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Review article

The Relationship between Methicillin Resistance and Biofilm Composition in *Staphylococcus Aureus*

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Abstract

Staphylococcus aureus (SA) is a common commensal bacterium and opportunistic pathogen in humans. Antibiotic-resistant SA strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA), have become increasingly prevalent in recent years. Another aspect of SA that makes it a successful pathogen is its ability to form biofilms. SA biofilms aid in the invasion of host tissues, and biofilms can form on the surfaces of implanted medical devices, leading to serious, persistent, and sometimes life-threatening infections. Biofilms also prevent effective antibiotic treatment and promote evasion of the host immune response. The biofilms formed by MRSA and by methicillin-susceptible SA (MSSA) are typically quite different, with MSSA biofilms usually composed of polysaccharides and MRSA biofilms typically composed of extracellular proteins and DNA. Interestingly, transfer of the *mec*A gene (confers resistance to methicillin) alone to a MSSA strain can result in a profound change in biofilm composition to reflect a MRSA biofilm phenotype. The mechanism for this switch in biofilm phenotypes is still an area of active investigation and may yield insights into how to combat this important human pathogen.

Introduction

Staphylococcus aureus (SA) establishes long-term colonization in 20-30% of the human population, and transient colonization in 60% [1-3]. Though carriage is usually asymptomatic, infection can cause a wide range of diseases including skin and soft tissue infection, bacteremia, pneumonia, and endocarditis [2,4-6]. SA is possibly the most common cause of food poisoning and the leading cause of death of any infectious agent in the United States of America and is a leading cause of hospital-associated infection throughout the developed world, being second only to *Clostridium difficile* in the United States [7-9]. Additionally, SA infection rates have increased in recent decades, including both hospital and community acquired infections [10]. Soft-tissue infections are estimated at 48.1 cases per 1,000 population [10]. Infections associated with SA can have mortality rates as high as 25% [11].

Virulence Factors and Antibiotic Resistance in SA

SA can carry a variety of virulence factors including leukocidins, hemolysins, enterotoxins, the super antigen TSST-1, protein A, and biofilm genes [6,12-15]. Antibiotic-resistant strains of SA were first detected shortly after the initial use of penicillin [2]. These first resistant strains produced a penicillinase and were still susceptible to the second-generation penicillins, such as methicillin, that were introduced in the early 1960s. However, resistance to these new drugs was reported within one year [2,8]. These strains of methicillin-resistant SA (MRSA) had acquired the *mec*A gene, which encodes an antibiotic-resistant transpeptidase which allows cell wall synthesis to carry on in the presence of beta-lactam antibiotics [8,16,17]. Though methicillin is no longer used clinically, the term MRSA is still used to describe any SA strain which carries *mec*A. MRSA, while originally only found in hospitals, was found to be common in the community outside of hospitals in the 1980s, leading to the terms hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA) to differentiate these genetically distinct families of MRSA [4,7]. The antibiotic vancomycin is used to treat MRSA infections as a last resort, though vancomycin-resistant SA (VRSA) strains have been reported in recent years [18,19]. Most clinical SA infections are transmitted via person-to-person contact,

in either hospital-acquired or community-acquired transmissions [4,20,21]. However, SA can also be transmitted to humans from direct contact with living livestock or through exposure to contaminated meats [22-25]. Our recent work has shown that SA isolated from meat products originating in animals fed antibiotics is significantly more resistant to multiple antibiotics compared to SA from meats obtained from animals raised in an antibiotic-free environment [26].

The antibiotic resistance gene of MRSA, *mec*A, was acquired as part of a mobile genetic element called the staphylococcal cassette chromosome *mec* (SCCmec). SCCmec is thought to have originated from a non-staphylococcal source, and twelve SCCmec variations have been described [27-29]. In addition to *mec*A, the SCCmec contains several genes of unknown function and what is known as the *ccr* gene complex, *ccr*AB and/or *ccr*C, all of which have roles in promoting site-specific recombination [27]. The gene *mec*A encodes the membrane-bound transpeptidase penicillin-binding protein 2A (PBP2a) which catalyzes peptidoglycan crosslinking during cell-wall synthesis [16]. This class of enzymes, which were named for their affinity for penicillin, are inhibited when bound by beta-lactams. PBP2a, however, has a uniquely lower affinity for beta-lactams, allowing PBP2a to carry on cell wall synthesis when the activity of the four native SA penicillin-binding proteins is blocked [28,30].

Some SCCmec types have a regulatory system in place consisting of a transcriptional repressor, a sensor-inducer, and an anti-repressor to control the expression of *mec*A, though most clinical MRSA strains appear to have a non-functional regulatory system [31]. High constitutive expression appears to be necessary to give the beta-lactam resistant phenotype associated with MRSA. Because of the slow response time of a functional regulatory system, strains without constitutive expression often appear susceptible to oxacillin in testing, even though they have a functional *mec*A gene [31,32]. The varied levels of beta-lactam resistance among MRSA strains is due to varied levels of *mec*A expression [5]. MRSAs can be classified into two categories based on resistance levels: heterogeneously resistant (HeR) and homogeneously resistant (HoR). HeR strains are those able to grow in oxacillin concentrations between 2 and 100 μg/mL while HoR strains can grow in oxacillin concentrations in excess of 100 μg/mL [5].

Biofilm formation by SA

SA forms robust biofilms, which are communities of cells that can be protected from antibiotic treatment and/or immune cell and immune factors. The cells in a biofilm are surrounded by an extracellular matrix – a network of biologically produced substances that hold the cells together and help them attach to surfaces [33]. SA biofilms are sticky conglomerations of cells surrounded by an extracellular matrix which provide protection from mechanical removal of cells, host immune responses (both innate and adaptive), and antibiotics, giving as much as a 6-log increase in cell viability over planktonic cells following antibiotic challenge [34-36]. Additionally, SA biofilm formation greatly increases the occurrence of horizontal gene transfer, contributing to the spread of antibiotic resistance [37]. SA biofilms are a major concern in hospitals, not just for the danger of infection of damaged host tissue, but also because of the ability of SA to form biofilms on implanted medical devices such as catheters, pacemakers, artificial heart valves, intravascular lines, and joint replacements [38]. Colonization of such devices can lead to serious, chronic infections that are difficult to treat [11,34].

SA biofilm formation is a highly organized process allowing for the formation of complex three-dimensional structures with channels that allow for the flow of nutrients to cells located deeper within the matrix [34,39]. The biofilms have tightly regulated growth patterns that regulate attachment to a surface, the growth and expansion of the biofilm, and detachment and spread to other sites [34,36]. These processes are regulated through quorum sensing, allowing the optimal cell density to be maintained by regulating the dispersal of cells for spread to new areas. During the growth phase of biofilms some cells will even undergo an apparently altruistic autolysis to provide neighboring cells with the materials necessary to construct the extracellular matrix, such as DNA [39,40].

Though there are some general characteristics shared amongst SA biofilms, the composition of the extracellular matrix from strain to strain can be drastically different [38,41,42]. In general, these varied extracellular matrix compositions are categorized into two classes based upon the presence of polysaccharide intercellular adhesin (PIA), forming both PIA-dependent and PIA-independent biofilms [5,38]. PIA-independent biofilms tend to have a matrix composed primarily of protein and extracellular DNA/eDNA. The biofilm class of any particular strain can be determined by a series of simple tests. Biofilms are grown in 96 well plates and then treated with proteinase K to degrade extracellular proteins; DNase to degrade eDNA; or sodium metaperiodate which oxidizes polysaccharide linkages. PIA-dependent biofilms are unaffected by proteinase K treatment and dispersed by sodium metaperiodate treatment, while PIA-independent biofilms are dispersed by proteinase K treatment or DNase and are unaffected by sodium metaperiodate [41,42]. While there have been thorough studies of the composition and genes associated with each class of biofilm [43-45] only an overview of the major components will be given here.

PIA-dependent SA biofilms

PIA-dependent biofilms are the classic biofilm type; they were the first studied and are what are usually described about

typical *S. aureus strains* [11,44,46]. Their extracellular matrix consists primarily of PIA, built from the polysaccharide poly-β(1-6)-N-acetylglucosamine, but it may also contain a variety of proteins, extracellular DNA (eDNA), and amyloid fibrils [44]. Many cytoplasmic proteins and genomic DNA may become associated with the extracellular matrix as cells undergo autolysis. This altruistic act of some cells, which is triggered through quorum sensing, provides the raw materials necessary to form the biofilm [34,39,40]. The eDNA, while not necessary for the structural integrity of PIA-dependent biofilms, is important for the formation of amyloid fibrils from phenol-soluble modulins, which contribute to biofilm stability [42,47]. The primary component of PIA-dependent biofilms is, of course, PIA. This polysaccharide is produced and assembled into the extracellular matrix by the products of the *icaADBC* operon. *icaA* encodes an N-acetylglucosaminyltransferase that synthesizes PIA; *icaD* produces a product that, while not fully understood, is known to increase the efficiency of *icaA*; *icaB* produces an N-deacetylase which partially deacetylates PIA; and *icaC* is involved in the exportation of PIA to the cell surface [44,48]. While several genes are known to influence the production of PIA the best characterized is *icaR*, a divergently transcribed repressor of the *ica* operon located just upstream of the *icaA* gene [48-50].

PIA-independent SA biofilms

PIA-independent biofilms are most notably characterized by the lack of PIA. These biofilms rely solely on extracellular proteins and eDNA for their structural integrity, a difference which can be seen by electron microscopy [38,41]. The reliance of eDNA in PIA-independent biofilms makes the *agr* quorum-sensing system, which triggers autolysis, vital in biofilm formation [40,51]. Once DNA is released it is thought to interact with cell surface proteins to bind cells one to another [38]. The primary proteins involved in the PIA-independent biofilm type are the membrane-bound fibronectin-binding proteins FnBPA and FnBPB [40]. The function of these proteins in biofilm formation appears to be redundant as either may be knocked out and biofilms will continue to form normally [52]. Other membrane-bound proteins, such as protein A and SasG have been shown to be involved in PIA-independent biofilm formation, though their specific functions and possible interactions with eDNA have yet to be studied [38,43]. Extracellular proteases are of crucial importance for PIA-independent biofilms. Protease production is limited during biofilm maturation but increased for biofilm dispersal to degrade the extracellular matrix and release the cells [35,51,53].

The link between antibiotic resistance and biofilm type in SA

Both MRSA and MSSA produce biofilms, but the biofilms they produce have been reported to be inherently different [38]. The only definite difference between a MRSA strain and a MSSA strain is the presence of the antibiotic resistance gene *mec*A. High-level expression of the *mec*A gene has been shown to repress the *ica* operon which is necessary for biofilm polysaccharide production [41], but the mechanism by which this occurs is unknown. In addition, the quorum-sensing *agr* system which is an important part of biofilm maturation and dispersion can also repress by *mec*A.

It has been observed that MRSA strains tend to produce a PIA-independent biofilm type while MSSA tend to produce PIA-dependent biofilms [5,38,41,43,54]. This trend is typically described in the context of homogeneously resistant (HoR) types of MRSA strains [5,38]. Pozzi et al transformed MSSA cells known to produce a PIA-dependent biofilm with a plasmid containing *mec*A. These cells were then put through a selection to isolate a HoR MRSA strain. A complete shift in biofilm type from PIA-dependent to PIA-independent was observed and *ica*A expression was drastically reduced. The plasmid was then cured (reverting back to MSSA) and the strain returned to a PIA-dependent biofilm type and *ica*A expression returned to normal. How this shift in biofilm type is accomplished is unknown. PBP2a (encoded by *mec*A) is membrane-bound and is not known to have any direct effect on transcription, yet mutation of its active site abolishes the effects on ica transcription. Furthermore, the repression of the *ica* operon was found to be *ica*R independent. It has been suggested that a change in cell wall architecture through the action of PBP2a may be responsible for this drastic shift in biofilm composition, yet this is still uncertain and more research into these mechanisms has yet to be done [5,38].

Biofilms are known to be hotspots for horizontal gene transfer (HGT). While biofilms likely contribute to antibiotic resistance gene acquisition in SA as well, this topic has not been explored much in the literature. Since eDNA is more common in MRSA biofilms as compared to MSSA biofilms, it is possible that MRSA biofilms have higher rates of HGT than MSSA biofilms. If true, this could potentially explain a mechanism by which MRSA has gained such high levels of antibiotic resistance.

Future perspective

The PIA-dependent biofilms typically seen in MSSA strains appear to be thicker and less penetrable than the PIA-independent biofilms seen with MRSA strains [38]. In our recent study, we showed that MSSA biofilms were not as affected by vancomycin, silver nanoparticles, or a combinatorial treatment as compared to MRSA biofilms [55]. Perhaps MSSA strains have evolved to produce a more robust biofilm which is less penetrable to antibiotics, since these isolates are inherently more susceptible to antibiotics. However, this hypothesis fails to explain why MRSA isolates would produce a less robust biofilm since immune pressure is likely similar for both MRSA and MSSA. It is possible that SA strains that develop both high-level antibiotic resistances combined with

strong/PIA-dependent biofilm formation are too virulent, and that natural selection prevents their widespread formation.

A better understanding of the control mechanisms of biofilm formation may allow for the discovery of new ways to disperse biofilms, and to promote weaker biofilm formation which may allow for more effective antibiotic therapy of SA infections.

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