-terminal hyperosmolarity resistence domain (Kuroda et al., 2008; Tanaka et al., 2008) have not been identified in the Embp. The overall structural organization of the Ebh protein seems to be more or less identical to Embp, but in

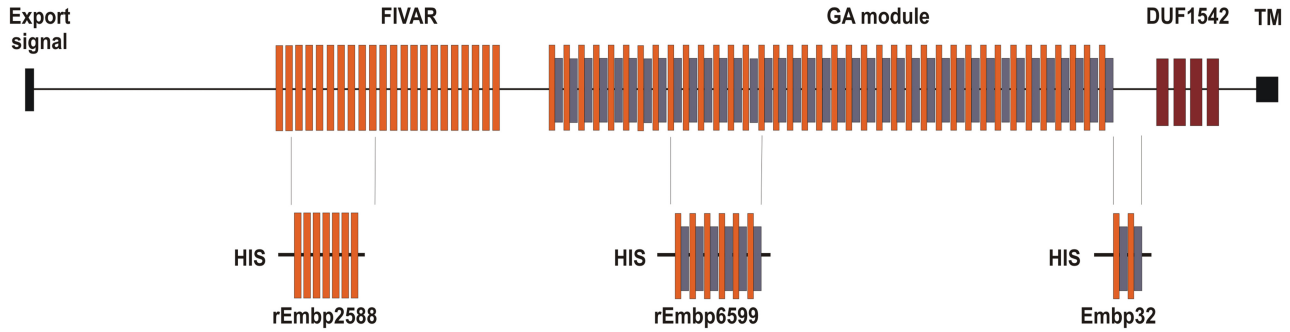

FIGURE 1 | Schematic representation of Embp and relative position of recombinant proteins for which functional data are available. Embp possesses an N-terminal YSIRK motif containing export signal, which is followed by a region of unordered conformation. The central part is comprised of the FIVAR or FIVAR-GA repeats, while the C-terminal end comprises up by four Domains of Unknown Function (DUF) and a putative transmembrane (TM) region (Christner et al., 2010). Embp32 is a recombinant Embp fragment (aa 9180–9421) from *S. epidermidis* NCTC11047 spanning four alternating FIVAR and FIVAR-GA repeats and that exhibits fibronectin binding capacity (Williams et al., 2002). rEmbp2588 (aa 2588–3187) contains seven FIVAR-only repeat units, while rEmbp6599 (aa 6599–7340) consists of six G-related albumin binding (GA) modules intercalated by FIVAR repeats. The rEmbp2588 and rEmbp6599 both bind to surface immobilized fibronectin (Christner et al., 2010).

detail gradual differences are apparent, e.g., only seven FIVAR motifs but 12 additional FIVAR-GA repeats and four additional DUF1542 repeats compared to Embp were predicted (Tanaka et al., 2008). In fact, it appears that among different *S. aureus* strains the number of repetitive modules (FIVAR and FIVAR-GA) within Ebh is variable, while available sequence data shows no variability of these features in Embp from *S. epidermidis* RP62A, ATCC12228 or 1585.

Crystallization of two 126 amino acid FIVAR-GA repeats from EbhA of *S. aureus* MU50 (termed EbhA-R7-R8) revealed a triple α-helical structure interconnected by a continuous alpha helical string displaying an elongated shape (Sakamoto et al., 2008; Tanaka et al., 2008). In fact, the corresponding FIVAR-GA repeat of Embp exhibits a very similar, if not identical structure (Büttner, Perbandt, Rohde, unpublished results). Additional preliminary structural analysis of repetitive FIVAR regions (Büttner, Perband, Rohde, unpublished results) or DUF1542 repeats (Linke et al., 2012) suggest that overall Embp constitutes an elongated rod-like conformation.

In collections of clinical significant *S. epidermidis* isolates *embp* was detected in more than 90% of strains (Rohde et al., 2004, 2007). In addition, evidence for the expression of Embp *in vivo* resulted from investigations showing the presence of anti-Embp antibodies in patients with confirmed *S. epidermidis* prosthetic joint infections (Mack, Büttner, Rohde, unpublished). Strikingly, using a flow cell model of biofilm formation, anti-Embp antibodies were shown to inhibit *S. epidermidis* 1457 biofilm formation (Lam et al., 2014), making Embp a potential candidate for preventive strategies (Götz, 2004).

Experimental evidence primarily suggested a role of Embp in primary attachment. Importantly, over-expression of Embp did not alter binding to unmodified polystyrene, but only boosted bacterial adherence to Fn-coated surfaces (Christner et al., 2010), and Embp–Fn interactions were necessary for biofilm accumulation on plastic surfaces that otherwise did not promote bacterial binding (Christner et al., 2010). Results from phage display suggested that FIVAR-GA repeats were relevant to the Fn-binding activity of Embp (Clarke et al., 2002; Williams et al., 2002). This assumption was later validated by biochemical analysis showing direct evidence for interactions between a recombinant protein containing FIVAR-GA repeats (Christner et al., 2010). In addition, these studies also found evidence that FIVAR-modules alone are capable of binding to immobilized Fn (Christner et al., 2010) (**Figure 1**). The vast majority of bacterial Fn-binding proteins bind to Fn via interactions with the N-terminal Fn type I domains (Bingham et al., 2008; Chagnot et al., 2012). This is especially true for the *S. aureus* Fn binding protein FnBPA (Meenan et al., 2007). Although the exact mechanism of Embp–Fn interaction awaits definitive molecular analysis, it is already clear that Embp uses an mechanism for Fn interactions independent of type I Fn modules, but involving Fn type III modules located at the C-terminus of Fn (Bustanji et al., 2003), most likely $FnIII_{12-14}$ (Christner et al., 2010). This type of interaction has only rarely been described in bacterial pathogens (Kingsley et al., 2004; Dabo et al., 2006).

In addition to its function in primary attachment, Embp is also functional as an intercellular adhesin. The intercellular adhesive properties and biofilm inducing activity of Embp was first detected in a laboratory derived strain *S. epidermidis* 1585v that, by a spontaneous chromosomal rearrangement, overexpressed a truncated isoform of Embp referred to as Embp1 (Christner et al., 2010). A transposon insertion within Embp1 resulted in abolished biofilm formation. By placing an inducible promoter in front of the wild-type *embp*, a biofilm inducing effect of full length Embp became apparent (Christner et al., 2010), proving the intercellular adhesive properties of Embp. Of notice, up-regulation of *embp* is also associated with resistance against uptake by professional phagocytes (Schommer et al., 2011). Studies on the overall impact of Embp on *S. epidermidis* cell wall assembly and its relation to immune-escape will shed light on the question if in this species, the giant protein carries similar functions as compared to Ebh in *S. aureus* (Cheng et al., 2014).

While at the time of its identification Embp appeared primarily as a factor mediating primary attachment, Aap was initially, and as already suggested by its designation, thought to confer

intercellular adhesion and thereby to contribute to biofilm accumulation. Aap is a covalently linked, cell wall associated protein consisting of an A- and a B-domain (Rohde et al., 2005; Gruszka et al., 2012; Conrady et al., 2013; Schaeffer et al., 2015). The 584 aa A domain harbors a N-terminal export signal, several imperfect, 16 amino acid repeats, and a globular 212 amino acid region with predicted α-helical and β-sheet content. The 212 amino acid region is highly conserved between Aap and its *S. aureus* ortholog SasG, and bioinformatical analysis predicts that this domain possesses lectin-like activity (Schaeffer et al., 2015). The B domain consists of a varying number of repetitive 128 amino acid repeats (Rohde et al., 2007). Variations of B repeats not only exist between independent *S. epidermidis* strains (e.g., reference strain RP62A possess 13 repeats, while *S. epidermidis* 1457 only harbors seven repeats) (Monk and Archer, 2007; Schaeffer et al., 2015), but are also encountered in clonally identical clinical isolates subsequently recovered during the course of device infections from individual patients (Rohde et al., 2007). This observation lead to the hypothesis that Aap B repeat variations could represent a mechanism contributing to *S. epidermidis* immune escape through modification of major cell surface epitopes (Rohde et al., 2007).

Aap can be detected on the bacterial cell wall, where it is most likely retained by covalent linkage to the peptidoglycan via its C-terminal gram-positive anchor region (Hussain et al., 1997; Rohde et al., 2005; Schommer et al., 2011; Conlon et al., 2014). A more detailed analysis using confocal microscopy demonstrated that within living, three dimensional *S. epidermidis* biofilms, Aap strictly localizes to the bacterial cell surface, while only minimal amounts are released into the biofilm matrix itself (Schommer et al., 2011). These results are underpinned and extended by electron-microscopic studies showing that Aap forms elongated fibers that project 120 nm away from the cell wall in localized tufts (Banner et al., 2007). Recently, using a structural biology approach, the molecular basis of this intriguing spatial organization was determined. An X-ray crystallography derived high resolution model of different recombinant proteins from the B region of *S. epidermidis* Aap (Conrady et al., 2013) or *S. aureus* SasG (Gruszka et al., 2012) showed that each B repeat consists of two regions, an approximately 80 aa G5 domain and an approximately 50 aa linker region referred to as E-region that interconnects repetitive G5 domains (Gruszka et al., 2012). The G5 domains each comprise two successive three-stranded β-sheets connected by triple-helix-like regions, while the E region is composed of two β-sheets (Gruszka et al., 2012; Conrady et al., 2013). E sequences fold cooperatively and form interlocking interfaces with G5 domains in a head-to-tail fashion, resulting in a contiguous, elongated, monomeric structure. Although E and G5 domains lack a compact hydrophobic core, G5 domain and multidomain constructs thereof have thermodynamic stabilities only slightly lower than globular proteins of similar size, explaining why Aap could form protruding fibers even under harsh environmental conditions (Gruszka et al., 2012; Conrady et al., 2013).

The functional importance of Aap for *S. epidermidis* biofilm formation was first recognized during studies in which chemically derived, biofilm-negative mutant M7 of *S. epidermidis* RP62A was analyzed (Hussain et al., 1997). Mutant M7 failed to express Aap on the cell surface, and antibodies raised against Aap were able to inhibit biofilm formation in biofilm-positive parent strain RP62A (Hussain et al., 1997). Later, Aap was independently picked up in experiments in which cell surface proteins of a clinically significant but biofilm-negative *S. epidermidis* wild-type strain 5179 were compared with those isolated from a laboratory derived, biofilm-positive revertant of that strain, referred to as 5179-R1. In protein preparations from 5179-R1 reduced amounts of full length Aap were detected, while in parallel, a shorter, roughly 140 kDa Aap isoform became apparent (Rohde et al., 2005). By using mass spectrometry and N-terminal sequencing evidence was created that the 140 kDa isoform mainly consists of repetitive B domain, mapping to aa 596 of the mature Aap protein. Rabbit antiserum raised against recombinantly expressed B domain inhibited biofilm formation by strain 5179-R1, not only directly supporting the idea of a functional involvement of Aap, but moreover indicating the crucial importance of B domain during this process. Indeed, genetic studies corroborated this hypothesis, showing that *in trans* expression of B domain in biofilm-negative *S. epidermidis* 1585 and surrogate host *S. carnosus* TM300 was sufficient to induce a biofilm-positive phenotype (Rohde et al., 2005). Importantly, expression of B domain did not alter the primary adherence properties, but resulted in cell aggregation, showing that in fact, Aap can be regarded as an intercellular adhesin (Rohde et al., 2005; Conrady et al., 2008; Geoghegan et al., 2010). Indeed, the importance of B domain for intercellular adhesion was also described for SasG in an *S. aureus* background (Geoghegan et al., 2010). The intercellular adhesive properties can be partially explained by Zinc-dependent homodimerization of B domains (Conrady et al., 2008, 2013), however, some evidence suggests the existence of additional, heterotypic interactions involved in Aap B domain mediated biofilm accumulation (Decker et al., unpublished results).

Importantly, expression of full length mature Aap is not sufficient to mediate intercellular adhesion during the second, accumulative phase of biofilm formation, but to become functionally active as an intercellular adhesin, Aap requires proteolytic processing, resulting in the removal of the A domain (Rohde et al., 2005; Geoghegan et al., 2010). Since Aap processing does not regularly occur under *in vitro* growth conditions (Rohde et al., 2005; Schaeffer et al., 2015), this phenomenon is a reasonable explanation for the finding that Aap-expressing *S. epidermidis* not necessarily form a biofilm (Rohde et al., 2007).

Although the intercellular adhesive Aap properties were recognized first, there is now mounting evidence supporting a significant role of Aap also in primary attachment to natural epithelial cells or artificial surfaces (Macintosh et al., 2009; Conlon et al., 2014; Schaeffer et al., 2015). Intriguingly, early work already pointed toward a role of domain A in this process, in which Aap B domain most likely is of only minor functional importance (Macintosh et al., 2009). Binding of Aap expressing *S. epidermidis* NCTC 11047 to squamous epithelial cells was partially inhibited by the addition of recombinant Aap A domain, as was binding of several additional, Aap-positive clinical *S. epidermidis* strains (Macintosh et al., 2009). Moreover, *in trans* expression of A domain in surrogate host *L. lactis* enabled the bacteria to

more efficiently adhere to corneocytes as compared to a *L. lactis* strain that expressed B domain alone (Macintosh et al., 2009), thus providing genetic evidence for a potential role of A domain in colonization of natural skin surfaces. In extension to the work of Macintosh and co-workers, the role of Aap A domain in primary attachment was further refined in two studies addressing the question as to which extent the A domain could also contribute to colonization of abiotic surfaces (Conlon et al., 2014; Schaeffer et al., 2015). In a clinical *S. epidermidis* isolate CSF41498, expressing an unprocessed mature, i.e., A domain containing Aap on the surface, attachment to polystyrene was almost completely abolished after deletion of *aap*. In addition, attachment was significantly inhibited by an antiserum raised against Aap A domain, whereas anti-Aap domain B antiserum had no significant impact on adherence capacities of that strain (Conlon et al., 2014). Intriguingly, deletion of *aap* in *S. epidermidis* 1457, expressing a processed Aap isoform devoid of the A domain, did not render the adherence capacities of that strain (Schaeffer et al., 2015). Moreover, anti-Aap A domain antiserum had no effect on binding to the surface of *S. epidermidis* 1457, supporting the idea that indeed, the presence of A domain, i.e., expression of a mature unprocessed Aap, is essential for Aap-mediated surface adherence. Indeed, *in trans* expression of full length Aap in 1457Δ*aap* resulted in improved bacterial binding (Schaeffer et al., 2015). Direct genetic evidence for an involvement of Aap A domain in staphylococcal plastic adherence is demonstrated by *in trans* expression of A domain fused to the C-terminal cell wall anchor in *S. carnosus* TM300, which significantly improved bacterial binding as compared to the wild-type strain or a strain expressing the Aap B domain (Schaeffer et al., 2015). Thus, a new picture of Aap evolved in which the protein plays a bifunctional role in both, the very early primary attachment phase as well as the later accumulative phases of biofilm formation, and that Aap's inherent functionalities are represented by separated and structurally distinct domains within the protein (Rohde et al., 2005; Macintosh et al., 2009; Conlon et al., 2014; Schaeffer et al., 2015).

MOLECULAR INTERACTIONS AND REGULATORY EVENTS DURING *S. EPIDERMIDIS* BIOFILM FORMATION

The detailed analysis of functional molecules contributing to *S. epidermidis* biofilm formation has clearly revealed that primary attachment as well as biofilm accumulation is mediated by redundantly organized factors with remarkable exclusive properties, but certainly broadly overlapping functional characteristics. The latter aspect is especially true for molecules active during the accumulative phase: production of PIA, Aap or Embp all efficiently induce cell aggregation, ultimately leading to assembly of a biofilm consortium. However, given the common observation that in clinical *S. epidermidis* isolates genes encoding for the PIA-synthesis machinery (*icaADBC*), *aap*, *embp*, and additional adherence-associated factors are all widely distributed, with a large percentage of strains carrying all three genes (Frebourg et al., 2000; Galdbart et al., 2000; Klug et al., 2003; Arciola et al., 2004; Rohde et al., 2004, 2007; Petrelli et al., 2006), the question is put forward if and how these mechanisms interact, and which consequences co-expression of distinct intercellular adhesins might have for *S. epidermidis* biofilm accumulation.

Indeed, the idea that distinct *S. epidermidis* intercellular adhesins cooperate during biofilm assembly is supported by epidemiological work identifying that strains being positive for *icaADBC* and *aap* appear to form stronger biofilms compared to strains being positive only for *icaADBC* or *aap* (Stevens et al., 2008). Bioinformatic analysis of *aap* and *embp* indeed suggests that direct interaction with PIA might be possible. The G5 domains of the Aap B domain are postulated to have N-acetylglucosamine binding activity (Bateman et al., 2005), and similarly, FIVAR regions of Embp are likewise predicted to have sugar binding potential (Christner et al., 2010), making the direct interaction between proteinacous intercellular adhesins and N-acetylglucosamine containing PIA possible. However, so far no experimental data are available that would support this important and interesting hypothesis. At present, it rather appears that parallel expression of specific intercellular adhesins introduces functional redundancy into biofilm accumulation, i.e., the intercellular adhesive properties of distinct adhesins functionally substitute for each other. Experiments, in which the susceptibility of biofilms of Embp-producing *S. epidermidis* strain 1585v against treatment with proteases was tested, revealed that, while being naturally sensitive against protease activity, additional *in trans* expression of *icaADBC* and PIA production protected the *S. epidermidis* 1585v biofilm from proteolytic breakdown (Christner et al., 2010). On the other hand, while PIA-dependent biofilms are readily disrupted by PIA-degrading enzyme DspB (Kaplan et al., 2004; Rohde et al., 2007), parallel expression of a proteinacous intercellular adhesin Embp and PIA rescued a biofilm-positive phenotype even in the presence of DspB (Christner et al., 2010). Functional substitution is also evident for Aap and PIA: the inactivation of *aap* in a PIA-producing genetic background had no apparent effect on biofilm formation in *S. epidermidis* 1457 as tested by conventional crystal violet biofilm assays or confocal laser scanning microscopy, probably because biofilm formation is already maximal in the various assay systems when PIA is expressed alone (Schaeffer et al., 2015). On the other hand, *in trans* expression of Aap B domain, being sufficient for induction of cell aggregation, induces biofilm formation in a PIA-negative, *icaADBC* mutant 1457-M10 (Henke and Rohde, unpublished results).

The interpretation of functional redundancy in intercellular adhesive molecules as a simple means allowing *S. epidermidis* to form as robust biofilms as possible, however, might be an inappropriate oversimplification of their actual functional importance during different *S. epidermidis* live styles, ranging between colonization and (foreign-material associated) host invasion. In that respect, it is important to acknowledge that a more detailed morphological analysis of PIA-, Aap- or Embp dependent biofilms revealed that these biofilm types differ significantly in their morphological properties. While in PIA-dependent biofilms, *S. epidermidis* cells are embedded into meshwork of PIA-containing extracellular matrix fibers, Aap production induces formation of densely packed cell layers that evenly cover the surface. In Embp-dependent biofilms, bacteria produce small amounts of Embp-containing extracellular matrix structures, however, these biofilms differ from PIA-dependent cell consortia by the lack of towers and clusters (Schommer et al., 2011). Overall,

PIA-dependent biofilms are significantly more stable against washing procedures as compared to protein-dependent biofilms, indicating their inherent, pronounced mechanical robustness. Thus, the specific biological properties of a given intercellular adhesin could constitute a way how *S. epidermidis* can cope with varying challenges during colonization and infection (Schommer et al., 2011). Analysis of invasive *S. epidermidis* strains from various types of infections supports the idea that in fact, the ability to differentially make use of specific intercellular adhesins equips *S. epidermidis* to specifically adapt to changing environments with potential fundamentally different requirements, e.g., presence of mechanical or osmotic stress, or exposure to effectors of the host immune system (Otto, 2014). Specifically, *S. epidermidis* strains from central venous catheter infections, i.e., a situation with significant exposure to mechanical stress and cellular and soluble factors of innate immunity (e.g., complement factors) are more likely to carry *icaADBC* compared to strains from prosthetic joint infections, i.e., an infection setting characterized by static conditions at the implant–tissue interface (Ziebuhr et al., 1997; Arciola et al., 2004; Rohde et al., 2004, 2007; Stevens et al., 2008; Mack et al., 2013).

Additional evidence suggesting that usage of specific intercellular adhesins indeed follows an adaptive program results from the observation that PIA-dependent biofilm formation on one hand, and Aap- and Embp-dependent biofilm formation on the other, are, at least under *in vitro* growth conditions, mutually exclusive (Rohde et al., 2005, 2007; Christner et al., 2010). In fact, under standard growth conditions in trypticase soy broth (TSB), neither Aap nor Embp-dependent biofilm formation would have been identified, since here Aap is not properly processed, while *embp* is not expressed at all (Rohde et al., 2005; Christner et al., 2010). The characterization of these intercellular adhesins only was possible by analysis of laboratory derived, spontaneous mutants or by introduction of artificial promoters allowing for inducible gene expression, respectively (Christner et al., 2010; Rohde et al., 2005). Thus, PIA- and Aap- or Embp-dependent modes of biofilm formation are obviously under the control of opposed regulatory circuits. For PIA-dependent biofilm formation, a very detailed picture of how *icaADBC* expression is integrated into a complex superimposed regulatory network has evolved. Others have recently reviewed this field in detail (Mack et al., 2004; Cue et al., 2012). In brief, several regulators of *icaADBC* expression and PIA synthesis were identified (Xu et al., 2006; Sadykov et al., 2011; Wang et al., 2011b), with sigma factor B and staphylococcal accessory regulator SarA being the most prominent (Fluckiger et al., 1998; Knobloch et al., 2001, 2004; Tormo et al., 2005b; Handke et al., 2007). Especially, negative regulator of *icaADBC* expression, IcaR, has been elucidated in great detail (Conlon et al., 2002). However, the regulation of PIA-independent mechanism of *S. epidermidis* biofilm formation remained obscure.

More recently, in an attempt to shed light onto potential negative regulators interfering with biofilm formation in clinical *S. epidermidis* isolates under *in vitro* conditions, a transposon mutant library established in biofilm-negative *S. epidermidis* 1585 was screened for biofilm-positive mutants (Christner et al., 2012). Independent biofilm-positive mutants were identified, and further analysis showed that all carried Tn*917* insertions in *sarA*. Intriguingly, inactivation of *sarA* induced a strong up-regulation of *embp* expression, and additional experimental work proved that Embp was necessary for 1585Δ*sarA* biofilm formation. Moreover, higher eDNA amounts were present in biofilms of 1585Δ*sarA*, and this finding was related to increased autolysis, itself being a result of over-production of metalloprotease SepA (Lai et al., 2007) and subsequent processing and functional activation of AtlE (Christner et al., 2012). Of notice, protease-mediated autolysin processing, augmented autolysis and subsequent eDNA release and biofilm formation has also been described in *Enterococcus faecalis* as part of a fratricidal mechanism (Thomas et al., 2008, 2009).

The results of the study by Christner and co-workers appear to contradict previous findings, showing that SarA is a positive regulator of *S. epidermidis* biofilm formation by augmenting *icaADBC* expression (Tormo et al., 2005b; Handke et al., 2007). However, a more detailed analysis of SarA function in PIA-positive background of *S. epidermidis* 1457 showed that in this strain, inactivation of *sarA* does not completely abolish biofilm formation (Handke et al., 2007). Even inactivation of *icaADBC* in 1457Δ*sarA* did not render the strain biofilm-negative, and in fact, the biofilm of 1457-M10Δ*sarA* is Embp- and eDNA-dependent (Christner et al., 2012). In conclusion, the study by Christner and co-workers establishes a key role for SarA in controlling the mode of biofilm formation in *S. epidermidis*: up-regulation of *sarA* shifts *S. epidermidis* toward production of a PIA-dependent type of biofilm, whereas down-regulation of *sarA* supports formation of PIA-independent types of biofilm formation (**Figure 2**). Clearly, the regulation involves transcriptional effects with direct consequences for intercellular aggregation (i.e., *embp* and *icaADBC* up- or down-regulation), but also post-translational mechanisms of regulation exerted via up- or down-regulation of metalloprotease SepA with subsequent proteolytic processing and functional modification of cell surface proteins (e.g., AtlE). Importantly, through the latter mode of action SarA could also influence Aap-dependent biofilm formation by boosting proteolytic removal and functional activation of Aap B-domain (**Figure 2**). In the future it will be of major interest as to which extent the SarA regulatory circuit and interrelated additional regulators, e.g., *agr* (Vuong et al., 2004b), *ssrAB* (Wu et al., 2015), *saeRS* (Lou et al., 2011), or *codY* (Batzilla et al., 2006) as well as the levels of proteolytic activity influence the balanced formation of PIA-dependent or -independent types of biofilm formation.

Certainly, the highly artificial character of studies related to the function of SarA makes it difficult to draw general conclusions on the relevance of this mechanism during *in vivo* biofilm accumulation. In that respect, it becomes clearer that that more appropriate models of biofilm formation are generally needed in order to reflect the *in vivo* situation during infection and colonization. These models must take into account the potential inappropriateness of TSB as a growth medium that obviously introduces a significant bias toward PIA-dependent biofilm formation into the experimental system. The finding that *embp*, but also additional factors involved in *S. epidermidis* biofilm formation (e.g., Fbe/SdrG) are only expressed in the presence of serum (Sellman et al., 2005, 2008; Christner et al., 2010), clearly argues

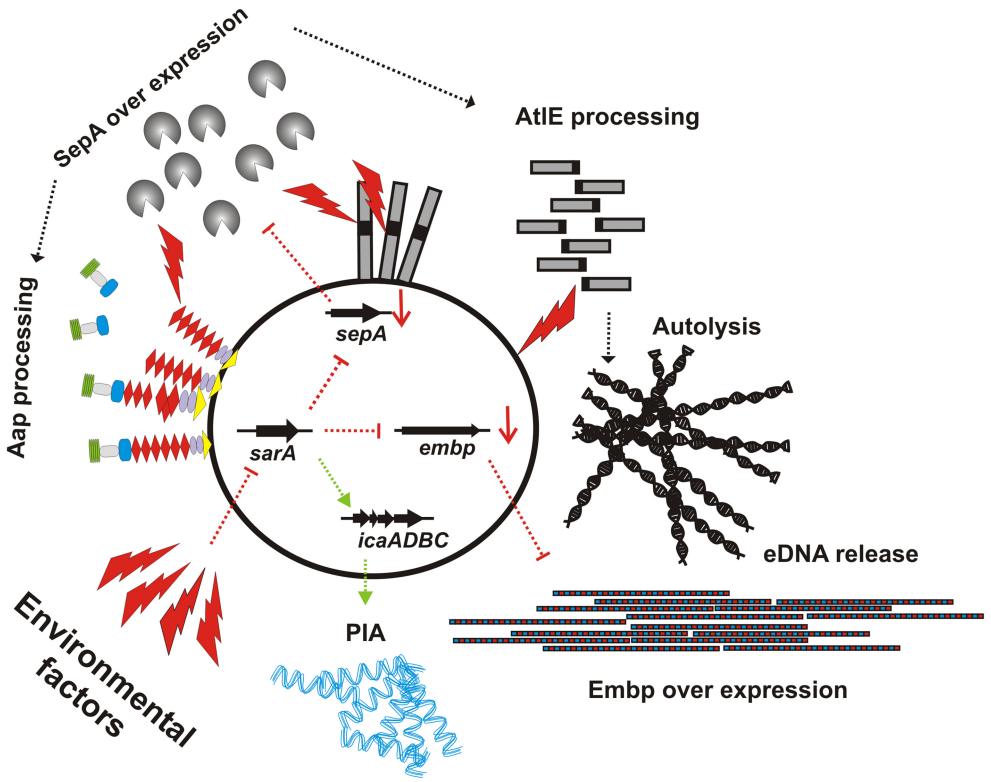

FIGURE 2 | Schematic representation of SarA effects on expression of independent intercellular adhesins. While in trypticase soy broth, *sarA* is expressed, leading to *icaADBC* expression and PIA dependent biofilm formation, down-regulation of *sarA* leads to an de-repression of *embp* expression, allowing for maintenance of a biofilm-positive phenotype despite *icaADBC* down-regulation and loss of PIA production. Parallel to *embp*, following inactivation of *sarA*, metalloprotease *sepA* is up-regulated, leading to increased AtlE processing, autolysis and subsequent eDNA release. Potentially, the over-production of SepA also contributes to Aap-dependent biofilm accumulation by proteolytically processing of the mature protein, resulting in the removal of it's A domain. Environmental (potentially also host) factors that repress *sarA* expression are unknown, as are the pathways via which *sarA* itself is regulated.

for experimental set-ups that mimic environmental conditions in ecological niches usually encountered by *S. epidermidis*, e.g., within the vestibulum nasi (Krismer et al., 2014) or on the skin (Ohnemus et al., 2007; Olson et al., 2014).

RELEVANCE OF *S. EPIDERMIDIS* BIOFILM FORMATION FOR THE PATHOGENESIS OF FOREIGN-MATERIAL ASSOCIATED INFECTIONS *IN VIVO*

In general, studies aiming at elucidating the clinical relevance of biofilm formation and the relative contribution of specific factors to foreign-material colonization and establishment of a chronic persistent infection are either studied using cell culture models (Vuong et al., 2004a,c; Schommer et al., 2011), *Caenorhabditis elegans* (Begun et al., 2007) or animal models of device infections, e.g., central venous catheter or prosthetic device infection models (Wang et al., 2011a; Odekerken et al., 2013; Scherr et al., 2014b; Schaeffer et al., 2015). In early studies on the importance of biofilm formation *in vivo* using animal models and genetically unrelated, biofilm-positive or biofilm-negative *S. epidermidis* isolates failed to demonstrate conclusive evidence that biofilm forming isolates are more virulent compared to biofilm-negative strains. However, subsequent studies using genetically defined, isogenic pairs of biofilm-positive wild-type and biofilm-negative mutants were more conclusive. In a subcutaneous catheter infection model in mice and a central venous catheter infection model in rats biofilm-positive, PIA-producing *S. epidermidis* 1457 was more virulent than its isogenic biofilm-negative transductant 1457-M10 (Rupp et al., 1999a,b). An AtlE-deletion mutant of this strain was also attenuated (Rupp et al., 2001). In a rat central venous catheter model, expression of *icaRADBC* in *icaADBC*-negative *S. epidermidis* strains also led to increased virulence (Li et al., 2005). A *Caenorhabditis elegans* infection model was used to study biofilm-positive *S. epidermidis* 9142, showing attenuation of an *icaA* insertion mutant compared to its isogenic, PIA producing parent strain 9142 (Begun et al., 2007). Virulence was restored to wild-type in the biofilm-negative mutant by complementation with cloned *icaADBC* (Begun et al., 2007). However, in a collection of *S. epidermidis* infective endocarditis isolates, PIA expression and pathogenicity for *C. elegans* was not closely associated (Monk et al., 2008). More recently, using a catheter infection model in which realistic colonization modalities were chosen (i.e., infection after catheter insertion and not usage of pre-colonized materials) the inactivation of *icaADBC* had no apparent effect on

colonization, while *aap* inactivation almost completely abolished the ability of *S. epidermidis* to establish an infection (Schaeffer et al., 2015).

A significant reason for the impaired pathogenicity of *icaADBC*-negative mutants in animals models is the improved ability of the innate immune system to clear biofilm-negative *S. epidermidis* (Schommer et al., 2011). There is significant experimental evidence from cell culture assays that indeed biofilm positive strain 1457 was less susceptible to killing by antimicrobial peptides and also displayed decreased phagocytosis and killing by polymorphonuclear granulocytes (PMNs) compared to its isogenic *icaA* mutant 1457-M10 (Vuong et al., 2004c). When *S. epidermidis* 1457 was either grown in a static biofilm or planktonic culture, the organism grown in a biofilm was less susceptible to phagocytic killing after opsonisation with normal human serum, as was an isogenic biofilm-negative *icaA*-insertion mutant (Kristian et al., 2008). PIA-dependent biofilm formation also interferes with host complement activation. Biofilm-positive wild-type bacteria pre-opsonised with normal human serum were more resistant to complement-dependent killing than the corresponding isogenic biofilm-negative bacteria (Kristian et al., 2008). There is, moreover, evidence that *S. epidermidis* biofilm formation interferes with phagocytic up-take and with pro-inflammatory activation of macrophages. This effect was irrespective of the intercellular adhesin used (Schommer et al., 2011). These phenotypes clearly could additionally contribute to the chronic persistent, low-grade inflammatory course of a *S. epidermidis* infection.

It is important to stress that, since *S. epidermidis* is an opportunistic pathogen, mechanisms of pathogenicity which are important in some types of device-related infection might be less crucial in others. For example, in the guinea pig tissue cage model (Zimmerli et al., 1982) there was no difference in virulence between a biofilm-positive wild-type *S. epidermidis* 1457 and its isogenic *icaA*-insertion mutant, and no difference between *icaADBC*-positive and -negative clinical isolates (Francois et al., 2003; Chokr et al., 2007). Nonetheless PIA was expressed *in vivo* in the tissue cages, and when animals were infected with both strains at the same time, the wild-type out-competed the mutant (Fluckiger et al., 2005). This may be because phagocytes are severely impaired in tissue cages (Zimmerli et al., 1984), masking the expected advantage of the wild-type.

S. epidermidis produces a number of pro-inflammatory peptides called phenol-soluble modulins (PSMs), which are produced in a strictly *agr*-controlled manner (Mehlin et al., 1999; Yao et al., 2005). PSM-δ rapidly lyses neutrophils, supporting the idea that the peptide is of relevance to the pathogenesis of *S. epidermidis*. However, PSM-δ is expressed only at low levels by *S. epidermidis* 1457, in line with a low overall cytolytic activity of *S. epidermidis* (Cheung et al., 2010). PSM-δ is expressed only at very low levels in *S. epidermidis* 1457 biofilms as compared to planktonic cells (Wang et al., 2011a). PSM-β peptides promote *S. epidermidis* biofilm structuring and detachment *in vitro* and dissemination of infection during catheter colonization *in vivo*, thereby providing the first mechanism of biofilm detachment in *S. epidermidis* (Wang et al., 2011a).

OUTLOOK AND FUTURE DIRECTIONS

Over the past two decades, significant progress has been made in our understanding of the specific pathogenic nature of *S. epidermidis* in foreign material-associated infections. A molecular picture evolved showing that *S. epidermidis* virulence is linked to biofilm formation, a phenotype that depends on a wide variety of different factors which carry distinct and tightly regulated functions during surface colonization and interactions with host immune responses. Thus, *S. epidermidis* biofilm research has reached a turning point, at which on one hand additional *in vitro* evidence for the involvement of dedicated mechanisms in surface colonization can easily be accumulated, but on the other hand the question of the *in vivo* relevance of a given factor or process certainly arises. Thus, a major future challenge will be to translate findings from highly artificial, simple *in vitro* biofilm analysis systems into complex (organ-) models that more appropriately reflect the *in vivo* infection settings. Moreover, it is of urgent importance to validate findings from *in vitro* models in relevant animal models of device infections. These approaches should involve not only state of the art molecular biology, biochemical and immunological methods but also time-resolved *in vivo* and *ex vivo* imaging technologies, allowing to create a more distinct picture of the invasive *S. epidermidis* life style in different and extremely variable environmental conditions. Using the new armament of technologies, including three-dimensional cell culture techniques and tissue engineering, efforts are necessary to study the role of *S. epidermidis* as a beneficial skin commensal more intensively.

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Streptococcus pneumoniae biofilm formation and dispersion during colonization and disease

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Streptococcus pneumoniae (the pneumococcus) is a common colonizer of the human nasopharynx. Despite a low rate of invasive disease, the high prevalence of colonization results in millions of infections and over one million deaths per year, mostly in individuals under the age of 5 and the elderly. Colonizing pneumococci form well-organized biofilm communities in the nasopharyngeal environment, but the specific role of biofilms and their interaction with the host during colonization and disease is not yet clear. Pneumococci in biofilms are highly resistant to antimicrobial agents and this phenotype can be recapitulated when pneumococci are grown on respiratory epithelial cells under conditions found in the nasopharyngeal environment. Pneumococcal biofilms display lower levels of virulence *in vivo* and provide an optimal environment for increased genetic exchange both *in vitro* and *in vivo*, with increased natural transformation seen during co-colonization with multiple strains. Biofilms have also been detected on mucosal surfaces during pneumonia and middle ear infection, although the role of these biofilms in the disease process is debated. Recent studies have shown that changes in the nasopharyngeal environment caused by concomitant virus infection, changes in the microflora, inflammation, or other host assaults trigger active release of pneumococci from biofilms. These dispersed bacteria have distinct phenotypic properties and transcriptional profiles different from both biofilm and broth-grown, planktonic bacteria, resulting in a significantly increased virulence *in vivo*. In this review we discuss the properties of pneumococcal biofilms, the role of biofilm formation during pneumococcal colonization, including their propensity for increased ability to exchange genetic material, as well as mechanisms involved in transition from asymptomatic biofilm colonization to dissemination and disease of otherwise sterile sites. Greater understanding of pneumococcal biofilm formation and dispersion will elucidate novel avenues to interfere with the spread of antibiotic resistance and vaccine escape, as well as novel strategies to target the mechanisms involved in induction of pneumococcal disease.

Keywords: biofilm, colonization, streptococcus, virus, virulence

BACKGROUND

Streptococcus pneumoniae colonizes the upper respiratory tract in humans. Colonization occurs on the mucosal surface of the nasopharynx during childhood and persists asymptomatically in healthy individuals into adulthood (Gray et al., 1981; Hogberg et al., 2007). Pneumococcal carriage rates are greater in children compared to adults, with approximately 20–50% carriage rate in children and 5–20% in adults in higher resourced countries while even higher rates are seen in resource poor settings where up to 90% of children and over half of adults are colonized (Gray et al., 1980; Revai et al., 2008; Huang et al., 2009; Pebody et al., 2009; Mackenzie et al., 2010; Korona-Glowniak and Malm, 2012; Adegbola et al., 2014). Despite a low attack rate, transition from asymptomatic colonization to disease occurs often enough that the pneumococcus remains a leading cause of acute otitis media, pneumonia, sepsis, and meningitis globally (Sleeman et al., 2006; O'brien et al., 2009; Black et al., 2010). In 2011, *S. pneumoniae* caused an estimated 2,858,000 severe pneumonia episodes and 411,000 deaths worldwide in children under the age of 5 (Walker et al., 2013). The burden of disease is highest in resource poor settings where the lack of nutrition, antibiotics, and vaccines make the population particularly susceptible to disease.

PROPERTIES OF PNEUMOCOCCAL BIOFILMS

INTRODUCTION

Biofilms are highly-structured communities of cells that produce an extracellular matrix and adhere to abiotic or biological surfaces (Costerton et al., 1999; Donlan and Costerton, 2002; Stoodley et al., 2002). Antibacterial resistance is an inherent characteristic of biofilms and the protective biofilm matrix enables evasion of host immune responses, facilitating persistence, and dissemination of bacteria (Costerton et al., 1999;

Donlan and Costerton, 2002; Chole and Faddis, 2003; Lewis, 2008; Sanchez et al., 2011b). In this context, resistance refers to an increased tolerance to antibacterials rather than a decreased susceptibility due to changes in the genome, such as mutations or obtaining antibiotic resistance genes. Pneumococcal colonization precedes disease and has been known to be more challenging to eradicate than invasive disease in patients as treatment with antimicrobial agents do not eliminate the majority of bacteria carried in the nasopharynx (Cohen et al., 1997, 1999; Dabernat et al., 1998; Dagan et al., 1998, 2001; Varon et al., 2000; Garcia-Rodriguez and Fresnadillo Martinez, 2002). Thus, a reasonable explanation for the decreased sensitivity of pneumococci to antimicrobial treatment during carriage is the formation of biofilm communities in the nasopharynx (Waite et al., 2001; Oggioni et al., 2006; Munoz-Elias et al., 2008; Trappetti et al., 2009; Sanchez et al., 2011b).

The original literature investigating pneumococcal biofilm formation *in vivo* detected biofilms during disease states such as otitis media, chronic rhinosinusitis, with some evidence for clustering of bacteria also during pneumonia (Hall-Stoodley et al., 2006; Sanderson et al., 2006; Hoa et al., 2009; Reid et al., 2009; Sanchez et al., 2011b). More recent data indicate that biofilm bacteria detected at disease sites represent asymptomatic colonization and, therefore, the presence of biofilms at sterile sites during disease presumably form a reservoir from which virulent bacteria may seed off under the right conditions, resulting in a role for biofilm bacteria in the disease process (Oggioni et al., 2006; Weimer et al., 2010; Sanchez et al., 2011b).

The vast majority of *in vitro* studies have been performed primarily on abiotic surfaces (Moscoso et al., 2006; Oggioni et al., 2006; Garcia-Castillo et al., 2007; Munoz-Elias et al., 2008; Domenech et al., 2009; Parker et al., 2009; Trappetti et al., 2009, 2011b,c; Sanchez et al., 2010, 2011a; Tapiainen et al., 2010; Camilli et al., 2011)., mimicking the classical models set up for organisms that confer problems in patients by producing biofilms on abiotic surfaces associated with medical devices. The extent of relevance these *in vitro* studies have *in vivo* is unclear as most of the biofilm formation experiments were conducted over short periods of time on abiotic surfaces that, as far as we know, are not major natural environments for the pneumococcal life cycle. For the same reason, *in vitro* studies on abiotic surfaces conducted for longer periods of time have unclear *in vivo* implications (Allegrucci et al., 2006; Allegrucci and Sauer, 2007; Vandevelde et al., 2014). Additional studies utilizing clinical isolates to study biofilms with longer biofilm formation times have been unable to show any association between the ability to produce *in vitro* biofilms on abiotic surfaces and *in vivo* virulence (Lizcano et al., 2010; Tapiainen et al., 2010). Furthermore, controversy exists in the literature regarding the correlation between biofilms grown *in vitro* on abiotic surfaces and their infectivity *in vivo* where investigators have suggested that biofilm bacteria are more likely (Trappetti et al., 2011b) or less likely (Sanchez et al., 2011b) to cause invasive disease. Our data at this point support the notion that biofilm bacteria are less virulent in invasive disease models (Marks et al., 2013). The virulence of biofilm bacteria will be covered in more depth in a separate review in this topic series by Orihuela et al. (Cross-reference to Orihuela review) (Gilley and Orihuela, 2014).

While these studies have been essential in building our understanding of pneumococcal accretion and biofilm formation, studies with more complex model systems that include physiological conditions and components modeling host–pneumococcal interactions have only recently shed more light on the phenotype of biofilm bacteria. In a study by Parker et al. bacteria recovered after adhering to epithelial cells had an increased ability to form biofilms on abiotic surfaces compared to bacteria with no previous exposure to epithelial cells (Parker et al., 2009). Also, Sanchez et al. found that biofilm bacteria grown on abiotic surfaces adhered better to epithelial cells than planktonic, broth grown bacteria (Sanchez et al., 2011b). These two studies, supported by studies in other human pathogens (Konkel et al., 1997; Sulaeman et al., 2012), demonstrate a relationship between epithelial cell adherence and biofilm formation, however, the studies have not investigated the role of this relationship during pneumococcal colonization.

FORMATION OF WELL-ORGANIZED AND STRUCTURED BIOFILMS DURING NASOPHARYNGEAL COLONIZATION

Researchers have speculated that pneumococci form biofilms in the nasopharynx *in vivo* (Waite et al., 2001; Oggioni et al., 2006; Munoz-Elias et al., 2008; Trappetti et al., 2009; Sanchez et al., 2011b). Recently, Marks et al. showed for the first time that pneumococci form highly structured biofilms during colonization of the murine nasopharynx (Marks et al., 2012a) BALB/c mice were inoculated intranasally with the pneumococcal strain EF3030, a clinical isolate known to be non-invasive and an efficient colonizer in murine models (Balachandran et al., 2002; Palaniappan et al., 2005; Shah et al., 2009). After 48 h, the pneumococcal carriage was 5×10^6 organisms per nasopharyngeal tissue, similar to other studies using EF3030 (Briles et al., 2003; Palaniappan et al., 2005; Shah et al., 2009). Scanning electron microscopy (SEM) images of excised nasopharyngeal tissue showed colonization on ciliated epithelium with a higher bacterial burden and increased biofilm density in posterior sections of the nasopharynx compared with the anterior sections (**Figure 1A**) (Marks et al., 2012a). In the anterior region we found pneumococcal single cells or diplococci scattered in the tissue. In contrast, aggregated and interconnected cells with tower and filamentous structures covered in extracellular matrix were observed in the posterior region layered on top of the ciliated epithelium. Other bacterial species were not identified in nasal tissues of the infected mice and no bacterial growth was observed in uninfected mice. These data have been confirmed by the Orihuela group that found biofilm formation on nasal septa during colonization of the murine nasopharynx (Blanchette-Cain et al., 2013). In their study, they found that biofilm formation during colonization required the CiaR/H two component system and that PsrP and SpxB had a major impact on bacterial aggregation, whereas CbpA, LuxS, and LytA had only modest effects.

BIOFILMS DISPLAY INCREASED RESISTANCE TO ANTIMICROBIAL AGENTS

Formation of bacterial biofilms confers greatly increased resistance to antimicrobial agents (Costerton et al., 1999; Donlan and Costerton, 2002; Chole and Faddis, 2003; Lewis, 2008; Sanchez

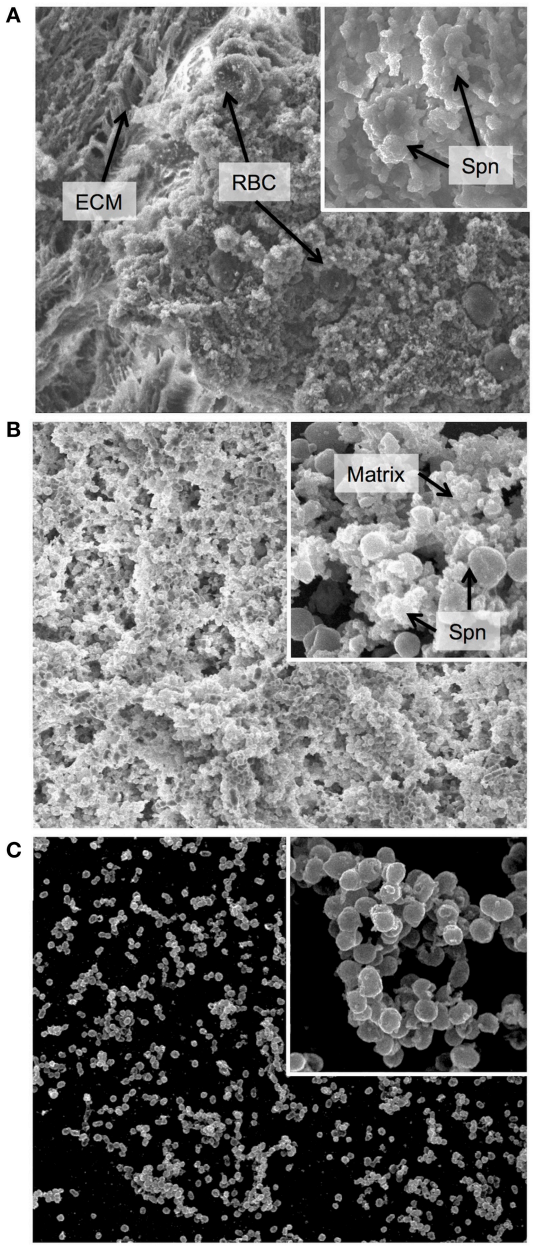

FIGURE 1 | Biofilm morphology. Scanning electron micrographs of biofilm communities formed **(A)** on the epithelial mucosa *in vivo*, **(B)** on epithelial cells grown *in vitro* and **(C)** on a glass substratum *in vitro*. The major image in each panel shows the biofilm at 2000x magnification and the insert in the upper right corner shows an increased magnification of 10,000x. ECM = extracellular matrix, used here instead of the more conventional EPS (extracellular polymeric substance) as secretion of specific polymeric substances have not yet been identified or characterized in *Streptococcus pneumoniae,* and the matrix is not well defined. RBC = red blood cell, Spn = *Streptococcus pneumoniae,* Matrix = biofilm matrix composed of extracellular substances and cellular debris. In general, the *in vivo* biofilms display a high degree of matrix formation that originated primarily from lysed bacterial cells and consists of cellular debris and DNA. Biofilms from *in vitro* cultures on epithelial cells show less encapsulation in matrix and more naked bacterial cells. However, biofilms formed on glass are much less developed with less biomass and almost no matrix formation.

et al., 2011b). The biofilm structure functions as a shield and protects the bacteria from the antimicrobials. Increased resistance, in this sense, has partly been attributed to a somewhat lower penetration of antibiotics into the biofilm structure but is probably equally or more associated with an adaptive phenotype shift of the biofilm bacteria (De Kievit et al., 2001; Drenkard and Ausubel, 2002; Nguyen et al., 2011; De La Fuente-Nunez et al., 2013). However, resistance to antimicrobial agents can be discussed as tolerance to antibiotics or as a result of acquired genes due to genetic exchange as will be discussed below in Section Role of Biofilm Formation during Pneumococcal Colonization. Biofilm bacteria constitute a heterogeneous population, with many bacteria in a more sessile state, having the "persister" phenotype described by Lewis (2008) or expressing other adaptive changes to resist environmental stressors (De La Fuente-Nunez et al., 2013).

This appears true also in pneumococcal biofilms. In our studies, treatment with gentamicin was used to test the functional and structural organization of the biofilm as the antibiotic is bactericidal against planktonic bacteria but does not penetrate well-organized biofilms effectively (Carmen et al., 2004; Abdi-Ali et al., 2006; Bartoszewicz et al., 2007). We also examined the effect of penicillin, a commonly used antibiotic, to provide clinically relevant results. Pneumococci closely associated with the murine nasopharyngeal tissue are highly resistant to gentamicin and penicillin while loosely associated bacteria are eradicated at a much lower concentration of antibiotics (Marks et al., 2012a), supporting the previous findings that showed a higher persistence of colonizing bacteria than those causing disease (Cohen et al., 1997, 1999; Dabernat et al., 1998; Dagan et al., 1998, 2001; Varon et al., 2000; Garcia-Rodriguez and Fresnadillo Martinez, 2002) and that biofilms are inherently more resistant to antibacterial agents (Costerton et al., 1999; Donlan and Costerton, 2002; Chole and Faddis, 2003; Lewis, 2008; Sanchez et al., 2011b). Enhanced resistance to aminoglycoside and beta-lactam antibiotics may also result from oxygen limitation as shown in *Escherichia coli* (Tresse et al., 1995, 1997). Altogether, these data suggest that biofilm formation during colonization may provide one mechanism that the pneumococci utilize to persist during antibiotic exposure in the human host.

MODELS TO STUDY BACTERIAL–HOST INTERACTIONS *IN VITRO*

A challenge in any study of host–bacterial interactions is to recapitulate *in vivo* findings using *in vitro* models. As described above (Section Introduction), the majority of work with pneumococcal biofilms has relied on *in vitro* model systems in which host-specific factors have not been included or examined. The nasopharyngeal environment contains a mucosal surface of respiratory epithelium and their secretions. This environment provides challenges to the bacterial organisms with low nutrient availability and also a lower temperature than the remaining body (approximately 32–34°C rather than 37°C) (Keck et al., 2000; Sahin-Yilmaz and Naclerio, 2011).

Our group has developed an *in vitro* model to simulate the upper respiratory tract, the site of pneumococcal colonization (Marks et al., 2012a). Biofilms grown on abiotic surfaces

were delayed in growth and had lower biomass and lacked structures seen in biofilms grown on epithelial cells or *in vivo* during nasopharyngeal colonization (**Figure 1**), suggesting that interactions with epithelial cells play an important role in biofilm formation (Parker et al., 2009; Sanchez et al., 2011b). Biofilms grown on live or fixed epithelial substrata formed complex biofilms with high biomass, similar matrix formation, general architecture and organization, and functional characteristics (e.g., antibiotic resistance) as biofilms formed *in vivo* in the mouse nasopharynx, thereby providing a suitable *in vitro* surrogate model for biofilm formation *in vivo* (**Figure 1B**). Both healthy human respiratory epithelial cells grown and differentiated in an air-liquid interphase and bronchial carcinoma cells support robust biofilm development (**Figure 1B**). This was not observed when biofilms were grown under the same conditions on plastic surfaces (**Figure 1C**). Moreover, the differences in structure and maturation also impacted on levels of gentamicin resistance; with biofilms grown on abiotic surfaces having decreased antibiotic resistance compared to biofilms grown on epithelial substrata. These phenotypic differences may indicate that abiotic surfaces lack important *in vivo* features to support optimal biofilm formation. There is one other group that has used epithelial cells as a substratum for biofilm formation. Vidal et al. used both paraformaldehyde-fixed HEp-2 epithelial and A549 lung carcinoma cells to produce static biofilms *in vitro* and also produced biofilms in a flow chamber bioreactor (Vidal et al., 2013). Consistent with our studies, the biomass of the biofilms was significantly higher in the presence of epithelial cells, and in their system more biomass was associated with the lung cells than the HEp-2 cells. Using this system, the authors were able to verify their earlier studies showing that both competence induction and autoinducer production is important for early biofilm formation (Vidal et al., 2011, 2013).

Environmental factors such as temperature and nutrient availability also impact biofilm formation. An environment of 34°C results in more dense and functional biofilms than biofilms formed at 37°C, measured both through morphology in SEM and by resistance to antimicrobial agents. These data suggest that the physiological temperature of the nasopharyngeal niche provides more optimal conditions to support biofilm formation. Finally, nutrient availability also impacts biofilm formation as nutrient-rich media did not support biofilm development as well as media containing fewer nutrients (Marks et al., 2012a,b).

CORRELATION BETWEEN BIOFILM FORMATION AND THE ABILITY FOR *IN VIVO* COLONIZATION

To validate the degree of correlation between our *in vitro* biofilm model and *in vivo* colonization, we compared the functional biofilm formation of pneumococcal strains with the bacterial burden during colonization of the same strains. The ability to form biofilms on epithelial cells directly correlated with the ability to colonize the murine nasopharynx (Marks et al., 2012a). Specifically, clinical isolates (EF3030 and BG8826) known to be effective colonizers of the murine nasopharynx (Lipsitch et al., 2000; Briles et al., 2003) formed more developed biofilms with higher biomass and biofilm-specific antibacterial resistance on epithelial cells than more invasive strains that are known to colonize the murine nasopharynx less effectively (D39, WU2, and SP670) (Benton et al., 1995; Briles et al., 2003; Mizrachi-Nebenzahl et al., 2003; Orihuela et al., 2003). Moreover, colonization-deficient strains in the D39 background that lacked virulence-associated factors, such as autolysin, pneumolysin, and PspC formed less structured, more antibiotic-sensitive biofilms, whereas pneumococci lacking PspA that is not associated with early colonization showed normal biofilm formation (Marks et al., 2012a). These differences were not observed in biofilms formed on abiotic surfaces.

ROLE OF BIOFILM FORMATION DURING PNEUMOCOCCAL COLONIZATION

Forming biofilms during colonization may serve several purposes for pneumococci. The biofilm provides a protective environment, in which the bacteria can adapt to coexist with the host by down-regulating factors involved in inducing inflammation in favor of factors used to scavenge nutrients from the harsh environment in the nasopharynx. This will be discussed more below in Section Distinct Phenotypic Properties of Dispersed Pneumococci. Another benefit of biofilm communities is the closeness of bacterial cells to each other as well as the proximity to DNA that makes up part of the extracellular matrix, providing an excellent environment to exchange genetic material to promote survival and adaptation to the host environment.

Pneumococci are highly competent organisms and their genome sequences show extensive signs of horizontal transfer of genetic material. The mechanism of competence initiation, DNA uptake and integration has been well studied in *S. pneumoniae* (Johnsborg and Havarstein, 2009) since the first observation of natural genetic transformation by Griffith (1928). Horizontal gene transfer is important for adapting to environmental stresses (Stewart and Carlson, 1986; Johnsborg et al., 2007), as it enables the acquisition of novel traits and the spread of antibiotic resistance (Majewski et al., 2000; Hakenbeck et al., 2001; Claverys et al., 2007). Previous studies have reported low levels of spontaneous DNA uptake and transformation in *S. pneumoniae* strains *in vivo* (Griffith, 1928; Ottolenghi and Macleod, 1963; Conant and Sawyer, 1967; Zhu and Lau, 2011). However, these *in vivo* studies were performed in the context of sepsis or other disease states where the level of biofilm formation is low. Additionally, most of the studies investigating natural transformation have used hypercompetent lab strains derived from Avery's experiments (Avery et al., 1944) or clinical isolates that require the addition of synthetic competence-stimulating peptide (CSP) (Pozzi et al., 1996; Wei and Havarstein, 2012).

Epidemiological studies suggest that colonizing bacteria rather than bacteria from invasive disease are the source of horizontal transfer or spread of antibiotic resistance between strains (Christenson et al., 1997; Nasrin et al., 1999; Ronchetti et al., 1999; Domenech et al., 2009) and that resistance selection occurs mainly in pneumococci colonizing young children, an age-group that has high carriage rates and exposure to antibiotics that consequently favor the selection of drug-resistance (Duchin et al., 1995; Samore et al., 2001; Brugger et al., 2009). This is supported by studies suggesting that natural transformation in the nasopharynx is facilitated by co-colonization of multiple pneumococcal

strains (Donkor et al., 2011; Leung et al., 2011). In addition, pneumococcal biofilm formation during colonization of the nasopharynx has been shown to up-regulate competence genes (Oggioni et al., 2006; Trappetti et al., 2011a).

INCREASED NATURAL TRANSFORMATION IN BACTERIA DURING COLONIZATION COMPARED WITH SEPSIS

To further study the *in vivo* signals and host conditions involved in increased natural transformation between strains *in vivo*, we performed experiments using our *in vitro* biofilm model as well as investigated transformation during colonization. When BALB/c mice were inoculated intranasally or intraperitoneally with equal numbers of *S. pneumoniae* strains SP670 (a clinical penicillin-resistant strain) and D39-C08P2 (a laboratory strain with an erythromycin cassette inserted downstream of the dihydrolipoamide dehydrogenase gene), natural transformation only occurred in bacteria colonizing the nasopharynx (Marks et al., 2012b). The transformation efficiency (the ratio of the number of double-resistant colonies over the total recovered population) indicated that colonizing bacteria in the murine nasopharynx showed a surprisingly high level of natural transformation (efficiency of $\sim 1 \times 10^{-2}$) whereas the natural transformation efficiency during sepsis was very low and similar to what has been presented in the literature (efficiency of 3×10^{-9}) (**Figure 2**). Thus, the transformation efficiency during colonization was approximately 10^7-fold higher than during sepsis. Also, sequential nasopharyngeal colonization, where one strain was inoculated and left to colonize the animals for 48 h before the other strain was added intranasally, had similar transformation efficiencies as when the strains were inoculated simultaneously (**Figure 2**). This model better mimics the natural, sequential acquisition of strains and the combined data is in agreement with epidemiological studies suggesting that colonizing bacteria are the predominant source of horizontal transfer of genes between strains (Christenson et al., 1997; Nasrin et al., 1999; Ronchetti et al., 1999; Doit et al., 2000).

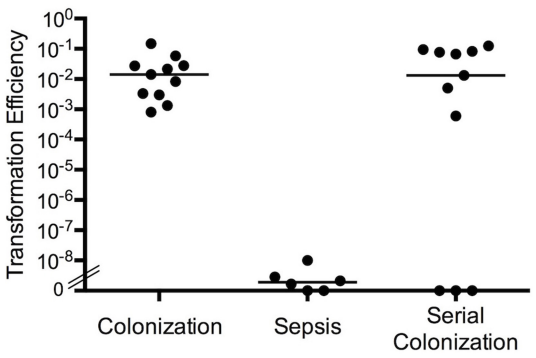

FIGURE 2 | Transformation efficiency in biofilm cultures and during nasopharyngeal colonization. Transformation efficiency of antibiotic resistance elements between *S. pneumoniae* strain SP670 (Pen^R) and D39-C08P2 (Erm^R). Mice were co-colonized with both strains at the same time, colonized sequentially with one strain added to the nares of mice 48 h prior to the second strain, or both strains were used for co-infection in a septicemia model in mice. Transformation efficiency was measured as the number of double-antibiotic resistant colonies divided by total recovery of bacteria from each condition.

The *in vivo* data could be corroborated *in vitro* using biofilms grown on epithelial cells at 34°C (Marks et al., 2012b). Seeding epithelial cells with equal numbers of the two antibiotic-resistant strains resulted in high numbers of double-resistant organisms. The highest transformation frequency was observed between 48 and 72 h after inoculation, which corresponded directly to the time points when the average competence gene expression in the biofilm population was highest. As our previous work demonstrated that pneumococcal biofilm formation occurs during nasopharyngeal colonization (Marks et al., 2012a) and transformation efficiency is increased in co-colonization or serial colonization compared to sepsis (Marks et al., 2012b), this suggests that biofilm formation plays a role in the increased genetic exchange seen during colonization. This is in agreement with another study showing more efficient gene transfer among streptococci in early biofilm structures (Wei and Havarstein, 2012). Moreover, several studies show that the matrix of most biofilms contains high concentrations of DNA that originate from lysis of bacterial cells in the biofilm (Thomas et al., 2009; Kiedrowski et al., 2011; Liu and Burne, 2011; Montanaro et al., 2011). Lysis can be obtained through autolysis but may also result from phage-mediated bacterial host lysis, enhancing pneumococcal biofilm development as measured by biomass and cell viability (Carrolo et al., 2010). In addition, it is known that the pneumococcal process of fratricide releases DNA from a subfraction of the population by triggered cell lysis due to competence development (Steinmoen et al., 2002). Biofilm growth may therefore provide an optimal environment for genetic exchange, further suggested by data where encapsulated strains that show no natural transformation *in vitro* during growth in broth can integrate resistance cassettes during biofilm growth with a transformation efficiency of 10^{-3} to 10^{-4} after addition of extracellular chromosomal DNA (1 μg/mL) without exogenous addition of CSP or antibiotic pressure (Marks et al., 2012b).

MECHANISMS OF INCREASED NATURAL TRANSFORMATION IN BIOFILMS

Induced competence

Biofilms have been shown to upregulate competence genes compared with broth-grown bacteria (Oggioni et al., 2006; Trappetti et al., 2011a). As mentioned above, it appears that competence is continuously upregulated during biofilm growth on epithelial cells. This does not necessarily mean that the total population of the biofilm is competent all the time. Rather, biofilms are heterogeneous and dynamic populations, suggesting that although the average competence gene expression in a biofilm is continuously high, this most likely reflects up- and down-regulation of competence in subpopulations within the biofilms. Under optimal biofilm-growth conditions, the constant presence of exogenous CSP did not increase the already high transformation efficiencies in biofilms (Marks et al., 2012b). However, when biofilms were formed under sub-optimal conditions such as in the presence of rich media (Todd-Hewitt medium containing yeast extract, THY) or at 37°C rather than 34°C, or on abiotic surfaces, addition of CSP significantly improved both biofilm formation and transformation efficiencies. The role of competence induction in biofilm formation is supported by several investigators that have

shown that inclusion of competence stimulating peptide increases the biomass of biofilms (Oggioni et al., 2006; Trappetti et al., 2011c). These differences were not seen in assays testing natural transformation during planktonic growth.

Capsule down-regulation

Capsule expression is affected by environmental factors (Selinger and Reed, 1979; Kim and Weiser, 1998; Weiser et al., 2001; Hammerschmidt et al., 2005) and phenotypic variation can occur in the transition from nasopharyngeal carriage to invasive disease (Waite et al., 2003). Transparent variants with thinner capsule are predominantly found during initial colonization while opaque strains with thicker capsule are found during invasive disease (Weiser et al., 1994; Cundell et al., 1995; Kim and Weiser, 1998; Kim et al., 1999). Increased capsule expression results in decreased transformation efficiency (Ravin, 1959) and only unencapsulated strains have been found to be naturally transformable in broth. We have found that the capsule locus is downregulated in biofilms compared with bacteria grown in broth (Marks et al., 2012b). Similar results have been presented in another study where biofilms grown on an abiotic substratum were compared with planktonic cultures (Hall-Stoodley et al., 2008). Altogether, these data suggest that capsule down-regulation during biofilm formation and colonization result in the increased transformation efficiency seen during biofilm growth.

Epithelial interactions

As the down-regulation of capsule was more pronounced when grown on epithelial cells than when biofilms formed on abiotic surfaces (Hall-Stoodley et al., 2008; Marks et al., 2012b), epithelial cells may play a major role in this regard. This is supported by a study from Hammerschmidt's laboratory showing that pneumococci downregulate their capsule when adhering to epithelial cells (Hammerschmidt et al., 2005). In our dual-strain biofilm studies, biofilms formed both on prefixed epithelial cells or glass displayed an elevated level of transformation efficiency (Marks et al., 2012b). However, the presence of a prefixed epithelial substratum resulted in a higher transformation efficiency than observed on glass. This further indicates the significance of bacteria–host interactions for optimal biofilm formation, which in turn potentiates effective transformation.

Nutrient availability

Other studies have shown that ion and nutrient concentrations play a role during transformation of planktonic pneumococcal cultures (Lacks and Greenberg, 1973; Chen and Morrison, 1987; Trombe, 1993). As previously mentioned, pneumococci grown in varying nutrient conditions show different abilities to form biofilms that correspond with their ability to promote transformation. Limited nutrients seems to be important for optimal biofilm formation as rich, complex media (THY) resulted in poor biofilm formation with low transformation efficiencies compared to biofilms formed in chemically defined media (CDM) (Marks et al., 2012b). Therefore, the nutrient environment seems to influence genetic exchange through its initial effects on biofilm formation.

Nasopharyngeal temperature

Temperature has been found to modulate competence development in pneumococci cultures (Lacks and Greenberg, 1973; Steinmoen et al., 2003) and studies of the role of temperature on transformation efficiency in broth cultures have indicated that transformation efficiency peaks around 32–34°C and decreases with increasing and decreasing temperatures (Hotchkiss, 1957). During colonization of the upper respiratory tract, pneumococci are exposed to temperatures of about 34°C, which are closer to the optimal temperature for transformation than is body temperature. Dual-strain biofilms consisting of strains with separate antibiotic-resistance markers were able to form at 37°C although with lower biomass than seen at 34°C (Marks et al., 2012b). However, temperature was extremely important for natural transformation in biofilms as no transformants could be recovered at the higher temperature while high transformation efficiency was seen at 34°C. For one strain pair, biofilms did not form at 37°C, whereas at 34°C this strain pair was able to form biofilms with high transformation efficiency. Similar results were seen in single-strain biofilms with the addition of exogenous DNA, although differences between the two temperatures were not as distinct. However, biofilms with comparable biomasses had similar transformation efficiencies.

Biofilm formation occurs during colonization of the nasopharynx by *S. pneumoniae*. This niche has specific growth conditions, including epithelial interactions, nutrient availability, and temperature that are optimal for the formation of biofilms. In contrast to planktonic growth, downregulation of capsule and induction of competence occurs in biofilms. Together these environmental factors are important both for pneumococcal biofilm formation *in vitro* and during nasopharyngeal colonization *in vivo*, as well as for the ensuing increased genetic exchange and natural transformation.

POPULATION DYNAMICS AND INCREASED FITNESS

Natural genetic transformation in the biofilm environment serves to increase the adaptation of the bacteria to a changing host environment and thus increases the fitness of the organism. Our mechanistic studies of biofilm-associated transformation revealed a separate mechanism whereby biofilms can promote fitness. We used a PspC- and PspA-negative strain (TRE121; erythromycin-resistant and tetracycline resistant) to investigate whether the *pspC* locus required for colonization (Balachandran et al., 2002) could be repaired if grown in the presence of the wild-type strain. Repair could then be observed by detecting only erythromycin-resistant bacteria carrying the PspA mutation as PspA is not required for early colonization. Mice inoculated with *pspA/pspC* null pneumococci alone were rapidly cleared. However, intranasal inoculation of a mixture of TRE121 and D39 pneumococci resulted in a population with the *pspC* gene (erythromycin resistant and tetracycline sensitive) repaired genetically and functionally. These studies supported that natural transformation during co-colonization can improve fitness by expanding the gene pool available for adaptation to the host environment (Marks et al., 2012b).

However, in the same experiment, where TRE121 were co-colonized with wild type D39, we were also able to isolate the

original PspC- and PspA-negative mutant, that when colonized alone was rapidly cleared. When performing our transformation experiments there seemed to be a trend, although not statistically significant, of increased biomass in multi-strain biofilms than single-species biofilms with equal inocula. Further investigations of dual-strain biofilms *in vitro* revealed that poor biofilm formers showed an increased biomass in the presence of good biofilm-forming strains. This increased fitness was not directly associated with acquisition of genetic factors as strains had the same colonization efficiency before and after co-colonization experiments. This fitness increase was also observed in co-colonization experiments with unencapsulated and encapsulated strains. In addition to providing an optimal environment for genetic exchange, co-colonization may also provide a haven for poorly colonizing strains when an effectively colonizing strain is also present. These data are supported by epidemiological studies showing the detection of rare serotypes or non-typeable pneumococci significantly more often in individuals colonized with multiple strains than with single strains (Brugger et al., 2010).

MECHANISM OF TRANSITION FROM ASYMPTOMATIC BIOFILM COLONIZATION TO DISSEMINATION AND DISEASE

Pneumococcal colonization of the nasopharynx is frequent in children (20–90%, with the higher numbers observed in resource-poor settings (Hill et al., 2006; Coles et al., 2011; Kwambana et al., 2011; Abdullahi et al., 2012)), and decreases, although not completely, in adulthood. Colonization always precedes infection (Kadioglu et al., 2008), however, the mechanism involved in the transition from biofilm colonization to disease is not entirely clear. Numerous studies have suggested that pneumococcal infection is associated with preceding or concomitant virus infections (Henderson et al., 1982; Chonmaitree et al., 1986, 2012; Kim et al., 1996; McCullers, 2006; Bakaletz, 2010; Pettigrew et al., 2011; Launes et al., 2012; Chertow and Memoli, 2013; Short et al., 2013) while other studies suggest that virus infections increase bacterial growth or dissociation from the nasopharyngeal tissue (Diavatopoulos et al., 2010; Vu et al., 2011). For example, Influenza A virus (IAV) is associated with an increased susceptibility to pneumococcal pneumonia (Morens et al., 2008; Shrestha et al., 2013; McCullers, 2014). IAV pathogenesis involves invasion and killing of respiratory epithelial cells, increased bacterial adhesion receptors in the respiratory niche, and suppression of immune responses to *S. pneumoniae* (McCullers and Bartmess, 2003; Sun and Metzger, 2008; Koppe et al., 2012; McCullers, 2014). Furthermore, IAV infection is associated with increased spread between infant mice, suggesting a role for IAV in release of pneumococci from biofilm colonization in order to spread between individuals (Diavatopoulos et al., 2010). While virus infection and host signals seem to influence nasopharyngeal biofilm communities, the exact mechanism(s) whereby transition from asymptomatic colonization to disease occur have been less studied.

INFLUENZA A VIRUS INFECTION PROMOTE BIOFILM DISPERSAL *IN VITRO* AND TRANSITION TO DISEASE *IN VIVO*

In vitro biofilm dispersal

Using IAV as a model system we have recently attempted to address the factors associated with disruption of biofilm colonization in the nasopharynx. Previous models with human respiratory epithelial cells (HRECs) have been limited by short coexistence times between the bacteria and epithelial cells (Hakansson et al., 1996; Marks et al., 2012a; Vidal et al., 2013). We recently developed a static biofilm model with live cultures of HRECs that survived with biofilm bacteria for up to 72 h and permitted the study of the role of virus infection on biofilm integrity (Marks et al., 2013).

Pneumococcal biofilms that were first formed on fixed HRECs were moved to live cells and were allowed to reestablish a biofilm for 24 h. At this time, IAV infection of the epithelial cells were performed. At 24 h after IAV infection, the total bacterial load did not differ between cells infected or not infected with virus. However, about 10-fold more bacteria were found in the supernatant than in the biofilm communities associated with the virus-infected epithelium. The increased bacterial numbers in the supernatant was found for several pneumococcal strains and was not associated with detachment of cells.

In vivo transition to disease

As IAV infection of epithelial cells *in vitro* results in release of bacteria from biofilms, we investigated the impact of IAV infection on pneumococcal colonization *in vivo* (Marks et al., 2013). Mice were colonized intranasally with EF3030 or D39 pneumococci for 48 h, the mice were then inoculated with IAV, and bacterial burden in various tissues was measured at days 1 and 5 post infection. EF3030 biofilms maintained stable colonization of the nasopharynx over 5 days, with a slightly higher level of colonization in the IAV-infected population. The increased colonization after IAV infection has been observed in earlier studies (Hirano et al., 1999; Tong et al., 2000; Garcia-Rodriguez and Fresnadillo Martinez, 2002; Diavatopoulos et al., 2010) and was recently shown to rely on increased growth of pneumococci due to increased availability of sialic acid from IAV neuraminidase activity (Siegel et al., 2014). Associated with the increased colonization, dissemination into the lungs and the middle ear of EF3030 increased over time in the presence of IAV. For D39, colonization was higher in the IAV population but total colonization decreased over time and, although IAV caused dissemination both into the lungs and the middle ear, the initial dissemination and bacterial burden decreased over time. These results showed that IAV infection could cause active egress of bacteria from biofilms and that those bacteria could disseminate in the host to otherwise sterile sites where they caused infection.

THE ROLE OF IAV-INDUCED CHANGES IN THE HOST ENVIRONMENT ON BIOFILM DISPERSAL AND TRANSITION TO DISEASE

In vitro biofilm dispersal

Upon IAV infection, a 10-fold increased ATP concentration was detected in the biofilm supernatant at 24 h, which was similar both in fold-change and in levels detected in the nasopharyngeal lavage fluid from mice infected with IAV for 24 h. Extracellular ATP as well as the recently described IAV-induced sympatomimetic response resulting in release of norepinephrine (NE) in the nasopharyngeal secretions constitutes well described "danger signals" potentially recognized by bacterial cells (Grebe et al., 2010; Xi and Wu, 2010). Additionally, symptomatic IAV infection

is likely to cause increased or changed nutrient availability in the nasopharynx and is usually accompanied with fever, two additional factors that were shown above to have a negative impact on biofilm formation and genetic transformation (Marks et al., 2012b). To avoid host cell-mediated responses, potential host agents induced by virus infection were applied exogenously to biofilms formed on fixed epithelia. The addition of NE, ATP, glucose, or HREC lysate induced dispersal of bacteria from tissue-attached biofilm communities into the supernatant, predominantly in the form of diplococci, a pneumococcal morphology previously found in the bloodstream or sputum of patients and animals (Tomasz et al., 1964). Exposure to febrile-range hyperthermia (FRH) at an elevated temperature of 38.5°C showed similar results and the combination of 38.5°C and HREC cell lysates showed additive effects, suggesting that during IAV infection the combined effect of the changing host environment likely produced the dispersal of bacteria from biofilms.

In vivo transition to disease

Similar to our *in vitro* studies above, host signals (ATP, NE, glucose, and FRH) resulted in dispersion of EF3030 and D39 from the nasopharynx and caused dissemination of pneumococci into the lungs or middle ear. These data show that host-derived inter-kingdom signals alone or in conjunction with IAV infection cause active dispersal of bacteria from the biofilm, which can subsequently disseminate to normally sterile sites and cause symptomatic infection (Marks et al., 2013). The mechanisms of IAV-induced transition from colonization to infection are depicted in **Figure 3**. The recognition of host factors by bacteria is an underexplored area (Hughes and Sperandio, 2008; Pacheco and Sperandio, 2009) where the main examples of how bacteria recognize the host environment is associated with sensory membrane kinases of two component systems and with a few examples known where bacteria can recognize and bind host-specific molecules such as cytokines. Future studies focused on understanding how bacteria recognize changes in their environment will be of great interest to understand host–pathogen interaction both during colonization and infection. A better knowledge of these mechanisms may help provide novel strategies to avoid transition to infection.

DISTINCT PHENOTYPIC PROPERTIES OF DISPERSED PNEUMOCOCCI

IN VIVO PHENOTYPE OF DISPERSED, PLANKTONIC, AND BIOFILM POPULATIONS

It has been shown that biofilm bacteria display lower virulence *in vivo* than broth-grown bacteria (Blanchette-Cain et al., 2013; Qin et al., 2013). However, the specific virulence phenotype of bacteria that are actively released from biofilms in response to a changing host environment (increased temperature, virus

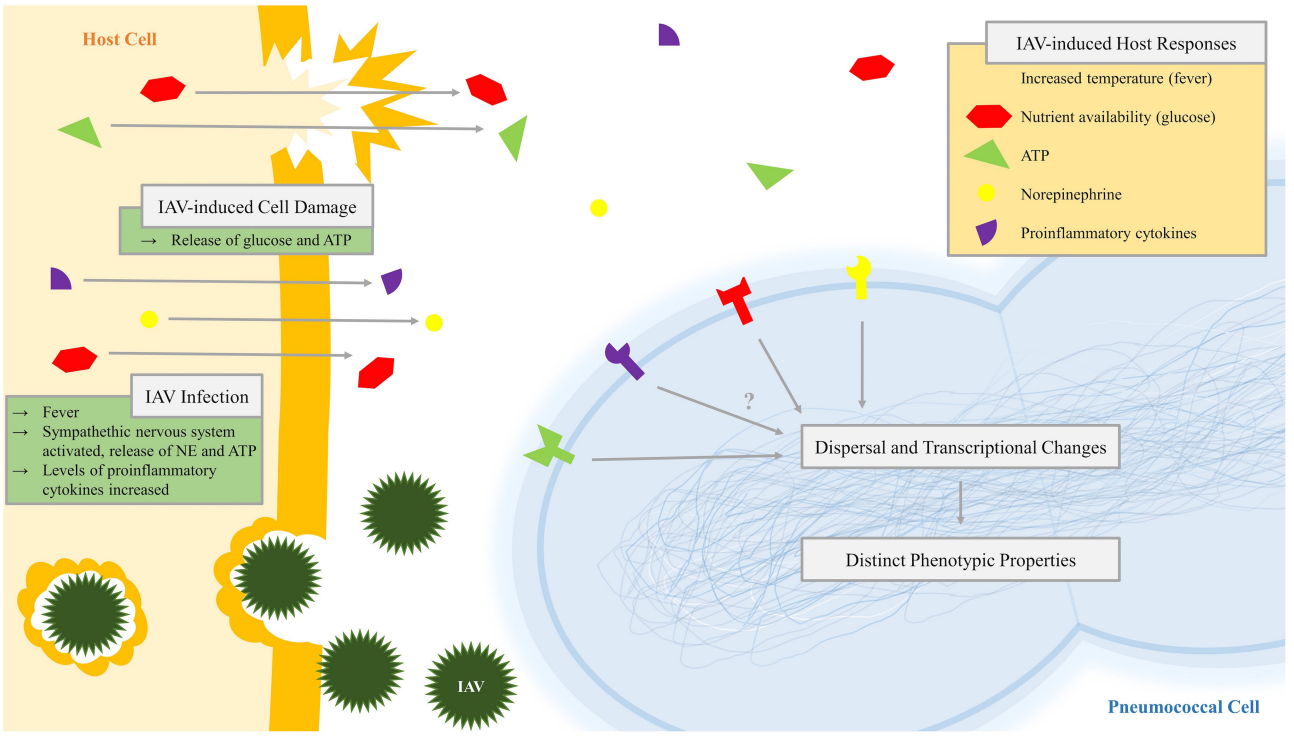

FIGURE 3 | Diagram of pneumococcal recognition of influenza-induced host responses leading to dispersal and transcriptional change. Infection with influenza A virus (IAV), which may include virus-induced host cell damage, leads to changes in the pneumococcal niche environment including virus-induced host responses such as increased temperature (fever), nutrient availability (glucose), extracytoplasmic ATP, norepinephrine, and proinflammatory cytokines. Pneumococci recognize these inter-kingdom signals by an unknown mechanism, leading to dispersal and changes in the transcriptome and resulting in pneumococcal populations with distinct phenotypic properties.

infection, etc.) has not been well described. Using an *in vivo* murine model of colonization and dissemination, we were able to confirm that actively-dispersed bacteria have a distinct phenotype from biofilm or planktonic, broth-grown bacteria (Marks et al., 2013). In general, the dispersed pneumococci were able to colonize the nasopharynx as well as the other populations, but disseminated into the lungs and middle ear at a higher degree than both planktonic, broth-grown bacteria and biofilm bacteria. This is in agreement with a previous study where opaque, broth-grown bacteria were able to translocate to the lungs and brain of mice while transparent, biofilm-derived bacteria remained in the nasopharynx (Trappetti et al., 2011b).

Actively-dispersed bacteria also induced a higher level of inflammation. Histological examination 7 days after colonization showed that mouse tissue infected with dispersed bacteria resulted in denudation of the epithelium, later supported in other studies (Blanchette-Cain et al., 2013), and had the presence of pronounced leukocyte infiltrates in the lungs and middle ear cavity. In agreement with the low bacterial load found in the tissues infected with biofilm-grown bacteria, there were no inflammatory infiltrates present. However, the nasal epithelium had shorter cilia compared to mock-infected mice. Mice inoculated with planktonic, broth-grown bacteria displayed a mixed phenotype, showing areas of epithelial denudation and some inflammation in the middle ear and lungs. Histological results were very similar between D39 and EF3030 pneumococci with the exception that no D39 bacteria were isolated from the middle ear.

After direct aspiration of bacterial populations into the lungs of mice, biofilm bacteria were cleared over time, induced minimal inflammation, and did not disseminate into the bloodstream. In contrast, temperature-dispersed biofilm bacteria caused high levels of bacterial burden in the lungs with three out of six mice challenged with EF3030 and all of the mice challenged with D39 showing pneumococcal dissemination into the bloodstream. Histological analysis of the lungs infected with dispersed bacteria showed a dense leukocyte infiltrate with hemorrhagic lesions, while planktonic, broth-growth bacteria showed a moderate bacterial burden in the lungs, resulting in moderate inflammation.

These phenotypes were not only specific to tissue infections. Intraperitoneal challenge with EF3030 or D39 biofilm bacteria resulted in rapid clearance of the bacteria from the bloodstream. D39 bacteria are well-characterized for their invasive potential (Smith et al., 2002) and resulted in a higher bacterial titer in the blood after 24 h. Actively-dispersed D39 bacteria showed an even more aggressive phenotype where the mice were more symptomatic and the majority of the mice had to be euthanized before 24 h based on becoming moribund (**Figure 4**). The phenotype was especially interesting when the strain EF3030 was used, as this strain when grown in broth failed to induce bacteremia and direct injection of 10^8 CFUs of EF3030 broth-grown bacteria were cleared within the 24 h. In contrast, injection of approximately 10^5 CFUs of EF3030 bacteria dispersed from biofilms after IAV infection or exposure to heat or extracellular ATP resulted in septicemia in all mice, with some mice becoming moribund before the end of the experiment (**Figure 4**). Interestingly, dispersed organisms caused a higher level of inflammation in the bloodstream and the animals became moribund at significantly lower bacterial levels in the blood. These results indicate that actively-dispersed pneumococci, that show high virulence and inflammatory potential, produce infection in animals that more closely resembles invasive pneumococcal disease in humans (Marks et al., 2013). Actively-dispersed pneumococci may thus provide a model that better mimics the physiological phenotype during human infection.

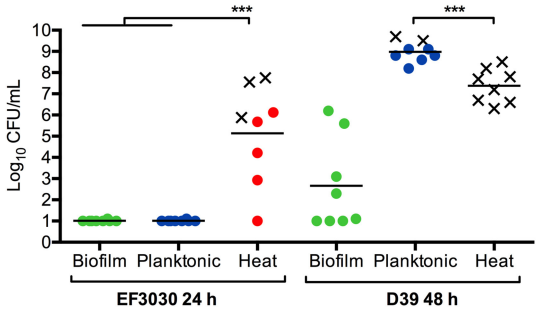

FIGURE 4 | Biofilm dispersion and infection. Mice were inoculated intraperitoneally with mechanically disrupted biofilm bacteria (Biofilm; green), broth-grown bacteria (Planktonic; blue), or heat-dispersed bacteria (Heat; red). Bacterial burden was measured in the blood after 24 h for EF3030 bacteria or 48 h for D39 bacteria or when mice became moribund. An X represents a mouse that was euthanized before the end of the experiment. $^{***}P < 0.001$.

DISPERSED BACTERIA ARE A DISTINCT POPULATION DIFFERENT FROM BIOFILM OR PLANKTONIC, BROTH-GROWN BACTERIA

The major differences in the virulence phenotype of biofilm bacteria, broth-grown bacteria, and actively-dispersed pneumococci suggest that these populations are distinct and likely have major differences in their transcriptional profiles. Previous studies have shown that some virulence genes are down-regulated in biofilm bacteria compared to broth-grown bacteria (Sanchez et al., 2011b) and that changes in the host environment results in alterations of pneumococcal transcriptional profiles (Orihuela et al., 2004b; Ogunniyi et al., 2012). These environmental signals include IAV-induced host responses such as rises in temperature (fever), nutrient availability, ion concentrations, and proinflammatory cytokines (Bakaletz, 2010; Grebe et al., 2010; Weiser, 2010) that trigger biofilm dispersal, leading to a distinct population of biofilm-dispersed bacteria showing an increased ability to disseminate and cause disease (Marks et al., 2013).

TRANSCRIPTIONAL DIFFERENCES IN ACTIVELY-DISPERSED PNEUMOCOCCI

To better understand the transcriptional profiles of dispersed bacteria, gene expression profiles of dispersed bacteria were compared to expression profiles in planktonic, broth-grown bacteria and to biofilm bacteria grown on fixed or live epithelial cells for 48 h using qRT-PCR of selected genes (Marks et al., 2013). Similar to previous work, competence genes were up-regulated in biofilm-grown bacteria compared to dispersed and planktonic, broth-grown populations while other genes involved in virulence, such as *cps* (capsule), *ply* (pneumolysin), *pavA* (adhesin), and *licD2* (opaque phenotype), were down-regulated during

biofilm growth, consistent with previous reports (Sanchez et al., 2011b; Marks et al., 2012b). More importantly, planktonic, broth-grown bacteria had different expression of *lytA*, *licD2*, and *pavA* compared with dispersed bacteria, the latter population showing significantly higher expression. These differences in gene expression suggests that the three populations are phenotypically distinct. Besides virulence differences, actively-dispersed pneumococci had a higher opaque to transparent ratio, adhered poorly to HRECs, but invaded and killed HRECs more effectively, as well as induced higher levels pro-inflammatory cytokine responses from the exposed HRECs (Marks et al., 2013).

In addition to the gene targeted RT-PCR approach, we used RNA-seq to obtain a global transcriptional profile among different samples, and identified complex alterations in the pneumococcal transcriptome in response to IAV-induced changes in the environment (Pettigrew et al., 2014). Among the actively-dispersed pneumococcal populations, IAV-induced dispersion had the most impact on the pneumococcal transcriptome compared to biofilm-grown bacteria. This was seen in both the fold-change and the number of differentially regulated genes. When combining the changes observed in IAV-, heat-, and ATP-dispersed populations of pneumococci, 90 differentially regulated genes were significantly changed in the same direction in at least two out of three dispersed populations compared to biofilm-grown bacteria. In general, carbohydrate metabolism, stress response, and known virulence factors were up-regulated in dispersed populations while genes associated with competence, amino acid metabolism, pyrimidine and purine metabolism, translation, and some regulatory genes were downregulated. These data correlate very well with a recent study demonstrating an increased expression of genes involved in cell wall biosynthesis, translation, and purine and pyrimidine metabolism in biofilm bacteria (Yadav et al., 2012). The data also correlate in part with a recent proteomics analysis that showed a changed metabolism in biofilm bacteria (Allan et al., 2014). However, as this analysis compared biofilms to planktonic, broth-grown bacteria that are very different in their transcriptional profile to actively dispersed bacteria, a direct comparison of the results are difficult to make.

Among the 20 out of 90 genes that were regulated in different directions in the dispersed populations, eight were genes involved in bacteriocin production and secretion. These genes were upregulated in IAV- and heat-dispersed pneumococci that showed the highest virulence in our murine model and were down-regulated in the ATP-dispersed population that showed the least virulent phenotype, suggesting a potential role of bacteriocins in virulence. Overall, similar patterns were seen in the comparison between actively-dispersed and planktonic, broth-grown bacteria.

The RNA-seq data showing differentially expressed genes involved in carbohydrate metabolism corresponded well with the direct measurement of glucose metabolism among the pneumococcal populations, with a higher production of intracellular ATP and lactate secretion (main product of pneumococcal glucose fermentation) in dispersed populations compared to biofilm-grown bacteria. In addition, biofilm bacteria had a lower baseline ATP level, suggesting low metabolic activity. Genes regulating carbohydrate metabolism have been associated with tissue-specific disease (Orihuela et al., 2004a; Iyer and Camilli, 2007; Ogunniyi et al., 2012), which is a similar pattern seen with the more virulent dispersed populations showing upregulation of genes associated with carbohydrate metabolism. However, there was not a direct correlation between glucose metabolism and virulence among the pneumococcal populations. In addition, there was variability in gene regulation and glucose metabolism among heat- and ATP-dispersed populations even though heat-dispersed pneumococci were more similar though not as virulent as IAV-dispersed while ATP-dispersed pneumococci were the least virulent dispersed population. These data indicate that virulence and transcriptional changes in response to environmental signals are complex.

CONCLUSIONS

Colonization by *S. pneumoniae* precedes disease and studies have shown that colonization is a necessary step in pneumococcal pathogenesis (Weiser, 2010; Simell et al., 2012). While there is evidence for the role of biofilms in disease (Hall-Stoodley et al., 2006; Sanderson et al., 2006; Hoa et al., 2009; Reid et al., 2009; Sanchez et al., 2010; Weimer et al., 2010; Sanchez et al., 2011b; Trappetti et al., 2011b; Blanchette-Cain et al., 2013), the role of biofilms in pneumococcal colonization has only recently been investigated. Asymptomatic colonization occurs within complex multicellular biofilm communities (Munoz-Elias et al., 2008; Marks et al., 2012a) while pneumococci from the blood and sputum exist as diplococci (Tomasz et al., 1964). Host–bacterial interactions are necessary for optimal biofilm formation displaying increased antibiotic resistance (Marks et al., 2012a). Furthermore, environmental conditions in this niche are important for increased genetic exchange and increased fitness either by expanding the genes available or through protective effects (Marks et al., 2012b). As summarized in **Figure 5**, these sessile, predominately transparent phase communities down-regulate virulence factors and show increased adherence, low invasiveness and toxicity to HRECs, and elicit low cytokine responses (Marks et al., 2013). Biofilm bacteria found during colonization are avirulent, but are a source of pathogenic bacteria upon signals from IAV-induced changes in the environment (Marks et al., 2013).

Respiratory viruses trigger host responses and signals resulting in changes in the niche environment, including nutrient availability, temperature, and ion concentration that play an important role in the pneumococcal transition from commensal bacteria to disease-causing pathogen (Marks et al., 2013) (see **Figure 4**). Furthermore, actively-dispersed pneumococci have distinct transcriptional profiles compared to biofilm or planktonic, broth-grown bacteria, showing upregulation of carbohydrate metabolism and bacteriocin production and down-regulation of genes associated with competence, amino acid metabolism, purine and pyrimidine metabolism, and other regulatory genes (Pettigrew et al., 2014). Dispersion may be an important survival strategy as exposure of asymptomatically colonized mice with host responses induced dissemination of pneumococci into the lungs and middle ear. Recognition of these host responses suggests that inter-kingdom signaling in an important mechanism of transition from asymptomatic colonizer to pathogen.

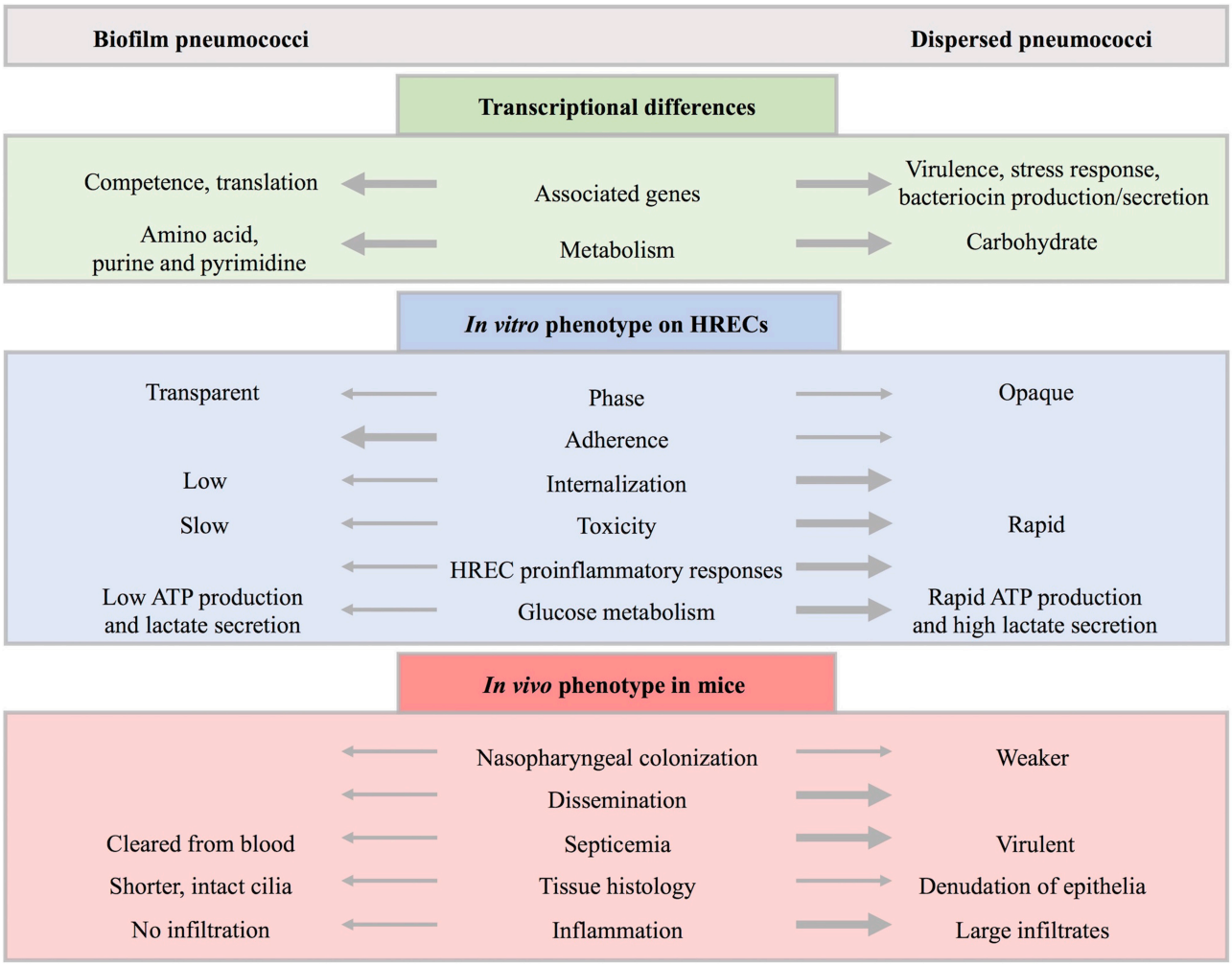

FIGURE 5 | Comparison of biofilm and dispersed pneumococcal populations. Biofilm-grown and biofilm-dispersed bacteria are distinct populations with different transcriptional profiles and phenotypic properties. In general, biofilm bacteria upregulate genes associated with competence while dispersed bacteria upregulate genes associated with virulence. Furthermore, genes associated with carbohydrate metabolism, bacteriocin production and secretion, stress response, and virulence factors are upregulated in dispersed populations compared to biofilm-grown bacteria while genes associated with colonization such as competence and fratricide, genes involved in amino acid metabolism, purine and pyrimidine metabolism, and translation are downregulated. This is in agreement with glucose metabolism assays where biofilm bacteria ineffectively produce ATP or secrete lactate in contrast to the rapid metabolism of glucose seen in actively-dispersed populations. In addition, biofilm bacteria are predominantly transparent in contrast to primarily opaque dispersed bacteria with upregulation of capsule expression. *In vitro* studies indicate that biofilm bacteria are less virulent and show increased adherence to human respiratory epithelial cells (HRECs). In contrast, dispersed bacteria are less adherent and have an increased ability to invade and kill HRECs with a higher induction of key cytokines involved in pro-inflammatory responses from exposed HRECs. *In vivo* studies show that both populations are able to colonize the murine nasopharynx, however, dispersed bacteria colonize more weakly and result in dissemination with a significantly higher bacteria load. In the mouse septicemia model, dispersed populations are virulent while biofilm bacteria are quickly cleared from the blood. When comparing infected mouse tissue, biofilm bacteria resulted in shorter, intact cilia with no inflammatory infiltration. This is in contrast to the denudation of epithelia and large inflammatory infiltrates seen in tissue infected with actively-dispersed bacteria.

FUTURE STUDIES

Our recently developed biofilm models have been instrumental in increasing our knowledge regarding pneumococcal colonization of the nasopharynx and the transition to invasive disease. Further understanding of pneumococcal biofilm formation will be important for addressing the spread of antibiotic resistance, serotype switching, vaccine escape, and protective effects in the context of co-colonization. In addition, biofilms have induced competence and capsule downregulation associated with increased transformation, which may be important for future models studying genetic exchange.

IAV-induced responses triggered the dispersion of a distinct population of pneumococci, suggesting that pneumococci recognize inter-kingdom signals. This model of pathogenesis with co-infection of IAV and pneumococci may be adapted to model co-infection and the transition to disease for other upper respiratory tract commensals that also experience increased virulence after IAV infection (e.g., *Staphylococcus aureus*). In addition, our

model could also be used to study a wider range of pneumococcal strains, such as clinical isolates that are not currently virulent in mouse models.

Future studies capitalizing on the RNA-seq data should focus on the role of carbohydrate metabolism, bacteriocin receptors, and other genes encoding surface proteins upregulated during invasive disease as these may represent novel targets for developing therapeutics. The transcriptional differences found between the pneumococcal populations explain the differences in virulence, however, future goals will involve understanding the mechanism involved in the induction of disease.

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Streptococcus pyogenes biofilms—formation, biology, and clinical relevance

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Streptococcus pyogenes (group A streptococci, GAS) is an exclusive human bacterial pathogen. The virulence potential of this species is tremendous. Interactions with humans range from asymptomatic carriage over mild and superficial infections of skin and mucosal membranes up to systemic purulent toxic-invasive disease manifestations. Particularly the latter are a severe threat for predisposed patients and lead to significant death tolls worldwide. This places GAS among the most important Gram-positive bacterial pathogens. Many recent reviews have highlighted the GAS repertoire of virulence factors, regulators and regulatory circuits/networks that enable GAS to colonize the host and to deal with all levels of the host immune defense. This covers *in vitro* and *in vivo* studies, including animal infection studies based on mice and more relevant, macaque monkeys. It is now appreciated that GAS, like many other bacterial species, do not necessarily exclusively live in a planktonic lifestyle. GAS is capable of microcolony and biofilm formation on host cells and tissues. We are now beginning to understand that this feature significantly contributes to GAS pathogenesis. In this review we will discuss the current knowledge on GAS biofilm formation, the biofilm-phenotype associated virulence factors, regulatory aspects of biofilm formation, the clinical relevance, and finally contemporary treatment regimens and future treatment options.

Keywords: *S. pyogenes*, biofilm, antibiotic resistance, virulence factors, transcriptional regulation

INTRODUCTION

Streptococcus pyogenes belongs to the serological group A among the streptococci (group A *Streptococcus*, GAS) and is an exclusively human pathogen. GAS causes significant disease worldwide and adds a large burden to national health care systems (Tan et al., 2014). An excellent compilation of data and estimates of the global burden of GAS diseases from 2005 revealed 616 million cases of pharyngitis, 111 million cases of pyoderma and at least 517,000 deaths due to severe invasive diseases and sequelae. This dataset is manifesting the important status of GAS among bacterial pathogens and is an impressive documentation of GAS impact on global mortality and morbidity (Bisno et al., 2005; Carapetis et al., 2005; Ralph and Carapetis, 2013).

Entry ports for GAS after person to person transmission are oral cavity, skin and wounds. In particular, mucosal membranes of the oropharynx and non-intact skin are preferred colonization sites (Cunningham, 2000; Tan et al., 2014). In otherwise healthy individuals, GAS usually causes mild and self-healing purulent infections of mucosal membranes and skin, such as pharyngitis, impetigo and pyoderma. In patients with predispositions such as immune-suppression, diabetes and related diseases, or specific HLA-DR (MHC class II cell surface receptor) subtypes, occasionally severe and invasive life-threatening diseases occur. Necrotizing fasciitis and streptococcal toxic shock syndrome belong to these disease manifestations with high morbidity and mortality rates. Antibiotic therapy is mandatory, even for uncomplicated primary infections, to prevent secondary autoimmune sequelae like rheumatic heart disease or glomerulonephritis (Cunningham, 2000).

GAS is well adapted to its human host, since it is equipped with a large set of virulence factors of all classes. The bacteria express surface proteins and secreted factors leading to (i) immunoglobulin and complement factor degradation (EndoS, Mac, C5a peptidase) and (ii) general complement inhibition (achieved by M protein, capsule expression and Sic), (iii) extracellular matrix and serum protein binding via multiple MSCRAMMS (microbial surface components recognizing adhesive matrix molecules) (M protein, Cpa, Eno, Epf, up to five different firbronectin-binding MSCRAMMS), (iv) dysregulation of coagulation (plasminogen/plasmin binding, streptokinase Ska activity), and (v) cytotoxic and cytolytic activity toward various host cell types (Nga, SLS, SLO). Depending on the presence of phage-related chromosomal islands as variable parts of the accessory genome in the different GAS serotypes, a variable number of superantigens (SpeA-J, SmeZ) is expressed and secreted (Banks et al., 2002; Spaulding et al., 2013).

The presence of individual genes encoding virulence factors is GAS serotype-specific and expression depends on environmental conditions. Transcriptional changes during GAS cultivation and pathogenesis were recently reviewed (Fiedler et al., 2010). A most recent review highlights the importance of GAS virulence factors for disease manifestation and pathogenesis (Walker et al., 2014). As successful pathogen, GAS tightly controls virulence factor gene expression to keep the number of exposed proteins for immune

recognition to a minimum. Regulation occurs on multiple levels including the activity of stand-alone transcription regulators and two component signal transduction systems (Kreikemeyer et al., 2003; Patenge et al., 2013), catabolite control (Almengor et al., 2007), control of mRNA decay (Bugrysheva and Scott, 2010), cis- or trans-acting regulation of small non-coding RNAs (Patenge et al., 2013), and quorum sensing (Jimenez and Federle, 2014). How these regulators interact under *in vitro*, *in vivo*-like, and host infection conditions, and how their activities are hierarchically clustered is currently studied intensively (McIver, 2009; Fiedler et al., 2010; Patenge et al., 2013). Information about these regulatory processes allows a better understanding of GAS pathogenic mechanisms and could identify novel levels for interference with anti-infectiva to prevent and/or cure GAS infections.

Apart from the well-studied GAS virulence traits and pathogenesis mechanisms, like host cell adherence/internalization, phagocytosis resistance, escape from phagocytic killing, host cell apoptosis induction and autophagy escape (Walker et al., 2014), the ability of GAS to form micro-colonies and matured biofilms *in vitro* and *in vivo* was just recently appreciated. Biofilms, due to their composition, physiology and physical parameters present a massive danger signal. The host immune defense interacts on all levels to attack these 3 dimensional foreign structures. Some of the above listed genes encoding virulence factors and regulators moved into the focus of GAS biofilm investigation and are discussed in this review.

Furthermore, the specific features of biofilms, i.e., the 3-dimensional structure, the matrix of extracellular polymeric substances, and the lower growth rates and differences in metabolism of the bacteria, cause problems in efficient antibiotic treatment of GAS organized in such structures. Therefore, in this review, we will also discuss potential alternatives to antibiotic treatment of GAS biofilms.

CLINICAL RELEVANCE OF GAS BIOFILMS

GAS was considered a classical extracellular human pathogen for a long time. Numerous studies have evaluated the potential of these bacteria to adhere to and internalize into almost all host cell types, a feature which was discussed as reason for the occurrence of recurrent GAS infections (Facinelli et al., 2001; Podbielski and Kreikemeyer, 2001). However, now it is under debate if recurrence is sufficiently explained by GAS host cell adherence/internalization or if GAS biofilms play a so far underappreciated role. Moreover, the question if GAS biofilms are clinically relevant needs to be addressed. Here we discuss this aspect with a careful look on terminology (microcolony vs. biofilm) and *in vitro* vs. *in vivo* observations and studies.

Particularly the *in vitro* biofilm phenotype was evaluated with isolate collections and for many of the clinically relevant/predominant GAS serotypes under static and flow conditions. In these studies, a significant heterogeneity of this phenotype was noted among strains of a particular serotype (Lembke et al., 2006). Another study revealed 90% of GAS serotypes, from invasive and non-invasive infections, to form biofilms, thereby supporting the notion that this is a trait of individual strains rather than a general serotype attribute (Baldassarri et al., 2006). Moreover, a reduced capacity to internalize into host cells in combination with macrolide-susceptibility was suggested as a strong reason for a biofilm-positive phenotype, as this is a means of protection from antibiotic treatment (Baldassarri et al., 2006). Together these and other facts suggested inclusion of biofilm phenotype data into epidemiological investigations of GAS (Köller et al., 2010).

Generally, two different entry ports could give rise to microcolony formation and the biofilm phenotype. First, GAS can enter new hosts via the oral cavity and establish in the upper respiratory tract. Here, in particular GAS pharyngitis is associated with antibiotic treatment failure leading to multiple infection episodes in affected patients (Facinelli et al., 2001; Podbielski and Kreikemeyer, 2001). Isolates from such cases have a higher tendency toward resistance against macrolide antibiotics in association with the presence of protein F1, a virulence factor supporting host cell internalization (Facinelli et al., 2001). This observation sustains the theory that GAS have an intracellular sanctuary where they persist and hide from eradication by antibiotic treatment and host defense mechanisms. Conley and colleagues rather related antibiotic treatment failure with biofilm formation capacity of GAS (Conley et al., 2003). They showed pharyngitis treatment failure patient isolates to have a biofilm-positive phenotype and increased MBEC (minimum biofilm eradication concentration) for all contemporary antibiotics used to treat acute pharyngitis cases. Moreover, GAS biofilms were found in tonsillar reticulated crypts, isolated from tonsillectomy material (Roberts et al., 2012). Thus, there is a clear link between GAS caused pharyngitis and biofilm formation capacity.

Second, also human skin acts as entry port for these pathogens. Skin from patients with impetigo and atopic dermatitis is a habitat for GAS microcolonies and biofilms (Hirota et al., 1998; Akiyama et al., 2003). Whether GAS microcolonies represent a specific physiological state with own existence or rather a prestage of "mature" biofilm is currently unclear. The latter is likely, as microcolonies are surrounded by a FITC-ConA stainable glycocalyx (Akiyama et al., 2003). Cho and Caparon clearly pointed out that GAS forms biofilm-like bacterial communities during soft tissue infection in zebrafish, which largely differ in gene expression patterns from GAS biofilms on abiotic surfaces (Cho and Caparon, 2005).

Further *in vivo* evidence from animal models revealed GAS biofilm formation during otitis media in a chinchilla middle ear infection model. However, biofilm formation was not strictly required for infectivity (Roberts et al., 2010a). In clinical studies in 37% of all non-severe recurrent acute otitis media (RAOM) cases in children GAS was identified as nasopharyngeal biofilm-producing otopathogen (Torretta et al., 2012). The final and most critical development step of biofilm lifestyle is the dispersal stage, which could transform mild and local infections into severe-disseminating diseases (Connolly et al., 2011a).

Apart from the accumulating data on GAS biofilm-mediated antimicrobial resistance toward contemporary antibiotics, leading to treatment failure and recurrent infection episodes, the fact that biofilm grown GAS are naturally competent and thus transformable with foreign DNA (Marks et al., 2014) is of major clinical relevance and concern.

CONCLUSION

Although the GAS biofilm phenotype just recently moved into the focus of research, quite a substantial number of studies collected *in vitro*, but more importantly, also *in vivo* evidence that this GAS lifestyle contributes to many diseases caused by GAS. As outlined above, there is in particular compiling data for a role of GAS biofilms during infection of oto-, nasopharyngeal-, and skin-localized human diseases. Thus, the question on the clinical relevance of GAS biofilms is no longer disputable. However, there is a strong requirement for intensification of research in this area and elucidation of better treatment options.

VIRULENCE FACTORS ASSOCIATED WITH THE GAS BIOFILM PHENOTYPE

To date, more than 50 virulence factors have been described in GAS. Their expression is tightly regulated and fine-tuned in dependence on growth phase and environmental conditions of the bacteria (Fiedler et al., 2010). Consequently, also the biofilm lifestyle of GAS is associated with a specific pattern of virulence factor expression that differs from that of planktonic GAS.

Since biofilm formation in general comprises at least three distinct stages—i.e., (i) initial adherence and microcolony formation, (ii) biofilm maturation with production of a matrix of extracellular polymeric substances (EPS), and (iii) detachment of sessile cells—it is not unexpected that virulence factor expression patterns differ in these respective stages (O'Toole et al., 2000).

TRANSCRIPTOME STUDIES ON GAS BIOFILMS

The only transcriptome study on GAS biofilms available so far has been carried out with the GAS HSC5 M14 serotype strain. Here, transcript levels in biofilm bacteria were compared to those of planktonic cells in the exponential and stationary growth phase. This analysis revealed an increased abundance of *speB* and *spd*/*mf* transcripts and a lower abundance of *ska* mRNA in biofilm GAS than in bacteria from the exponential phase of planktonic cultures. While M protein expression was more or less constant in biofilm formation, the capsule biosynthesis genes were slightly induced in the maturation phase (Cho and Caparon, 2005). Cho and Caparon propose that in later stages carbohydrate metabolism and capsule biosynthesis are essential to establish a solid biofilm with bacteria encased in a robust matrix of extracellular polysaccharides (Cho and Caparon, 2005). In *S. pyogenes*, the main sugar components in the matrix are L-glucose and D-mannose (Shafreen et al., 2011).

The data of this study are somewhat contradictory to a later work, where expression of several virulence genes in biofilm and planktonic bacteria of the M3 GAS strain MGAS315 has been assessed by qPCR. In contrast to the work of Cho and Caparon, biofilms and planktonic bacteria were exposed to keratinocytes. When grown as biofilms on live keratinocytes for 48 h, down-regulation of genes for streptolysins (*sagA*, *slo*), hyaluronic acid capsule biosynthesis (*hasA*), M-protein (*emm3*) and the cysteine protease SpeB (*speB*), was observed while competence-associated *com* genes were upregulated in comparison to planktonic cells exposed to epithelial cells (Marks et al., 2014).

With these limited data sets of only two GAS serotypes and the differential setup of the biofilm assays in the studies of Cho and Caparon (2005) and Marks et al. (2014) it is not possible to deduce any biofilm specific transcriptome yet. Similar studies including more serotypes are needed. Having in mind that the ability to grow in biofilms is rather a strain specific trait and not a GAS serotype attribute (Baldassarri et al., 2006; Köller et al., 2010) it is questionable if a general biofilm transcriptome can be elucidated.

MSCRAMMS

It has early been recognized that GAS biofilm formation is largely varying between different strains. Certain GAS strains are able to bind to abiotic polystyrene surfaces, while other strains need matrix or serum protein coated surfaces to establish biofilms or are unable to produce biofilms at all (Conley et al., 2003; Lembke et al., 2006). Obviously, adhesive surface structures are needed in GAS biofilms to mediate autoaggregation and attachment of the bacteria to the biotic or abiotic surface (Manetti et al., 2007; Courtney et al., 2009; Oliver-Kozup et al., 2011, 2013). E.g., various GAS strains, representing 8 *emm* types, likely associated with at least 6 different FCT (fibronectin-binding, collagen-binding, T-antigen)-types, have been shown to lose their ability to form biofilms when treated with trypsin, thereby removing trypsin-sensitive surface proteins (Courtney et al., 2009).

Biofilm formation ability of GAS strains seems to be associated with certain M- and FCT-types. This indicates that adhesion and co-aggregation processes mediated by M- or M-like proteins and/or FCT region-encoded pili are essential for the successful establishment of biofilms (Lembke et al., 2006; Manetti et al., 2007, 2010; Edwards et al., 2008; Köller et al., 2010).

To date, 9 different types of FCT regions with distinct architectures are described in GAS (Kratovac et al., 2007; Falugi et al., 2008). The FCT region encoded core pilus operon comprises genes for a pilus backbone protein, at least one matrix protein binding ancillary protein, sortases (SrtB/SrtC2), and a signal peptidase (Kratovac et al., 2007; Kreikemeyer et al., 2011). There is clear evidence hinting at an association between FCT type and biofilm formation (Köller et al., 2010; Manetti et al., 2010). While FCT type 1 strains were shown to be generally good biofilm formers, independent of media or pH-conditions, FCT-9 strains are poor biofilm formers under all conditions tested so far. In strains with other FCT types, e.g., FCT-2, FCT-3, FCT-5, and FCT-6, biofilm production depends on culture conditions and is triggered by low pH, e.g., caused by sugar metabolism in unbuffered media (**Figure 1**). FCT type 4 strains show an inhomogeneous response to environmental conditions with respect to biofilm formation. Generally, M28 and M89 strains tested were poor biofilm formers under all conditions, while M12 strains showed a medium and pH dependent biofilm formation (Köller et al., 2010; Manetti et al., 2010). The specific differences in involvement of pilus structures in biofilm formation in different FCT-type strains might in part be attributed to the high diversity in amino acid sequence of the pilus backbone proteins, which can vary among as well as within FCT-types (Falugi et al., 2008). E.g., T6 type pilus backbone proteins, which can be found in M6/FCT-type 1 strains, seem to strongly promote biofilm formation (Kimura et al., 2012). It has also been demonstrated that ancillary pilus

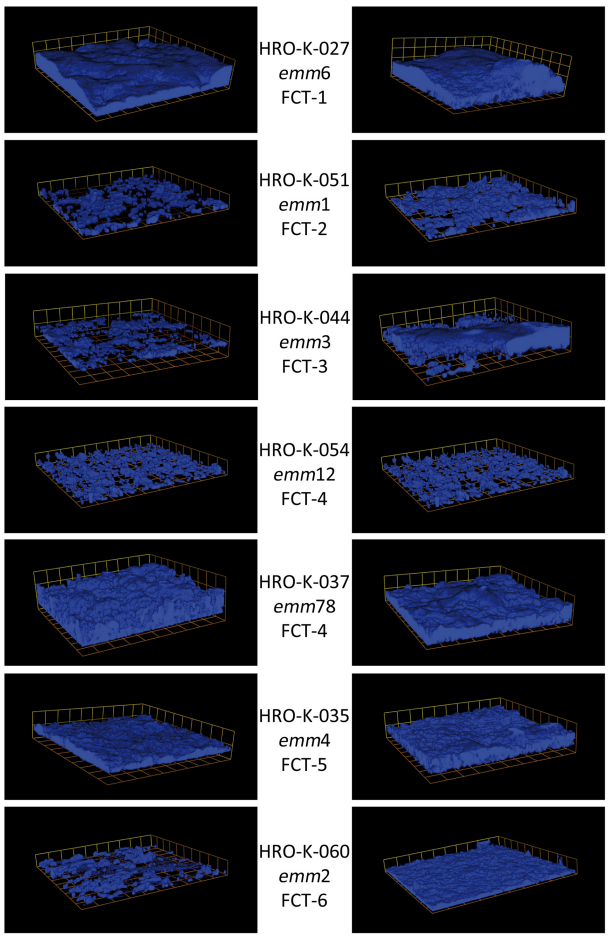

FIGURE 1 | Three-dimensional images of biofilms of various GAS *emm*/FCT types grown in C-medium in the absence or presence of glucose. Cells were stained with Alexa Fluor 647 and visualized via Confocal Laser Scanning Microscopy (CLSM); magnification 600 times; box size 13.8 × 13.8 μm. **Left panel**: non-supplemented C-medium. **Right panel**: C-Medium supplemented with 30 mM glucose. Strain names, *emm*-types, and FCT types are given in the middle column.

proteins such as Ancillary protein 1 are necessary for biofilm formation (Becherelli et al., 2012).

FCT-region encoded pili are obviously necessary for biofilm formation, but other factors are needed as well, since several GAS strains expressing functional pili in moderate levels are still unable to form biofilms. In contrast, GAS strains with low pilus expression or with defective pili are generally poor biofilm formers (Manetti et al., 2007). As mentioned above, these additional factors are most likely M- and M-like proteins, but also other surface adhesins such as the fibronectin binding proteins PrtF2 and Scl1, or the AgI/II type polypeptide AspA (Cho and Caparon, 2005; Luo et al., 2008; Courtney et al., 2009; Maddocks et al., 2011; Oliver-Kozup et al., 2013). It has been proposed that M- or M-like proteins are needed for LTA-stabilization, thereby increasing hydrophobicity on the GAS surface, which facilitates autoaggregation and adhesion to biotic and abiotic surfaces and consequently biofilm formation (Cho and Caparon, 2005; Courtney et al., 2009). This is supported by the finding that most M- and M-like protein defective mutants show decreased biofilm formation and lower hydrophobicity compared to their wild type parent strains (Cho and Caparon, 2005; Courtney et al., 2009).

The cell wall anchored adhesion AgI/II type polypeptide AspA has been shown to mediate GAS M28 biofilm formation on saliva coated surfaces (Brady et al., 2010; Maddocks et al., 2011, 2012; Hall et al., 2014). AgI/II type proteins bind to salivary glycoproteins. In GAS M28 AspA is proposed to mediate biofilm formation by direct protein-protein interaction with the salivary glycoprotein gp-340 (Maddocks et al., 2011). This is supported by the fact that AspA deficient mutants of GAS M28 show an about 50% reduced biofilm mass when cultivated on gp-340 or saliva-coated surfaces. On uncoated polystyrene surfaces biofilms of AspA mutants resembled those of the cognate WT strains (Maddocks et al., 2011).

The collagen-like protein Scl1 binds cellular fibronectin and also mediates biofilm formation (Caswell et al., 2010; Oliver-Kozup et al., 2011, 2013). M3 strains intrinsically harboring a *scl1* gene with a mutation that results in production of a truncated Scl1 protein where shown to be unable to form biofilms on abiotic surfaces. M41, M28, and M1 strains with *scl1* deletions showed a decreased biofilm formation compared to their cognate wild types. *S. pyogenes* Scl1 expressed on the surface of the heterologous host *Lactococcus lactis* enables biofilm formation of this bacterium (Oliver-Kozup et al., 2011). Considering its specific binding to cellular fibronectin, Scl1 might be of special importance for microcolony/biofilm formation of GAS in wounds (Oliver-Kozup et al., 2013).

Apparently, a critical amount (or number?) of surface associated adhesive structures is necessary to mediate initial adherence and autoaggregation of the bacteria. As mentioned above, structures potentially involved are pili, M- or M-like proteins, PrtF2, Scl1, or AspA. The importance of the respective structures in different GAS strains/M-serotypes/FCT-types obviously differs. Also regulatory mechanisms are not ubiquitous among GAS strains. While in some strains environmental signals such as a low pH are needed to induce biofilm formation, other strains produce biofilms in a pH-independent manner. It is likely that some of the adhesive structures are more important to mediate the initial adherence to abiotic surfaces while others will be crucial for adherence to biotic structures. For sure the latter will be of more significance in the patient. A concerted investigation of all surface structures mentioned above in terms of the involvement in biofilm formation on biotic surfaces is still missing.

SpeB AND OTHER SECRETED ENZYMES

The streptococcal pyogenic exotoxin B (SpeB) and other secreted enzymes are associated with the biofilm lifestyle of GAS. SpeB as a secreted cysteine protease is suspected to degrade peptides that stabilize the biofilm matrix (Roberts et al., 2010a,b; Connolly et al., 2011a,b). It has been shown that in the M1 serotype strain MGAS5005 SpeB activity is negatively correlated with biofilm production. While regulator mutants with high SpeB production (MGAS5005Δsrv) are completely unable to form biofilms, deletion of *speB* in these strains reconstitutes the biofilm phenotype

of the wild type strain. Also chemical inhibition of SpeB activity increases biofilm masses of MGAS5005Δsrv (Roberts et al., 2010a,b). Furthermore, the external addition of active SpeB to MGAS5005 cultures significantly inhibits biofilm formation (Roberts et al., 2010b).

In line with these data, downregulation of *speB* expression has been observed in biofilms of an M3 serotype strain compared to planktonic bacteria (Marks et al., 2014).

Next to SpeB, other secreted enzymes might be critical for biofilm formation. Proteases and nucleases that potentially degrade components of the extracellular matrix need to be suppressed to maintain the structural integrity of GAS biofilms. While in other streptococci such as *S. intermedius*, *S. mutans*, or *S. pneumoniae*, extracellular DNA (eDNA) is a structural component of biofilm matrix, there is no direct prove for eDNA in GAS biofilms yet (Whitchurch et al., 2002; Montanaro et al., 2011; Domenech et al., 2012). However, increased secretion of enzyme degrading matrix components at a certain point in time might promote dispersion of GAS biofilms and thereby facilitate distribution of GAS within the host. This hypothesis is supported by the data of Cho and Caparon, who found high *speB* expression levels in mature biofilms compared to planktonic bacteria of the HCS5 M14 GAS strain (Cho and Caparon, 2005).

CAPSULE

The role of the hyaluronic acid capsule in GAS biofilms is not entirely clear yet. There are somewhat contradictory observations described by different groups. Cho and Caparon observed a slight induction of capsule biosynthesis gene (*has*-operon) transcripts in GAS strain HSC5 during biofilm maturation. Furthermore, they found a mutant defective of capsule biosynthesis to be unable to form a solid biofilm under flow conditions, while under static conditions the biofilm masses were unaffected by this mutation (Cho and Caparon, 2005). Marks and colleagues on the other hand described a decrease in *hasA* transcription in biofilms of MGAS315 (M3) on keratinocytes in comparison to keratinocyte-exposed planktonic bacteria (Marks et al., 2014). Furthermore, there is indirect evidence that capsule production inhibits biofilms, since it has been shown for several GAS strains that *covS* deletion leads to an increased capsule production but also to lower biofilm biomasses (Sugareva et al., 2010). Initially, a thick capsule might mask adhesive surface structures, thereby preventing adhesion and co-aggregation of the bacteria, which would probably rather inhibit biofilm formation. In later stages of biofilm maturation, capsule production could be involved in establishing a robust biofilm matrix, as suggested by Cho and Caparon (2005).

CONCLUSION

From all studies introduced above the M protein as one major virulence determinant of GAS seems to support biofilm formation, but secreted proteins and capsule could impair biofilm formation. However, it has to be kept in mind that the data discussed here are all based on *in vitro* experiments that most likely only poorly resemble the *in vivo* situation. Most of the experimental data are based on biofilms formed on abiotic plastic surfaces, sometimes coated with matrix or serum proteins. Only few studies analyzed biofilms grown on epithelial cells (Fiedler et al., 2013; Marks et al., 2014). To our knowledge, device-associated GAS biofilms have never been described in patients. Furthermore, although GAS microcolony formation in the oropharynx has been observed (Diaz et al., 2011; Roberts et al., 2012; Torretta et al., 2012; Woo et al., 2012), it is yet not known whether complex biofilms—as they can be obtained *in vitro*—are actually occurring in patients. Therefore, taken together, the relevance of these *in vitro* data for real infections in patients remains unclear.

REGULATORY ASPECTS OF GAS BIOFILMS

The biofilm lifestyle is associated with broad transcriptional changes, affecting the expression levels of about 25% of the GAS genes (Cho and Caparon, 2005). Several transcriptional regulators were shown to be involved in and crucial for the establishment and maintenance of biofilms. From the data available to date, three major regulatory processes can be deduced that facilitate the biofilm lifestyle of GAS:

(i) Peptide pheromone based quorum sensing mediated by the short hydrophobic peptides SHP2/SHP3 (Chang et al., 2011).
(ii) Repression of secreted and surface associated enzymes such as the cysteine protease SpeB and other proteases and nucleases (Dmitriev et al., 2008; Roberts et al., 2010a; Connolly et al., 2011a; McDowell et al., 2012).
(iii) Induction of surface associated autoaggregative and adhesive structures such as M- and M-like proteins and the FCT region encoded pilus (Cho and Caparon, 2005; Luo et al., 2008; Manetti et al., 2010).

The major players and the regulatory network contributing to GAS biofilm formation are summarized in **Figure 2**.

QUORUM SENSING

Quorum sensing mechanisms are crucial for biofilm formation in many organisms. In GAS, four different ways of inter- and intraspecies communication are described, i.e., Rgg-, Sil-, lantibiotics-, and LuxS/Autoinduer-2-dependent processes (Jimenez and Federle, 2014).

In GAS, biofilm formation is associated with peptide-pheromone based quorum sensing mediated by the short hydrophobic peptide (SHP) pheromones SHP2 and SHP3. These peptide pheromones are encoded downstream of two genes encoding for the Rgg-like transcriptional regulators Rgg2 and Rgg3, respectively (Chang et al., 2011; Federle, 2012; Lasarre et al., 2013; Aggarwal et al., 2014). The propeptides are secreted and processed to the mature peptide pheromones SHP2C8 and SHP3C8, which are taken up into GAS via the oligopeptide permease Opp. The transcription of both peptide pheromone genes *shp2* and *shp3* is inhibited as long as Rgg3 is bound to the respective promoters. SHP2C8 and SHP3C8 bind to Rgg3 and Rgg2, leading to a dissociation of Rgg3 from and binding of Rgg2 to the *shp2* and *shp3* promoters. In a positive feedback loop, this induces the expression of *shp2* and *shp3* (Chang et al., 2011; Aggarwal et al., 2014). In GAS M49 NZ131 it has been shown that SHP2/3 dependent activation via Rgg2 induces biofilm production, while

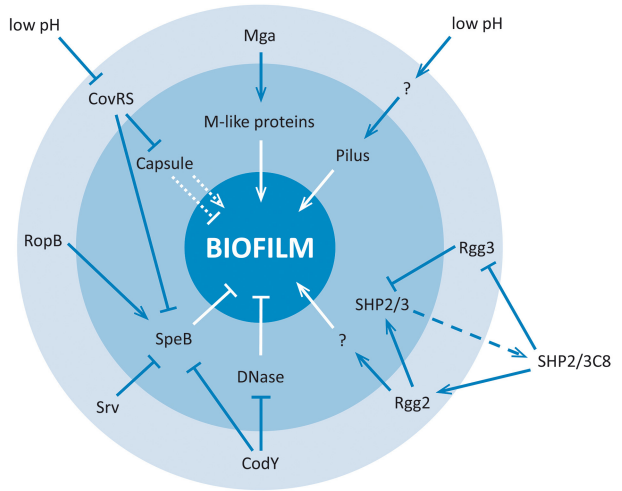

FIGURE 2 | Regulatory network involved in GAS biofilm formation. Arrow heads indicate direct or indirect induction, blocked lines indicate direct or indirect repression, dashed lines indicate export out of the bacterial cell, and dotted lines indicate ambiguous effects. Outer circle (light blue): transcriptional regulation level; Inner circle (darker blue): biofilm-associated virulence factors; Outside: environmental conditions and quorum sensing peptides influencing the biofilm phenotype. "?" stands for unknown Regulator/regulatory mechanism.

Rgg3 represses biofilms via repression of SHP2/3 production. It is not known to date, which transcriptional changes are caused by the SHP2/3 dependent activation of Rgg2 and inactivation of Rgg3 that finally lead to biofilm formation. Furthermore, it has not been elucidated yet whether this system also controls biofilm formation in other GAS strains, but *in silico* analyses show that Rgg2 and Rgg3 are present in all GAS strains (Chang et al., 2011).

Two of the other above-mentioned quorum sensing systems of GAS have been associated with the GAS biofilm lifestyle as well. For an M18 strain it could be shown that a SilC deletion mutant was significantly impaired in biofilm formation (Lembke et al., 2006). Furthermore, there are hints that LuxS is involved in the control of SpeB production and *emm* gene expression, which could influence biofilm formation (Lyon et al., 2001; Marouni and Sela, 2003; Siller et al., 2008; Beema Shafreen et al., 2014). Both of the latter QS systems have not been investigated in the context of GAS biofilm in detail yet. For more details on GAS quorum sensing please refer to a current review by Jimenez and Federle (Jimenez and Federle, 2014).

TRANSCRIPTIONAL REGULATORS OF SpeB AND OTHER SECRETED ENZYMES

Since SpeB activity leads to dispersal of biofilm structures and prevents biofilm formation in GAS, repression of *speB* transcription is necessary for successful biofilm establishment (Doern et al., 2009). Therefore, regulators involved in transcription of *speB* also control biofilm formation in GAS. Transcriptional regulation of SpeB is quite complex and involves direct and indirect actions of numerous GAS regulators, as recently reviewed by Carroll and Musser (2011). Positive regulators directly acting at the promoter of the *speB* gene are RopB, another member of the Rgg-regulator family also referred to as Rgg1 (Chaussee et al., 1999; Neely et al., 2003; Dmitriev et al., 2008; Hollands et al., 2008), and the sugar metabolism regulator CcpA (Kietzman and Caparon, 2010; Shelburne et al., 2010). Consequently, deletion of the *ropB* gene leads to lower *speB* expression and an increased biofilm formation as shown in the M49 NZ131 strain (Chang et al., 2011). To our knowledge, for CcpA an influence on biofilm formation has not been elucidated yet.

The CovR (aka CsrR) response regulator of the CovRS two component system probably binds directly to the *speB* promoter as well, acting as a transcriptional repressor (Miller et al., 2001). Consequently, repression of *speB* transcription by CovR enables GAS biofilm formation. CovRS influence on biofilm formation seems to be serotype or even strain dependent. It has been shown that deletion of the sensor kinase CovS leads to decreased biofilm formation in most strains tested. However, for some M6 strains an increased biofilm formation has been observed in CovS deletion strains (Hollands et al., 2010; Sugareva et al., 2010). Furthermore, it was shown that a mutant of the HSC5 strain lacking the CovR response regulator is unable to form biofilm at all (Cho and Caparon, 2005).

Another virulence-associated regulator, Srv, is involved in control of of *speB* expression via indirect mechanisms (Reid et al., 2004; Doern et al., 2009; Roberts et al., 2010a; Connolly et al., 2011a).The deletion of *srv* in the M1T1 strain MGAS5005 leads to an increased activity of SpeB and therefore to loss of the biofilm phenotype (Reid et al., 2006; Doern et al., 2009). In Western Blot analyses SpeB could not be detected in MGAS5005 biofilms after 24 h growth, whereas in the *srv* deletion mutant high amounts of SpeB are present in cultures after 24 h growth (Doern et al., 2009). The Srv mediated repression of SpeB activity is not restricted to the MGAS5005 strain, which has a naturally occuring mutation that leads to an inactive CovS sensor kinase. The effects of Srv on SpeB and biofilm production have also been observed for other GAS strains, although effects of *srv* deletion are not as drastic in those strains as they are in MGAS5005 (Connolly et al., 2011a).

Another regulator potentially involved in biofilm formation is CodY, a regulator involved in the response to nutrient deprivation in many gram positive bacteria (Sonenshein, 2005). CodY deletion mutants were shown to have a reduced biofilm formation capacity of GAS in chemically defined medium (McDowell et al., 2012). This effect probably also results from the indirect CodY-mediated repression of the production of SpeB and other secreted proteases and nucleases (McDowell et al., 2012).

TRANSCRIPTIONAL REGULATION OF BIOFILM-RELEVANT MSCRAMMS

The transcriptional regulation of GAS surface associated adhesins has been subject to extensive investigations and the regulatory networks have often been reviewed in the past (Kreikemeyer et al., 2003; Hondorp and McIver, 2007; McIver, 2009; Fiedler et al., 2010). Nevertheless, only few of the regulators involved have been investigated with respect to their impact on biofilm formation. Since biofilm formation is apparently associated with the pilus and the M-protein family, it is quite obvious that transcriptional regulators influencing the expression of the FCT region encoded pilus genes and the *emm* gene should influence biofilm formation in GAS. Mga is the major stand-alone transcriptional

positive regulator of *emm* and *emm*-like genes (Hondorp and McIver, 2007). Consequently, Mga inactivation leads to a loss of autoaggregation and biofilm formation capacity in GAS (Cho and Caparon, 2005; Luo et al., 2008). Regulation of Mga itself is very complex and was recently reviewed (Hondorp and McIver, 2007; Patenge et al., 2013).

For some strains, i.e., those harboring an FCT-2, -3, or -4 type pilus encoding region, one of the major environmental signals driving biofilm formation is the external pH, as shown exemplarily for an FCT type 3 strain in **Figure 3**. In these strains, pilus expression is induced under acidic conditions. In contrast, FCT-1 strains produce pH-independent biofilms and do not show any pH-dependent differences in pilus gene expression (Köller et al., 2010; Manetti et al., 2010). The regulator(s) mediating the pH-driven expression of the pilus genes are not known yet. It is likely that the FCT-region encoded RofA-like regulators RofA or Nra might be involved, although this has not been experimentally proven yet (Kreikemeyer et al., 2002, 2011).

Conclusion

Environmental signals such as low pH and critical levels of peptide pheromones initiate complex regulatory circuits leading to biofilm formation in GAS. The details in environmental triggers, transcriptional changes, and regulators involved seem to be strain-specific and are not completely understood yet.

TREATMENT OF GAS BIOFILMS—CLINICAL AND EXPERIMENTAL ASPECTS

Penicillin remains the most important therapeutic agent to medicate GAS skin and upper respiratory tract infections such as impetigo, erysipelas, cellulitis, tonsillitis, pharyngitis, or scarlet fever (Bisno et al., 2002; Shulman et al., 2012). Fortunately, there is no confirmed case of beta-lactam resistant GAS to date. Patients with penicillin intolerance can be treated with cephalosporins or macrolides such as erythromycin, azithromycin or clarithromycin instead. Of note, increasing numbers of infections with macrolide-resistant strains are reported, ranging up to 38% of erythromycin resistant isolates (Syrogiannopoulos et al., 2001; Sauermann et al., 2003; Richter et al., 2005; Ardanuy et al., 2010; Rubio-Lopez et al., 2012). This leads to a decrease in suitability of this agent class for calculated treatment of local GAS infection.

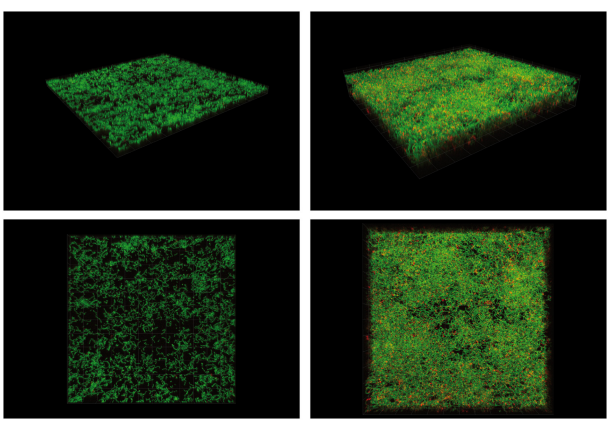

FIGURE 3 | Confocal Laser Scanning micrographs of 24 h *emm3*/FCT-3 GAS strain HRO-K-044 biofilms cultured in alkalined or acidified C-medium. Cells were stained with live/dead dye containing Syto9 and Propidiumiodide. Magnification 630 times; box size: 19.8 × 19.8 µm. **Left panel:** mature biofilm grown in C-medium with initial pH of 8.5. **Right panel:** mature biofilm grown in C-medium with initial pH of 6.5. Upper row: 45° perspective. Lower row: top view.

Severe systemic GAS infections such as sepsis, necrotizing fasciitis or streptococcal toxic shock like syndrome (STSS) are most commonly treated utilizing penecillin in combination with clindamycin. The combination with clindamycin proved to be beneficial to prevent GAS toxin production during therapy (Zimbelman et al., 1999; Russell and Pachorek, 2000).

Although there seems to be no naturally occurring penicillin resistant GAS isolates, it was stated that in about 30% of GAS infections the pathogens are not completely eradicated despite adequate antibiotic therapy and general susceptibility of the GAS isolates (minimal inhibitory concentration [MIC] $\leq$ 0.25 mg/L), potentially resulting in recurrent infections and persistent carriage (Conley et al., 2003; Baldassarri et al., 2006; Ogawa et al., 2011). There are two explanations discussed in the literature. On the one hand, intracellular persistence of GAS could prevent successful eradication of the bacteria by antibiotic treatment of the patients (Podbielski and Kreikemeyer, 2001). On the other hand, biofilms are discussed to be a cause for treatment failure. It was shown that GAS isolates organized in biofilm-structures could suffer penicillin concentrations up to 400 mg/l (minimum biofilm eradication concentration [MBEC] $\geq$ 400 mg/L). This exceeds the usual MICs by far, displaying clinical resistant levels (Conley et al., 2003; Baldassarri et al., 2006; Lembke et al., 2006; Shen et al., 2013). Hence, GAS organized in biofilm structures are able to survive antibiotic treatment that is adequate for planktonic GAS. To reach a better clinical outcome and lower treatment failure rates, it would be necessary to adapt treatment of GAS infections to achieve an effective degradation of biofilm structure.

EXPERIMENTAL APPROACHES OF GAS BIOFILM TREATMENT

Degradation of bacterial biofilm structures can be achieved by treatment with endolysins. This has been reported for several gram positive pathogens, e.g., *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Bacillus antracis*, *Streptoccus suis* (Loeffler et al., 2001; Schuch et al., 2002; Becker et al., 2008; Domenech et al., 2011). Even species with a high tendency to multiple antibiotic resistances can be efficiently killed by such lysins.

For degradation of GAS biofilms, the streptococcal-specific bacteriophage C1 encoded bacteriophage lysin C (PlyC) is of special interest. It was shown that this multimeric N-acetylmuramoyl-L-alanine amidase hydrolyzes GAS cell walls and eliminates bacterial cells *in vitro* and *in vivo* (Krause, 1957; Fischetti et al., 1971; Raina, 1981; Loeffler et al., 2001; Nelson et al., 2001, 2006; Köller et al., 2008). Shen and colleagues furthermore demonstrated PlyC to degrade both GAS biofilm structures and biofilm associated cells efficiently, thereby affecting GAS biofilms significantly more than penicillin (Shen et al., 2013). These abilities make PlyC a reasonable candidate supplement

for therapeutic treatment of GAS infections. However, more *in vitro*, *in vivo*, and clinical studies are needed to elucidate the applicability of PlyC for GAS biofilm eradication in patients.

Next to this endolysin, other substances with known broad antibacterial properties have been shown to inhibit GAS biofilms. Of special interest could be manuka honey that is available as sterilized medical grade honey (medihoney) for topic wound treatment. Maddocks and others described medihoney to inhibit the expression of *sof* and *sfb1* genes encoding for fibronectin binding streptococcal surface proteins, resulting in reduced human tissue binding and biofilm formation capacity (Maddocks et al., 2012). It has to be considered that these findings have been reported only for one clinical isolate representing an M28 serotype (MGAS6180; M28). It remains to be seen if these effects are transferable to other strains/*emm* genotypes, since there are remarkable differences of the regulatory networks reported as discussed above.

It has furthermore been shown that the fatty acid messenger cis-2-decenoic acid produced by *Pseudomonas aeruginosa* can induce dispersion of biofilms of GAS and other bacteria. If such substances could be administered in patients in combination with conventional antibiotics, this would probably lead to eradication of GAS biofilm associated infections (Davies and Marques, 2009).

Beside the chemical treatment of mature GAS biofilms, also probiotic effects of physiological bacteria on GAS biofilm formation have been reported (Guglielmetti et al., 2010a,b; Fiedler et al., 2013). It has been shown that bacteria physiologically colonizing the upper respiratory tract, such as *Streptococcus oralis* and *Streptococcus salivarius*, protect epithelial cells from GAS adherence. This observation indicates a role of these bacteria in host health. It was reported that *S. oralis* could induce protection of eukaryotic cells even without largely binding to the cells or producing bacteriocins affecting GAS (Fiedler et al., 2013). Further on, *S. salivarius* was shown to provide host cell protection against GAS by forming an impermeable biofilm so the host epithelial cells are inaccessible for initial GAS tissue colonization (Fiedler et al., 2013). Other authors stated *S. salivarius* K12 to antagonize GAS growth by expressing the lantibiotics salivarin A2 and B subsequently influencing GAS biofilm formation (Di et al., 2013, 2014).

CONCLUSION

The standard antibiotic medication for patients with GAS infections is not sufficient to eradicate GAS biofilms. Alternative or additional therapeutics are currently investigated. Phage lysin C represents the most promising candidate for clinical application. However, more efforts are needed in developing treatment strategies to prevent extensive and repeated antibiotic treatment in patients with biofilm associated recurrent GAS infections.

OUTLOOK AND FUTURE CHALLENGES

Due to the high tolerance of GAS biofilms toward antibiotics, GAS biofilms are likely to be associated with antibiotic treatment failure in patients. Therefore, the major future challenge will be the development of new therapeutic strategies to prevent the extensive use of antibiotics on patients with recurrent GAS biofilm associated infections. As can be seen from this review, we are just at the beginning of understanding the GAS biofilm phenotype and its relevance for GAS pathogenesis. Therefore, extensive further studies on the biological processes involved in GAS biofilm formation are necessary.

A major question in this context is which environmental factors trigger GAS biofilm formation. It is highly likely that apart from carbon source, external pH, and peptide pheromone levels, host innate immune responses trigger GAS biofilm development *in vivo*. Furthermore, more efforts are needed to decipher the role of individual virulence factors and gene regulation circuits in GAS biofilm development *in vivo*, since most of the current knowledge is based on *in vitro* data.

Since the GAS biofilm formation capacity is very strain specific, it will be important to include the determination of the biofilm phenotype of GAS strains into epidemiological investigations. Particularly the relation of the biofilm phenotype to other parameters frequently studied in GAS epidemiology, e.g., *emm*- or FCT-type, antibiotic resistance or presence/absence of certain virulence factors, needs to be elucidated. Ideally, such studies will lead to phenotypic profiles that will allow deducing the potential of GAS isolates for biofilm formation.

Such tools would facilitate the specific treatment of patients with recurrent infection potentially associated with GAS biofilms. The most promising candidates for clinical application in GAS biofilm eradication in patients are specific phage lysins such as PlyC, since they have excellent MBEC values. Research in this area should be intensified toward application in clinical practice.

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Pneumococci in biofilms are non-invasive: implications on nasopharyngeal colonization

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Streptococcus pneumoniae (the pneumococcus) is an opportunistic pathogen that colonizes the human nasopharynx asymptomatically. Invasive pneumococcal disease develops following bacterial aspiration into the lungs. Pneumococci within the nasopharynx exist as biofilms, a growth phenotype characterized by surface attachment, encasement within an extracellular matrix, and antimicrobial resistance. Experimental evidence indicates that biofilm pneumococci are attenuated vs. their planktonic counterpart. Biofilm pneumococci failed to cause invasive disease in experimentally challenged mice and *in vitro* were shown to be non-invasive despite being hyper-adhesive. This attenuated phenotype corresponds with observations that biofilm pneumococci elicit significantly less cytokine and chemokine production from host cells than their planktonic counterparts. Microarray and proteomic studies show that pneumococci within biofilms have decreased metabolism, less capsular polysaccharide, and reduced production of the pore-forming toxin pneumolysin. Biofilm pneumococci are predominately in the transparent phenotype, which has elevated cell wall phosphorylcholine, an adhesin subject to C-reactive protein mediated opsonization. Herein, we review these changes in virulence, interpret their impact on colonization and transmission, and discuss the notion that non-invasive biofilms are principal lifestyle of *S. pneumoniae*.

Keywords: *Streptococcus pneumoniae*, biofilms, colonization, virulence, transmission

INTRODUCTION

Streptococcus pneumoniae (the pneumococcus) is a leading cause of community-acquired pneumonia (CAP), sepsis, and meningitis throughout the world despite the existence of multiple effective vaccines (Bennett et al., 2014). This Gram-positive, encapsulated bacterium asymptomatically colonizes the human nasopharynx where carriage can last for months (Gray et al., 1980). In susceptible individuals, usually the very young and elderly, aspiration of pneumococci can lead to pneumonia and subsequently invasive pneumococcal disease (IPD). At any given time approximately 40% of children and 15% of adults are colonized (Crook et al., 2004; Huang et al., 2009). Annual global IPD burden is roughly 14.5 million cases resulting in 800,000 deaths in children under the age of 5 and a case fatality rate surpassing 20% in the elderly (O'Brien et al., 2009; Heron, 2012; Naucler et al., 2013).

S. pneumoniae in sputum and blood samples from individuals with IPD are primarily in the form of lancet-shaped diplococci; the same morphology observed when grown planktonically in media. Growth as diplococci or short chains is now recognized to help *S. pneumoniae* evade stochastic alternative pathway mediated complement deposition and opsonophagocytosis (Dalia and Weiser, 2011). Within the past 15 years it has become evident that the pneumococcus also forms biofilms *in vivo* during nasopharyngeal colonization (**Figure 1**) and otitis media (Hoa et al., 2009; Reid et al., 2009). Biofilms are aggregates of surfaced attached bacteria encased within an extracellular matrix (ECM). The ECM, which *in vivo* is composed of host factors, polysaccharides, and extracellular DNA, is now understood to protect bacteria from the host immune system and desiccation (Moscoso et al., 2006); the latter being important during pneumococcal fomite transmission (Walsh and Camilli, 2011). Importantly, biofilm pneumococci have been shown to be decisively less virulent than their planktonic counterparts. This review focuses on how *S. pneumoniae* modulates its virulence during biofilm formation and why this may promote long-term, asymptomatic colonization. We also discuss the increasingly evident role biofilms play during pneumococcal transmission on fomites.

BIOFILM PNEUMOCOCCI ARE AVIRULENT

Given the importance of biofilms in recalcitrant infections and for *S. pneumoniae* in the middle ear during otitis media (Reid et al., 2009; Chauhan et al., 2014), initial studies examining pneumococcal biofilms sought to associate the ability to form biofilms with enhanced virulence (Munoz-Elias et al., 2008; Lizcano et al., 2010). However, the ability to form biofilms *in vitro* could not be linked to the anatomical site from which a clinical isolate was obtained (i.e., nasopharynx of an asymptomatic carrier or blood from individual with IPD), nor the ability of the isolate to cause bacteremia in an infectious mouse model (Hall-Stoodley et al., 2008; Lizcano et al., 2010). Importantly, these and other

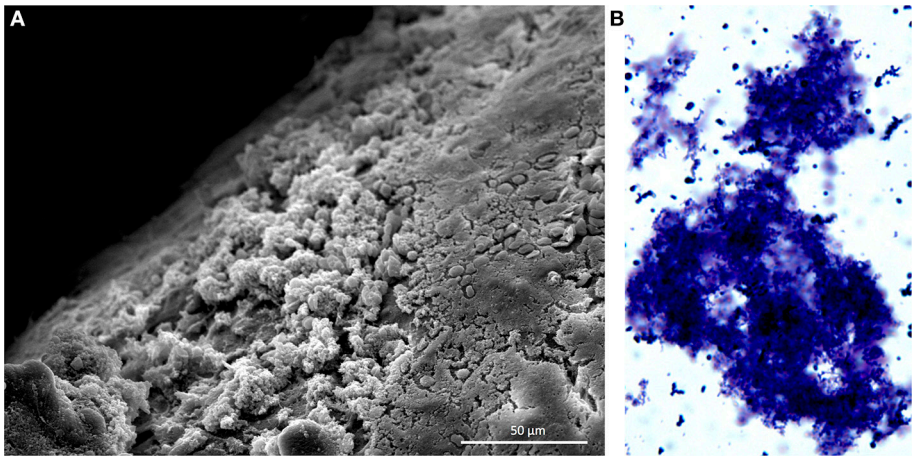

FIGURE 1 | Pneumococcal biofilms form in the nasopharynx. (A) Scanning electron microscopy image of *S. pneumoniae* biofilms formed on the nasal septum of a mouse. Mice were experimentally colonized 7 days prior. Biofilms are the non-contiguous aggregates on the left. **(B)** *S. pneumoniae* biofilm aggregate in nasopharyngeal lavage fluid. Sample was collected from mouse 14 days after experimental colonization. Pneumococci were stained with crystal violet and visualized with a light microscope at 400X. Image credit: Krystle Blanchette.

studies have shown that *in vitro* biofilm formation was most enhanced for mutants that lacked capsular polysaccharide (CPS) (Moscoso et al., 2006). CPS mutants are avirulent due to their inability to prevent opsonophagocytosis (Hyams et al., 2010). Thus, the fact that unencapsulated mutants form more robust biofilms suggested a direct disconnect between pneumococcal biofilm formation and its propensity for invasive disease.

To directly test if pneumococci within biofilms were virulent, Sanchez et al. intratracheally challenged mice with equal colony forming units (CFU) of a virulent serotype 4 isolate grown to exponential (mid-logarithmic) phase in media or as a 3-day biofilm in a continuous flow-through reactor. They observed that only mice infected with planktonic pneumococci progressed to bacteremia while most of those challenged with biofilm pneumococci had negative blood cultures (**Figure 2A**) (Sanchez et al., 2011b). Studies by Blanchette-Cain et al. showed that pneumococci grown as a biofilm were hyper-adhesive yet uninvasive when tested *in vitro* on Detroit-562 pharyngeal epithelial cells (**Figure 2B**) (Blanchette-Cain et al., 2013). Marks et al. had similar results and showed that pneumococci grown as biofilms on fixed and live NCI-H292 bronchial epithelial cells neither invaded nor were internalized. Of note, Marks et al. showed that pneumococci recently dispersed from a biofilm due to an inflammatory signal, such as viral infection, were hyper-virulent with substantially greater capacity to cause invasive disease in mice than either biofilm pneumococci or pneumococci grown for a sustained period planktonically (Marks et al., 2013). Why recently dispersed pneumococci are more virulent than their sustained planktonic counterparts is not immediately clear, albeit two possibilities are that these bacteria carry biofilm ECM components that enhance their adhesive capacity, and major changes in gene expression profiles (Pettigrew et al., 2014). This observation helps to explain why viral infection is an established risk factor for the development of pneumococcal pneumonia (Brundage, 2006; McCullers, 2006).

REDUCED CAPSULAR POLYSACCHARIDE DURING BIOFILM GROWTH

CPS is the principal virulence determinant for *S. pneumoniae* and exists in >90 identified serotypes (Bennett et al., 2014). In addition to resisting opsonophagocytosis (Melin et al., 2010), the negative or neutral charge of CPS plays an important role in helping the pneumococcus evade entrapment in mucus (Nelson et al., 2007). The necessity of CPS for IPD is exhibited by the fact that all invasive strains of *S. pneumoniae* are encapsulated whereas unencapsulated pneumococci are infrequent and usually only associated with topical eye infection (Barker et al., 1999).

Multiple investigators have reported an inhibitory role for CPS during *in vitro* biofilm formation with capsule deficient mutants forming substantially more robust biofilms than their encapsulated parent strain (Moscoso et al., 2006; Qin et al., 2013). Allergucci and Sauer showed that biofilms formed by a serotype 3 isolate were in large part composed of spontaneous mutants deficient in CPS related genes (Allegrucci and Sauer, 2007). Marks et al. have added evidence that this may occur *in vivo* by showing that unencapsulated pneumococci form more robust biofilms on the surface of epithelial cell monolayers (Marks et al., 2012). In fact, the presence of a capsule was shown to inhibit unencapsulated pneumococci from forming robust biofilms in mixed *in vitro* cultures (Domenech et al., 2009). Yet, CPS production is required for efficient *in vivo* colonization (Shainheit et al., 2014), indicating that during colonization the pneumococcus must strike a balance between CPS hindrance of biofilm formation and resistance to host defense.

Gene expression analyses using qRT-PCR and microarrays have shown that genes within the CPS operon were downregulated during *in vitro* biofilm formation vs. planktonic growth (Oggioni et al., 2006; Sanchez et al., 2011b). Moreover, the amount of capsule detected and the enzymes responsible for CPS production were substantially lower for biofilm vs. planktonic grown pneumococci as detected by ELISA and MALDI-TOF

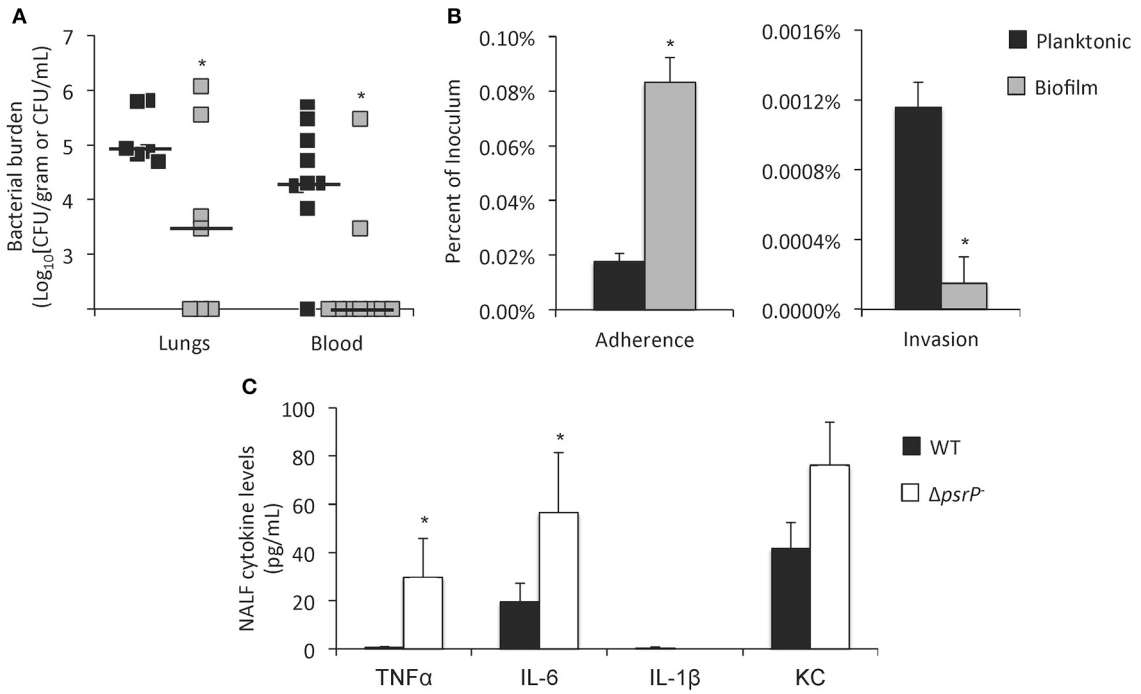

FIGURE 2 | Biofilm pneumococci are less virulent and elicit a weaker immune response than their planktonic counterparts. (A) Bacterial titers in the lungs and blood of BALB/c mice challenged intratracheally with 10^5 CFU of planktonic or biofilm derived *S. pneumoniae* (each square = individual mouse; $n = 6$–8). From: Sanchez et al. (2011b). **(B)** Percentage of planktonic and biofilm derived *S. pneumoniae* that attached and invaded Detroit-562 pharyngeal cells *in vitro*. Percentages were calculated from the total inoculum. **(C)** Cytokine levels in nasopharyngeal lavage fluid (NALF) of colonized mice. Mice were challenged with wild type (WT) and PsrP-deficient ($\Delta psrP^-$) *S. pneumoniae* and NALF collected 7 days later. Note that $\Delta psrP^-$ does not form biofilms during colonization ($n = 5$). Panels **(B,C)** from: Blanchette-Cain et al. (2013). Asterisks denote a statistically significant difference ($P < 0.05$).

(Sanchez et al., 2011a,b). In agreement with dynamic changes in CPS production, pneumococci reduce capsule thickness once in contact with epithelial cells (Hammerschmidt et al., 2005). This is supported by microarray gene analysis of cells in contact with respiratory epithelial cells *in vitro* (Orihuela et al., 2004b). Thus, biofilm pneumococci reduce levels of CPS making them more susceptible to phagocytosis following aspiration.

PHASE VARIATION

S. pneumoniae oscillates between an opaque phase variant that produces high levels of CPS and low levels of cell wall teichoic acid, and a transparent phase variant with low CPS and high cell wall teichoic acid (Weiser et al., 1994). The basis for phase variation is now understood to be epigenetic, with alternate methylation patterns on genes (Manso et al., 2014). Due to negative selection for the transparent phase by phagocytes, opaque variants predominate in the blood (Kim and Weiser, 1998). In contrast, the transparent phenotype is better able to adhere to cells and thus predominates in the nasopharynx (Weiser et al., 1994). Of note, Sanchez et al. have shown that *in vitro* biofilms are primarily composed of the transparent variant, despite the seed cultures used to initiate the biofilm being mostly opaque (Sanchez et al., 2011b).

In its transition from opaque to transparent, the pneumococcus loses virulence potential while enhancing its ability to adhere to host cells. As discussed, loss or a reduction in CPS enhances susceptibility to opsonophagocytosis yet is required for the exposure of surface adhesins that mediate bacterial attachment to host cells (Ring et al., 1998). Critically, cell invasion occurs for planktonic but not biofilm pneumococci (Blanchette-Cain et al., 2013). The increased amount of teichoic acid carried by the transparent variant also makes it subject to recognition by C-reactive protein (CRP), resulting in activation of complement (Kim et al., 1999). However, phosphorylcholine residues present on teichoic acid allow the pneumococcus to bind to the host ligand platelet-activating factor (PAFr) receptor on host cells (Cundell et al., 1995).

Despite loss of capsule and increased exposure of teichoic acid, pneumococci in biofilms are resistant to opsonophagocytosis (Yuste et al., 2007). One reason for this includes that CRP binding to phosphorylcholine is competed with by members of the choline-binding protein family (Mukerji et al., 2012), such as the adhesin Choline-binding protein A (CbpA) which is upregulated during transparent phase growth as well as in biofilms (Sanchez et al., 2011b). CbpA is also a key inhibitor of complement deposition through its binding to Factor H and complement component C3 (Cheng et al., 2000; Dave et al., 2001). Another choline-binding protein that plays a key role in complement inhibition includes Pneumococcal surface protein A (PspA), which prevents classical complement activation in a C1q dependent manner (Tu et al., 1999; Yuste et al., 2007; Mukerji et al., 2012). Importantly, the opaque variant has been suggested to play a

critical role in the formation of the ECM (Trappetti et al., 2011). Of note, gene expression studies for biofilms and transparent pneumococci do not entirely overlap. Thus, phase variation is an important aspect of pneumococcal biofilm formation but is not entirely responsible for its phenotype.

DOWNREGULATION OF METABOLIC PROCESSES AND MODULATED VIRULENCE GENE EXPRESSION

Antimicrobial resistance is one of the defining properties of biofilms and has been extensively documented for biofilm pneumococci, particularly in the context of recurring otitis media (Stewart and Costerton, 2001; Hall-Stoodley et al., 2008). Why, the ability of an isolate to form well-structured biofilms *in vivo* was correlated with resistance to high concentrations of gentamycin (Marks et al., 2012). Enhanced resistance to antimicrobials in biofilm pneumococci may be due to a decrease in metabolic rate, which also confers resistance to antimicrobials targeting cell wall, protein synthesis, and DNA replication. The ECM also serves as an inhibitor or off-target for antimicrobials. This topic is extensively reviewed elsewhere (Domenech et al., 2012). Once aspirated, a reduced metabolic rate would presumably impair the ability of biofilm pneumococci to respond in a timely fashion to hostile host factors present in the lower respiratory tract.

Along such lines, planktonic and biofilm *S. pneumoniae* are now recognized to have distinct protein and gene expression profiles. Using qRT-PCR, Oggioni et al. showed that the gene expression profile of virulence-associated genes of different strains isolated from the blood were more similar to that of planktonic growth in broth, whereas the same strain isolated from the lungs, brain, or nasopharynx of infected mice was more similar to that of *in vitro* biofilms (Oggioni et al., 2006). Microarray analysis of *in vitro* grown planktonic vs. biofilm pneumococci showed that biofilm pneumococci downregulated genes involved in protein synthesis, energy production, metabolism, CPS production; along with the virulence genes that encode the pneumococcal pilus, which has been shown to be an invasin (Barocchi et al., 2006), and the pore-forming toxin pneumolysin (Sanchez et al., 2011b). Pneumolysin has been demonstrated to be required for systemic bacteremia and host cell damage and inflammation (Orihuela et al., 2004a; Mitchell and Dalziel, 2014), thus its down regulation would most likely compromise virulence. Yet pneumolysin has also been shown to contribute toward *in vitro* biofilm formation (Shak et al., 2013). Thus, and like that for CPS, pneumolysin production is most likely fine-tuned to strike a balance with the host during colonization. In contrast, the genes encoding the adhesins PsrP, PavA as well as the previously discussed CbpA, were detected as being upregulated during biofilm growth (Sanchez et al., 2011b; Qin et al., 2013). These proteins may play a role in intra-species aggregation such as observed during *in vivo* biofilms, either by binding to other pneumococci directly or through bridging molecules such as fibronectin (Blanchette and Orihuela, 2012). The, why biofilm pneumococci do not invade cells remains unclear.

Mass spectroscopy (MS) based identification of proteins isolated from biofilm and planktonic cell lysates confirm profound differences between these two physiological growth states (Allegrucci et al., 2006; Sanchez et al., 2011a). One important caveat to this approach is that pneumococcal biofilms are in part composed of dead pneumococci and proteomic studies don't distinguish between proteins from live bacteria or those dead bacteria that have accumulated within the biofilm. When alive, these dead bacteria may have had a substantially different proteome. Nonetheless, and in agreement with microarray studies, MS of biofilm and planktonic cell lysates by Sanchez et al. found that the frequency of peptides corresponding to enzymes involved in protein synthesis and processing, energy metabolism, CPS production, and proteins involved in transcription, regulation and DNA binding, as well as the virulence determinants enolase, pyruvate oxidase (produces hydrogen peroxide), and pneumolysin were less frequent in biofilm lysates than planktonic lysates (Sanchez et al., 2011a). The extent to which major differences occur in the proteome is further highlighted by the finding that antiserum from humans who recovered from IPD robustly recognized proteins in planktonic cell lysates but not biofilm cell lysates when tested by Western blot (Sanchez et al., 2011a). This provides evidence that the *in vivo* antigen protein profiles for colonization vs. invasive disease are considerably different, and that the overall productions of factors that mediate a response to a host or subvert the host response are altered.

HOST RESPONSE TO BIOFILM PNEUMOCOCCI

Only recently have investigators begun to examine how the host responds to biofilm pneumococci. Studies by Blanchette-Cain et al. have shown that biofilm pneumococci elicit significantly less Interleukin (IL)-6 and IL-8 from Detroit-562 pharyngeal epithelial cells than planktonic cultures. Similarly, biofilm pneumococci elicited less IL-6, IL-1β, and TNFα, from J774A.1 macrophages. *In vivo*, mutant pneumococci lacking the biofilm determinant PsrP, and thus unable to form *in vivo* biofilms, elicited greater TNFα, IL-6, IL-1β, and KC production in the nasopharynx of 7-day colonized mice vs. its parent strain (**Figure 2C**) (Blanchette-Cain et al., 2013). This was credited to the reduced tissue invasiveness of biofilm pneumococci, but as indicated may also involve reduced production of the toxin pneumolysin. Importantly, pneumococci may also actively suppress the host response in a way that has not yet been determined. For example studies have shown that Group B Streptococcus interacts with Siglec-5 and this dampens the host response (Carlin et al., 2009). Future studies examining this possibility are warranted.

Given the fact that the majority of individuals are colonized asymptomatically, we speculate that non-invasive pneumococci within biofilms promote long-term colonization and transmission through less vigorous activation of the innate immune response and therefore a delay in the onset of the adaptive response and their clearance. Yet, direct evidence for this is lacking with intranasal challenge of mice with PsrP-deficient or other mutants that are biofilm deficient not resulting in reduced bacterial titers in the nasopharynx when measured by CFU or qRT-PCR (Blanchette-Cain et al., 2013). Thus, studies are warranted to determine what the true physiological advantage of this immunoquiescent phenotype actually is and if it impacts the number of bacteria in the nasopharynx or long-term carriage.

BIOFILMS AS SOURCE OF FOMITE TRANSMISSION AND CONCLUSIONS

Multiple studies have shown that biofilm-derived pneumococci are more resistant to desiccation than their planktonic counterparts (Walsh and Camilli, 2011), with viable cells isolated from fomites over a period ten times longer than planktonic (Marks et al., 2014). Additionally, viable pneumococci have been recovered from a variety of desiccated surfaces in a day care setting: hands, books, and both hard and soft toys. Importantly, desiccated pneumococci recovered from fomites still retain colonization capabilities in a murine model, even with a normal inoculum (Walsh and Camilli, 2011; Marks et al., 2014). As is shown in **Figure 1B**, the pneumococcal aggregates can be sloughed from the nasopharynx and these aggregates most likely are a vehicle for transmission, possibly providing the bacteria with moisture and nutrients for an extended period outside the body. We point out that dispersed bacteria, as shown by Marks et al. (2013), are planktonic and may be a second vehicle for transmission following an inflammatory episode such as virus infection.

Most individuals carrying *S. pneumoniae* are colonized asymptomatically, thus the biofilm state is the major form by which the pneumococcus interacts with its host. Herein we have discussed how biofilm pneumococci are distinct from their planktonic counterparts. Specifically, pneumococci downregulate CPS, enhance expression of adhesins, shift toward the transparent phenotype, and lower the expression of metabolic processes and key virulence determinants that elicit a robust host response. Therefore, biofilm pneumococci seem to be exquisitely honed to the colonization phenotype at the expense of the invasive phenotype. There are many questions that remain to be answered; for example, direct evidence that the immunoquiescent phenotype confers a colonization advantage is lacking. This may be due to limitations in the current model systems and/or our ability to quantify bacteria *in vivo*. Additionally, does the pneumococcus rely on dispersal of biofilm aggregates or the spread of highly invasive biofilm dispersed planktonic pneumococci as the principle method for transmission, or are both effective? Perhaps different strains rely differently on these transmission methods. There are also infections that seem to be a mix of biofilms and planktonic bacteria, for example during otitis media. How these two physiological states impact the course of disease is unclear and warrants attention. In summary, a myriad of functional reasons can and do exist for why biofilm pneumococci are less virulent. A better understanding of the short-term survival and long-term evolutionary advantages would substantially enhance our understanding of pneumococcal biology, and may permit us to develop novel targets for bacterial clearance.

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Streptococcus mutans-derived extracellular matrix in cariogenic oral biofilms

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Biofilms are highly structured microbial communities that are enmeshed in a self-produced extracellular matrix. Within the complex oral microbiome, *Streptococcus mutans* is a major producer of extracellular polymeric substances including exopolysaccharides (EPS), eDNA, and lipoteichoic acid (LTA). EPS produced by *S. mutans*-derived exoenzymes promote local accumulation of microbes on the teeth, while forming a spatially heterogeneous and diffusion-limiting matrix that protects embedded bacteria. The EPS-rich matrix provides mechanical stability/cohesiveness and facilitates the creation of highly acidic microenvironments, which are critical for the pathogenesis of dental caries. In parallel, *S. mutans* also releases eDNA and LTA, which can contribute with matrix development. eDNA enhances EPS (glucan) synthesis locally, increasing the adhesion of *S. mutans* to saliva-coated apatitic surfaces and the assembly of highly cohesive biofilms. eDNA and other extracellular substances, acting in concert with EPS, may impact the functional properties of the matrix and the virulence of cariogenic biofilms. Enhanced understanding about the assembly principles of the matrix may lead to efficacious approaches to control biofilm-related diseases.

Keywords: biofilms, dental caries, *Streptococcus mutans*, extracellular matrix, spatial heterogeneities, mechanical stability, exopolysaccharides, eDNA

Biofilms are highly dynamic and structured communities of microbial cells that are firmly attached to a surface and enmeshed in a three-dimensional (3D) extracellular matrix of polymeric substances such as exopolysaccharides (EPS), proteins and nucleic acids (Branda et al., 2005; Flemming and Wingender, 2010). The extracellular matrix produced by specific microorganisms promotes microbial adhesion and cohesion while also hindering diffusion (Flemming and Wingender, 2010). It essentially provides a 3D scaffold for biofilm development, helping to shape spatial, metabolic and microenvironmental heterogeneities (Stewart and Franklin, 2008; Mann and Wozniak, 2012; Wozniak and Parsek, 2014). Thus, the matrix is critical for the existence of biofilm lifestyle and full expression of virulence by bacterial and fungal pathogens. Consequently, matrix builders (i.e., the microorganisms that produce or process polymeric substances) play a key role in the development of biofilms.

Many infectious diseases in humans are caused by virulent biofilms, including those occurring in the mouth. Among them, dental caries continues to be one of the most ubiquitous and costly biofilm-dependent oral diseases worldwide, which compromise the health and well-being of children and adults alike (Marcenes et al., 2013). This disease results from complex interactions between specific oral microorganisms, host factors and diet that promote the establishment of caries-producing (cariogenic) biofilms on tooth surfaces (Selwitz et al., 2007; Russell, 2008). The assembly of cariogenic biofilms is a prime example of how pathogenic bacteria such as *Streptococcus mutans* orchestrate the development of virulent biofilms on (tooth) surfaces, as an extracellular matrix assembles (as reviewed in Hamada and Slade, 1980; Loesche, 1986; Bowen and Koo, 2011). EPS are the main constituents of the matrix in cariogenic biofilms and are recognized as essential virulence factors associated with dental caries (Yamashita et al., 1993; Mattos-Graner et al., 2000; Vacca Smith et al., 2007). Nevertheless, other constituents such as extracellular DNA (eDNA) and lipoteichoic acids (LTA) have been also found in high amounts in the matrix of cariogenic biofilms.

The microbial composition and structural organization of cariogenic biofilms are not static but rather change dynamically (Marsh, 2003). In the complex oral microbiome, *S. mutans* is not always the most numerous species; many organisms are equally acidogenic and aciduric (Takahashi and Nyvad, 2011; Valm et al., 2011; Mattos-Graner et al., 2014). However, *S. mutans* is a major matrix producer and can rapidly modulate the formation of cariogenic biofilms when dietary sucrose and starch are present (Firestone et al., 1982; Marsh, 2003; Ribeiro et al., 2005; Paes Leme et al., 2006). Sucrose serves as substrate while

starch hydrolysates act as acceptors for EPS (glucans and fructans) synthesis by *S. mutans* glucosyl- and fructosyltransferases (Gtfs and Ftfs) (Fu and Robyt, 1991; Bowen and Koo, 2011). Moreover, *S. mutans*-released Gtfs are constituents of the pellicle and synthesize glucans *in situ*, promoting local colonization of *S. mutans* and other organisms; Gtfs also bind to surface of other oral microorganisms converting them into glucan producers (as reviewed in Bowen and Koo, 2011). Thus, the production of EPS on surfaces enhances local accumulation and clustering of microbes on teeth. As the biofilm develops, the EPS formed *in situ* enmeshes and surrounds the microorganisms while forming an insoluble matrix facilitating the assembly of spatially heterogeneous yet cohesive 3D multicellular structures (as reviewed in Koo et al., 2013).

The spatial heterogeneities shaped by EPS synthesis form a complex 3D matrix architecture and create environmental and protective niches within biofilms that can directly modulate caries pathogenesis. Available evidence suggests there is a substantial limitation of diffusion into and out of the biofilm due to the presence of insoluble EPS-rich matrix, which could facilitate acid accumulation and hinder neutralization by buffering saliva that surrounds the teeth, as reviewed recently (Bowen and Koo, 2011; Koo et al., 2013) and thereby it will not be discussed here. Furthermore, EPS from *S. mutans* may be charged due to the incorporation of LTA (Kuramitsu et al., 1980; Rölla et al., 1980; Vickerman and Jones, 1992) and possibly eDNA (see later). The presence of negatively charged EPS appears to affect the penetration (and antimicrobial activity) of positively charged chlorhexidine into biofilms (Hope and Wilson, 2004). The detailed mechanisms involved in limiting diffusion remain to be elucidated. Furthermore, little is known about how secreted metabolites and proteins migrate from producing microorganisms within the matrix of intact biofilms. It is noteworthy that polysaccharide within plaque-biofilms is not evenly distributed, and its density is enhanced at the tooth interface (Reese and Guggenheim, 2007), which could affect mass transport and diffusion properties across the biofilm structure (Thurnheer et al., 2003; Robinson et al., 2006). Recently, Xiao et al. (2012) showed the importance of the manner by which the EPS matrix is assembled three-dimensionally and how it is spatially arranged with the bacterial cells to create compartmentalized pH microenvironments, while conferring protection to bacteria against chlorhexidine locally within intact biofilm architecture.

In parallel, sugars are fermented by *S. mutans* and other acidogenic organisms embedded in the matrix, facilitating the formation of highly acidic microenvironments (pH 4.5–5.5) (Vroom et al., 1999; Xiao et al., 2012; Guo et al., 2013). The low pH niches induce EPS synthesis while cariogenic organisms such as *S. mutans* prosper (Quivey et al., 2000; Lemos and Burne, 2008; Smith and Spatafora, 2012). As the environmental acidic stress further increases, the microbial diversity is reduced in favor of a highly acid-tolerant and acidogenic microbiota (Takahashi and Nyvad, 2011). Consequently, local acidity ensures continuous biofilm accretion and acid-dissolution of adjacent tooth enamel, leading to the onset of dental caries. Altogether, the creation of localized microenvironments, delineated by a diffusion-limiting matrix, has profound effects on the architecture, metabolism and expression of virulence of biofilm as a whole. Although the immediate cause of enamel dissolution is certainly acid production, the absence of the "sheltering" effect of the biofilm matrix would minimize the ability of acids to demineralize in the presence of saliva. The insoluble EPS-rich matrix produced by *S. mutans* is a unique virulence feature of this species that helps to set it apart from other acidogenic and aciduric species.

Importantly, well-established biofilms become recalcitrant to antimicrobials and difficult to remove from surfaces (Hall-Stoodley et al., 2004; Marsh et al., 2011; Stewart, 2014). Historically (and currently), mechanical removal of plaque-biofilm by tooth brushing and dental flossing, in addition to fluoride use, have been the standard measures to prevent dental caries. Thus, enhanced understanding of how biofilms can be disrupted and removed from the surface of attachment could lead to improved strategies to eradicate them. The EPS formed on surfaces and further development of polymeric matrix may be responsible for the mechanical properties of cariogenic biofilms, such as adhesive strength and cohesiveness.

The presence of glucans enhances local adhesion strength of *S. mutans* on apatitic surfaces (Schilling and Bowen, 1992; Tsumori and Kuramitsu, 1997; Cross et al., 2007), while development of a glucan-rich matrix and cell-glucan adhesions are essential for the structural integrity of the biofilm 3D architecture (Banas and Vickerman, 2003; Lynch et al., 2007; Xiao et al., 2012). In addition, the viscoelastic properties of *S. mutans* biofilms are similar to those of organic polymers (Vinogradov et al., 2004). Thus, the mechanical properties of biofilms may be determined by the EPS composition and degree of branching, and spatial distribution of EPS-rich matrix, which in turn modulate the three-dimensionality of the biofilm architecture. A variety of biophysical methods, ranging from rheometry (Klapper et al., 2002; Towler et al., 2003), uniaxial compression to fluid flow (Körstgens et al., 2001; Busscher and van der Mei, 2006), and atomic force spectroscopy (Cross et al., 2007; Das et al., 2011) have been applied to characterize the mechanical properties of bacterial adhesion and biofilm formation. However, it remains unclear how EPS modulate adhesive and cohesive forces of the matrix, which are essential for the mechanical stability and surface attachment of biofilms, particularly in cariogenic biofilms where the EPS matrix plays a critical scaffolding role.

Recently, we investigated the mechanical stability and surface detachment of mature *S. mutans* biofilms using rheometry and shear stress-based methods. A rheometer measures the changes in rigidity and viscoelasticity of biofilms, which are key parameters of their mechanical properties (Vinogradov et al., 2004; Cense et al., 2006; Cheong et al., 2009). However, biofilm's response to fluid shear stress (deformation and detachment) is also recognized as relevant for its mechanical stability (Klapper et al., 2002). We developed a device that generates a diverse range of shear stress to assess how incremental increases in shear cause distinctive pattern of biofilm removal and detachment from saliva-coated hydroxyapatite (sHA) surfaces (which mimics pellicle-coated teeth) (Hwang et al., 2014). We used the shear-inducing device with EPS-digesting enzymes to examine the influence of the matrix on mechanical stability of well-established *S. mutans* biofilms (Hwang et al., 2014).

The data revealed a two-phase biofilm removal profile from the sHA surface following measured applications of increasing shear stresses. We observed an initial bulk elimination that was proportional to the amount of shear stress applied, followed by increased resistance to removal of the remaining biomass close to the surface. Confocal fluorescence imaging showed a thick and dense basal layer of EPS adjacent to the sHA surface, which could enhance biofilm anchoring to the surface and thereby contribute to the increased resistance to shearing. Indeed, degradation of the EPS via non-biocidal glucanohydrolase (dextranase) caused a major disruption on the ability of the biofilm to withstand mechanical removal from the surface, greatly facilitating its removal and further surface detachment (Hwang et al., 2014). Strikingly, large sections of the biofilms were completely detached from the sHA surface upon application of shear stress on dextranase-treated biofilms. Thus, both the content and spatial distribution of EPS-rich matrix influence the mechanical stability and surface attachment of intact *S. mutans* biofilms.

To further investigate the role of EPS-matrix on the mechanical stability of the biofilms, the rheological properties of biofilms treated with EPS-digesting enzymes were examined using rheometry on intact biofilm samples (Mert and Campanella, 2008; **Figure 1**). Intact *S. mutans* biofilms presented higher storage modulus (31,718 ± 3,440 Pa) than loss modulus (3,775 ± 450 Pa), indicating that *S. mutans* biofilm has a viscoelastic (solid-like) behavior and is highly structured (Hwang et al., 2014), in agreement with previous studies (Vinogradov et al., 2004; Cense et al., 2006). The contribution of EPS matrix to the viscoelasticity and rigidity of *S. mutans* biofilm was assessed by treating biofilms with two distinct glucanohydrolases: mutanase, which hydrolyzes α-(1→3) glucosidic linkages and branch points in GtfB and GtfC-derived insoluble glucans, and dextranase, which digests α-(1→6) (and branch points) present in GtfB/C- as well as in GtfD-derived glucans (Guggenheim, 1970; Hayacibara et al., 2004). The insoluble glucans produced by GtfB and C are essential for the assembly of a 3D extracellular matrix scaffold and localized pH microenvironments in cariogenic biofilms (Xiao et al., 2012), which may explain their role in the expression of *S. mutans* virulence *in vivo* (Tanzer et al., 1985; Yamashita et al., 1993) as well their association with caries activity in humans (Mattos-Graner et al., 2000; Vacca Smith et al., 2007).

Digestion of EPS matrix with dextranase or mutanase caused more than a two-fold reduction in the storage modulus (**Figure 1**) compared to untreated biofilms, while the combination of both glucanohydrolase led to a 3-fold reduction (vs. untreated biofilms). Treatment with glucanohydrolases substantially changes biofilm rigidity, which greatly facilitated removal of mature biofilms (Hwang et al., 2014). These observations indicate that the digestion of α-(1→6) and α-(1→3) glycosyl linkages in the matrix structure severely impacts biofilm cohesiveness and stability. Indeed, the 3D biofilm structure eventually collapsed when the biofilms were incubated with the EPS-digesting enzymes for a prolonged period (>5 h). Altogether, the ability of mature *S. mutans* biofilms to withstand mechanical clearance is associated at least in part by the amounts, spatial distribution and structural rigidity of the exopolysaccharides-rich matrix.

These findings have clinical relevance in the pathogenesis of dental caries and development of novel antibiofilm approaches. The shear rate in the oral cavity due to salivary flow is relatively low (Bourne, 2002), which could explain why EPS-mediated biofilm build-up persists on tooth surfaces and is difficult to detach under salivary flow. Thus, novel devices to enhance mechanical removal of biofilms can be developed based on knowledge about the biophysical properties of the biofilm. Recently, a prototype AirFloss instrument that generates high shear stress locally was capable of removing sucrose-grown *S. mutans* biofilms from the interproximal space (Rmaile et al., 2014). Furthermore, enzymes or compounds capable of altering the viscoelastic properties of the biofilm could be effective to prevent biofilm-dependent diseases (Daniels et al., 2010; Kostakioti et al., 2013; Nguyen et al., 2014). The possibility of using glucanohydrolases as therapeutic approach against dental caries has been explored (e.g., Bowen, 1972; Guggenheim et al., 1980) despite limitations in the clinical setting, possibly due to

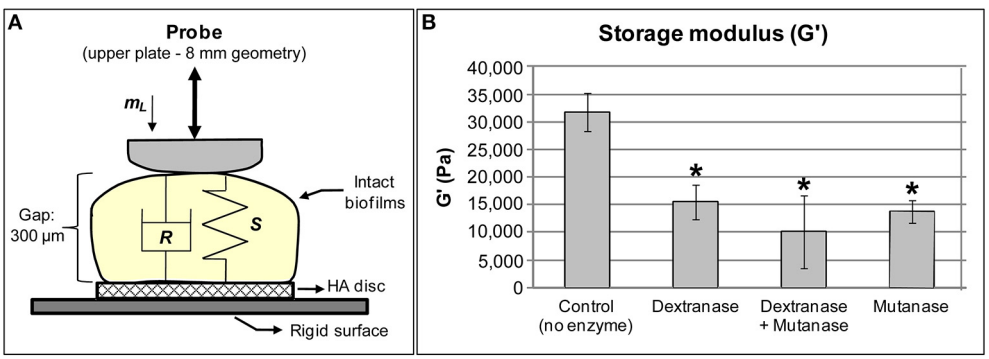

FIGURE 1 | *Streptococcus mutans* biofilms subjected to rheometry analysis. (A) Close-up view of biofilm samples placed between the upper and bottom plates of the rheometer. Stiffness, S, indicates rigidity of the sample whereas damping, R, is related to viscosity of the samples. m_L is the initial load applied to the sample during measurement. Gap: the size was standardized at 300 µm because the 3D structure of biofilm is not homogeneous, and 300 µm was the average maximum thickness of 115 h-old biofilms grown in 1% sucrose. **(B)** *Storage modulus* of *S. mutans* biofilms incubated with 2 units of each enzyme (* $P < 0.05$ vs. control), using small amplitude oscillatory shear experiments performed within the linear viscoelastic region at 1 Hz. All treatments with glucanohydrolases modified the rheological properties, specifically decreased the *storage modulus* of treated biofilms.

lack of retention in the mouth and/or enzyme degradation by proteolysis in saliva (Hull, 1980). Nevertheless, the concept of digesting/removing or changing the structure of EPS matrix to control biofilms is certainly attractive. Thus, new enzymes or novel approaches to deliver or retain them in active form should be devised.

Clearly, EPS play a critical role in the assembly and virulence of cariogenic biofilms by providing a 3D scaffold that shape the microenvironment and ensure mechanical stability. Yet, they are not alone. The production and release of both eDNA and LTA by *S. mutans* is also highly induced by sucrose, starch and increased acidity (Ciardi et al., 1977, 1981; Jacques et al., 1979; Rölla et al., 1980; Hardy et al., 1981; Perry et al., 2009; Klein et al., 2010). However, their functional roles in matrix assembly remain poorly understood.

eDNA AND LTA ALSO INFLUENCE THE STRUCTURE AND STABILITY OF CARIOGENIC BIOFILM MATRIX

eDNA and LTA may contribute to the assembly of the matrix by enhancing glucan synthesis (Kuramitsu et al., 1980; Chiu and Baker, 1994) and promoting bacterial binding to surfaces (Ciardi et al., 1977; Vickerman and Jones, 1992; Das et al., 2010). eDNA is often a byproduct of autolysis (Steinberger and Holden, 2005; Allesen-Holm et al., 2006; Perry et al., 2009), but it can be also secreted via microvesicles or membrane vesicles (Liao et al., 2014). eDNA enhances *S. mutans* adhesion to glass surfaces by creating thermodynamically favorable conditions for bacterial adhesion and surface aggregation due to acid-base interactions (Das et al., 2010). Moreover, eDNA builds and strengthens the matrix by interacting with exopolysaccharides within *Myxococcus xanthus* biofilms, where acidic pH enhances the binding between eDNA and EPS (Hu et al., 2012). These findings may be relevant to cariogenic biofilms.

Dietary sucrose and starch enhance release of eDNA into the matrix in high quantities due to upregulation of *lytTS* genes (and the *ccpA* gene) during *S. mutans* biofilm formation (Klein et al., 2010). The two component system *lytTS* is required to activate expression of *lrgAB* genes that are part of *S. mutans* arsenal to control autolysis and biofilm formation (Ahn et al., 2010). The expression of *lytTS* and *lrgAB* is regulated by availability of carbohydrates via CcpA (Ahn et al., 2010). In addition, the *gtfB* expression is also upregulated in biofilm growing in the presence of sucrose and starch (Klein et al., 2009, 2010). Thus, eDNA interacting with GtfB, may have important roles in EPS production, *S. mutans* colonization and biofilm matrix assembly (Klein et al., 2010; Liao et al., 2014).

eDNA increased glucan synthesis by GtfB adsorbed on saliva-coated hydroxyapatite (sHA) and on *S. mutans* cell surfaces (but not on *Streptococcus gordonii*), while being incorporated into glucan structure (**Figure 2**). Moreover, a larger number of *S. mutans* cells bound to the glucan formed on apatitic surface in presence of eDNA than did on glucan without eDNA, while no effects were observed on bacterial binding to sHA (Liao et al., 2014; **Figure 2**). eDNA interspersed with glucans may provide enhanced binding sites for *S. mutans* colonization. Strikingly, an opposing trend was observed for *S. gordonii* (a commensal non-cariogenic organism), which adhered more avidly on sHA with eDNA than on glucan-coated surface either with or without eDNA (**Figure 2**). Therefore, eDNA may contribute to cariogenic biofilm initiation by increasing EPS (glucans) synthesis *in situ* that display selective and enhanced binding capacity to *S. mutans*.

Furthermore, as biofilms develop, eDNA appears to be incorporated into the matrix because the addition of DNAse disrupted the structural integrity of the biofilms (Klein et al., 2010; Liao et al., 2014), suggesting that eDNA play an important role in biofilm matrix structure. Further biofilm characterization confirmed that eDNA is indeed an essential component of the matrix, which is found interconnected with glucans (**Figure 2**). It is apparent that eDNA is released not only from cell lysis, but also through membrane vesicles during different phases of biofilm development (Liao et al., 2014). Deficiency of protein secretion and membrane protein insertion machinery components (i.e., Ffh, YidC1, and YidC2) can reduce the amount of eDNA in the matrix (Liao et al., 2014). Thus, eDNA together with GtfB-derived glucans may facilitate accumulation of cariogenic bacteria while assembling highly structured and cohesive biofilm matrix.

Lipoteichoic acids may be also relevant for matrix assembly and biofilm formation. LTA from *S. mutans* is abundant in the pellicle and induces insoluble glucan synthesis (Ciardi et al., 1977; Kuramitsu et al., 1980; Rölla et al., 1980). LTA may enhance bacterial binding to tooth surfaces, and affect the composition of the matrix (Kuramitsu et al., 1980; Rölla et al., 1980), particularly when sucrose and starch are available. LTA is anchored to the cell membrane (Ellwood and Tempest, 1972; Neuhaus and Baddiley, 2003) and can be released to the extracellular milieu during cell wall remodeling. The mechanism of LTA synthesis was described for a few species but not for *S. mutans* (Reichmann and Gründling, 2011; Denapaite et al., 2012). Our BLAST analyses showed that *S. mutans* gene SMU.775 (a hypothetical protein) is a homolog to *ltaS* gene that encodes the LTA synthase enzyme of *Staphylococcus aureus*. The *dltABCD* operon is required for addition of D-alanine residues to LTA (Neuhaus and Baddiley, 2003); these residues affect adhesion, biofilm formation and cariogenicity of *S. mutans* (Spatafora et al., 1995, 1999; Gross et al., 2001; Götz, 2002). Furthermore, the proteins encoded by genes *dltA*, *dltD* and SMU.775 were most abundant when the matrix is being actively constructed during the initial phases of biofilm formation (Klein et al., 2012).

It is apparent that eDNA and LTA may act in concert with EPS, directly modulating the assembly, structural organization and functional properties of the matrix during cariogenic biofilm formation; alterations in these processes can alter the matrix composition impacting biofilm assembly and virulence potential. Furthermore, the production of *S. mutans* eDNA and LTA is enhanced in the presence of other organisms (Klein et al., 2012). Thus, other species may contribute to the matrix construction, either by directly modulating *S. mutans* expression of gene products involved with eDNA and LTA export, or by releasing LTA and eDNA themselves into the matrix. The mechanisms that triggers the release of eDNA and LTA and how these byproducts are incorporated into the biofilm matrix remain unclear, and studies are in progress to investigate their structural and functional roles in cariogenic biofilms.

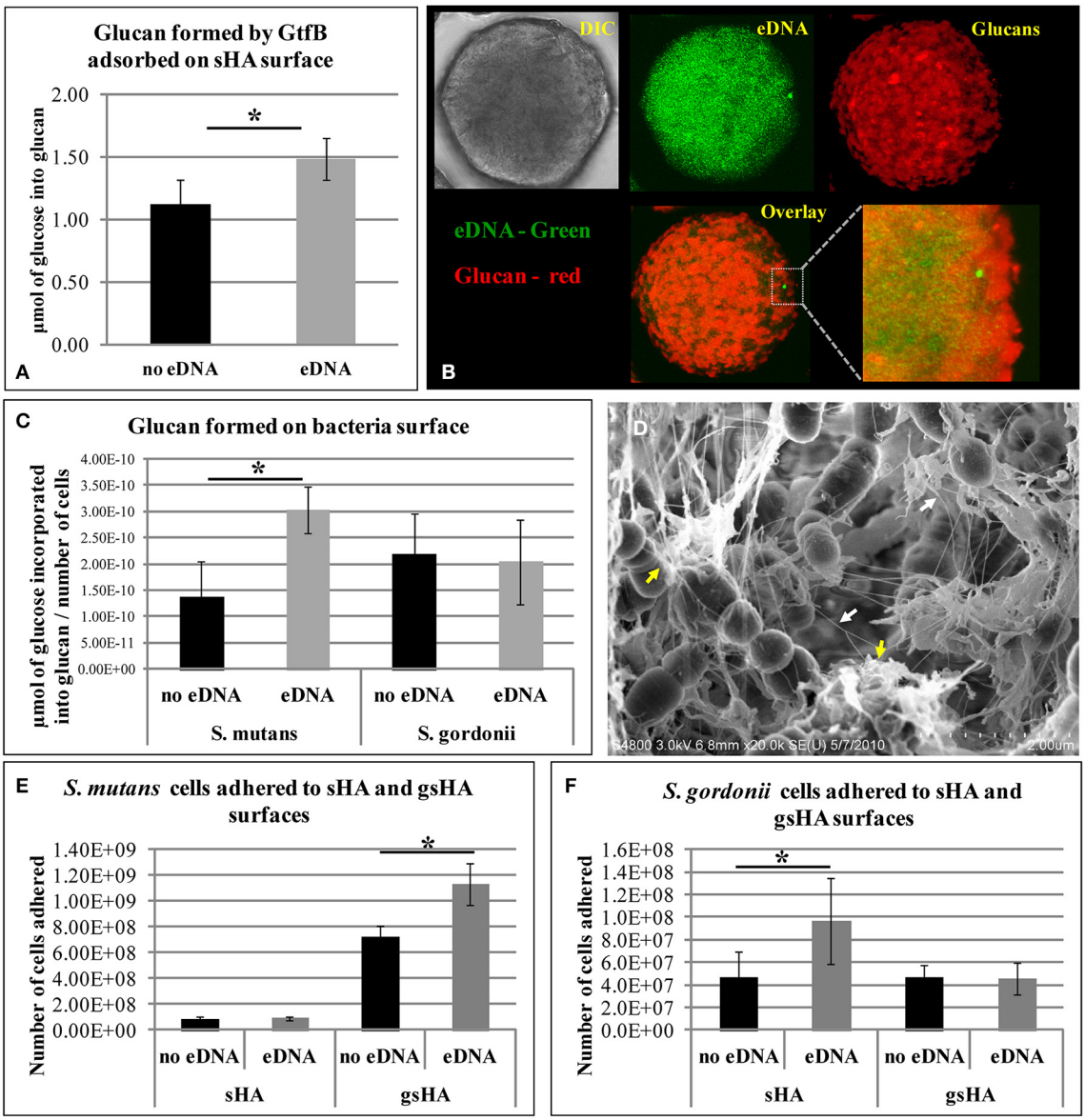

FIGURE 2 | Influence of eDNA on glucans synthesis and bacterial adhesion. (A) Glucan formed by GtfB adsorbed to sHA surface. **(B)** This panel of images shows the sHA bead in DIC (gray), in green is the eDNA associated to surface (which presents a punctuated distribution pattern), and red are the glucans formed. Overlay and close-up image show eDNA interspersed with glucans. **(C)** Glucan produced by GtfB adsorbed to *S. mutans* and *S. gordonii* cells. **(D)** FE-SEM analysis of *S. mutans* biofilms on apatitic surface. Images highlight interaction of nanofibrous eDNA (white arrows) and wool-like glucans (yellow arrows). Bacterial adhesion to apatitic surfaces in presence and absence of eDNA are shown for *S. mutans* **(E)** and *S. gordonii* **(F)**. sHA: saliva-coated hydroxyapatite; gsHA: glucan formed on saliva-coated hydroxyapatite. An asterisk (*) denotes $p < 0.05$. Note: **(D,E)** were kindly provided by Dr. Zezhang (Tom) Wen (School of Dentistry, Louisiana State University Health Sciences Center, New Orleans, Louisiana, USA).

CONCLUSIONS AND FUTURE PERSPECTIVE

The matrix in cariogenic biofilms has roles far beyond providing bacterial binding sites and holding microbial cells together. It provides a diffusion-limiting 3D scaffold that shapes the spatial and microenvironmental heterogeneities, helping to create a myriad of acidic pH and protective niches, while modulating the mechanical properties of the biofilm. These properties are critical for the persistence, dynamic microbial composition changes and expression of virulence of cariogenic biofilms. In this context, it is conceivable that the primary role of *S. mutans* in the pathogenesis of dental caries resides with its ability to assemble an insoluble polymeric matrix and not simply with numerical superiority or acidogenicity. Furthermore, the presence of other organisms in the biofilms may also contribute to the matrix assembly. Clearly, enhanced understanding about the extracellular matrix biology and its functional properties may lead to enhanced ways to prevent dental caries. For example, how other components of the matrix (such as eDNA and LTA) are associated with glucans,

and how together affect the microenvironmental and mechanical properties of cariogenic biofilm need further clarification. At the same time, measurement of biophysical properties associated with resistance to mechanical clearance may be additional factors to be considered when searching for effective antibiofilm therapeutics. The results of such investigations may have relevance beyond the mouth, as matrix and microenvironmental niches hinder drug efficacy in other biofilm-associated diseases and industry related issues.

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Molecular determinants of staphylococcal biofilm dispersal and structuring

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Staphylococci are frequently implicated in human infections, and continue to pose a therapeutic dilemma due to their ability to form deeply seated microbial communities, known as biofilms, on the surfaces of implanted medical devices and host tissues. Biofilm development has been proposed to occur in three stages: (1) attachment, (2) proliferation/structuring, and (3) detachment/dispersal. Although research within the last several decades has implicated multiple molecules in the roles as effectors of staphylococcal biofilm proliferation/structuring and detachment/dispersal, to date, only phenol soluble modulins (PSMs) have been consistently demonstrated to serve in this role under both *in vitro* and *in vivo* settings. PSMs are regulated directly through a density-dependent manner by the accessory gene regulator (Agr) system. They disrupt the non-covalent forces holding the biofilm extracellular matrix together, which is necessary for the formation of channels, a process essential for the delivery of nutrients to deeper biofilm layers, and for dispersal/dissemination of clusters of biofilm to distal organs in acute infection. Given their relevance in both acute and chronic biofilm-associated infections, the Agr system and the *psm* genes hold promise as potential therapeutic targets.

Keywords: *Staphylococcus aureus*, *Staphylococcus epidermidis*, biofilm, phenol-soluble modulins, medical devices

INTRODUCTION

First described in 1878, staphylococci are Gram-positive microorganisms that are implicated in human skin and soft tissue infections, blood stream infections including valvular and device-associated infective endocarditis, osteomyelitis, pneumonia, and infections involving other implanted medical devices (Lowy, 1998). Staphylococci are further classified based on coagulase designation, into coagulase-positive staphylococci, comprising mostly the important human pathogen *Staphylococcus aureus*, and the coagulase-negative staphylococci (CoNS) (Kloos and Schleifer, 1986). Of the CoNS, *Staphylococcus epidermidis* is most commonly isolated from human infections (Vuong and Otto, 2002). In the era of implantation of medical devices, many staphylococcal species have emerged as important pathogens, primarily due to their ability to form deeply seated microbial communities, referred to a biofilms, on the surfaces of native tissues and implanted medical devices (Costerton et al., 1999; Otto, 2008). Because these microbial communities are shielded from the effects of antimicrobial therapy and the host immune system, medical therapy involving infections of implanted medical devices can be particularly challenging (Hoiby et al., 2010). Often complete explantation of the implanted medical devices in conjunction with prolonged courses of antimicrobial therapy are necessary in curative approaches, incurring additional risks to patients and excess cost to the health care system.

Within the past decades, research efforts have led to important advances in the understanding of the molecular determinants of these microbial communities, implicating exopolysaccharides, proteins, and extracellular DNA (eDNA) in the formation of the extracellular biofilm matrix. Enzymes that degrade these molecules have been discussed as potential effector molecules of biofilm structuring and dispersal. However, much of these insights have been gleaned *in vitro*. Only recently have molecular tools enabled the optimization of *in vivo* models in the study of staphylococcal biofilm-associated infections (Joo and Otto, 2012). This review focuses on key *in vitro* and *in vivo* experiments that have led to current understanding of the determinants of staphylococcal biofilm structuring and dispersal.

STAGES OF BIOFILM DEVELOPMENT

Current literature models biofilm development in three stages: (1) attachment, (2) proliferation/formation of the matured biofilm, and (3) detachment/dispersal (O'Toole et al., 2000; Otto, 2013) (**Figure 1**). Based on work in *Pseudomonas aeruginosa*, these three stages have been further sub-categorized to include a total of five stages, but such further differentiation has not yet been made for staphylococcal biofilm development

(Sauer et al., 2002). During attachment, staphylococcal surface-attached proteins, mostly so-called microbial components recognizing adhesive matrix molecules (MSCRAMMs) establish non-covalent interactions with host tissue or host protein that coat device surfaces (Patti et al., 1994; Otto, 2008). As discovered by *in vitro* research, several other surface molecules such as teichoic acids may also be important in the direct attachment to abiotic surfaces (Gross et al., 2001), which is, however, not believed to have an important role during the *in vivo* infection of indwelling medical devices. Following attachment, proliferation and maturation of the biofilm ensues, with the production of an extracellular matrix consisting of the staphylococcal biofilm exopolysaccharide polysaccharide intercellular adhesin (PIA) (Mack et al., 1996), teichoic acids, proteins and eDNA (Joo and Otto, 2012). During this stage, channels and mushroom-shaped structures form to facilitate nutrient delivery to deeper layers of the biofilm (O'Toole et al., 2000; Otto, 2008). The last stage of biofilm development is characterized by the detachment of biofilm clusters and the dissemination of these clusters to distal sites (O'Toole et al., 2000; Otto, 2008, 2013).

During the second and third stages of biofilm development, it is thought that disruption of intercellular adhesive forces is necessary for the formation of channels and mushroom-shaped structures, and also for biofilm detachment/dissemination (Otto, 2013). Previous work has implicated proteases (Boles and Horswill, 2008), nucleases (Mann et al., 2009; Sharma-Kuinkel et al., 2009; Kiedrowski et al., 2011; Beenken et al., 2012), and a family of staphylococcal proteins called phenol-soluble modulins (PSMs) (Wang et al., 2011; Periasamy et al., 2012b) in this role. However, of these proposed effector molecules, to date, only PSMs have been consistently demonstrated to facilitate staphylococcal biofilm maturation and dispersal through both *in vitro* and *in vivo* models (Otto, 2013).

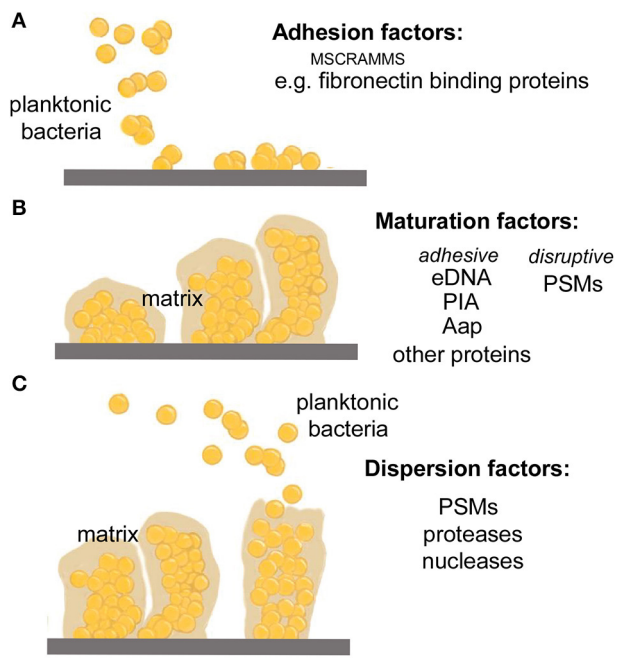

FIGURE 1 | Stages of biofilm development. (A) During the attachment phase, planktonic bacteria adhere to a biotic surface, such as human tissue or a human matrix-covered indwelling device, by non-covalent interactions between human matrix proteins and dedicated bacterial surface binding proteins (mostly, MSCRAMMs). **(B)** After attachment is accomplished, biofilm cells multiply producing an extracellular biofilm matrix that is composed of a variety of macromolecules, including specific exopolysaccharides (in many staphylococci, PIA), eDNA, teichoic acids, and a series of proteins such as the fibril-forming accumulation-associated protein, Aap. Furthermore, the biofilm develops a structured form with channels and mushroom-like towers, which is dependent on the disruptive forces of the PSM structuring molecules discussed in this review. **(C)** In the last phase of biofilm development, clusters of bacteria or single bacteria may detach from the biofilm in a process also called dispersal or sloughing. This process is stimulated by mechanic forces (such as under flow), the PSM surfactants, and by enzymes that degrade biofilm matrix molecules such as nucleases and proteases. The relevance of the latter mechanism for infection is unclear.

PSMs IN BIOFILM STRUCTURING AND DISPERSAL

First described in 1999 in *S. epidermidis* (Mehlin et al., 1999), PSMs are a family of small peptides (~21–44 amino acids long), with amphipathic, α-helical secondary structures and surfactant-like properties (Mehlin et al., 1999; Peschel and Otto, 2013; Cheung et al., 2014a) (**Figure 2**). The smaller, ~20 amino acid peptides are grouped into the α-class and the longer ~44 amino acid peptides into the β-class of PSMs. In particular the α-class peptides are cytotoxic to many cell types, and work by non-specific membrane damage, while the β-class peptides lack cytotoxicity (Wang et al., 2007). All PSMs are pro-inflammatory by activation of the formyl peptide receptor 2 (FPR2) on human immune cells (Wang et al., 2007; Kretschmer et al., 2010).

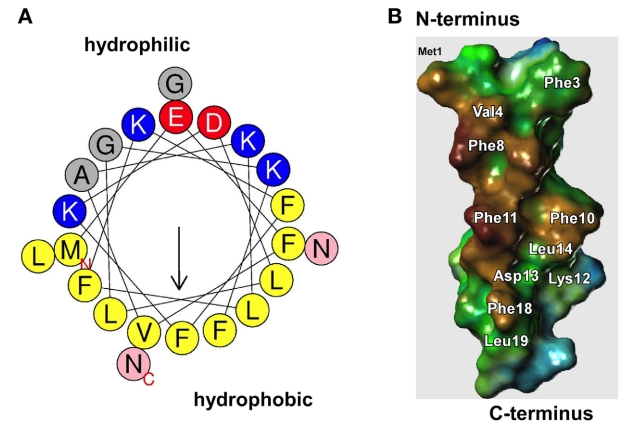

FIGURE 2 | Surfactant properties of PSMs. (A) All PSMs form amphipathic α-helices. In the α-type PSMs, the helix stretches over virtually the whole peptide, while the longer β-type PSMs contain an α-helical part at their C-terminus. The graph shows an α-helical wheel presentation of PSMα3. Hydrophilic and hydrophobic amino acids occupy opposite sides of the helix, giving the helix strongly amphipathic character. **(B)** Model of PSMα3 structure (modeled after the known structure of δ-toxin that was determined by NMR studies). The hydrophobic side is shown. Replacement of amino acids on the hydrophobic side, mainly of large hydrophobic residues such as phenylalanine, leads to impaired biofilm structuring capacity.

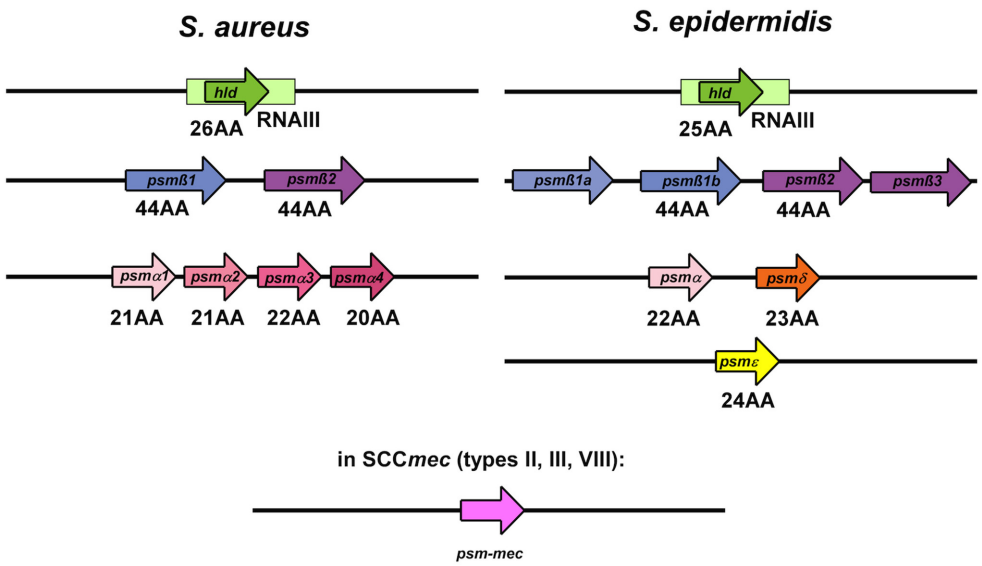

FIGURE 3 | PSM genes in *S. aureus* and *S. epidermidis*. PSMs are known to occur in a variety of staphylococci, but only in *S. aureus* and *S. epidermidis* were *psm* genes identified and gene products analyzed in a systematic manner. The graph shows the genetic arrangement of *psm* genes in *S. aureus* and *S. epidermidis*. The *hld*/RNAIII, *psm*β, and *psm*α/*psm*δ loci show strong similarity between *S. aureus* and *S. epidermidis*, suggesting that they are evolutionarily related. The *psm-mec* gene is encoded on SCC*mec* mobile genetic elements present in similar form in *S. aureus* and *S. epidermidis*.

S. epidermidis β-PSMs (Wang et al., 2011) and all of the *S. aureus* PSMs (Periasamy et al., 2012b) (**Figure 3**) have been shown to be key effector molecules in biofilm structuring and dissemination (Otto, 2013). Other *S. epidermidis* PSMs may have similar roles, but this is awaiting the rather difficult construction of *psm* gene deletion mutants in *S. epidermidis* and their investigation. The general mechanism by which PSMs contribute to biofilm structuring and dispersal is believed to be the disruption of non-covalent (electrostatic or hydrophobic) interactions between biofilm matrix macromolecules (Otto, 2013). While the physico-chemical properties of PSMs strongly favor that notion, direct evidence for such a mechanism is difficult to achieve. Some evidence is derived from an alanine screen of the PSMα3 peptide, whose capacity in biofilm detachment was most strongly impaired when large hydrophobic residues were exchanged for alanine (Cheung et al., 2014b) (**Figure 2B**). Notably, PSMs must be produced during biofilm growth for structuring and dispersal to take effect. External addition of PSMs to already formed biofilm does not disrupt biofilms (Wang et al., 2011), most likely because the physico-chemical mechanism by which PSMs work is not sufficient to disrupt the covalent bonds in macromolecular networks of, for example, exopolysaccharide that surrounds cells in a mature biofilm.

S. EPIDERMIDIS β-PSMs

S. epidermidis produces six PSM peptides, PSMα, PSMβ1, PSMβ2, PSMδ, PSMε, and δ-toxin (Wang et al., 2007). Next to δ-toxin, β-PSMs are the primary PSMs produced in *S. epidermidis* (Yao et al., 2005; Cheung et al., 2010), and seem to be key players in biofilm structuring and dispersal (Wang et al., 2011). They are encoded by the *psm*β operon, which also encodes a gene, *psm*β3, whose gene product does not appear to be produced or secreted (Yao et al., 2005; Cheung et al., 2010). Some strains have a duplication of the *psm*β1 gene, resulting in higher relative production of that PSMβ peptide. *In vitro*, the role of β-PSMs as effector molecules in *S. epidermidis* biofilm structuring and dispersal seems to be concentration-dependent (Wang et al., 2011). At medium concentrations, PSMβ peptides promoted biofilm formation, by providing the disruptive forces necessary for the formation of channels and thus formation of a mature biofilm. However, at higher concentrations, PSMβ caused biofilm detachment, thereby inhibiting overall biofilm extension. Of note, this mechanism was independent of the type of biofilm (protein- vs. exopolysaccharide-dependent) examined (Wang et al., 2011). This suggests that differential concentration allows the same effector molecule to play disparate roles in the earlier proliferative stage involving formation of the matured biofilm as well as the subsequent detachment/dispersal stage.

In a murine model of indwelling catheter-related infection, when compared to its isogenic *psm*β deletion mutant, the wild-type strain was noted to promote biofilm dissemination to the lymphatic system and the distal organs of the infected animals. Moreover, when compared to mice treated with control serum alone, those treated with anti-PSMβ antibodies had lower burden of dissemination of infection to their distal organs (Wang et al., 2011). These results support *in vitro* findings observed at higher concentrations of PSMβ, and recapitulate the role that PSMβ peptides play in *S. epidermidis* biofilm detachment and dissemination.

S. AUREUS PSMs

S. aureus produces four PSMα peptides that are encoded in the *psm*α operon, two PSMβ peptides that are encoded in the *psm*β operon, and δ-toxin that is encoded by RNAIII (Wang et al.,

2011; Peschel and Otto, 2013). When mutants in the *psm*α, *psm*β, and *hld* (the gene coding for δ-toxin) loci in clinically relevant community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains (DeLeo et al., 2010) were examined in *in vitro* and *in vivo* models, all classes of *S. aureus* PSMs were implicated in biofilm structuring and detachment (Periasamy et al., 2012b). (The *hld* mutant was constructed by introducing an altered start codon, abolishing only translation of *hld*, not to interfere with the function of RNAIII.) *In vitro*, under both static and dynamic growth conditions, all isogenic *S. aureus psm* mutants produced thicker biofilms, demonstrated less channel formation, and had smoother surfaces than the wild-type (Periasamy et al., 2012b). It is remarkable that removal of any one class of PSMs resulted in a significant effect on the biofilm phenotype, indicating that presence of all PSMs is needed for efficient biofilm structuring and dispersal. Interestingly, the biofilm-enhancing effect was not additive, as in a complete *psm* deletion strain (*psm*α/*psm*β/*hld*), biofilm formation was not stronger than in the single *psm* deletion mutants, a result that yet remains unexplained. It may be due to the fact that in the absence of all PSMs, a beneficial effect on biofilm structuring and maturation, as seen with low concentrations of PSMβ peptides in *S. epidermidis* (Wang et al., 2011), is completely abolished. Furthermore, as in *S. aureus* PSMβ peptides are only produced at very low concentrations, the considerable impact on biofilm dispersal and structuring that was found with the *S. aureus psm*β deletion mutant is particularly remarkable (Periasamy et al., 2012b). Why biofilm dissemination/dispersal is thus most prominently seen within the β-subclass of PSMs remains unclear. It is plausible, but remains speculative, that since PSMβ is less cytotoxic than PSMα or the δ-toxin, the observed effects might be attributable to specialization of this subclass to the role of promotion of biofilm detachment/dissemination (Otto, 2013).

In murine models of *S. aureus* catheter infection, the isogenic *psm* triple deletion mutant (*psm*α/*psm*β/*hld*) demonstrated notably decreased infection dissemination to the surrounding tissues and the lymphatic system when compared to the wild-type strain (Periasamy et al., 2012b), confirming the role that PSMs play in the detachment/dispersal stage of *S. aureus* biofilm development. Here, in contrast to the *in vitro* results, the effect was additive, with the total *psm* deletion mutant showing a more pronounced phenotype than the single *psm* mutants. However, it needs to be stressed that in the case of the PSMα peptides, survival in organs may also be affected by their functions in evasion of innate host defense mechanisms.

PSM-mec

PSM-mec is a PSM that—in contrast to all other characterized PSMs—is encoded on a mobile genetic element, namely staphylococcal cassette chromosomes (SCC) *mec* elements of types II, III, and VIII (Queck et al., 2009; Chatterjee et al., 2011). Its impact on biofilm formation *in vitro* is modest; *S. aureus psm-mec* mutants only show slightly decreased capacity to form biofilms and increased aggregation compared to the isogenic wild-type strain (Queck et al., 2009). These phenotypes are likely caused by a combination of the direct impact of PSM-mec on biofilm formation, which is negative (at concentrations in the physiological range of ∼20–100 μg/ml), in accordance with that of other PSMs, and the negative impact that the *psm-mec* RNA has on the production of other *S. aureus* PSMs (Kaito et al., 2013; Cheung et al., 2014c).

DO PSM FIBRILS HAVE A ROLE IN *IN VIVO* BIOFILM FORMATION?

It has been reported that some PSMs form amyloid-like fibrils *in vitro* and that *psm* mutants show less *in vitro* biofilm formation due to the lack of those fibrils (Schwartz et al., 2012). However, PSM fibrils were only observed in a specific growth medium. Notably, the theoretical impact that PSM fibrils have on the biofilm phenotype is essentially opposite to that facilitated by their biofilm-disruptive forces. *In vivo* results support the relevance of the latter mechanism during infection (Wang et al., 2011; Periasamy et al., 2012b), as described above.

REGULATION OF PSMs BY THE Agr SYSTEM

In staphylococci, the production of PSMs is controlled by the accessory gene regulator (Agr) system, a quorum-sensing mechanism that controls gene expression according to bacterial cell density (Novick et al., 1993; Vuong et al., 2004a; Wang et al., 2007). The *agr* locus contains the *agrA, C, D*, and *B genes* (RNAII transcript) and RNAIII which contains the *hld* gene that encodes the PSM δ-toxin (Novick et al., 1995) (**Figure 4**).

The Agr system regulates cell density-dependent gene expression using two proteins that comprise a classical two-component system, the sensor histidine kinase AgrC and the response regulator AgrA, and two proteins, AgrD and AgrB, which represent the structural and maturation proteins of the extracellular signal called auto-inducing peptide (AIP) (Novick and Geisinger, 2008). AIP binds to AgrC and activates (phosphorylates) the DNA-binding regulator AgrA, leading to the transcription of RNAIII and RNAII. This activation is dependent on the extracellular concentration of AIP, which signals cell density. As AIP thus promotes its own production, the circuit provides autofeedback, leading to a rapid change of gene expression at a certain cell density. In contrast to all other targets of Agr, which include a series of positively regulated toxins and negatively regulated surface proteins that are controlled by RNAIII, expression of the *psm* operons is stimulated by direct binding of AgrA to their promoters (Queck et al., 2008). Control of PSMs by quorum-sensing thus likely preceded the link of other Agr targets to the system via the development of RNAIII around the gene encoding the PSM δ-toxin during evolution. This underlines the key role PSMs are believed to play both in the commensal and infectious lifestyles of staphylococci (Periasamy et al., 2012a).

Since the *psm* operons are under strict control by AgrA, *S. aureus* isogenic *agr* mutants have been shown to produce thicker biofilms (Vuong et al., 2000, 2003) and demonstrated less channel formation and smoother surfaces than the wild-type (Periasamy et al., 2012b). Furthermore, the phenotype of the total *S. aureus psm* deletion mutant (*psm*α/*psm*β/*hld*) was observed to be very similar to that of an *agr* mutant (Periasamy et al., 2012b). Moreover, expression of *agr* and *psm* were noted to be most

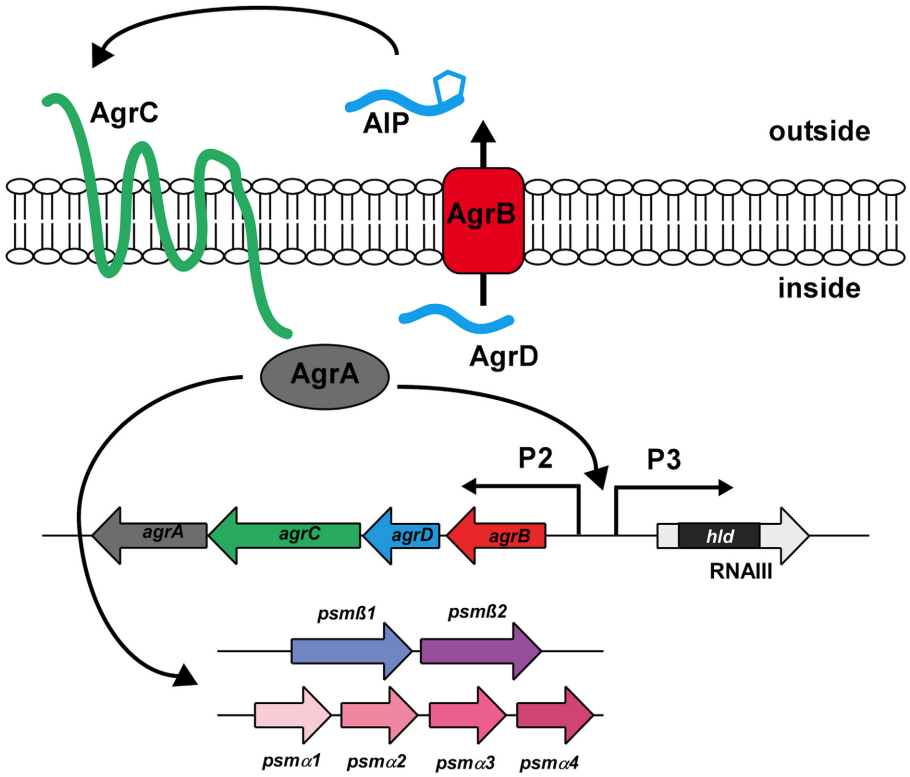

FIGURE 4 | The Agr quorum-sensing system. The Agr system is an auto-regulatory system controlling gene expression in response to increasing cell density. It consists of the structural gene coding for the extracellular signal (AgrD), which is post-translationally modified and exported via AgrB. Upon reaching a certain threshold concentration, the AgrD AIP triggers auto-phosphorylation of the histidine kinase AgrC, which in turn leads to phosphorylation and activation of the DNA-binding response regulator AgrA. AgrA binding activates transcription from the AgrP2, AgrP3, *psmα*, and *psmβ* promoters. Agr targets other than PSMs are regulated by RNAIII, the regulatory RNA surrounding the *hld* (δ-toxin) gene.

prominent within the outer layers of the biofilm, the site of active biofilm expansion and dissemination. These findings indicate that PSMs are the key effector molecules of quorum-sensing dependent biofilm structuring and detachment in *S. aureus* (Periasamy et al., 2012b).

It is thought that up-regulation of the Agr system, leading to increased production of PSMs, favoring biofilm maturation and detachment, might have important roles in acute infection (Vuong et al., 2004a; Wang et al., 2007). Whereas, mutation of the Agr system or *psm* genes, favors the development of extensive and compact biofilms (Wang et al., 2011) that have lost the capacity to disseminate, a situation that may be of benefit in localized chronic biofilm-associated infections (Joo and Otto, 2012). In fact, *agr* mutants were frequently isolated from such infections (Vuong et al., 2004b; Traber et al., 2008).

Because in addition to the PSMs, the *agr* locus regulates a series of other cytotoxic toxins, such as leukocidins and α-toxin, it has been proposed that control of this locus might serve as a therapeutic target (Ji et al., 1997; Otto, 2004; Wright et al., 2005; Kong et al., 2006). However, the benefit of such intervention remains undefined with respect to biofilm-associated infections, as interference with *agr* inhibits dissemination of biofilm to distal targets (Wang et al., 2011) but also favors localized biofilm formation (Wang et al., 2011).

PROTEASES AND NUCLEASES

Several proteins and eDNA have been implicated in *in vitro* staphylococcal biofilm formation; and thus, proteases and nucleases were found to contribute to biofilm structuring and dispersal *in vitro* (Boles and Horswill, 2008; Mann et al., 2009; Sharma-Kuinkel et al., 2009; Kiedrowski et al., 2011; Beenken et al., 2012). This is discussed in depth elsewhere in this review series. However, to date, *in vitro* findings have not been confirmed *in vivo*, or *in vitro* and *in vivo* findings have yielded conflicting results (Beenken et al., 2012). Furthermore, in contrast to PSM-mediated mechanisms, protease-dependent biofilm structuring is strain-dependent as proteins are premier determinants of biofilm formation only in a subset of strains (O'Neill et al., 2007). Therefore, the exact roles of degradative enzymes in staphylococcal biofilm structuring and dissemination/dispersal remain to be clearly delineated.

BIOFILM DEVELOPMENT: REFLECTING THE "ORIGINAL ROLE" OF PSMs IN THE COMMENSAL LIFESTYLE OF STAPHYLOCOCCI?

As, for example, the exceptionally direct mode of quorum-sensing control over PSM expression indicates, PSMs have a key and evolutionarily early role in staphylococcal pathogenesis (Queck et al., 2008; Periasamy et al., 2012a). The surfactant-based mechanism

of biofilm structuring and detachment may be similar to the role that PSMs have during the commensal lifestyle of staphylococci on the human skin. In addition to structuring biofilm-like agglomerates in places like sebaceous glands, where staphylococci often reside, PSMs may also facilitate the acquisition of nutrients by emulsification and promote a means to spread over surfaces by surfactant-mediated "sliding" activity. On soft agar surfaces, PSMs have indeed been shown to promote such sliding activity (Tsompanidou et al., 2011, 2013).

CONCLUSION

In summary, among the effector molecules that have been proposed as molecular determinants of staphylococcal biofilm dispersal and structuring, only PSMs have been demonstrated to be relevant in *S. aureus* and *S. epidermidis* biofilm-associated infection under both *in vitro* and *in vivo* settings. Under strict regulation by the global regulator Agr, PSMs are believed to enable the disruption of non-covalent forces in the biofilm matrix based on their amphipathic structure, to form channels that are necessary for the delivery of nutrients to deeper levels of the biofilm, and provide the disruptive forces necessary for the detachment of clumps of biofilm to distal sites.

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Staphylococcus aureus biofilms: recent developments in biofilm dispersal

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Staphylococcus aureus is a major cause of nosocomial and community-acquired infections and represents a significant burden on the healthcare system. *S. aureus* attachment to medical implants and host tissue, and the establishment of a mature biofilm, play an important role in the persistence of chronic infections. The formation of a biofilm, and encasement of cells in a polymer-based matrix, decreases the susceptibility to antimicrobials and immune defenses, making these infections difficult to eradicate. During infection, dispersal of cells from the biofilm can result in spread to secondary sites and worsening of the infection. In this review, we discuss the current understanding of the pathways behind biofilm dispersal in *S. aureus*, with a focus on enzymatic and newly described broad-spectrum dispersal mechanisms. Additionally, we explore potential applications of dispersal in the treatment of biofilm-mediated infections.

Keywords: biofilm, dispersal, protease, nuclease, stringent response, *Staphylococcus aureus*

STAPHYLOCOCCUS AUREUS BIOFILMS AND INFECTION

Staphylococcus aureus is a Gram-positive human commensal that persistently colonizes the anterior nares of approximately 20–25% of the healthy adult population, while as many as 60% are intermittently colonized (Eriksen et al., 1995; Hu et al., 1995; Kluytmans et al., 1997; Ellis et al., 2014). Studies have linked *S. aureus* nasal colonization to an increased risk of infection (Dall'Antonia et al., 2005; Ellis et al., 2014). As evidence, 65% of people with *S. aureus* infections are colonized with the same strain, whereas the percentage jumps to 80% in nosocomial infections (Weinstein, 1959; von Eiff et al., 2001; Wertheim et al., 2004). The infections that result are quite diverse, and can include acute infections, such as bacteremia and skin abscesses, that are generally caused by planktonic cells through the production of secreted toxins and exo-enzymes (Gordon and Lowy, 2008). In contrast, chronic infections are associated with a biofilm mode of growth where *S. aureus* can attach and persist on host tissues, such as bone and heart valves, to cause osteomyelitis and endocarditis respectively, or on implanted materials, such as catheters, prosthetic joints, and pace makers (Parsek and Singh, 2003; Kiedrowski and Horswill, 2011; Barrett and Atkins, 2014; Chatterjee et al., 2014). Implanted materials become coated with host proteins upon insertion, and the matrix-binding proteins on the surface of *S. aureus* facilitate attachment to these proteins and development of a biofilm (Cheung and Fischetti, 1990; Francois et al., 1996). In cases of infected medical devices, removal of the device is often necessary to treat the infection (Darouiche, 2004).

A biofilm is defined as a sessile microbial community in which cells are attached to a surface or to other cells and embedded in a protective extracellular polymeric matrix. This mode of growth exhibits altered physiologies with respect to gene expression and protein production (Parsek and Singh, 2003; Archer et al., 2011; Kiedrowski and Horswill, 2011). Biofilm developmental stages have been defined by many and can be divided into at least three major events: initial attachment, biofilm maturation, and dispersal (**Figure 1A**). During initial attachment, an individual planktonic cell will reversibly associate with a surface, and if the cell does not dissociate, it will bind irreversibly to the surface. Attachment is mediated through surface proteins, referred to as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Foster et al., 2014). During infection, these proteins play major roles in attachment to host factors such as fibrinogen, fibronectin, and collagen. Biofilm maturation occurs through cell division and the production of the extracellular polymeric matrix. The composition of the biofilm matrix varies between strains, but in general can contain host factors, polysaccharide, proteins, and extracellular DNA (eDNA) (Montanaro et al., 2011; Cue et al., 2012; Foster et al., 2014). Following biofilm accumulation, cells within the biofilm can reactivate to a planktonic state through dispersal (Boles and Horswill, 2011). The major mechanisms of *S. aureus* dispersal will be explored in this review.

Growth in a biofilm plays an important role during infection by providing a defense against several clearance mechanisms. The biofilm matrix can impede the access of certain types of immune defenses, such as macrophages, which display incomplete penetration into the biofilm matrix and "frustrated phagocytosis" (Scherr et al., 2014). Additionally, biofilm cells display increased tolerance to antibiotics (de la Fuente-Nunez et al., 2013). In contrast to heritable antibiotic resistance mechanisms, biofilm-associated antibiotic tolerance is a transient state in which normally susceptible bacteria enter an altered physiology that decreases sensitivity. When these cells disperse and reenter a planktonic state, they regain normal antibiotic sensitivity (Singh et al., 2009). One suggested mechanism for this phenomenon is that the biofilm matrix blocks access to actively growing

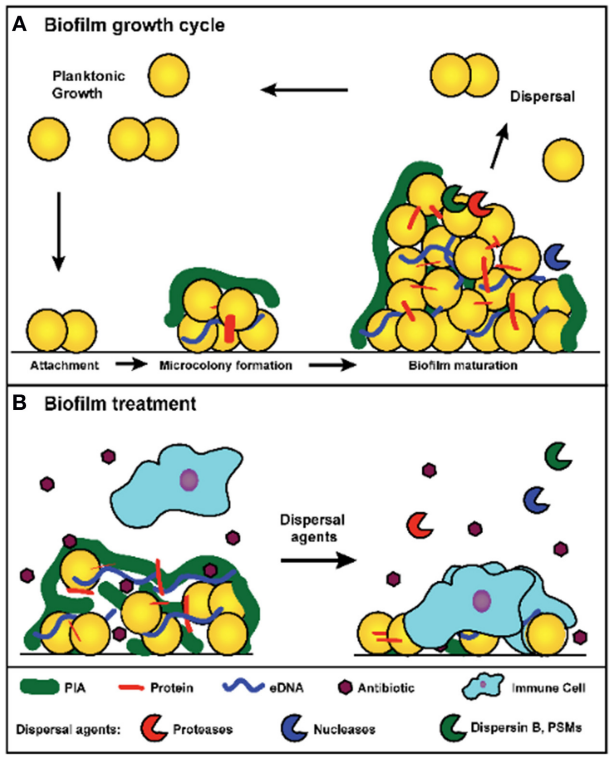

FIGURE 1 | (A) Model of *S. aureus* biofilm growth cycle. In summary, upon coming into contact with a surface, planktonic cells attach through surface-associated proteins. Following attachment, cells divide and begin production of the extracellular matrix, which leads to the formation of a microcolony. As cell division continues, biomass accumulates and a mature biofilm is formed. Environmental signals within the biofilm trigger the activation of dispersal mechanisms, and upon dispersal, cells re-enter a planktonic growth state and can seed new sites for biofilm formation. **(B)** Treatment of a *S. aureus* biofilm. Antibiotic exposure will kill susceptible planktonic cells and metabolically active cells near the surface of the biofilm. However, persister cells and metabolically dormant cells within the biofilm survive and remain protected from immune defenses by the biofilm matrix. Treatment with dispersal agents increases the effectiveness of antibiotic penetration and promotes clearance. Antibiotic sensitive cells within the biofilm are exposed and killed after degradation of the matrix, and the antibiotic tolerant cells (such as persisters) survive and are susceptible to the immune system.

cells within the biofilm by decreasing antibiotic diffusion rates. However, this mechanism is dependent on the type of antibiotic, as certain antibiotics are capable of penetrating the biofilm (Singh et al., 2010). An alternative proposal is that antibiotic tolerance is due to the development of physiologically dormant persister cells that form stochastically during biofilm growth (Lewis, 2010). Due to their decreased metabolic activity, they are inherently resistant to antibiotics. Furthermore, persister cells develop at greater rates within a biofilm than within actively growing planktonic cultures (Singh et al., 2009). As such, they are thought to play a large role in the recalcitrance of biofilm-associated infections.

Beyond offering resistance to clearance mechanisms, biofilms also play an important role in the progression of chronic diseases. Following the establishment of a biofilm, individual cells can disperse from the original biofilm and either seed new sights of infection or mediate an acute infection such as sepsis (Costerton et al., 1999). The role played by the *S. aureus* quorum sensing system during dispersal supports this model (Boles and Horswill, 2008; Lauderdale et al., 2010). Dispersal has been the focus of many recent studies due to its importance in chronic infections and the biofilm model of growth, and an analysis of major dispersal mechanisms has led to the development of dispersal-mediated treatment options for biofilm infections (Kaplan, 2010; Boles and Horswill, 2011). This review discusses the major mechanisms for *S. aureus* biofilm dispersal. In addition, it analyzes the potential for developing dispersal-mediated treatments for biofilm infections (**Figure 1B**).

THE *STAPHYLOCOCCUS AUREUS* BIOFILM MATRIX

The *S. aureus* biofilm matrix is a complex glue that encases all of the cells in the mature structure, and it is thought to be composed of host factors, secreted and lysis-derived proteins, polysaccharide, and eDNA. The contribution of each of these factors depends heavily on the strain background and on environmental conditions (Fitzpatrick et al., 2005; Abraham and Jefferson, 2012). Furthermore, the effectiveness of many dispersal mechanisms is dependent on the matrix composition (Chaignon et al., 2007; Izano et al., 2008). A brief background on the major components of the biofilm matrix and factors involved in generating these components will be provided.

A major constituent of the biofilm matrix is polysaccharide intercellular adhesin (PIA), also known as polymeric N-acetylglucosamine (PNAG) (O'Gara, 2007). PIA is an important component in both *S. aureus* and *S. epidermidis* biofilms that is produced by enzymes encoded in the *icaADBC* locus. PIA is composed of β-1,6-linked *N*-acetylglucosamine polymer, and the proteins encoded in the *ica* locus are responsible for the synthesis, export, and modification of PIA. The PIA polymer plays an important role in the structural integrity of biofilms *in vitro* and *in vivo*, although numerous studies have identified *S. aureus* strains capable of forming *ica*-independent biofilms (Beenken et al., 2003; Fitzpatrick et al., 2005; Toledo-Arana et al., 2005; Lauderdale et al., 2009; Brooks and Jefferson, 2014). The matrix components of these biofilms were later identified as proteins and eDNA (O'Neill et al., 2007, 2008; Rhode et al., 2007; Boles et al., 2010), which function as intercellular adhesins in the absence of PIA.

Many proteins have been implicated as important components in attachment and biofilm matrix development. These include surface-associated proteins such as protein A, fibrinogen-binding proteins (FnBPA and FnBPB), *S. aureus* surface protein (SasG), biofilm-associated protein (Bap), and clumping factor B (ClfB) (Cucarella et al., 2001; Corrigan et al., 2007; O'Neill et al., 2008; Merino et al., 2009; Geoghegan et al., 2010; Abraham and Jefferson, 2012). Many of these factors play a role both in attachment and accumulation. In addition, secreted proteins such as extracellular adherence protein (Eap), and beta toxin (Hlb) play a role in biofilm maturation (Huseby et al., 2010; Sugimoto et al., 2013). However, the importance of individual proteins varies largely between strains (Artini et al., 2013). For example, Bap-dependent biofilms have not been identified in any human isolates, and as such it is more likely that Bap plays a role in bovine

mastitis (where it was originally identified) than in human diseases (Lasa and Penades, 2006). In addition to dedicated matrix proteins, intracellular proteins have been identified within the biofilm matrix. These proteins are likely released by cell lysis and nonspecifically incorporated into the matrix (Foulston et al., 2014). The relative importance of lysis-derived proteins is not yet understood.

The most recently identified and appreciated biofilm matrix component is eDNA. Due to the negative charge of the DNA polymer, eDNA potentially acts as an electrostatic polymer that anchors cells to a surface, to host factors, and to each other. Early biofilms are most sensitive to DNase treatment, suggesting that eDNA may be important during attachment (Mann et al., 2009). eDNA is produced through the autolysis of a subpopulation of the biofilm cells (Thomas and Hancock, 2009), and this altruistic suicide is mediated through the activity of murein hydrolases, encoded by the *atl* and *lytM* genes. Murein hydrolases degrade peptidoglycan and typically play an important role during cell wall rearrangements and cell division. Increased expression of these enzymes allows for autolysis in *S. aureus*. Autolysis is regulated through the activity of two operons, *cidABC* and *lrgAB*, that function together in a manner similar to bacteriophage holin/antiholin systems (Sadykov and Bayles, 2012). CidA, the holin in this system, oligomerizes in the cell membrane and results in the formation of a pore that is utilized for the transport of the murein hydrolase. LrgAB functions as the antiholin and prevents the activity of CidA. Studies have indicated that the regulation of autolysis is tied to micro-environmental niches that form within a biofilm, such as the hypoxic conditions found near the base of the biofilm (Moormeier et al., 2013).

There are some reported examples of interactions between eDNA and specific proteins within the biofilm. The best characterized example in *S. aureus* is beta toxin (Huseby et al., 2010), which is a secreted neutral sphingomyelinase capable of lysing erythrocytes and lymphocytes. However, it is structurally related to the DNaseI superfamily of proteins and is able to bind DNA. Beta toxin forms insoluble oligomers upon binding DNA that could serve as a bridge to hold the biofilm structure together. Deletion of the *hlb* gene correlates with a reduction in biofilm formation in both *in vitro* and *in vivo* models. Additional studies have implicated that proteins with non-specific DNA-binding activity may be important matrix components in multiple bacterial species, as antibodies against IHF, a common member of the DNABII family of proteins, are capable of disrupting existing biofilms in *in vitro* and *in vivo* models (Goodman et al., 2011; Novotny et al., 2013).

BIOFILM DISPERSAL MECHANISMS

The primary biofilm dispersal strategy utilized by *S. aureus* is the production of various exo-enzymes and surfactants to degrade the extracellular polymeric matrix. The effectiveness of individual mechanisms is highly dependent on the matrix composition of the *S. aureus* strain in question (Chaignon et al., 2007; Kiedrowski et al., 2011). In general, mechanisms utilizing the enzymatic self-destruction of either protein and/or eDNA in the matrix are less effective at dispersing polysaccharide-dependent biofilms. In contrast, the mechanisms specifically targeting PIA are ineffective against polysaccharide-independent biofilms. In this review, the dispersal mechanisms targeting each matrix component will be discussed, with an emphasis on self-targeting enzymatic mechanisms (**Table 1**), and two recently described fundamental processes with biofilm dispersing activity will also be covered. Non-specific mechanisms, such as the surfactant activity of phenol-soluble modulins (PSMs), are effective against most *S. aureus* biofilms and are reviewed elsewhere (Peschel and Otto, 2013).

ENZYMATIC DISPERSAL MECHANISMS

Protease-mediated dispersal

S. aureus produces 10 secreted proteases, including seven serine proteases (SspA and SplA-F), two cysteine proteases (SspB and ScpA), and one metalloprotease (Aur) (Shaw et al., 2004). The role of proteases in biofilm dispersal was initially characterized during the analysis of *S. aureus* strains deficient in the global regulators *sarA* and *sigB* (Bronner et al., 2004) that were unable to form biofilm (Beenken et al., 2003; Trotonda et al., 2005; O'Neill et al., 2008). Characterization of these mutants revealed that the observed biofilm phenotypes resulted from elevated protease activity levels (Tsang et al., 2008; Lauderdale et al., 2009; Marti et al., 2010; Zielinska et al., 2012; Mootz et al., 2013). The high protease activity results in the degradation of important matrix proteins and destabilization of the biofilm (Zielinska et al., 2012). This phenotype could be reversed by the deletion of multiple protease genes or the addition of protease inhibitors (McGavin et al., 1997; Tsang et al., 2008; Mootz et al., 2013). The ability of the V8 serine protease (SspA), the staphopains (SspB and ScpA), and aureolysin (Aur) to disrupt biofilms have been demonstrated (**Table 1**), with the relative importance of each varying between strains and conditions. The V8 serine protease can degrade FnBPs and Bap (McGavin et al., 1997; O'Neill et al., 2008; Marti et al., 2010), and aureolysin can degrade ClfB and Bap to mediate biofilm disruption (Marti et al., 2010; Abraham and Jefferson, 2012). While the staphopains can disrupt the biofilm matrix, no target proteins have yet been characterized (Mootz et al., 2013). Additional targets such as Atl, Spa, and SasG have been proposed, but have not been linked to individual proteases (Lauderdale et al., 2009; Kolar et al., 2013). Despite the identification of some specific matrix proteins as targets for degradation, the large number of proteases and potential matrix protein targets will require proteomic analysis to dissect the complex mechanism behind protease-mediated dispersal.

The production of proteases is positively regulated through the *S. aureus* quorum sensing system, *agr* (Thoendel et al., 2011). The *agr* system is activated upon detection of an autoinducing peptide (AIP) that is encoded and produced by the *agr* operon. The AIP is detected by a two-component system that regulates virulence through the production of a regulatory RNA, RNAIII. The *agr* system regulates the virulence state of the cell by activating the production of secreted toxins and enzymes and the down-regulation of surface factors. The *agr* system induces the expression of both proteases and PSMs, which act as surfactants to disperse biofilms (Peschel and Otto, 2013). Thus, activation of the *agr* system can result in a shift from a biofilm state to a planktonic state of growth. This has been demonstrated through

Table 1 | Biofilm dispersal mechanisms.

Dispersal agent	Mechanism	References	Specific factor
Proteases	Degradation of proteinaceous matrix components	McGavin et al., 1997; O'Neill et al., 2008	V8 protease (SspA)
		Mootz et al., 2013	Staphopains (Cysteine Proteases)
		Abraham and Jefferson, 2012	Aureolysin (Aur)
		Marti et al., 2010	Aur, SspA
		Lauderdale et al., 2010; Shukla and Rao, 2013	Proteinase K
		Beenken et al., 2003; Trotonda et al., 2005; Tsang et al., 2008; Zielinska et al., 2012	*sarA* regulation
		Lauderdale et al., 2009	*sigB*, *agr* regulation
agr activation by AIP	Expression of agr regulated factors (proteases and PSMs)	Yarwood et al., 2004; Boles and Horswill, 2008; Lauderdale et al., 2010	AIP
Phenol-soluble modulins	Surfactant-mediated dispersal	Peschel and Otto, 2013	PSMs
S. epidermidis Esp	Degradation of proteinaceous matrix components; inhibition of autolysis through Atl degradation	Iwase et al., 2010; Chen et al., 2013; Sugimoto et al., 2013	Esp
Nucleases	Degradation of eDNA	Kiedrowski et al., 2011	Nuc
		Kiedrowski et al., 2014	Nuc2
Dispersin B	Degradation of polysaccharide matrix components	Kaplan et al., 2004; Donelli et al., 2007	DisB
D-amino acids	Protein synthesis inhibition in *B. subtilis*, unknown in *S. aureus*	Kolodkin-Gal et al., 2010; Hochbaum et al., 2011; Leiman et al., 2013; Sanchez et al., 2013	D-amino acids
Stringent response inhibition	Unknown	de la Fuente-Nunez et al., 2014; Reffuveille et al., 2014	Peptide 1018

the addition of AIP to existing biofilms, which results in complete dispersal (Boles and Horswill, 2008; Lauderdale et al., 2010), and through the use of fluorescent reporters, which demonstrated that cells detach from the biofilm after *agr* activation (Yarwood et al., 2004).

In addition to native *S. aureus* proteases, recent studies have indicated that the production of non-native proteases may impact *S. aureus* biofilm growth in bacterial communities. The serine protease Esp produced by *S. epidermidis* has been shown to disperse *S. aureus* biofilms (Sugimoto et al., 2013). This was first identified when it was observed that *S. aureus* colonization rates of the human nares negatively correlate with colonization rates of *esp* positive *S. epidermidis* (Iwase et al., 2010). Following this discovery, it was shown that Esp is able to cleave an array of *S. aureus* proteins, including Eap, FnBPA, and Atl (Chen et al., 2013; Sugimoto et al., 2013). The mechanism of Esp-mediated dispersal is thus two-fold: Esp degrades matrix proteins important for intercellular adhesion and prevents the release of eDNA by degrading murein hydrolase.

Nuclease-mediated dispersal

S. aureus produces two extracellular nucleases, referred to here as nuclease (Nuc) and nuclease2 (Nuc2) (Tang et al., 2008). The production of the major secreted Staphylococcal nuclease, also known as micrococcal nuclease or thermonuclease, is conserved across most clinical isolates and is produced *in vivo*. A recent study utilized this fact and developed a nuclease-specific probe for imaging *S. aureus* infections (Hernandez et al., 2014). Nuclease is regulated by the global regulator *sigB* and the SaeRS two-component system (Kiedrowski et al., 2011; Olson et al., 2013), and the expression of *nuc* is greatly reduced during biofilm growth conditions, suggesting that Nuc may play a role in the biofilm growth cycle (Olson et al., 2013).

Two major roles have been proposed for Nuc during infection, the disruption of neutrophil extracellular traps (NETs) and modulating biofilm development. It has been shown that the expression of nuclease results in reduced biofilm formation *in vitro*, while a *nuc* mutant displays enhanced biofilm formation (Mann et al., 2009; Kiedrowski et al., 2011). These phenotypes correlate with levels of eDNA accumulation during biofilm growth, where lack of nuclease results in the preservation of high molecular weight eDNA (Mann et al., 2009; Kiedrowski et al., 2011). This agrees with an earlier study that found a minimum size of 11 kb fragments was necessary for biofilm integrity (Izano et al., 2008). The second role proposed for nuclease during infection is the evasion of NETs. NETs are a newly discovered killing

mechanism utilized by neutrophils against bacterial infections. Activated neutrophils secrete nuclear DNA at the site of infection to entrap bacteria and enhance bacterial killing. Nuclease is able to degrade NETs and promote resistance against killing by neutrophils (Berends et al., 2010; Thammavongsa et al., 2013). The relative importance of each activity during infection has not yet been explored. Overall, *in vivo* studies indicate that *nuc* mutants are attenuated during infection (Berends et al., 2010; Olson et al., 2013). However, it is unclear whether this attenuation results from a reduced ability to disperse from a biofilm and disseminate to new sites, an increased susceptibility to killing by neutrophils, or the inability to scavenge nucleotides in the host. It is possible that all these functions of nuclease are important during infection.

In contrast to nuclease, the function of Nuc2 is still relatively unknown. This is in part due to the difficulty in studying Nuc2 in wild type backgrounds, as its activity is masked by Nuc. A recent study has shown that Nuc2 is a membrane-bound nuclease with an extracellular catalytic domain. Nuc2 activity is detectable in a *nuc* mutant, but the activity is very low (Kiedrowski et al., 2014). This is likely due to low expression levels, as mechanistic studies demonstrated that the Nuc2 catalytic domain is functional. Addition of purified Nuc2 was able to partially disperse existing biofilms, suggesting that Nuc2 could play a role in localized dispersal during infection. This localized dispersal could result in the formation of channels within the biofilm or supplement Nuc activity in high flow environments (such as those seen during endocarditis) where Nuc would be unable to accumulate. However, further studies will be necessary to determine the function of Nuc2 *in vivo*.

Dispersin B-mediated dispersal

The enzyme dispersin B isolated from *Actinobacillus actinomycetemcomitans* is able to disperse polysaccharide-dependent *Staphylococcus epidermidis* and *S. aureus* biofilms (Kaplan et al., 2004). Dispersin B disrupts the biofilm by hydrolyzing the glycosidic linkages of PIA. No homolog of dispersin B has been identified in the *S. aureus* genome so it is unlikely the organism utilizes this mechanism for dispersal during biofilm growth. However, treatment of biofilms with dispersin B does result in increased susceptibility to antimicrobials (Donelli et al., 2007). Thus, dispersin B could be developed as a potential anti-biofilm treatment.

BROAD-SPECTRUM DISPERSAL MECHANISMS

D-amino acids

It has been reported that D-amino acids produced during late stationary phase induce biofilm dispersal in multiple bacteria, including *S. aureus* (Kolodkin-Gal et al., 2010). The role of D-amino acids in dispersal was initially discovered in *Bacillus subtilis*. The proposed mechanism behind this dispersal was the incorporation of D-amino acids into the peptidoglycan, resulting in a failure to attach the major matrix protein, TasA, to the cell wall. Subsequently, this resulted in decreased intercellular adhesion via the detachment of existing TasA fibers. This dispersal mechanism was tested in additional bacterial species, including *S. aureus* and *P. aeruginosa*, where a similar phenotype were observed (Hochbaum et al., 2011).

However, a recent study has found that the effect of D-amino acids observed in *B. subtilis* was due to a strain specific mutation in the *dtd* gene (Leiman et al., 2013). *dtd* encodes a D-tyrosyl-tRNA deacylase and is responsible for preventing the misincorporation of D-amino acids into protein. As such, the D-amino acid biofilm dispersal effect observed in the *dtd* mutant was due to a growth defect caused by interference with protein synthesis. The impact of D-amino acids on *S. aureus* biofilm is therefore unclear and requires further investigation. However, D-amino acids may still offer clinical applications for the prevention of biofilm infections. It has been shown that pre-treatment of polymeric surfaces with D-amino acids reduces *S. aureus* biofilm formation *in vitro* (Hochbaum et al., 2011; Sanchez et al., 2013).

Stringent response

The stringent response is a general bacterial system triggered by nutrient starvation that allows cells to adapt to stressful conditions, such as those seen during infection (Srivatsan and Wang, 2008). During nutrient starvation, the alarmone ppGpp is produced by RelA/SpoT homologs and elicits regulatory changes that switch the cell to a metabolically inactive state. Studies have linked the stringent response to virulence and biofilm formation in multiple bacterial species (Lemos et al., 2004; Nguyen et al., 2011; Vogt et al., 2011; Chavez de Paz et al., 2012; He et al., 2012; Wexselblatt et al., 2012; Sugisaki et al., 2013). In *S. aureus*, evidence suggests the stringent response plays a role during infection (Geiger et al., 2010), but its impact on biofilm has not been extensively studied.

A recent study identified a synthetic cationic peptide capable of dispersing biofilms in a large number of clinically relevant bacterial pathogens, including *S. aureus*, without inhibiting planktonic growth (de la Fuente-Nunez et al., 2014). The peptide affected both Gram-negative and Gram-positive organisms, implicating that the peptide was targeting a general bacterial process. Further investigation determined that the peptide was inhibiting the stringent response through a direct interaction with ppGpp that resulted in the degradation of the alarmone. This result indicates that the metabolic state of the cell plays some role in dispersal. Additional research will be necessary to explore the role of stringent response in *S. aureus* biofilm dispersal.

IMPLICATIONS FOR CLINICAL TREATMENT OF BIOFILM INFECTIONS

Biofilm dispersal has drawn interest as a potential means of treating persistent *S. aureus* infections. The intentional dispersal of a biofilm coupled with antibiotic therapy would expose and kill metabolically active cells and render any remaining persister cells vulnerable to the immune system (**Figure 1B**). Increased antibiotic susceptibility has been observed with most dispersal agents, including many industrially produced enzymes such as dispersin B, proteinase K, and DNaseI (Lauderdale et al., 2010; Kaplan et al., 2012b; Shukla and Rao, 2013; Reffuveille et al., 2014). The efficacy of dispersal-mediated treatments could potentially be improved by the inclusion of a drug targeting persister cells (Conlon et al., 2013). In addition to the treatment of existing infections, dispersal mechanisms could be utilized in the prevention of biofilm formation associated with implanted medical devices. Several

studies have found that pretreatment of polymeric surfaces with dispersing agents can reduce biofilm formation *in vivo* (Donelli et al., 2007; Sanchez et al., 2013). The slow release of dispersal agents from the implanted device should prevent biofilm accumulation and facilitate clearance of the bacteria by the immune system. While these approaches sound promising, there are several concerns that have not yet been thoroughly addressed. First, induced dispersal could result in acute infections if the antibiotic fails to eradicate the released cells. Sub-inhibitory concentrations of certain antibiotics have been linked to enhanced *agr* activation (Joo et al., 2010), which could accelerate an acute response. Sub-inhibitory concentrations of β-lactams have also been linked to the induction of eDNA release and biofilm formation (Kaplan et al., 2012a), which could be counter-productive when coupled with a dispersal agent. Embolisms resulting from the release of cell clumps embedded in matrix components represent another major concern. Studies will need to address these challenges before dispersal agents are tested in a clinical setting.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The ability to form a biofilm is an important virulence determinant for the persistence of *S. aureus* chronic infections. In this review, we focused on the strategies utilized by *S. aureus* to escape from a biofilm through dispersal and disseminate to other body sites. Ongoing research continues to improve our understanding of the exo-enzymes and surfactants that degrade the biofilm matrix and release cells into the surrounding environment. The enzymes that have drawn the most attention are the secreted cysteine proteases (staphopains), V8 serine protease (SspA), and nuclease (Nuc). The relative importance of each enzyme will depend on the strain-specific composition of the biofilm matrix. The proteases and surfactant molecules are under *agr* quorum-sensing control, and activation of this regulatory system is a known dispersing mechanism.

Going forward, additional studies are necessary to fill specific knowledge gaps. The targets of the major proteases (V8, Aur, staphopains) are still not fully described, although some candidate surface proteins, like the FnBPs and ClfB, have been identified. The function of Nuc in biofilm dispersal has not been examined in detail. It is likely other exo-enzymes, such as hyaluronidase and lipases, are also important in dispersal mechanisms, but have not been fully investigated in biofilm studies (Rosenthal et al., 2014). In addition to the matrix-degrading mechanisms, it is possible that D-amino acids and the stringent response may play a role in dispersal, but further work is needed to better characterize these mechanisms. Perhaps the area of greatest need is confirming dispersal mechanisms in relevant animal models of infection and testing the efficacy of dispersal agents in treating biofilm infections. Additionally, coupling these agents with antibiotic therapy to facilitate clearance of a recalcitrant infection has received little attention. Overall our knowledge of enzymatic dispersal mechanisms has expanded in recent years, but many details still remain unclear. Further work on the topic will allow for the development of better treatment options for biofilm-mediated diseases.

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Methicillin resistance and the biofilm phenotype in *Staphylococcus aureus*

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Antibiotic resistance and biofilm-forming capacity contribute to the success of *Staphylococcus aureus* as a human pathogen in both healthcare and community settings. These virulence factors do not function independently of each other and the biofilm phenotype expressed by clinical isolates of *S. aureus* is influenced by acquisition of the methicillin resistance gene *mecA*. Methicillin-sensitive *S. aureus* (MSSA) strains commonly produce an *icaADBC* operon-encoded polysaccharide intercellular adhesin (PIA)-dependent biofilm. In contrast, the release of extracellular DNA (eDNA) and cell surface expression of a number of sortase-anchored proteins, and the major autolysin have been implicated in the biofilm phenotype of methicillin-resistant *S. aureus* (MRSA) isolates. Expression of high level methicillin resistance in a laboratory MSSA strain resulted in (i) repression of PIA-mediated biofilm production, (ii) down-regulation of the accessory gene regulator (Agr) system, and (iii) attenuation of virulence in murine sepsis and device infection models. Here we review the mechanisms of MSSA and MRSA biofilm production and the relationships between antibiotic resistance, biofilm and virulence gene regulation in *S. aureus*.

Keywords: biofilm, LPXTG proteins, Atl, PIA, *mecA*, methicillin resistance, c-di-AMP, *Staphylococcus aureus*

BIOFILM FORMATION BY STAPHYLOCOCCI AND ITS MEDICAL SIGNIFICANCE

Implantable medical devices have revolutionized modern healthcare; however attachment to these devices by surface adhering bacteria resulting in biofilm formation and device related infections (DRIs) substantially impact patient morbidity and mortality. Biofilms formed by staphylococci have for many decades been recognized as the most frequent cause of biofilm-associated infections with *Staphylococcus epidermidis* and *S. aureus* being among the most common etiologic agents of DRIs (Davies, 2003; Otto, 2013). Infections associated with biofilms are difficult to treat because the biofilm matrix and phenotypic characteristics of the bacteria confer resistance to the host immune response and the action of antimicrobial drugs (O'Gara and Humphreys, 2001).

All implanted medical devices are susceptible to colonization by staphylococci and staphylococcal biofilm-associated infections have been associated with devices ranging from implanted catheters to prosthetic heart valves, cardiac pacemakers, contact lenses, cerebrospinal fluid shunts, joint replacements and intravascular lines (Donlan and Costerton, 2002; Hall-Stoodley et al., 2004). Damaged host tissue is also a risk factor for developing biofilm-associated infection. *S. epidermidis* and *S. aureus*, which are part of the normal skin flora are opportunistic pathogens that can cause deep-seated skin infections for example in burn or post-operative wounds (Hammond et al., 2011). They are also known to form biofilms on damaged heart valves, leading to the development of life-threatening infective endocarditis (Claret et al., 2007; Nethercott et al., 2013).

S. epidermidis, unlike the more virulent *S. aureus*, relies almost exclusively on its ability to colonize and form biofilms on implanted medical devices to cause clinical infections (O'Gara and Humphreys, 2001; Christner et al., 2012) and a strong correlation has been reported between the pathogenicity of *S. epidermidis* and its ability to colonize implanted medical devices (Vuong and Otto, 2002). The ability of *S. aureus* to form biofilms on implanted medical devices or damaged host tissue is also a key virulence factor for this pathogen, especially in healthcare settings where antibiotic usage is high and such biofilm formation represents a survival mechanism for the bacteria (Høiby et al., 2010). Among the most studied mechanism of biofilm formation is the production of the *icaADBC* operon-encoded polysaccharide intercellular adhesion (PIA) or poly-N-acetylglucosamine (PNAG) by both *S. aureus* and *S. epidermidis* (O'Gara, 2007; Joo and Otto, 2012). However, biofilm formation independent of the *ica* operon has also been described in both *S. epidermidis* and *S. aureus* (Hussain et al., 1997; Fitzpatrick et al., 2005; Tormo et al., 2005; Qin et al., 2007; O'Neill et al., 2008; Schroeder et al., 2009; Shahrooei et al., 2009; Geoghegan et al., 2010).

In this review, we describe different mechanisms used by methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) isolates to form biofilm and how the acquisition or loss of methicillin resistance impacts on, not only the

biofilm phenotype of *S. aureus*, but also on global gene regulation and virulence.

BIOFILM FORMATION BY METHICILLIN-SENSITIVE *S. AUREUS*

The first described mechanism of *S. aureus* biofilm formation involved PIA/PNAG production (Cramton et al., 1999, 2001) that had earlier been described in *S. epidermidis* (Mack et al., 1994; Heilmann et al., 1996a,b; Heilmann and Götz, 1998). PIA is a glycan of β-1,6-linked 2-acetamido-2-deoxy-D-glucopyranosyl residues with a net positive charge that promotes intercellular aggregation and attachment of cells to inert surfaces (Rohde et al., 2010). The *ica* operon consists of four biosynthesis genes, *icaA*, *icaD*, *icaB,* and *icaC* and a divergently transcribed repressor, *icaR* (Conlon et al., 2002; Götz, 2002). The majority of research on the activity of the *ica* locus has been conducted in *S. epidermidis* but there is high nucleotide sequence homology between the *ica* loci of *S. epidermidis* and *S. aureus* and 78% identity at the amino acid level (Cramton et al., 1999). An early study demonstrated that a transposon mutation in the *ica* operon of *S. epidermidis* impaired biofilm formation and PIA production (Heilmann et al., 1996b). Subsequent studies showed that *S. aureus* also commonly uses *icaADBC*-encoded PIA as a mechanism of biofilm formation (Cramton et al., 1999, 2001).

Carriage of the *ica* locus is a characteristic of most clinical *S. aureus* strains (Cramton et al., 1999; Fowler et al., 2001; Rohde et al., 2001). One of the first reports of PNAG-independent *S. aureus* biofilm production was described in bovine mastitis isolates, which formed biofilm mediated by the biofilm associated protein (Bap) (Cucarella et al., 2001, 2004). An early study with the human *S. aureus* isolate UAMS-1, also revealed an *icaADBC*-independent biofilm phenotype under *in vitro* and *in vivo* conditions (Beenken et al., 2004).

Investigations into PIA-dependent and PIA-independent mechanisms of biofilm formation identified a correlation between methicillin susceptibility and biofilm in *S. aureus* (Fitzpatrick et al., 2005; O'Neill et al., 2007, 2008; Houston et al., 2011). This relationship between the biofilm phenotype and β-lactam susceptibility was first investigated in *S. epidermidis* by Mempel et al. who reported that variable levels of PIA production were significantly associated with different levels of β-lactam susceptibility in phenotypic variants (Mempel et al., 1994, 1995). The significant association between methicillin susceptibility in *S. aureus* and *ica*-dependent biofilm formation was first reported when PIA production was found to be essential for biofilm formation by MSSA but not MRSA (O'Neill et al., 2007). MSSA biofilms are significantly induced in growth media supplemented with NaCl, which is known to activate *ica* operon expression (Fitzpatrick et al., 2006; O'Neill et al., 2007). Furthermore, MSSA biofilms are susceptible to sodium metaperiodate treatment (which oxidizes polysaccharide bonds) and are resistant to treatment with proteinase K (O'Neill et al., 2007). Deletion of the *ica* locus abolished biofilm-forming capacity among clinical MSSA isolates that are amenable to genetic manipulation, as did mutation of the staphylococcal accessory regulator *sarA* (Valle et al., 2003; O'Neill et al., 2007).

Building on an original finding that four clinical MRSA isolates were capable of producing *icaADBC*-independent biofilm (Fitzpatrick et al., 2005), our laboratory reported differences in the environmental regulation of biofilm formation among 32 *S. aureus* isolates from intensive care units (15 MSSA and 17 MRSA strains) and found that NaCl-induced, PIA-dependent biofilm was more likely to be associated with MSSA biofilm formation (Fitzpatrick et al., 2006). A follow-up study of a large collection of 212 *S. aureus* isolates from device-related infections (114 MRSA and 98 MSSA) representing five clonal complexes (CC5, CC8, CC22, CC30, and CC45) further demonstrated that MSSA strains were more likely to produce NaCl-induced, PIA-mediated biofilm whereas MRSA biofilm was induced in media supplemented with glucose and was PIA-independent (O'Neill et al., 2007).

BIOFILM FORMATION BY MRSA STRAINS

The *ica* locus was found to be redundant for MRSA biofilm formation (O'Neill et al., 2007). Unlike the NaCl-induced biofilm expressed by MSSA clinical isolates, biofilm formation by MRSA isolates was significantly more likely to be induced by the addition of glucose to the growth medium, which is associated with the acidification of the culture media (O'Neill et al., 2007). MRSA biofilms are resistant to sodium metaperiodate treatment but are susceptible to proteinase K treatment, implicating protein adhesins in this biofilm phenotype (O'Neill et al., 2007).

As observed in MSSA isolates, mutation in *sarA* also abolished biofilm formation by clinical MRSA isolates (Beenken et al., 2004; O'Neill et al., 2007). SarA is a known repressor of four major extracellular proteases, namely SspA, SspB, Aur, and ScpA (Karlsson et al., 2001) and it is proposed that impaired glucose-induced biofilm formation by MRSA *sarA* mutants may be associated with upregulation of protease activity, which can inhibit *S. aureus* biofilm production (Marti et al., 2010).

A deletion in the accessory gene regulator (*agr*) system enhanced biofilm formation by clinical MRSA strains but had no significant effect on biofilms formed by MSSA strains (O'Neill et al., 2007). In contrast to *sarA*, *agr* positively regulates expression of the four major proteases (Shaw et al., 2004). Additionally, mutations in the *agr*-regulated Aur metalloprotease and the SplABCDEF serine proteases increased biofilm formation and reduced detachment from established biofilms (Boles and Horswill, 2008). Similarly, the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) enhanced biofilm formation, further implicating increased protease activity as the mechanism of *agr*-mediated dispersal from biofilms (Boles and Horswill, 2008). The positive impact of an *agr* mutation on MRSA biofilm formation is largely consistent with other research showing that deletion of *agr* can enhance *S. aureus* biofilm formation (Vuong et al., 2000; Beenken et al., 2003), whilst a separate study demonstrated that *agr* deletion does not significantly alter *icaADBC* expression or PIA production (Vuong et al., 2003). Interestingly, the reduced pH of cultures grown in media supplemented with glucose, was associated with repression of the *agr* system (Regassa et al., 1992). Furthermore, activation of the *agr* system was shown to initiate biofilm dispersal (Boles and Horswill, 2008). The

agr locus encodes two divergent transcripts, RNAII and RNAIII driven by the P2 and P3 promoters, respectively (Novick et al., 1993). RNAII comprises four genes, *agrBDCA,* that encode for the synthesis of secreted autoinducing peptide AIP. At threshold concentrations, AIP binds to the sensor histidine kinase AgrC, which phosphorylates AgrA and activates the P2 and P3 promoters, resulting in upregulated *agr* expression and increased transcription of the RNAIII effector molecule. Exogenous addition of AIP to biofilms induced dispersal from established biofilms by upregulating the *agr* system and subsequently increasing protease production (Boles and Horswill, 2008). The enhanced biofilm phenotype observed in *agr* mutants is also attributed to the loss of production of the RNAIII-encoded delta-toxin, which has surfactant properties (Vuong et al., 2000, 2004). Biofilm detachment by agr^+ strains is proposed to require the formation of a film of amphipathic delta-toxin molecules expressed at the biofilm/fluid interface which can inhibit the hydrophobic interactions between bacterial cell surfaces, thus lowering surface tension and causing cell detachment from the biofilm matrix (Vuong et al., 2004).

ROLE OF LPXTG-ANCHORED SURFACE PROTEINS IN MRSA BIOFILM FORMATION

S. aureus expresses 28 surface proteins, 21 of which are predicted to contain LPXTG binding motifs required for normal display on the cell surface (Ton-That et al., 2000; Mazmanian et al., 2001). Anchoring of these LPXTG motif-containing proteins to peptidoglycan is catalyzed by Sortase, an extracellular transpeptidase encoded by the *srtA* gene (Mazmanian et al., 2000). A *srtA* mutation affected biofilm accumulation by clinical MRSA strains from three clonal complexes and had no impact on *ica*-dependent biofilms formed by MSSA strains (O'Neill et al., 2008). As noted earlier Bap was the first LPXTG-anchored surface protein implicated in *ica*-independent *S. aureus* biofilm formation but is rarely found in human isolates (Cucarella et al., 2001; Tormo et al., 2005).

The fibronectin-binding proteins (FnBPs) were subsequently shown to play an important role in *ica*-independent biofilm formation by human MRSA isolates (O'Neill et al., 2008; Shanks et al., 2008; Vergara-Irigaray et al., 2009). The FnBPs are multifunctional surface proteins with an N-terminal A domain and a C-terminal wall spanning LPXTG-anchoring domain separated by tandem repeats involved in binding to fibronectin (Schwarz-Linek et al., 2003; Meenan et al., 2007). Both FnBP proteins, FnBPA and FnBPB, are involved in the accumulation phase of MRSA biofilm formation in hospital and community isolates under static and flow conditions, with each individual protein capable of complementing the biofilm defect of a double *fnbpAB* mutant (O'Neill et al., 2008; McCourt et al., 2014). The FnBPs do not contribute to PIA-mediated biofilm formation by clinical MSSA strains (O'Neill et al., 2008). Expression of the FnBPs was constitutive in a MRSA isolate producing an FnBP-dependent biofilm but restricted to the exponential growth phase in MSSA isolates that form PIA biofilms (Geoghegan et al., 2013). The N-terminal of the FnBPA protein, specifically residues 166–498 comprising the N2 and N3 subdomains of the A domain, has been implicated in the biofilm phenotype via a Zn(2+)-dependent mechanism (O'Neill et al., 2008; Geoghegan et al., 2013). A separate study from the Lasa laboratory, demonstrated that MRSA strain 132 was capable of producing either acid stress-induced FnBP-dependent biofilm or osmotic stress-induced exopolysaccharide-mediated biofilm (Vergara-Irigaray et al., 2009). Furthermore, the 132 *fnbpAB* mutant displayed significantly impaired colonization and biofilm formation compared to the isogenic 132 *ica* mutant in a murine model of subcutaneous catheter-related infection (Vergara-Irigaray et al., 2009). Recently the formation of bacterial biofilm-like aggregates in human synovial fluid by MRSA and MSSA strains was attributed to expression of the FnBPs as well as the fibrinogen binding proteins ClfA and ClfB, identifying biofilm and in particular LPXTG-wall anchored proteins as important virulence determinants in staphylococcal joint infections (Dastgheyb et al., 2014).

In MRSA strains where the FnBPs have not (yet) been implicated in protein dependent biofilm, a number of other LPXTG proteins may be important. The accumulation associated protein (Aap), which is 54% identical to the SasG protein of *S. aureus* (Corrigan et al., 2007) was first identified in *S. epidermidis* and is known to play a well-defined role in the biofilm phenotype (Hussain et al., 1997; Rohde et al., 2005; Conrady et al., 2008, 2013; Conlon et al., 2014; Schaeffer et al., 2015). SasG, which is found in approximately 50% of human clinical isolates, has also been implicated in the intercellular accumulation phase of *ica*-independent biofilm formation (Corrigan et al., 2007; Sung et al., 2008). SasG promotes intercellular aggregation via homo-oligomerization of the SasG protein (Kuroda et al., 2008) with biofilm formation promoted by the repeated B domains of the SasG protein (Geoghegan et al., 2010). The B repeat regions promote *ica*-independent biofilm formation in a Zn(2+)-dependent manner through the formation of extended fibrils on the cell surface (Geoghegan et al., 2010; Gruszka et al., 2012). Protein A also promotes polysaccharide-independent biofilm but does not need to be anchored to the cell wall via its LPXTG motif to do so, with exogenous Protein A capable of promoting biofilm accumulation (Merino et al., 2009). However, the role or otherwise of SasG and protein A to staphylococcal biofilm-associated infections has not yet been investigated. Readers are referred to a companion review in this series focusing on staphylococcal proteinaceous biofilms by Pietro Speziale.

ROLE OF THE MAJOR AUTOLYSIN AND EXTRACELLULAR DNA IN MRSA BIOFILM FORMATION

The major autolysin, Atl, which is involved in daughter cell separation, cell wall homeostasis and peptidoglycan turnover (Yamada et al., 1996; Biswas et al., 2006) plays a role in the early stages of *ica*-independent biofilm formation by clinical MRSA strains (Houston et al., 2011). This bifunctional peptidoglycan hydrolase was first implicated in the primary attachment of *S. epidermidis* to polystyrene (Heilmann et al., 1997; Biswas et al., 2006), with *atlE* mutants exhibiting significantly attenuated virulence in a rat central venous catheter infection model (Rupp et al., 2001). AtlE of *S. epidermidis* also possesses vitronectin-binding activity implicated in AtlE-mediated biofilm formation on plasma protein-coated polymer surfaces (Heilmann et al., 1997). Clinical

MRSA strains that produce *ica*-independent biofilms require Atl for primary attachment to polystyrene surfaces (Houston et al., 2011).

The major autolysin of *S. aureus* is expressed as a 137.5 kDa pro-protein which is proteolytically processed to yield a 3.1 kDa signal sequence, a 17.6 kDa propeptide and two enzymatically active regions, a 63.3 kDa *N*-acetylmuramyl-L-alanine amidase enzyme and a 53.6 kDa endo-β-*N*-acetylglucosaminidase enzyme (Oshida et al., 1995). Catalytic activity of the amidase region is required for PIA-independent proteinaceous biofilm formation by hospital acquired MRSA (HA-MRSA) strains (Houston et al., 2011). The protease inhibitor PMSF, which increases the release of extracellular autolytic enzymes (Fournier and Hooper, 2000), and polyanethole sodium sulfanate, which inhibits autolytic activity without impairing growth (Wecke et al., 1986; Yabu and Kaneda, 1995), both prevented biofilm formation by HA-MRSA strains indicating a vital role for both the unprocessed Atl protein and the active amidase and glucosaminidase enzymes in early MRSA biofilm formation (Houston et al., 2011).

Construction of enzymatically inactive point mutations within the active regions of Atl revealed Atl-mediated cell lysis and the release of extracellular DNA (eDNA) by both the amidase and glucosaminidase regions as the mechanisms of Atl-mediated biofilm formation (Bose et al., 2012). Several studies have reported a role for eDNA in the *ica*-independent healthcare associated and community acquired MRSA (CA-MRSA) biofilm phenotypes (Izano et al., 2008; Lauderdale et al., 2010; Houston et al., 2011). In *S. aureus*, addition of DNase I to the culture media inhibited PIA-independent biofilm formation by HA-MRSA but did not significantly disperse mature MRSA biofilms implicating eDNA in the attachment and/or early stage of biofilm development (Houston et al., 2011). In the CA-MRSA strain USA300, the secreted thermostable nuclease enzyme, Nuc has also been shown to negatively impact on biofilm formation (Kiedrowski et al., 2011). In a mouse model of catheter infections, however, mutations in *nuc* and a second nuclease gene *nuc2* of the UAMS-1 MSSA strain were associated with reduced biofilm (Beenken et al., 2012) indicating that additional studies with MSSA and MRSA isolates, are needed to fully elucidate the role of these enzymes and extracellular nucleic acid in the biofilm phenotype *in vivo*.

A study of *ica*-independent mechanisms of biofilm formation by *S. aureus* revealed that altered levels of autolysis were associated with defective biofilm production (Boles et al., 2010). Recently cytoplasmic proteins released during the stationary phase of growth, which may be a consequence of autolysis, have been shown to be part of the biofilm matrix of *S. aureus* HG003 (Foulston et al., 2014). Interestingly the release of cytoplasmic proteins during stationary phase appears to be in response to decreasing pH, which can be triggered by the addition of excess glucose to the growth media (Foulston et al., 2014) and it is worth noting that these same growth conditions promote Atl/FnBP-mediated MRSA biofilm formation (O'Neill et al., 2008). A simplified model of MSSA and MRSA biofilm mechanisms together with scanning electron micrographs of SH1000 (MSSA) and BH1CC (MRSA) biofilms is presented in **Figure 1**.

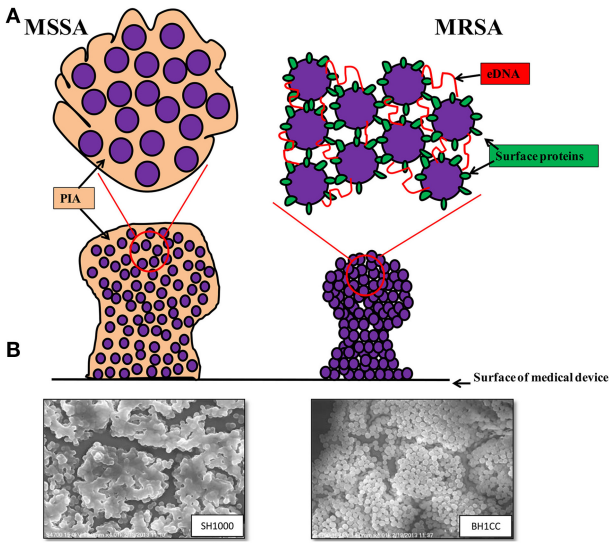

FIGURE 1 | (A) Graphic representations of the biofilm phenotypes expressed by MSSA and MRSA. MSSA strains form *ica*-dependent, PIA-mediated biofilms whereas MRSA strains form biofilms independent of PIA and require surface proteins such as the fibronectin binding proteins, Atl-mediated cell lysis and eDNA for colonization of surfaces and biofilm accumulation. **(B)** Scanning electron micrographs (3500 × magnification) of biofilms formed by MSSA strain SH1000 grown in BHI media supplemented with 4% NaCl (left) and MRSA strain BH1CC grown in BHI supplemented with 1% glucose (right).

HOW DOES METHICILLIN RESISTANCE AFFECT THE BIOFILM PHENOTYPE AND VIRULENCE?

METHICILLIN RESISTANCE AND BIOFILM

The mechanism(s) underpinning the association between the MRSA and MSSA biofilm phenotypes and utilization of polysaccharide and protein adhesins, respectively remains unclear. However, the level of resistance to beta-lactam antibiotics appears to be important for the biofilm phenotype. For instance, excision of the SCC*mec* element from the hospital MRSA strain BH1CC was associated with reduced FnBP-mediated biofilm forming capacity, presumably due to up-regulated protease activity (Pozzi et al., 2012; Rudkin et al., 2012). The relationship between methicillin resistance and biofilm is further complicated by the observation that MRSA strains can express either low level heterogeneous resistance (HeR) to methicillin or high-level, homogeneous resistance (HoR) (Keaton et al., 2013; Mwangi et al., 2013). Carriage of the *mecA* gene alone does not confer the HoR phenotype and additional genetic events are also needed (Pozzi et al., 2012; Mwangi et al., 2013; Dordel et al., 2014). In the laboratory isolation of homogeneously resistant strains from HeR strains is readily achieved by plating cell suspensions on media supplemented with high concentrations of oxacillin, the clinically used derivative of methicillin (100 μg/ml) (Pozzi et al., 2012; Dordel et al., 2014). In clinical isolates of MRSA the *ica* locus is present and expressed but PIA does not appear to be produced (O'Neill et al., 2007). In an engineered derivative of the laboratory strain 8325-4 carrying the *mecA* gene on a plasmid and expressing HoR, the *ica* operon was repressed >300-fold compared to its MSSA

parent and was associated with a switch from PIA-dependent to proteinaceous biofilm (Pozzi et al., 2012). In contrast expression of HeR in 8325-4 did not significantly alter the biofilm phenotype (Pozzi et al., 2012). Extracellular protease activity was reduced in the 8325-4 HoR strain and 8235-4 HoR biofilms were dispersed with proteinase K implicating a protein adhesin in this phenotype (Pozzi et al., 2012). However, because this biofilm phenotype was independent of PIA/PNAG, the major autolysin or any of the LPXTG cell wall anchored proteins whereas clinical MRSA isolates express an FnBP/eDNA dependent biofilm, it appears that expression of homogeneous methicillin resistance is associated with at least two distinct biofilm phenotypes. It remains to be determined whether PBP2a expression exerts direct or indirect (e.g., via altered cell wall architecture) effects on biofilm production. In a murine device infection model 8325-4 and 8325-4 HoR were recovered in similar numbers from implanted catheters, suggesting that PIA/PNAG and protein adhesin-mediated biofilms may be equally effective for device colonization *in vivo* (Pozzi et al., 2012).

OXACILLIN RESISTANCE AND VIRULENCE

High level PBP2a expression in MRSA is associated with repression of the Agr quorum sensing operon (Pozzi et al., 2012; Rudkin et al., 2012). Agr repression in MRSA is directly linked to *mecA* expression and the subsequent changes in cell wall architecture are accompanied by reduced cytotoxin production and attenuated virulence. The repression of Agr blocks the co-ordinated switch-on of toxin and enzyme secretion during the latter stages of bacterial growth *in vitro*, leaving the cell arrested in the surface protein expression stage of the Agr regulatory cycle (Rudkin et al., 2012). As noted above, increased surface protein expression and repression of extracellular protease production are also consistent with the protein-mediated MRSA biofilm phenotype (Pozzi et al., 2012).

Murine infection model studies showed that mice infected with the 8325-4 HoR strain were more likely to survive than mice infected with 8325-4 (Pozzi et al., 2012). The 8325-4 HoR strain did not significantly disseminate beyond the surrounding peri-catheter tissue whereas high numbers of the 8235-4 MSSA strain were recovered from the kidneys, liver and spleen (Pozzi et al., 2012). In a murine bacteremia model, mice infected with Δ*SCCmec* and Δ*mecA* mutants of the HA-MRSA strain BH1CC consistently lost more weight during the course of infection and suffered >60% mortality compared to the wild type (Rudkin et al., 2012). These data have led to the hypothesis that MRSA strains have sacrificed virulence for high level antibiotic resistance. Supporting this idea, the infectivity and lethality of MRSA strains was also reduced in guinea pig and murine models of infection compared to MSSA strains (Mizobuchi et al., 1994). Interestingly, MRSA and MSSA isolates were equally virulent in immunocompromised animals (Mizobuchi et al., 1994). Furthermore, a study of 104 patients with *S. aureus* bacteremia found that MSSA bacteremia correlated with significantly higher rates of infective endocarditis than MRSA (Abraham et al., 2004). Similarly, MSSA infections have been associated with a significantly higher illness severity score from invasive disease than MRSA clones (Wehrhahn et al., 2012).

The phenol soluble modulin *mec* (*psm-mec)* locus located adjacent to *mecA* on type II SCC*mec* elements promotes biofilm formation in MRSA strains (Kaito et al., 2011, 2013) and has also been implicated in reduced virulence of MRSA strains in murine models of infection (Kaito et al., 2008, 2011; Queck et al., 2009). The *psm-mec* locus was directly implicated in inhibiting translation of the *agrA* transcript (Kaito et al., 2013). Interestingly carriage of *psm-mec* was only associated with HA-MRSA strains and was absent in the more virulent CA-MRSA isolates and mutation or deletion of the locus from HA-MRSA increased virulence capacity in murine infection models (Kaito et al., 2013). In addition to PSM-*mec*, phenol soluble modulins (PSMs) generally have surfactant qualities and are known to be centrally involved in biofilm structuring and detachment, including dissemination of biofilm-associated infection (Wang et al., 2011; Periasamy et al., 2012). In this context, repression of the Agr system including PSM-*mec* in MRSA is consistent with enhanced biofilm formation.

Oxacillin resistance has pleiotropic effects on *S. aureus*, changing the biofilm phenotype, altering global gene regulation, reducing toxin production and ultimately reducing virulence. However, the importance of MRSA strains as pathogens in healthcare and community setting reflects the high degree of adaptation of *S. aureus* to methicillin resistance and the associated cellular changes. The attenuated virulence associated with high level resistance in HA-MRSA strains, is consistent with their confinement within healthcare settings (Collins et al., 2010; Rudkin et al., 2012), whereas the resistance/virulence equilibrium in CA-MRSA strains has clearly supported their ability in infect otherwise healthy individuals.

NUCLEOTIDE SIGNALING AND METHICILLIN RESISTANCE

The mechanisms underpinning the relationship between antibiotic resistance and virulence in *S. aureus* remain to be elucidated but studies over recent years implicate nucleotide signaling in these phenotypes. Mutation of the *S. aureus* diadenylate cyclase gene *dacA*, which reduced c-di-AMP levels, resulted in the conversion of a HoR MRSA to a HeR strain (Dengler et al., 2013). Conversely mutations in the *gdpP*-encoded c-di-AMP phosphodiesterase, which resulted in increased c-di-AMP levels were accompanied by HoR to methicillin (Corrigan et al., 2011; Pozzi et al., 2012) or increased tolerance to beta-lactam antibiotics (Griffiths and O'Neill, 2012). In *Bacillus subtilis*, the activity of the GdpP homolog YybT is strongly repressed by the stringent response alarmone ppGpp, which is synthesized by the RelA enzyme (Rao et al., 2010) and Mwangi et al. reported that activation of the stringent response and constitutive ppGpp production was accompanied by homogeneous methicillin resistance (Mwangi et al., 2013). Proteomic analysis has revealed that aminoacyl-tRNA biosynthesis was repressed by subinhibitory concentrations of oxacillin in both MSSA and MRSA (Liu et al., 2014). Reduced levels of aminoacyl-tRNA may trigger the stringent response, which is normally activated by amino acid starvation and specifically the concomitant reduction in charged tRNA levels. A recent genomics analysis of HoR strains derived from a range of clinical HeR isolates identified mutations in 27 genes and 3 intergenic regions, most of which are potentially involved in the

stringent response (Dordel et al., 2014). Collectively these data implicate the stringent response in the HoR phenotype and may suggest that ppGpp-mediated repression of GdpP activity leads to reduced c-di-AMP levels and in turn modulates resistance to beta-lactam antibiotics, biofilm and virulence. Identification of c-di-AMP targets may help elucidate the mechanism c-di-AMP-controlled beta-lactam resistance and initial studies to identify c-di-AMP target proteins identified in *S. aureus* and *Listeria monocytogenes* suggest a pleiotrophic role for this nucleotide second messenger (Corrigan et al., 2013; Sureka et al., 2014).

CONCLUDING THOUGHTS

Biofilm formation is a key virulence factor of staphylococci and distinct mechanisms are employed by MSSA and MRSA for biofilm formation. Clinical MSSA strains predominantly form biofilm dependent on the *icaADBC* operon and PIA production whereas MRSA strains form biofilms independent of PIA (O'Neill et al., 2007). There are important roles for LPXTG-anchored surface proteins, the major autolysin and eDNA during MRSA biofilm formation in the absence of PIA (O'Neill et al., 2008; Houston et al., 2011). Acquisition of methicillin resistance appears to repress polysaccharide-type biofilm production and promote formation of proteinaceous-type biofilms (Pozzi et al., 2012). While the different mechanisms of biofilm formation employed by MSSA and MRSA do not appear to impair the ability of either to colonize implanted biomaterial *in vivo*, the acquisition of methicillin resistance by HA-MRSA is associated with an overall downregulation of virulence gene expression (Pozzi et al., 2012; Rudkin et al., 2012, 2014). Despite this, HA-MRSA strains remain a significant cause of morbidity and mortality for hospitalized patients perhaps reflecting their successful adaptation to a specific niche within the hospital setting where there is a large pool of immunocompromised individuals that often require implanted medical devices and intensive antibiotic treatments (Pozzi et al., 2012). Understanding how MRSA has remained a successful pathogen in the hospital environment, will inform the development of novel therapeutics. Indeed treating MRSA strains with oxacillin may still have therapeutic potential (Rudkin et al., 2014). In CA-MRSA strains, the lower levels of PBP2a expression compared to HA-MRSA is associated with maintenance of high level toxin production. Using oxacillin to increase PBP2a expression can repress Agr activity and secretion of cytolytic toxins, indicating therapeutic potential (Rudkin et al., 2014). Further investigations to assess the global impact of oxacillin treatment on MRSA gene expression are needed but these data illustrate how advances in our understanding of antibiotic resistance and biofilm might be exploited in the development of new strategies to better prevent and treat *S. aureus* infections.

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Biofilm formation by *Streptococcus agalactiae*: influence of environmental conditions and implicated virulence factors

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Streptococcus agalactiae (Group B *Streptococcus*, GBS) is an important human pathogen that colonizes the urogenital and/or the lower gastro-intestinal tract of up to 40% of healthy women of reproductive age and is a leading cause of sepsis and meningitis in the neonates. GBS can also infect the elderly and immuno-compromised adults, and is responsible for mastitis in bovines. Like other Gram-positive bacteria, GBS can form biofilm-like three-dimensional structures that could enhance its ability to colonize and persist in the host. Biofilm formation by GBS has been investigated *in vitro* and appears tightly controlled by environmental conditions. Several adhesins have been shown to play a role in the formation of GBS biofilm-like structures, among which are the protein components of pili protruding outside the bacterial surface. Remarkably, antibodies directed against pilus proteins can prevent the formation of biofilms. The implications of biofilm formation in the context of GBS asymptomatic colonization and dissemination to cause invasive disease remain to be investigated in detail.

Keywords: streptococcal infections, *Streptococcus agalactiae*, biofilms, group B streptococcus, pili/fimbriae/curli

INTRODUCTION

The beta-hemolytic Gram-positive *Streptococcus agalactiae* (Group B Streptococcus, GBS) is often encountered in the gastro-intestinal and the genital tract of healthy women as part of the normal flora. From this site, the bacteria can reach the newborn through the birth canal and cause sepsis and/or meningitis (Gibbs et al., 2004; Dando et al., 2014). GBS is also an important cause of morbidity and mortality in the elderly and in immuno-compromised adults. Primary manifestations of adult GBS disease include bacteremia, skin and soft tissue infections, pneumonia, osteomyelitis and urinary tract infections (Edwards et al., 2005; Skoff et al., 2009). GBS can also colonize the mammary gland of ruminants, where it is able to survive for long periods causing clinical and sub-clinical mastitis (Keefe, 1997).

GBS colonization and infection of target tissues requires the capacity of these bacteria to adhere and to persist in mucosal epithelial surfaces. In this habitat, the formation of biofilm-like communities could facilitate microbial survival and proliferation by enhancing resistance to host defenses and nutrient deprivation.

The present review summarizes recent studies investigating the capacity of GBS to form biofilm-like structures *in vitro*, how this mode of growth is affected by environmental conditions, and the contribution of adhesin virulence factors.

EXPERIMENTAL EVIDENCE OF GBS BIOFILM FORMATION AND INFLUENCE OF ENVIRONMENTAL CONDITIONS

Initial evidence suggesting that GBS could be implicated in the formation of biofilms came from studies by Donlan and Costerton where GBS bacteria were found on intrauterine devices in association with other known biofilm formers such as *Staphylococcus aureus* and *Staphylococcus epidermidis* (Donlan and Costerton, 2002).

Macroscopic assays were set up to investigate the biofilm forming capacity of GBS strains belonging to different lineages (Rinaudo et al., 2010). According to these type of assay, bacteria are cultured under static conditions in the wells of plastic tissue culture plates and, after several washes, microbial three-dimensional structures are stained with crystal violet or similar compounds (O'Toole et al., 2000).

More restrictive experimental methods have recently been set up to better discriminate between GBS weak and strong biofilm formers. One of these approaches mimics fluid circulation in the host by using flow conditions in laminar chamber systems (Konto-Ghiorghi et al., 2009). An alternative multiwell-based protocol is based on plate incubation under shaking and removal of non-attached bacteria by extensive washing, followed by replacement of the growth medium (D'Urzo et al., 2014).

Environmental conditions are known to strongly influence the capacity to form biofilm by many bacterial species (Froeliger and Fives-Taylor, 2001; Moscoso et al., 2006; Manetti et al., 2007). Several studies have investigated GBS *in vitro* biofilm formation using one of the above described methods and different growth media, with contrasting results. For instance, Kaur et al. (2009), Borges et al. (2012), and Yang et al. (2012) investigated biofilm production under neutral and acidic pH conditions. They found larger biofilm amounts at pH 6.5 compared to pH 4.2, probably due to poor bacterial growth at low pH. By contrast, Ho et al. (2013) found that low pH induced biofilm formation in nutrient-limited chemically defined medium (M9YE) and not in rich media like Todd-Hewitt Broth (THB). Konto-Ghiorghi et al. (2009) reported that a uniform biofilm was produced only on

Luria Broth and RPMI 1640 supplemented with 1% glucose. The need for glucose for GBS biofilm formation was confirmed by Rinaudo et al. who also demonstrated that this sugar does not affect planktonic bacterial growth (Rinaudo et al., 2010). Previous studies had shown that in the GBS related species *Streptococcus pyogenes* the glucose biofilm enhancing effect was the direct result of acidification due to metabolic production of organic acids (Manetti et al., 2010). Evidence that acidic pH and not glucose concentration was the environmental signal driving GBS biofilm formation *in vitro* was obtained by D'Urzo et al. (2014). The authors tested a wide panel of strains in both buffered or non-buffered nutrient-rich (THB) and nutrient-limited (RPMI) media, in the presence or absence of glucose. Strong biofilm formation was observed only in glucose-containing non-buffered media and in low pH media even in the absence of glucose. In an *in vivo* setting, exposure of GBS to the acidic milieu of the vagina could be the signal sensed by the bacteria to grow in a sessile mode in this site. In this context, temporal shifts in GBS loads were recently observed in a mouse model of vaginal colonization (Carey et al., 2014), and have also been reported in humans (Hansen et al., 2004). It is tempting to speculate that pH variations and consequently GBS biofilm formation on epithelial cells could affect GBS carriage fluctuations.

Therefore, the discrepancies between the different studies in the observed capacity of GBS strains to form biofilm-like structures *in vitro* can possibly be explained by the use of different types of assays and growth conditions.

CONTRIBUTION OF PILI AND OTHER SURFACE VIRULENCE FACTORS TO GBS BIOFILM FORMATION

Long filamentous structures protruding from the surface of Gram-positive bacteria were discovered in the last decade (Ton-That and Schneewind, 2004; Soriani and Telford, 2010). These structures resemble the pili found in Gram-negative bacteria, although in Gram-positives pilus protein components are linked by covalent bonds.

Pili in *S. agalactiae* were discovered during screening of multiple genomes for surface-exposed protein antigens as possible vaccine targets (Lauer et al., 2005). By SDS-PAGE, the pilus polymers appeared as a ladder of bands ranging from 150 kDa to beyond the resolution of the gels, while immune electron microscopy revealed long appendages protruding outside the capsule that covers the bacterial surface (**Figure 1**). The genes encoding the GBS pilus machinery are clustered in three related genomic islands (Islands PI-1, -2a, and -2b) located in two separate loci flanked by direct repeats and conserved genes. All islands contain three genes encoding the pilus components, i.e., one backbone protein essential for pilus assembly (BP) and two accessory proteins (AP1 and AP2), plus two genes encoding sortase enzymes catalyzing the covalent linkage of the pilus proteins into long polymers (Dramsi et al., 2006; Rosini et al., 2006). The pilus proteins of PI-1 and PI-2b differ by very few amino acids, while PI-2a is more variable with seven alleles described both for the BP and the AP1 presenting sequence identities between 48–98% for BP and 87–98% for AP2. Remarkably, mouse immunization with BP-1, BP-2b, and AP1-2a conferred protection against infection with a large panel of virulent strains, and at least one of the three islands is present in all GBS (Margarit et al., 2009; Madzivhandila et al., 2011; Martins et al., 2013).

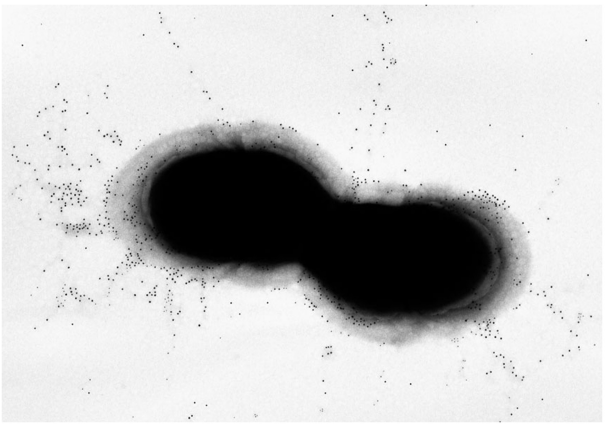

FIGURE 1 | Pilus Island-2a. Immunogold transmission electron microscopy of GBS 515 grown to exponential phase. Bacteria were incubated with polyclonal sera raised against the corresponding pilus 2a backbone variant obtained as recombinant protein, and labeled with secondary antibodies conjugated with 10 nm gold nanoparticles.

The discovery of pili in Gram-positive pathogens raised the question on the possible role of these highly surface-exposed structures in host colonization and infection. Pioneering evidence for their involvement in cell adhesion and biofilm formation was obtained in *S. pyogenes* (Manetti et al., 2007).

Studies using GBS isogenic mutants lacking pilus 2a components or the sortase enzymes responsible for pilus polymerization and cell wall attachment, indicated a role of pili in host cell contact and in the formation of multi colony three-dimensional structures on abiotic surfaces (Konto-Ghiorghi et al., 2009; Rinaudo et al., 2010). These biofilm-like structures were also analyzed by confocal laser scanning microscopy where the wild-type GBS515 strain bearing pilus variant 2a and an isogenic mutant unable to assemble pili, were seeded on glass polylysine-coated coverslips and stained with a fluorescent dye. As shown in **Figure 2**, the wild-type strain formed structured multilayered aggregates of surface-adherent bacteria resembling a mature biofilm, while the deletion mutant did not.

Remarkably, antibodies directed against the backbone of pilus 2a and its main ancillary protein inhibited the formation of these biofilm-like structures in a dose dependent manner, while antibodies against the small ancillary protein located at the pilus base near the cell wall, did not show any effect (Rinaudo et al., 2010). The same study investigated biofilm formation by 289 GBS clinical isolates using the above described crystal violet assay under static conditions. A correlation between the high surface exposure of pilus 2a and the biofilm formation phenotype was observed.

In the more recent study by D'Urzo et al., the formation of biofilm-like structures *in vitro* by 389 GBS isolates was investigated under more stringent conditions to better discriminate between weak and strong biofilm formers (see above). Also in this case, a high variability among strains was observed both in pilus expression and in the capacity to form biofilms, even when

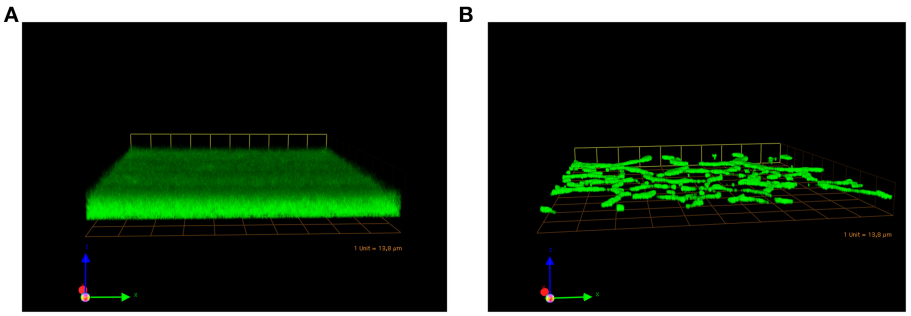

FIGURE 2 | Pilus Island-2a involvement in the formation of biofilms *in vitro*. Confocal scanning laser microscopy micrographs of biofilm development by GBS 515 **(A)** and its mutant derivative containing an in-frame deletion in the pilus backbone gene (Rosini et al., 2006) **(B)**. Bacteria were grown on glass coverslips under static conditions at 37°C for 72 h, fixed and stained with SYTO-9 prior to confocal analysis (magnification 60x).

they belonged to the same serotype or MLST phylogenetic lineage. A subset of serotype III strains belonging to the hyper virulent lineage ST-17 harboring both pilus 1 and 2b was shown to form stronger biofilms than all other tested strains, particularly at low pH (D'Urzo et al., 2014). Importantly, ST-17 strains are the most frequent cause of late-onset neonatal infections (Tazi et al., 2010). The proteins responsible for the higher capacity of this ST-17 subset of strains to form biofilms have not yet been identified, and could be possible targets to prevent colonization/disease of this hypervirulent lineage.

Park et al. investigated the phenotype of the CsrRS two-component regulatory system knockout, and showed an increase capacity of CsrRS mutant bacteria to adhere to host cells and to form biofilm-like structures on plate (Park et al., 2012). This regulatory effect of CsrRS on bacterial adherence and biofilm formation correlated with the expression of multiple surface adhesins but not of Pilus 1, excluding a role of this pilus variant in biofilm formation in the investigated isolate. The same and other authors identified BsaB/FbsC as a protein adhesin involved in biofilm formation and regulated by the CsrRS system (Buscetta et al., 2014; Jiang and Wessels, 2014); different from the pilus proteins, FbsC expression appeared slightly downregulated in a CsRS dependent manner at acidic versus neutral pH.

CONCLUSIONS

Similar to other Gram-positive pathogens colonizing the human host, Group B *Streptococcus* can form multicellular communities that are expected to facilitate its persistence under environmental stress conditions. A number of *in vitro* studies have demonstrated that GBS forms biofilm-like structures on abiotic surfaces. Yet, the presence in these structures of the extracellular matrix typical of bacterial biofilms has not been investigated in this species. Additional studies are also needed to confirm the relevance of biofilm formation *in vivo*. If this would be the case, the involved virulence factors could constitute new therapeutic and preventive targets against this important human pathogen.

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Protein-based biofilm matrices in Staphylococci

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Staphylococcus aureus and *Staphylococcus epidermidis* are the most important etiological agents of biofilm associated-infections on indwelling medical devices. Biofilm infections may also develop independently of indwelling devices, e.g., in native valve endocarditis, bone tissue, and open wounds. After attachment to tissue or indwelling medical devices that have been conditioned with host plasma proteins, staphylococcal biofilms grow, and produce a specific environment which provides the conditions for cell–cell interaction and formation of multicellular communities. Bacteria living in biofilms express a variety of macromolecules, including exopolysaccharides, proteins, extracellular eDNA, and other polymers. The *S. aureus* surface protein C and G (SasC and SasG), clumping factor B (ClfB), serine aspartate repeat protein (SdrC), the biofilm-associated protein (Bap), and the fibronectin/fibrinogen-binding proteins (FnBPA and FnBPB) are individually implicated in biofilm matrix formation. In *S. epidermidis*, a protein named accumulation-associated protein (Aap) contributes to both the primary attachment phase and the establishment of intercellular connections by forming fibrils on the cell surface. In *S. epidermidis*, proteinaceous biofilm formation can also be mediated by the extracellular matrix binding protein (Embp) and *S. epidermidis* surface protein C (SesC). Additionally, multifunctional proteins such as extracellular adherence protein (Eap) and extracellular matrix protein binding protein (Emp) of *S. aureus* and the iron-regulated surface determinant protein C (IsdC) of *S. lugdunensis* can promote biofilm formation in iron-depleted conditions. This multitude of proteins intervene at different stages of biofilm formation with certain proteins contributing to biofilm accumulation and others mediating primary attachment to surfaces. This review examines the contribution of proteins to biofilm formation in Staphylococci. The potential to develop vaccines to prevent protein-dependent biofilm formation during staphylococcal infection is discussed.

Keywords: *Staphylococcus*, biofilm, cell wall-anchored proteins, extracellular proteins, homophilic interactions

INTRODUCTION

Staphylococcus aureus and *Staphylococcus epidermidis* cause a broad spectrum of diseases in humans ranging from soft tissue infections and abscesses in organ tissues to osteomyelitis, endocarditis, and toxic shock syndrome. It is not surprising that these bacteria, especially *S. aureus*, encode a large array of virulence factors that enable the organisms to infect different tissues within the host. Both species display a strong capacity to form biofilms, which are functional multilayered communities of microrganisms adhering to a surface embedded in a self-synthesized extracellular matrix. Biofilm infections are important clinically because bacteria in biofilms exhibit recalcitrance to antimicrobial compounds and persistence in spite of sustained host defenses. The development of a bacterial biofilm is a complex, multifactorial process and can be divided into three phases which involve specific molecular factors: attachment, accumulation/maturation, and detachment/dispersal (O'Toole et al., 2000; Otto, 2013). Initial attachment can occur on inert or biotic surfaces. Attachment of Staphylococci to an abiotic surface, such as the naked plastic or metal surface of an indwelling medical device, is dependent on the physico-chemical characteristics of the device and bacterial surface components such as the accumulation-associated protein (Aap) (Conlon et al., 2014), autolysins AtlA (Houston et al., 2011; Bose et al., 2012) and AtlE (Rupp et al., 2001) or wall teichoic (WTA) and lipoteichoic acids (LTA) (Gross et al., 2001). Primary attachment to a biotic surface in host tissues and synthetic surfaces coated with plasma proteins, such as fibronectin, fibrinogen, and vitronectin, is governed by cell wall-anchored (CWA) proteins including clumping factors A and B and the fibrinogen/fibronectin-binding proteins FnBPA and FnBPB from *S. aureus* or the fibrinogen-binding protein SdrG/Fbe from *S. epidermidis* (Vaudaux et al., 1995). Once attachment to tissue or matrix-covered devices is accomplished, staphylococcal biofilms grow by proliferation and production of a scaffolding extracellular matrix. Until recently the only known matrix components were polysaccharide intercellular adhesin (PIA), also known as poly-N-acetyl-glucosamine (PNAG) (Mack et al., 1996), and extracellular DNA (eDNA) (Montanaro et al., 2011). PIA, which has a net positive charge, may promote intercellular interactions by binding to the negatively charged surfaces of bacterial cells.

It is now recognized that several staphylococcal surface proteins can also promote the accumulation phase in an *ica*-independent manner (Foster et al., 2014). Thus, CWA proteins mediate primary attachment and also promote intercellular adhesion and biofilm accumulation and maturation (**Figure 1**). This is followed by the dispersal phase where the biofilm structure is disrupted by enzymatic degradation of matrix components, most notably by proteases (Boles and Horswill, 2008), nucleases (Sharma-Kuinkel et al., 2009; Kiedrowski et al., 2011; Beenken et al., 2012), and a group of small amphiphilic α-helical peptides, known as phenol-soluble modulins (PSMs) functioning as surfactants (Wang et al., 2011; Periasamy et al., 2012).

This review will focus on the role of surface proteins in biofilm formation, with particular emphasis on the recent discoveries that several CWA proteins promote accumulation by specific homophilic interactions.

CWA PROTEINS

The surfaces of staphylococcal cells are decorated with a variety of CWA proteins that are anchored to peptidoglycan by the enzymatic activity of sortases (Foster et al., 2014) (**Figure 2**). The precise repertoire of CWA proteins varies among strains. *S. aureus* can express up to 24 different CWA proteins whereas coagulase-negative Staphylococci such as *S. epidermidis* and *S. lugdunensis* express a smaller number. Moreover, the expression of CWA proteins can be altered by growth conditions. For example, some proteins are expressed only under iron-limited conditions, whereas others are found predominantly on cells in the exponential or stationary phases of growth.

Secretory signal sequences that are located at the amino termini direct the translated proteins to the secretory (Sec) apparatus in the membrane and are cleaved during secretion. At their carboxyl termini, each of these proteins has a characteristic sorting signal, which facilitates their covalent anchorage to peptidoglycan. The housekeeping sortase A anchors the majority of CWA proteins which have the LPXTG motif within their sorting signal. In contrast, sortase B of *S. aureus* and *S. lugdunensis* anchors Isd proteins which have sorting signals with the motif NPQxN/P and which are only expressed under iron-restricted conditions (Foster et al., 2014).

It has been proposed recently (Foster et al., 2014) that CWA proteins be classified primarily based on structural and functional considerations (**Figure 2**). The microbial surface component recognizing adhesive matrix molecules (MSCRAMM) family comprises proteins with tandemly-linked IgG-like folds in the N-terminal A region. In the archetypal MSCRAMMs SdrG, ClfA, and ClfB the N2 and N3 subdomains are sufficient to promote binding to ligands by the dock, lock, and latch (DLL) mechanism. Linking the A region to the cell wall-anchoring domain are serine-aspartate dipeptide repeats of varying length in the case of the Clf–Sdr subfamily, or tandem repeats of fibronectin binding domain in the case of FnBPs. The Sdr proteins have additional 110–113 residue B repeats located between the A region and the SD repeat region that act as rigid rods to project the A domain further from the cell surface (Foster et al., 2014).

Near iron transporter (NEAT) motif proteins are involved in heme capture from hemoglobin and help bacteria to survive in the host, where iron is restricted. The defining characteristic of Isd

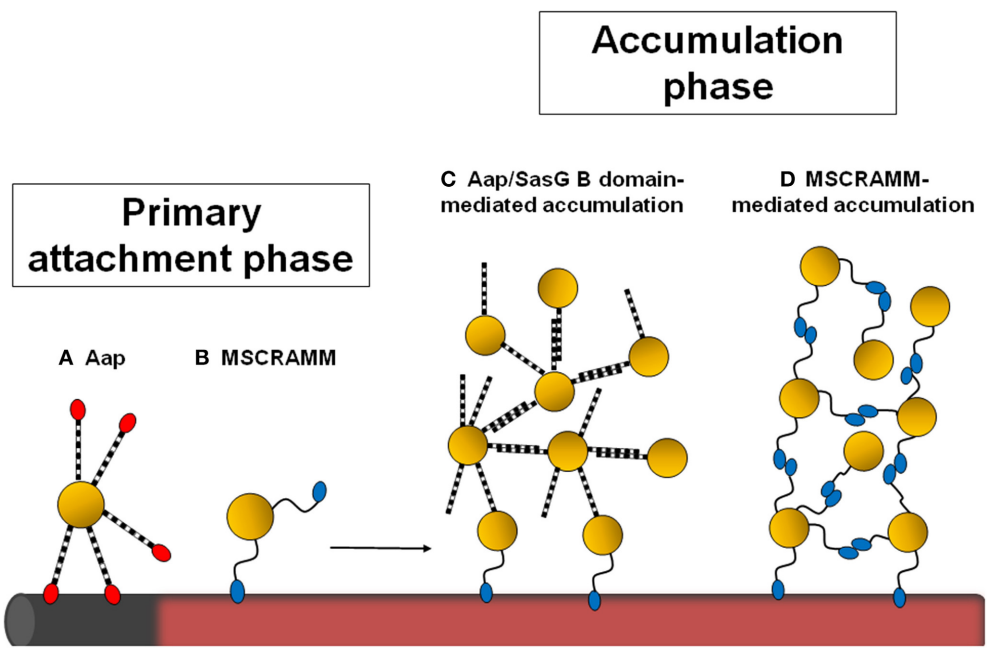

FIGURE 1 | Schematic diagram showing the stages of staphylococcal biofilm formation influenced by cell wall-anchored proteins. Staphylococci can attach to the naked surface of a foreign device (shown in gray) or to a device that has become coated with host plasma components (pink). The Aap A domain (in red) promotes primary attachment to uncoated surfaces **(A)**. Attachment to plasma-coated surfaces is mediated by MSCRAMMs **(B)**. If the Aap/SasG A domain is removed by proteolytic cleavage, the B region can promote intercellular accumulation **(C)**. Alternatively, homophilic interactions between staphylococcal MSCRAMMs on different cells mediate biofilm accumulation **(D)**.

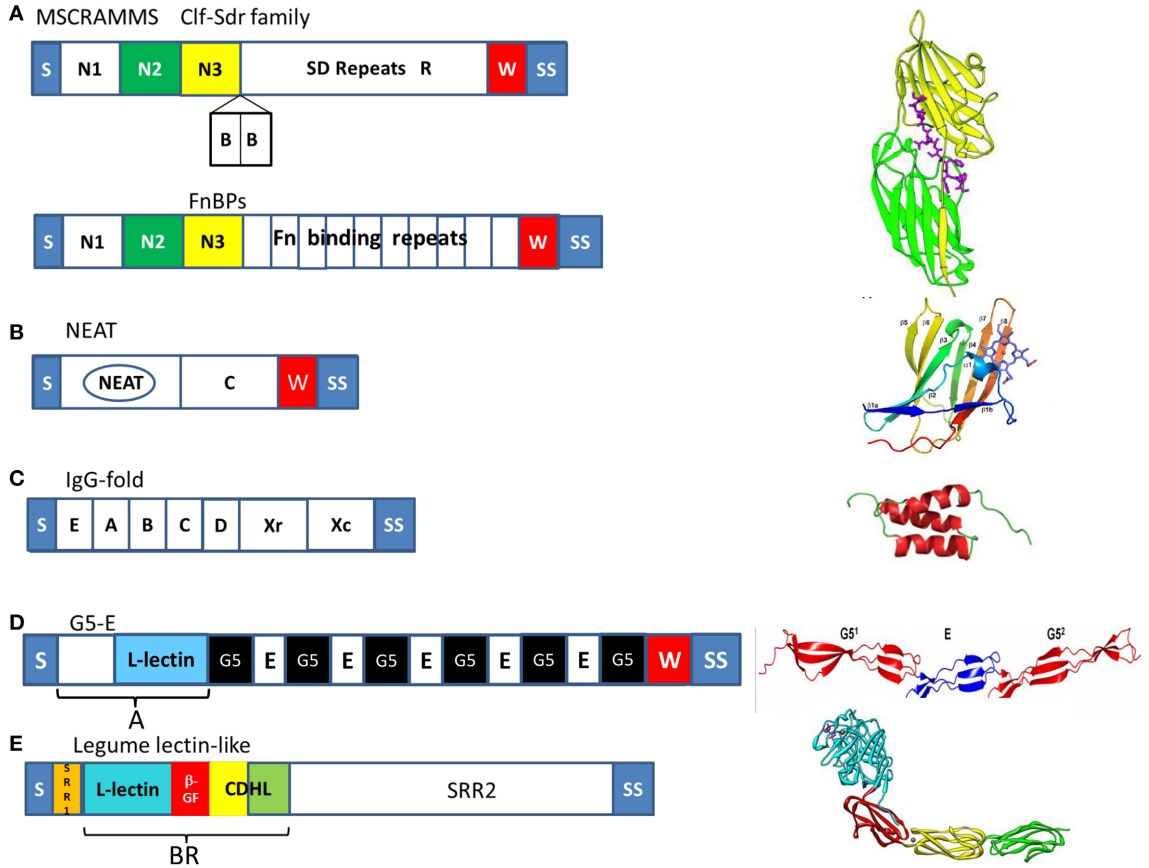

FIGURE 2 | Organization of the major families of cell wall-anchored proteins of Staphylococci. The diagrams to the left show the organization of the proteins into subdomains and on the right the structure of the defining subdomain; tandemly arrayed IgG-like folds N2 (green) and N3 (yellow) of MSCRAMMs, a NEAT motif in Isd proteins, triple helical bundles in protein A, G5-E repeats in Aap and SasG, and the BR domain of SraP comprising a legume-like lectin domain (cyan), a β-grasp fold domain (β-GF, red), and two cadherin-like domains (CDHL, yellow and green). Common features of CWA proteins are S, secretory signal sequence, W, wall spanning region and SS, the sorting signal.

CWA proteins is the presence of one or more NEAT motifs, which bind either hemoglobin or heme (Hammer and Skaar, 2011). The CWA Isd proteins also have functions other than those involved in heme transport.

Protein A is a multifunctional CWA protein that is ubiquitous in *S. aureus*. At the N terminus, protein A contains four or five homologous modules (known as EABCD), each of which consists of single separately folded three-helical bundles that can bind to several distinct ligands. Located between this region and the cell surface is region Xr, which is composed of octapeptide repeats that are highly variable in number, followed by a constant region Xc (Foster et al., 2014).

The serine-rich adhesin for platelets SraP has a complex N-terminal domain comprising short serine-rich repeats followed by a B region (BR) that is subdivided into four subdomains, a legume-like (L-type) lectin domain that is responsible for adhesion to glycoproteins containing N-acetyl neuraminic acid, a β-grasp fold domain and two cadherin-like domains (Yang et al., 2014).

S. aureus surface protein G (SasG) is closely related to Aap of *S. epidermidis*. Both proteins have repeated G5 domains separated by 50-residue sequences known as E regions (Gruszka et al., 2012; Conrady et al., 2013). The G5-E domains of SasG and Aap share 64% amino-acid identity. At the N-termini of the proteins are N-terminal A domains (Roche et al., 2003). Within the A domains of SasG and Aap is a L-type lectin domain.

EVIDENCE FOR THE INVOLVEMENT OF CWA PROTEINS IN BIOFILM

IDENTIFICATION

The first step in investigating an unknown mechanism of biofilm formation is to determine if the matrix is composed of protein and/or polysaccharide by incubating an established biofilm with a protease such as trypsin or with periodate which oxidizes glucose-containing polysaccharides. A reduction in the integrity of the biofilm by protease treatment is a clear indication of the involvement of protein. The absence of the *ica* genes required for biosynthesis of the PNAG/PIA and/or a lack of detectable extracellular polysaccharide on the cell surface is consistent with a novel, perhaps protein-dependent, mechanism.

The morphology of cells visualized by scanning electron microscopy in a PIA/PNAG biofilm matrix is quite distinct from

biofilm involving proteins. In the former, cells are embedded in copious extracellular material while cells from a protein-dependent biofilm are in close contact without a detectable extracellular matrix (Vergara-Irigaray et al., 2009).

Site-specific mutagenesis offers a clear-cut method for determining if a CWA protein is involved. Loss of biofilm in a null mutant defective in sortase A or sortase B suggests the involvement of a CWA protein. Systematic inactivation of genes encoding individual CWA proteins will identify the individual component(s). A mutant defective in a single CWA protein might not give a completely defective phenotype because two or more proteins might contribute. For example, in the case of FnBP-dependent biofilm, inactivation of both FnBPA and FnBPB was required to eliminate biofilm formation completely (O'Neill et al., 2008; Vergara-Irigaray et al., 2009).

Transposon mutagenesis followed by identification of the inactivated gene showed that the Bap protein was involved in biofilm formation by *S. aureus* bovine mastitis strain V329 (Cucarella et al., 2001). This approach could also suggest a role for non-covalently anchored proteins as well as identify potential regulators controlling expression of biofilm-associated proteins.

Once a CWA protein has been identified by mutagenesis or is suspected from other evidence, the gene can be cloned into a plasmid vector and used to complement the mutation or to express the protein in a surrogate host, either a different strain of the same species that naturally lacks the gene in question or in a heterologous host such as *S. carnosus* or *Lactococcus lactis*. If the gene is placed under the control of a regulatable promoter then the concentration of the inducer can be used to control the density of biofilm.

Many CWA proteins are composed of several distinct domains (**Figure 2**). Further genetic manipulation can help identify the domain involved in biofilm. Staphylococcal cells expressing SasG B repeats but not the A domains still formed biofilm whereas cells expressing A domains but lacking B repeats did not (Geoghegan et al., 2010). This strongly implicated the B repeats in biofilm formation. Subdomain N2 of the A region of SdrC was also implicated by a similar approach (Barbu et al., 2014).

Individual subdomains can be cloned and expressed as recombinant proteins. Inhibition of biofilm formation by incubation of the growing culture with purified recombinant proteins provided evidence for the role of the B domains of SasG/Aap and the N2 region of SdrC (Geoghegan et al., 2010; Barbu et al., 2014). Antibodies raised against individual subdomains have also been used to inhibit biofilm formation and to support studies with recombinant proteins and expression of truncates.

HOMOPHILIC INTERACTIONS

Specific homophilic interactions between CWA proteins expressed on different cells are likely to be an important mechanism of cell–cell accumulation during biofilm development (**Figure 1**). The ability of purified recombinant CWA protein to bind to bacterial cells expressing the protein on their surface provided preliminary evidence for homophilic interactions for SraP (Sanchez et al., 2010), IsdC (Missineo et al., 2014), and SasG (Geoghegan et al., 2010) mediated biofilm accumulation.

If the CWA proteins can engage in homophilic interactions during biofilm accumulation, purified recombinant proteins should be able to form dimers in solution. This is certainly the case for Aap/SasG, IsdC, SraP, and SdrC (Conrady et al., 2008; Geoghegan et al., 2010; Missineo et al., 2014; Yang et al., 2014).

Phage display screening first identified the putative interaction domains within the N2 subdomain of SdrC (Barbu et al., 2014). M13 phages expressing a random 12 amino acid peptide library were panned against the recombinant SdrC A domain and two consensus peptides within subdomain N2 were identified (Barbu et al., 2014) (**Figure 3**).

Proof of the mechanism of homophilic binding and identification of residues involved will be provided by the X-ray crystal structure of the dimers formed in solution. This has been achieved with the cadherin-like domains of SraP (Yang et al., 2014) and the G5-E (B repeats) of Aap (Conrady et al., 2013) (**Figure 3**).

AaP/SasG

The Aap and SasG proteins of *S. epidermidis* and *S. aureus*, respectively, have very similar structural and functional organization. Aap can promote either the primary attachment or accumulation phase of biofilm formation depending on the strain being studied (**Figure 1**). Primary attachment is mediated by the N-terminal A domain (Conlon et al., 2014) while the B regions mediate biofilm accumulation (Rohde et al., 2005). The A region must be removed by proteolysis (Aap) or by limited digestion within the B repeat region (SasG) to allow the B domains to interact and promote biofilm accumulation (Rohde et al., 2005; Geoghegan et al., 2010). The proteins have 5–17 B repeats, each comprising nearly identical 78 residue G5 subdomains followed by an E spacer region of 50 residues (**Figure 2**). G5 domains are characterized by five conserved glycine residues, and they adopt a β–triple helix–β-like fold. In general, proteins that comprise highly similar domains in a tandem arrangement are prone to misfolding. As the amino acid sequence of each G5 domain is identical, it is thought that alternating individually folded G5 and E regions is a mechanism to prevent protein misfolding (Gruszka et al., 2012).

The G5-E repeats can undergo a Zn^{2+}-dependent homophilic interaction to form an antiparallel twisted cable (**Figure 3**). The structural basis was studied using short repeat segments but molecular modeling indicated that five repeats are required for complete twists to form (Conrady et al., 2013) (**Figure 3**). This is consistent with the finding that biofilm formation by SasG requires at least five repeats to be expressed on the cell surface (Corrigan et al., 2007).

The *S. epidermidis* strain CSF41498 requires Aap for primary attachment to surfaces (Conlon et al., 2014). In strain CSF41498, the A domain is not processed by proteases. Thus, Aap is capable of promoting either the primary attachment or accumulation phase of biofilm formation depending on whether the A domain has been removed by proteases (Conlon et al., 2014; Schaeffer et al., 2014).

SraP

The N-terminal BR domain of SraP forms a rigid rod-like structure that projects the lectin-binding region away from the cell

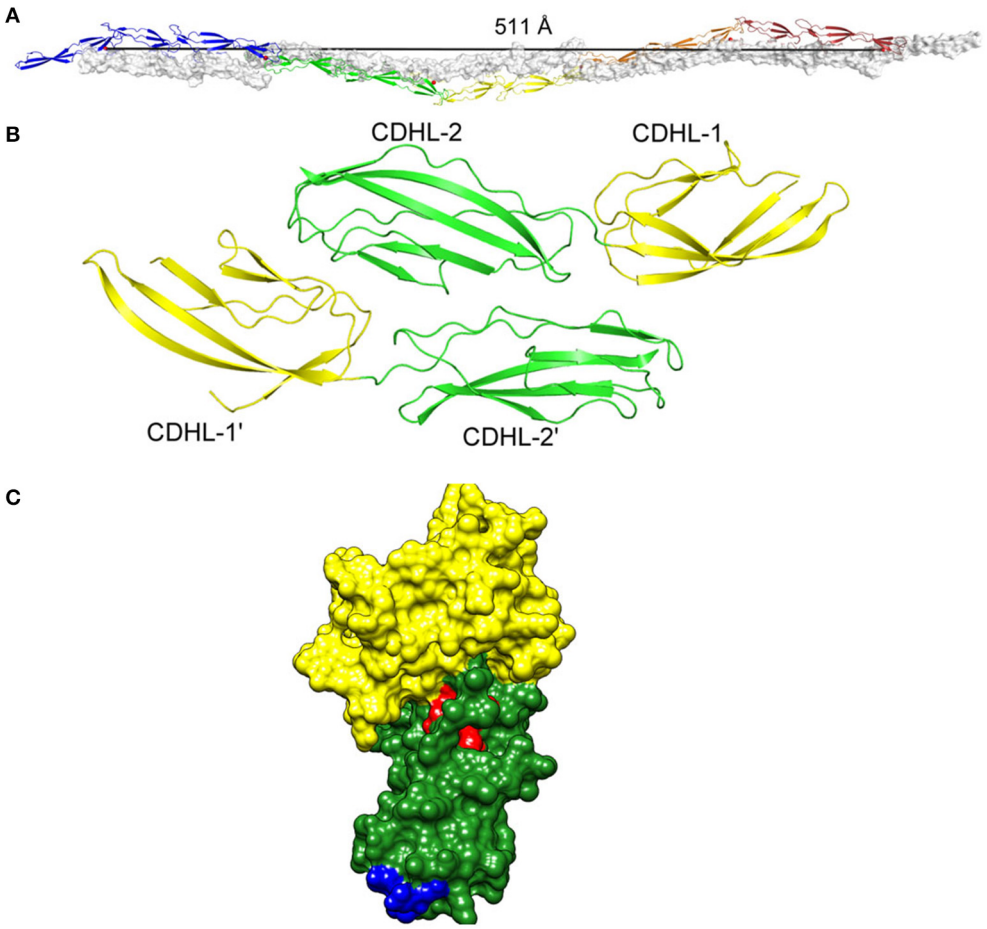

FIGURE 3 | (A) Homophilic interactions between cell wall-anchored proteins. Five repeated G5-E domains of Aap form an anti-parallel twisted rope. **(B)** The SraP cadherin-like domain dimer. **(C)** The N2 (green) and N3 (yellow) subdomains of the A region of SdrC modeled on the crystal structure of ClfA with the residues involved in forming the homophilic interaction domains shown in red and blue. Figure adapted from Conrady et al. (2013).

surface (Yang et al., 2014). As well as promoting adhesion to neuraminic acid-containing glycoproteins, the BR region is predicted to promote biofilm formation by a homophilic interaction between the pairs of cadherin-like domains. The crystal structure of dimers showed that CDHL-2 binds across the junction of the tandemly arrayed CDHL-1 and -2 (Yang et al., 2014) (**Figure 3**).

FnBPs, SdrC, AND ClfB

Several MSCRAMM proteins can promote biofilm accumulation. In each case the N-terminal A domain is responsible. For FnBPs the binding region was further localized to the N2N3 subdomains (Geoghegan et al., 2013), and in the case of SdrC, subdomain N2 (Barbu et al., 2014). The N2N3 subdomains engage in binding to ligands by the DLL mechanism. However, DLL is not involved in FnBPA-mediated biofilm because a strain expressing a variant of FnBPA lacking the latching peptide still formed biofilm (Geoghegan et al., 2013). Furthermore, an asparagine residue in the FnBPA ligand binding trench that is crucial for ligand binding by DLL could be substituted without reducing biofilm formation (Geoghegan et al., 2013).

In certain strains of *S. aureus* growing in Ca^{2+}-depleted conditions biofilm formation depends upon ClfB. This is unique in that other biofilm mediators are inhibited by Ca^{2+} depletion (Abraham and Jefferson, 2012). SasG, Aap, and FnBP-dependent biofilm formation requires Zn^{2+} (Conrady et al., 2008; Geoghegan et al., 2010, 2013). In contrast, Mn^{2+} inhibits SdrC dimerization and SdrC-dependent biofilm formation (Barbu et al., 2014).

During biofilm growth, expression of genes encoding a number of CWA proteins, including ClfB and FnBPA, is increased (Resch et al., 2005). It is possible that expression of biofilm-associated genes is influenced differently by regulatory factors in clinical isolates from biofilm-associated infection and laboratory strains of *S. aureus*. For example, HA-MRSA strains are frequently genotypically or phenotypically *agr* negative (Fowler et al., 2004; Rudkin et al., 2012). In addition it is likely that proteases modulate surface protein-dependent biofilm formation. ClfB-mediated biofilm formation has been demonstrated for strains where the aureolysin-encoding gene is inactivated so it is possible that this phenotype is restricted to strains harboring loss of function mutations in the *aur* gene (Abraham and Jefferson, 2012).

BIOLOGICAL SIGNIFICANCE

Flow cell systems are a superior method for analysing the contribution of proteins to biofilm formation *in vitro* compared to using static microtiter plate assays. The development of biofilm can be monitored over time. The importance of FnBPs in biofilm formation by HA- and CA-MRSA (O'Neill et al., 2008; Vergara-Irigaray et al., 2009; McCourt et al., 2014) and Aap in biofilm formation by *S. epidermidis* (Conlon et al., 2014; Schaeffer et al., 2014) has been demonstrated using flow cells. It will be important to determine if other proteins can support biofilm formation under flow conditions which more closely mimic the *in vivo* situation.

In the case of FnBPs and Aap, the importance of protein-dependent biofilm formation has been proven *in vivo*. FnBP-deficient mutants colonized catheters implanted in mice poorly while the absence of the *ica* operon had no effect (Vergara-Irigaray et al., 2009). Aap-deficient mutants colonized indwelling catheters less well than the wild-type strain in a rat model of catheter-related infection (Schaeffer et al., 2014). It will be important to test mutants deficient in other factors implicated in protein-dependent biofilm in animal models of foreign body infection.

OTHER CWA PROTEINS INVOLVED IN STAPHYLOCOCCAL BIOFILM FORMATION

This section reviews other CWA proteins with reported roles in biofilm formation but where the mechanistic basis is less well understood (summarized in **Table 1**). The first surface protein identified that could induce biofilm development was Bap of *S. aureus*. Bap is a large protein of 2276 amino acids whose core region consists of 13 identical tandem repeats of 86 residues. Bap promotes biofilm formation in staphylococcal strains isolated from mammary glands in ruminants suffering from mastitis (Cucarella et al., 2001; Arrizubieta et al., 2004).

Embp, a giant protein located in the cell wall of *S. epidermidis* and with potential functional similarity to large homologous proteins produced by other pathogenic bacteria such as *S. aureus*, mediates binding of *S. epidermidis* to surface attached fibronectin so is likely to constitute the first step of biofilm formation on conditioned surfaces. The Found In Various Architectures (FIVAR) region of Embp mediates binding of *S. epidermidis* to surface attached fibronectin, while the binding site in fibronectin for Embp was assigned to the fibronectin domain type III12 (Christner et al., 2010).

Table 1 | Surface proteins implicated in staphyococcal biofilm formation.

Surface protein	Biofilm static[a]	Biofilm flow[b]	Strains	Mutant[c]	Expression in surrogate host[d]	Over-expression[e]	Primary attachment[f]	Accumulation[g]	Homophilic interaction[h]
S. aureus FnBPs	+	+	MRSA 123 HA-MRSA CC22, CC8 USA300 LAC	+	+ SA		+	+	(+)
SdrC	+	NT	Newman	+	+ LL		+	+	+
SasG	+	NT	8325-4, SH1000		+ SA	+	−	+	(+)
SasC	+	NT			+ SC	+	+	+	NT
ClfB	+	NT	10833 Newman	+	+ LL		NT	+	NT
Spa	+	+				+	NT	+	NT
SasX	+	NT	ST239	+	NT		+	+	NT
SraP	+	NT	ISP479C	+	NT		NT	+	+
Bap	+	+	Bovine V329	+	+ SA		+	+	NT
S. epidermidis									
Aap	+	NT	RP62a 5179	+	+ SE		NT	+	+
Embp*	+	NT	1585 1457	+	NT	+	+ via Fn	+	NT
S. lugdunensis									
IsdC	+	NT	N920143	+	+ LL		+	+	+

NT, not tested.

[a] Biofilm formation in a standard microtiter dish format with bacteria grown statically.

[b] Biofilm formation measured under flow conditions.

[c] The role of the surface protein was demonstrated by studying isogenic mutants.

[d] The role of the surface protein was demonstrated by expression of the cloned gene in a surrogate host (SA, S. aureus; LL, L. lactis; SC, S. carnosus; SE, S. epidermidis).

[e] Over-expression of the protein using a multicopy plasmid in a surrogate host, or from the chromosomal gene in a host strain with regulatory mutations leading to high level expression.

[f] Primary attachment to unconditioned plastic surfaces [or following conditioning with fibronectin (Fn)].

[g] Accumulation phase measured.

[h] Homophilic interaction proven, or (in parenthesis) strongly suspected.

**Embp is not sortase-anchored. It lacks a consensus C-terminal sorting signal and is removed from cells by boiling or sonication.*

SasC represents another *S. aureus* CWA protein factor that is involved in cell aggregation and biofilm formation. Expression of full-length SasC or its N-terminal domain, which contains a FIVAR motif, mediates the formation of bacterial aggregates, increased attachment to polystyrene, and enhanced biofilm formation (Schroeder et al., 2009).

Overproduction of protein A by *S. aureus* was shown to be responsible for bacterial aggregation and biofilm formation (Merino et al., 2009). Moreover, exogenous addition of synthetic protein A or bacterial supernatants containing protein A can also promote biofilm development. Protein A-mediated biofilm formation was inhibited by addition of serum or immune IgG (Merino et al., 2009). However, it should be noted that the ability of protein A to promote biofilm formation was only ever demonstrated in a laboratory strain engineered to over express the protein.

A recent study of strain ST239, a dominant MRSA strain in the Far East, showed that expression of SasX, a protein that confers virulence in mouse skin and lung infection, may cause bacterial aggregation and promote biofilm formation (Li et al., 2012).

It is unclear whether aggregation and biofilm formation by these different CWA proteins is the result of homophilic interactions between two identical molecules expressed on the surface of neighboring cells. It is possible that these proteins mediate heterophilic interactions with other surface proteins or with non-proteinaceous cell wall structures.

CYTOPLASMIC AND SECRETED PROTEINS ALSO CONTRIBUTE TO BIOFILM FORMATION

Although several CWA proteins of Staphylococci have been identified as important components of the biofilm, the composition of the extracellular matrix still remains uncertain. Recently, it has been reported that the biofilm matrix is largely composed of cytoplasmic proteins that reversibly associate with the cell surface in response to decreasing pH during biofilm formation (Foulston et al., 2014). Additionally, proteins present in the secretome contribute to the composition and formation of staphylococcal biofilm. Eap and Emp are secreted proteins which are non-covalently attached to the *S. aureus* cell surface and have previously been implicated in a number of aspects of *S. aureus* pathogenesis (Chavakis et al., 2005). They are involved in biofilm formation under low-iron growth conditions (Johnson et al., 2008).

The unprocessed wall-anchored major autolysin Atl of *S. aureus* facilitates initial attachment to surfaces in the early events of the FnBP-dependent biofilm phenotype (Houston et al., 2011). Proteolytical cleavage of Atl to the amidase and glucosaminidase domains leads to cell lysis, eDNA release, and cell accumulation. Following these early biofilm events, the FnBPs are required for biofilm maturation (Houston et al., 2011).

Alpha-toxin is a secreted haemolytic toxin which plays an integral role in *S. aureus* biofilm formation. An *hla* mutant was unable to colonize plastic surfaces fully under both static and flow conditions. Thus, it has been proposed that that α-toxin plays a role primarily in cell–cell interactions during biofilm formation (Caiazza and O'Toole, 2003) although the mechanistic basis of this is unclear.

The β-toxin is a neutral sphingomyelinase that lyses erythrocytes and kills proliferating human lymphocytes. It plays a key role in the establishment of *S. aureus* biofilms. This toxin forms covalent cross-links to itself in the presence of DNA, producing an insoluble nucleoprotein matrix *in vitro,* and strongly stimulates biofilm formation *in vivo* as demonstrated by a role in causation of infectious endocarditis in a rabbit model (Huseby et al., 2010).

Amyloid fibrils can also form part of an *S. aureus* biofilm. The PSMs can in certain conditions aggregate to form amyloid fibrils on the surface of the bacterium (Schwartz et al., 2012). Similarly, the signal peptide of the *S. aureus* quorum-sensing molecule AgrD forms amyloid-like aggregates (Schwartz et al., 2014). These fibril structures contribute to overall stability of the biofilm.

PREVENTION OF BIOFILM FORMATION BY ANTIBODIES AGAINST CWA PROTEINS

Targeting those processes that occur early in biofilm development and dispersal represents an attractive strategy to interfere with biofilm formation. Considering that many CWA proteins generate a potent immune response, the use of staphylococcal surface-exposed proteins as vaccines represents a promising way to eradicate biofilm formation both *in vitro* and *in vivo*. Several studies have been performed to investigate whether immunization with CWA protein domains can induce protection against biofilm development.

Polyclonal (Rohde et al., 2005) and monoclonal antibodies (Hu et al., 2011) specific to Aap inhibited biofilm formation by strains that develop an Aap-dependent biofilm. Similar inhibitory effects on FnBP-promoted biofilm formation were observed when incubating MRSA strains with Fab fragments recognizing region A of FnBPA (O'Neill et al., 2008). Active vaccination with a recombinant truncated SesC inhibited *S. epidermidis* biofilm formation in a rat model of subcutaneous foreign body infection. Moreover, antibodies to SesC were shown to be opsonic by an *in vitro* opsonophagocytosis assay (Shahrooei et al., 2012). Polyclonal antibodies targeting the phosphonate ABC transporter substrate binding protein (PhnD) inhibited both *S. epidermidis* and *S. aureus* biofilms (Lam et al., 2014). PhnD-specific antibodies blocked biofilm development at the initial attachment and aggregation stages and also served to enhance neutrophil binding, motility, and biofilm engulfment, supporting the concept that PhnD may be a good target for biofilm intervention strategies performed either by vaccination or through passive administration of antibodies (Lam et al., 2014). PSMs have surfactant-like characteristics and the soluble peptides participate in structuring/destructuring biofilms (Wang et al., 2011; Periasamy et al., 2012). In contrast to this, fibrils comprising PSM aggregates preserve the integrity of the biofilm (Schwartz et al., 2012). Antibodies against PSM peptides inhibited bacterial spread from indwelling medical devices suggesting that interference with biofilm detachment mechanisms may prevent dissemination of biofilm-associated infections (Wang et al., 2011).

Brady et al. (2011) identified immunogenic cell wall proteins expressed during an *S. aureus* biofilm infection and used a quadrivalent vaccine, including four of the identified antigens (glucosaminidase, an ABC transporter lipoprotein, a conserved hypothetical protein, and a conserved lipoprotein) combined

with antibiotic therapy and demonstrated reduced *S. aureus* biofilm formation on infected tibias using a chronic osteomyelitis model (Brady et al., 2011).

Finally, Gil et al. (2014) found that a common core of secreted proteins (exoproteome) is contained in both exopolysaccharide-based and protein-based biofilm matrices. Intradermal administration of an exoproteome extract of an exopolysaccharide-dependent biofilm induced a humoral immune response and reduced the number of bacterial cells inside a biofilm and on the surrounding tissue using an *in vivo* model of mesh-associated biofilm infection (Gil et al., 2014).

Altogether, these studies demonstrate the potential of biofilm matrix exoproteins and CWA proteins as promising targets for antibody-mediated strategies against staphylococcal biofilm formation.

CONCLUSIONS

Several staphylococcal surface proteins can support biofilm formation. Representative isolates from the major lineages of MRSA form protein-dependent biofilm *in vitro* suggesting that this is likely to be of medical relevance (O'Neill et al., 2007). The importance of FnBP- and Aap-dependent biofilm formation has been demonstrated *in vivo* using animal models of foreign body infection (Vergara-Irigaray et al., 2009; Schaeffer et al., 2014). It will be important to determine how widespread FnBP-mediated biofilm formation is among *S. aureus* strains from different genetic backgrounds. Biofilm formation by certain isolates of HA-MRSA from CC8 and CC22 and CA-MRSA of the USA300 lineage (CC8) is dependent on FnBPs (O'Neill et al., 2008; McCourt et al., 2014). Studies to assess the contribution of FnBPs to biofilm formation should be extended to all major classes of CA- and HA-MRSA. It will also be important to establish if other surface proteins contribute to biofilm accumulation in clinically relevant strains and to study their role using animal models of infection.

Further insights into the mechanistic basis of surface protein-mediated biofilm formation will inform the design of specific inhibitors of the protein–protein interactions involved in biofilm accumulation. Small molecules or peptides could be used to prevent or treat biofilm-associated infection.

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