Environmental factors modulate biofilm formation by *Staphylococcus aureus*

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**Abstract**

Biofilm formation on indwelling medical devices represents an exclusive evasion mechanism for many pathogenic bacteria to establish chronic infections. *Staphylococcus aureus* is one of the major bacterial pathogens that are able to induce both animal and human infections. The continued emergence of multiple drug-resistant *S. aureus*, especially methicillin-resistant *S. aureus*, is problematic due to limited treatment options. Biofilm formation by *S. aureus* complicates the treatment of methicillin-resistant *S. aureus* infections. Therefore, elucidating the mechanisms of biofilm formation in this pathogen is important for the development of alternative therapeutic strategies. Various environmental and genetic factors contribute to biofilm formation. In this review, we address the environmental factors and discuss how they affect biofilm formation by *S. aureus*.

**Keywords**

*Staphylococcus* aureus, MRSA, biofilm, antibiotics, environmental conditions

**Significance and formation of biofilms by *Staphylococcus aureus***

Biofilm formation of pathogenic bacteria in medical devices plays an important role in the development of chronic infections. The increased use of medical implants in health care is strongly associated with elevated biofilm-related infections. *S. aureus* is one of the most frequent agents causing hospital-acquired catheter-related bloodstream infections (CRBSI) associated with biofilm formation.¹ In 2017, approximately 120,000 cases of *S. aureus* bloodstream infections occurred and led to approximately 20,000-associated deaths in the United States.²
Multiple drug-resistant bacterial infections present a rapidly growing threat to public health worldwide. In the United States, hospital-acquired methicillin-resistant *S. aureus* (HA-MRSA) isolates have been responsible for 40%–80% of all hospital-acquired *S. aureus* infections, causing approximately 11,000 deaths in 2011. Community-acquired-MRSA (CA-MRSA), a more virulent form, has spread worldwide, causing severe skin and soft tissue infections. *S. aureus* is also one of the main pathogens that cause bovine mastitis, incurring an estimated US$2 billion annual loss to the US dairy industry. Livestock-associated-MRSA (LA-MRSA) was identified in food animals and in retail meats worldwide. The emergence of MRSA and vancomycin-resistant *S. aureus* (VRSA) increases treatment complications of CRBSI-associated infections. Public health concern stems from limited available options for treating MRSA infections. Beta-lactam class antibiotics, such as methicillin, have been successfully used in the treatment of *S. aureus* infections. However, numerous isolates of this pathogen have acquired resistance to these drugs soon after their introduction for treatment. Although the Food and Drug Administration’s (FDA) Guidelines require more restricted and judicious use of medically important antibiotics in livestock to curtail the emergence of more resistant strains, this is clearly not sufficient to prevent the evolution of new and more virulent progeny.

The formation of bacterial biofilm involves multiple steps, including attachment, proliferation, and detachment during infection (Figure 1). The initial attachment most likely occurs via binding of bacterial surface proteins or receptors to host matrix proteins, such as fibrinogen, fibronectin, and vitronectin. During the

![Figure 1. Schematic representation of biofilm formation during infection.](image)
proliferation phase, biofilm-forming cells embed within a protective extracellular matrix composed of mainly extracellular polysaccharide intercellular adhesion (PIA), teichoic acids, protein, and DNA. The matrix components form a barrier to protect bacteria from being killed by antibiotics or clearance by host defense mechanisms.\(^8\) The detachment process of staphylococcal cells from the biofilm body remains elusive, although some evidence suggests that production of the aliphatic delta-toxin complex might be involved in the release of staphylococcal cells from biofilms.\(^9\)

In order to adapt to variable habitats, \textit{S. aureus} has evolved a series of two-component signal regulators that enable the bacteria to sense its immediate surroundings and to modulate bacterial cellular responses and the expression of virulence genes. In many cases, the response regulators modulate target gene expression directly as a transcriptional activator and/or repressor. \textit{S. aureus} is able to protect itself in response to stressed conditions by switching to alternative lifestyles, such as in a biofilm.\(^10\)–\(^13\) These conditions are complicated, and some environmental factors associated with biofilm formation are discussed below.

**Effect of environmental conditions on biofilm formation**

Several environmental factors have been demonstrated to affect the ability of \textit{S. aureus} to form biofilm as follows:

**Composition of culture media**

A variable phenotype of biofilm formation can be observed in different culture media.\(^14\) It has been reported that the Luria-Bertani broth (LB) medium was the optimum medium for the biofilm formation compared to tryptic soy broth (TSB) supplemented with 2\% glucose (TSBglu) and brain heart infusion (BHI).\(^15\) However, Singh and co-workers showed that BHI broth was more effective for biofilm formation than TSB.\(^16\) Supplementation with glucose, sucrose, and sodium chloride could significantly increase biofilm formation.\(^16\)

Arce Miranda and co-workers found that biofilm formation by \textit{S. aureus} was improved under an aerobic condition in thioglycolate medium compared to TSB medium, with lower reactive oxygen species (ROS) and nitric oxide (NO) production.\(^17\) This indicates that ROS, reactive nitrogen intermediates (RNI) and its downstream derivatives may affect biofilm development.\(^17\) It was suggested that the formation of biofilm may induce cellular stress, which in turn affects bacterial growth under different conditions and generates ROS and RNI, and consequently decreases the extracellular matrix under unfavorable conditions.\(^17\)

Ions in medium can influence biofilm formation by \textit{S. aureus} since the staphylococcal cells exhibit a moderate hydrophobic property. The ability of \textit{S. aureus} to initially adhere to polystyrene was reported to be associated with the ionic strength of the culture medium.\(^18\) It was found that the depletion of iron in the culture medium enhanced biofilm formation, while the addition of iron repressed the
development of biofilm in a Fur-independent mechanism. Low iron conditions induced biofilm formation independently of the overproduction of PNAG polysaccharide. Furthermore, staphylococcal Emp and Eap proteins regulated by Fur, were found to be important for biofilm formation by *S. aureus* Newman strain in low iron conditions. Other regulators, including Sae, Agr, and SarA, also play critical roles in biofilm formation under iron-restricted conditions. Although it has been demonstrated that the expression of the *ica* operon is required for biofilm formation in iron-depleted conditions, it was shown that the depletion of iron decreased biofilm formation and PIA production in *S. aureus* strain 113. Moreover, iron chloride (FeCl₃·6H₂O) was able to enhance the biofilm formation by *S. aureus* Xen 31 strain in a dose-dependent manner. This suggested the importance of iron for some *S. aureus* isolates to develop a biofilm. Salicylic acid (SAL) is a major metabolite of aspirin that is able to bind iron and form a complex. It was reported that SAL was able to repress the production of CodY and then turn on the expression of the *ica* operon, which could profoundly affect polysaccharide composition in the biofilms of *S. aureus*. Together, these findings indicate that the impact of iron on biofilm formation is dependent on the genetic background of *S. aureus* isolates.

Calcium impacts the biofilm architecture of *S. aureus*. Altering Ca²⁺ concentrations affected the thickness and topography of biofilm. The role of calcium in biofilm formation may differ between different *S. aureus* isolates. The Newman strain of *S. aureus* had a limited ability to form biofilms when cultured in the presence of calcium chelators. In contrast, *S. aureus* strain 10883 could generate robust biofilms in the presence of calcium chelators. It seems that calcium may mediate biofilm formation through interactions with a surface adhesin clumping factor B (ClfB). In strain 10833 with a null mutation of clfB, the addition of calcium chelators in the culture medium abolished the phenomenon of elevated biofilm formations.

Our laboratory has also observed the strain-dependent effect on biofilm formation by *S. aureus* in the same culture medium (Figure 2, unpublished data). Different *S. aureus* strains showed variable capacities to form biofilms in the TSB medium supplemented with 3% NaCl and 0.5% glucose, indicating the role of genetic background in biofilm formation.

**Osmotic pressure**

Changing osmotic pressure in the culture medium has been reported to affect the ability of *S. aureus* to form biofilms. This phenomenon is associated with changes in the expression of genes contributing to the modulation of biofilm formation, including *icaA*, *sarA*, *rbf*, and *sigma B*. The addition of NaCl enhanced aggregation, biofilm stability, and strength in a dose-dependent manner, with an increased expression of the *ica* operon at 4% and 6% NaCl. It was found that the addition of 7% NaCl could increase biofilm formation by a foodborne *S. aureus* strain harboring *icaA*, and could also induce the biofilm development in an
It was suggested that NaCl-enhanced biofilm formation is more prevalent among methicillin-sensitive *S. aureus* (MSSA) isolates than MRSA isolates. It was also reported that the addition of 4% NaCl in the BHI medium didn’t affect the capacity of biofilm formation for some *ica*-positive MRSA. NaCl could decrease biofilm formation for some *S. aureus* isolates directly or indirectly by inducing overproduction of the Sigma B factor. These findings indicate that a more complicated regulatory network is involved in the response to osmolality stress during biofilm formation. Contrary to these findings, Singh and co-workers reported no significant effect of NaCl on biofilm formation by *S. aureus* isolated from clinical sources. However, when the NaCl was combined with glucose, and sucrose, a significant increase of biofilm formation was noted.

The presence of glucose in the culture medium was found to promote biofilm formation in a dose-dependent manner. When combined with NaCl in the TSB medium, glucose was able to enhance biofilm formation. The reason why the combination of glucose and sodium chloride can increase biofilm formation
has been hypothesized to be that glucose might be phosphorylated by glucokinase to form glucose-6-phosphate, which subsequently enters the anabolic phase and favors biofilm formation.\textsuperscript{40}

**Biofilm culture plates**

Pretreatment of wells in culture plates or flow chamber with human plasma was found to affect the biofilm formation by some \textit{S. aureus} isolates.\textsuperscript{41,42} The presence of human plasma in the BHI improved the reproducibility and capacity of \textit{S. aureus} to form biofilms in both flow chambers under controlled shear flow and in static well plates.\textsuperscript{43} The biofilms of most tested \textit{S. aureus} isolates exhibited a thicker biomass in the presence of plasma and glucose in the culture medium.\textsuperscript{44} A fibrous-like structure and fibrin could be observed in the biofilm matrix formed by \textit{S. aureus} in the presence of plasma.\textsuperscript{44–46} \textit{S. aureus} produces coagulase that converts plasma fibrinogen into an insoluble fibrin clot, which in turn may promote the capacity of \textit{S. aureus} to adhere to various surfaces.\textsuperscript{47,48} Adhesion is the first step in biofilm formation. Relevant factors, including extracellular matrix proteins that bind to bacterial adhesins can thus affect the ability of \textit{S. aureus} to attach to the surface in the presence of plasma during culture (Figure 1).\textsuperscript{49} It was found that the coagulase was required for biofilm formation of \textit{S. aureus} during skin infection.\textsuperscript{46} Therefore, it was postulated that the bacterial driven deposition of fibrin may play a key role in biofilm formation during bacterial growth with plasma.\textsuperscript{44}

It has been shown that \textit{S. aureus} RN4220 was able to form biofilm on a hydrophilic, negatively charged polystyrene surface when cultured in both TSB and BHI media.\textsuperscript{31} When changing the culture surface to untreated, more hydrophobic polystyrene material, the same organism was unable to form biofilms under the same culture conditions.\textsuperscript{31} \textit{S. aureus} had a higher capacity to develop biofilms on hydrophilic surfaces, such as glass and stainless steel, compared to hydrophobic surfaces, such as polypropylene and polystyrene.\textsuperscript{38} It was suggested that the hydrophobicity profoundly affected bacterial attachment or detachment to the surface during biofilm formation.\textsuperscript{38,50} Contrarily, it was reported that \textit{S. aureus} ATCC 12600, a slime producing strain, was able to develop a lower density of biofilms on hydrophilic surfaces compared to hydrophobic surfaces.\textsuperscript{51}

The smoothness of material surfaces influences biofilm development. \textit{S. aureus} was able to generate early-stage biofilm colonies after 7 days and form mature biofilms after 14 days on a smooth poly(dimethyl siloxane) elastomer (PDMSe) surface.\textsuperscript{52} However, the organism could not develop early biofilm colonies until day 21 on the topographical surface (Sharklet AF surface), which is an engineered surface microtopography based on the skin of sharks.\textsuperscript{52} It was found that more \textit{S. aureus} isolates were able to form biofilm on the polystyrene surface than on stainless steel and rubber surfaces, whereas none of the tested strains were able to generate biofilms on silicone.\textsuperscript{53} Interestingly, under the condition of a sub-lethal dose of vancomycin, VRSA could form a thicker biofilm layer on hydrophobic silicon and nylon surfaces, which contained unsaturated, saturated, and cyclic fatty acids, as
compared to hydrophilic glass surfaces that only contained straight-chain saturated fatty acids. It is possible that the exposure to vancomycin may influence cell wall structures, which consequently affects the ability of VRSA to interact with extracellular fatty acids on different hydrophobic surfaces.

**Aerobic conditions**

It was observed that MRSA strains generated weaker biofilms during aerobic growth compared to those grown in CO₂-rich conditions. Oxygen regulated cid expression spatially and temporally. Cid, in coordination with lrg, mediated bacterial cell death in biofilm development of S. aureus. A hypoxic condition could elevate cell lysis and biofilm formation by increasing expression of autolysin, AtlA, and by inhibiting teichoic acid production. Autolysin-induced lysis enabled bacterial cells to release DNA, a critical component of the biofilm matrix, to promote biofilm formation. It was found that the srrAB two-component system regulated bacterial cell lysis and biofilm formation in response to the accumulation of reduced menaquinone. A sub-atmospheric condition not only inhibited bacterial growth, biofilm development, and wood biofilm, it also decreased the production of virulence factors and biofilm components, such as α-hemolysin, PIA, and extracellular adhesins and DNA.

The role of NO in the biofilm formation of S. aureus relies on its levels. A high concentration of NO inhibited biofilm development, whereas a lower or sub-physiological concentration of NO stimulated biofilm formation. In a thioglycolate medium, microaerobic conditions of growth led to less biofilm formation than in aerobic growth. The sustained exposure of NO-releasing nanoparticles alleviated biofilm thicknesses and decreased bacterial viability both in vitro and in vivo in a rat central venous catheter model of infection.

**Acidity and alkalinity of the culture medium**

The formation of biofilms requires appropriate levels of acidity in a bacterial culture medium. Neither a highly acidic (pH 3) nor a strong alkaline (pH 12) condition could benefit biofilm development of S. aureus. Weak acidic pH conditions promoted biofilm formation compared to a basic pH. However, S. aureus produced more stable biofilms at the physiological related pH environments. This supported the finding that the addition of extra glucose in the medium could enhance the biofilm formation of MRSA.

The citrate exhibited two distinct functions on bacterial growth and biofilm formation. The first was that addition of a low concentration of sodium citrate increased biofilm formation in an ica independent manner. Citrate was able to stimulate the expression of FnB and FnB, two major fibronectin-binding proteins important for biofilm formation of CA-MRSA. Several other intermediates of tricarboxylic acid cycle promoted biofilm formation under low sodium citrate conditions. The graRS two-component system is required for citrate-
induced cell-to-cell interactions. The second finding was that a high citrate condition was bactericidal to the organism.

**Physical culture conditions**

Alteration of shaking magnitude and fluctuation levels may influence the biomass, density, and thickness of biofilms when cultured. It has been reported that increasing of sheer magnitude and fluctuation levels promoted the biofilm tolerance to vancomycin and ciprofloxacin.

Physiologically relevant and low-fluid-shear environments may benefit an attachment-independent biofilm development. Castro and co-workers found that bacterial cells also exhibited a slow growth rate, produced fewer virulence factors, and initiated alternative metabolic pathways during down-regulating RNA chaperone Hfq. Static culture conditions enabled SCCmec IV MRSA strains to generate more biomass than those SCCmec I/III MRSA isolates, and the biomass of SCCmec II MRSA biofilms was remarkably less than that of SCCmec I/III MRSA biofilms. However, the opposite phenomena were observed under dynamic conditions for these MRSA isolates. It was suggested that the flow conditions could be beneficial for bacterial growth and the formation of the biofilm matrix.

**Antibiotics-induced stress**

Exposure to sub-lethal levels of β-lactam antibiotics was found to cause bacterial aggregation and biofilm formation by some *S. aureus* strains through autolysin-induced bacterial lysis and release of DNA to form a fundamental matrix. The presence of a sub-inhibitory concentration of oxacillin was also reported to induce the icaA-dependent PIA, promoting the biofilm formation of most MRSA isolates. Moreover, low levels of clindamycin induced a stress response at a transcriptional level through sigma B and up-regulated major biofilm-associated genes, such as *atlA, lrgA, agrA, psm, fnbA*, and *fnbB*, and consequently enhanced the capacity of *S. aureus* to develop biofilms. VRSA and vancomycin-intermediate resistant *S. aureus* (VIRS) biofilm formation was remarkably elevated after exposure to vancomycin. After exposure to sub-inhibitory vancomycin, VRSA was capable of developing biofilms on nylon and silicon indwellings. Vancomycin-enhanced biofilm formation was attributed to the *cidA*-mediated autolysis and release of extracellular DNA, and PIA production. Chang and co-workers have reported that vancomycin enhanced VRSA biofilm formation via up-regulation of *atlA* and *sarA* genes. In contrast to VRSA and VIRS biofilms, vancomycin impeded autolysis of vancomycin-sensitive *S. aureus* (VSSA) during biofilm formation.
Other factors

Alcohol is another factor that could support biofilm formation by *S. aureus*. S. aureus cells within alcohol-induced biofilms were found to maintain viability, but at reduced rates. Alcohol-enhanced biofilm formation probably resulted from its induction of icaA and icaD expression. However, the combination of alcohol and ethylenediaminetetraacetic acid was able to alleviate biofilm production with unknown mechanisms. Some other factors that affect bacterial biofilm formation include culture temperature and duration.

Conclusion

*S. aureus* is an important pathogen that causes a variety of infections. The formation of biofilms on indwelling medical devices enables *S. aureus* to evade host immune responses and establish chronic infections. The ability to develop biofilms varies among different *S. aureus* isolates. Multiple environmental factors, including nutrients, antibacterial agents, pH, shearing force, temperature, and so on are able to induce stress responses and can profoundly affect the life cycle stages of biofilm formation, including initial attachment, maturation, and detachment.

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