

Review

Extracellular Enzymes, Pathogenity and Biofilm Forming in Staphylococci

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The pathogenicity of *S.aureus* strains is related to features like its adherence, various toxins, enzymes, structural and extracellular factors. In our study, the relationship between biofilm formation and lipase, protease, urease activity were investigated in *S. aureus* strains isolated from various clinical specimens sent to our microbiology laboratory. Congo red agar was used to detect biofilm production. The lipolytic activity of all strains was evaluated on Tween 20 agar. The proteolytic activity of the strains was evaluated by Skim Milk Agar. Christensen Urea agar was used to determine the urease activities of all strains. Slime factor and biofilm formation are pathogenicity factors as well. 101 (57.7%) of 175 clinical isolates were negative for biofilm formation while 74 (42.3%) samples were positive according to the phenotypic assessment of colony morphology on CRA. The relationship between biofilm formation and lipase, protease and urease activity of all the isolates are researched by using Spearman's correlation coefficient. There was an evident relation between biofilm formation with lipase activity ($r=0.195$, $p=0.10$) while protease ($r=0.001$, $p=0.99$) and urease ($r=0.06$, $p=0.4$) activity were not found related.

Keywords: Biofilm; Lipase; Pathogenicity; Protease; *S.aureus*; Ürease.

INTRODUCTION**What is biofilm?**

A biofilm is a compacted assemblage of microorganisms enclosed in a matrix primarily composed of polysaccharide, and attached on a surface. Biofilms have been found on a variety of surfaces such as indwelling medical devices, industrial water system pipes or aquatic systems in the natural environment. The microbial organisms growing in a biofilm are physiologically distinct from their planktonic counterparts.^{1,2} Biofilm formation has been recognized as a protective mode of cell growth which allows for survival in hostile environments, and also under certain circumstances, such as nutrient deprivation. Biofilm dispersal in the form of clumps plays an important role in helping the cells to colonize new niches.³ At present, the general resistance of biofilms has been explained by several possible mechanisms.^{4,5} First, the biofilm matrix might react with superoxides, neutralized charged metals or dilute antimicrobial agents to generate sub-lethal concentrations. Moreover, resistant phenotypes referred to as “*persisters*”, which have been found in a biofilm, contribute to the resistance. Whether these are indeed a Stoodley unique resistant phenotype or are simply the most resistant cells remains unclear.^{5,6}

Biofilm Development

The process of biofilm formation Recent advances have been made to show that biofilm development experiences a multiple-stage and differentiated process rather than a simple, uniform step. Five sequential regulated stages have been proposed for biofilm formation.^{6,7} During

the first two stages, the cells loosely adhere to surfaces. Further, the attached cells aggregate together and form micro-colonies; subsequently, mature biofilm develops on surfaces in stages three and four.^{7,8} Then, under certain circumstances, the biofilm cells are shed off, return to the mobile mode characterized in stage five.⁸ The cells eventually attach to a surface when conditions are appropriate, start a new cycle of biofilm formation.^{1,9-12}

Biofilm Formation in *Staphylococcus Aureus*

Biofilm formation in *S. aureus* experiences a similar process to that of *S. epidermidis*; it begins with the initial reversible bacterial adherence to a surface by some non-specific adhesion, followed by an irreversible bacterial specific attachment mediated mainly by an array of MSCRAMMS.^{13,14} Then a mature biofilm is developed characterized by multilayered bacterial cells stuck together and producing extracellular polymeric substances (EPS).¹⁵ In circumstances such as nutrient deprivation, or under heavy shear forces, detachment of clumps of the biofilm bacteria occurs.^{3,7} The released bacterial clumps start to attach to new niches and initiate a new cycle of biofilm formation.³ Polysaccharide *Intercellular Adhesin* (PIA) mediated biofilm formation in *S. aureus*. Polysaccharide *intercellular adhesin* was initially purified from *S. epidermidis*. It was identified in *S. aureus* later and shown to have a similar function. Since the structure of the N-acetylglucosamine residues in *S. aureus* is shown totally succinylated, it was designated As *Poly-N-Succinyl B-1, 6-Glucosamine* (PNSG).¹⁶ Polysaccharide intercellular adhesin has been defined as an important virulence factor for *S. epidermidis* pathogenicity in various foreign-body animal infection

models.¹⁷ Biofilm production has been shown to play a major role in the pathogenesis of infection caused by *Staphylococcus aureus*.^{17,18} The biofilm formation is the leading cause of the pathogenesis of *S. aureus* associated with biomaterial infections.^{17,18} In *S. Aureus Polysaccharide Intercellular Adhesin* (PIA) was encoded by *icaA* and *icaD* genes.^{16,18,19} Production of PIA Biofilms are communities of microorganisms that are attached to each other and/or biotic or abiotic surface, are embedded in a self-produced extracellular matrix, and show markedly reduced susceptibility to antimicrobial agents.⁷ It is estimated that the majority of chronic infections and most device-related infections are biofilm-associated.^{2,3,17,18} However, biofilm infections are difficult to diagnose and extremely difficult to treat.¹⁹

S. AUREUS PATHOGENICITY

Staphylococcus aureus is a virulent pathogen that is currently the most common cause of infections in hospitalized patients.⁹ The success of *S. aureus* as a pathogen and its ability to cause such a wide range of infections are the result of its extensive virulence factors.⁹ The structural characteristic of biofilms that has the greatest impact on the outcome of chronic bacterial infections, such as native valve endocarditis, is the tendency of individual microcolonies to break off and/or detach when their tensile strength is exceeded.⁸ Urease is needed in the urea cycle and in the metabolism of amino acids to degrade urea to form CO₂ and NH₃.¹⁷ The resulting ammonium and/or ammonia (depending on the pH of the cells) is toxic for the host cells and might accumulate in and outside the bacterial cells.^{14,15} Bacterial proteases secreted into an infected host may exhibit a wide range of pathogenic potentials. Staphylococci, in particular, *Staphylococcus aureus* are known to produce several extracellular proteases, including serine-, cysteine- and metalloenzymes.^{18,19} In our study, the presence of lipase, protease and urease enzymes in *S. aureus* strains isolated from various clinical specimens sent to our microbiology laboratory were investigated. The properties of adherence depend on properties such as various toxins, enzymes, structural and extracellular factors. Slime factor production and biofilm formation are also pathogenicity factors. Staphylococci have been shown to be able to adhere to medical devices. *S. aureus* and *S. epidermidis* are the most frequently isolated agents associated with medical device-related infections.¹⁵⁻¹⁷ *Staphylococcus aureus* is a virulent pathogen that is currently the most common cause of infections in hospitalized patients.^{9,12}

EXTRACELLULAR ENZYMES OF STAPHYLOCOCCUS AUREUS

Staphylococcus species secretes many extracellular active substances, such as coagulase, hemolysin, nuclease, phosphatase, lipase, proteases, fibrinolysin, enterotoxins, and toxic shock syndrome toxin.^{20,21} These proteins are known as virulence factors that cause disease in animal and animals.²¹ The report, it was verified for the production of Lipase from among the 25 isolates, 15(60%) of isolates produce the Lipase production.²² Most of the known Staphylococcal lipases are produced by pathogenic members of the genus, i.e. *Staphylococcus aureus* and *S. epidermidis*. Lipase interferes with the phagocytosis of the infectious lipase-producing *S. aureus* cells by host granulocytes, thus indicating a direct involvement of lipase in pathogenesis.^{20,21} The success of *S. aureus* as a pathogen and its ability to cause such a wide range of infections are the result of its extensive virulence factors.² The structural characteristic of biofilms that has the greatest impact on the outcome of chronic bacterial infections, such as native valve endocarditis, is the tendency of individual microcolonies to break off and/or detach when their tensile strength is

exceeded.³ Lipolytic activity was determined by using the method.⁹ Urease is needed in the urea cycle and in the metabolism of amino acids to degrade urea to form CO₂ and NH₃.⁴ The resulting ammonium and/or ammonia (depending on the pH of the cells) is toxic for the host cells and might accumulate in and outside the bacterial cells.^{4,5} Proteolytic activity was assayed.¹⁰ The overnight broth culture was spotted into 1% skim milk agar and incubated at 37°C for overnight. After the incubation period, the clear zone of hydrolysis was observed. The presence of a transparent zone around the colonies indicated protease activity bacterial proteases secreted into an infected host may exhibit a wide range of pathogenic potentials. Staphylococci, in particular, *Staphylococcus aureus*, are known to produce several extracellular proteases, including serine-, cysteine- and metalloenzymes.^{6,7} Their insensitivity to most human plasma protease inhibitors and, even more, the ability to inactivate some of these make the proteases potentially harmful.^{6,8} In our study, the presence of lipase, protease and urease enzymes in *S. aureus* strains isolated from various clinical specimens sent to our microbiology laboratory were investigated.

MATERIAL AND METHODS

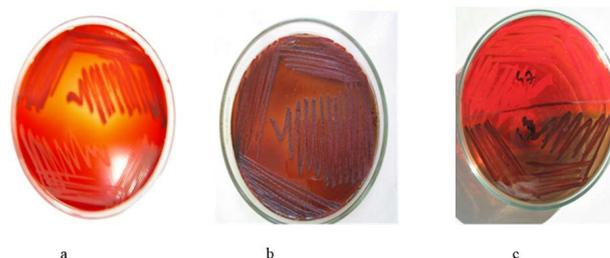
Biofilm formation and lipase, protease, urease activity were investigated in *S. aureus* strains isolated from various clinical specimens sent to our microbiology laboratory, Denizli, Turkey. Methicillin resistance was not evaluated in this article.

Investigation of Biofilm Formation

Qualitative detection of biofilm formation of these isolates was performed using *Congo Red Agar* (CRA), according to (Freeman et al., 1989) Isolates were streaked onto the agar to obtain single colonies and incubated overnight at 37 °C aerobically and a further 24 hours at room temperature. The interpretation of results followed (Freeman et al., 1989) and (Arciola et al., 2002).^{23,24}

Congo red agar was used to detect biofilm production.²³ Congo reddish agar medium was prepared to contain 10 g of agar, 50 g of sucrose, 37 g of brain-heart infusion vial and 0.8 g of Congo red. Cultures made in such a way that a single colony fell on these mediums were incubated overnight at 37 °C, followed by incubation of the cultures for 48 hours at room temperature. *S. epidermidis* ATCC 12228, which does not produce biofilms, and *S. epidermidis* ATCC 35984, which produces strong biofilms, were used as controls. Cultures cultured in Congo red agar medium at 37 °C overnight and after 48 hours incubation at room temperature after 48 hours of reddish-black, rough, dry, transparent colony-forming biofilm positive, pinkish-red, flat and central dark (ox-eye view) colony biofilm was considered negative (Figure 1).

Figure 1. Evaluation of biofilm formation by Congo red agar method [a-biofilm negative, b-biofilm positive, c-biofilm negative (upper) and biofilm positive (bottom)]

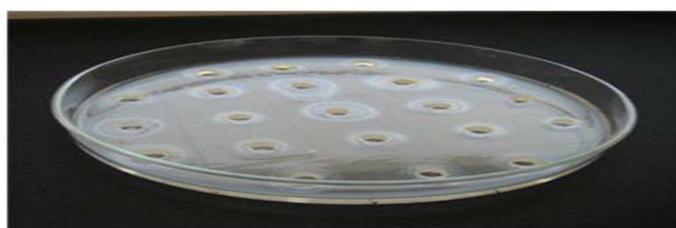


SCREENING FOR EXTRACELLULAR ENZYMES

Investigation of Lipolytic Activities

The lipolytic activity of the isolates was determined onto Tween 20 agar.^{18,25} The experiments were carried out in The lipolytic activity of all strains was evaluated on Tween 20 agar (containing 10g peptone, 5g NaCl, 0.1g CaCl₂, 20g agar and 1ml Tween 20) per liter. Produced by incubation at 37 °C overnight in Brain heart medium (pH 7.5) containing 10g of peptone, 5g of yeast extract, 5g of NaCl, 1g of K₂ HPO₄ .3H₂ O. Subsequently, strains diluted 1: 100 in Brain hart medium were inoculated into 20 µl of the wells opened with sterile glass pipette onto Tween 20 agar. The plates were evaluated after 72 hours incubation at 37 °C. The presence of lipolytic activity was detected around the inoculation by the appearance of halo formation, depending on whether the tween was a line-shaped precipitate(Figure 2).

Figure 2. Lipolytic activity of S. aureus strains on Tween 20 agar



Investigation of Proteolytic Activities

The proteolytic activity of the strains was evaluated by the agar plate method. For this, Skim Milk Agar (SMA) containing 1% skim milk, 1% tryptone, 0.5% yeast extract, 0.5% NaCl and 1.5% agar was used. 20 .µl of supernatant from 1:100 diluted samples were added to sterile diluted wells in SMA. Plates were assessed after being incubated for 48 hours at 37 °C. The presence of proteolytic activity was determined by the occurrence of opaque zones (halo formation) around the wells due to casein hydrolysis.²⁶⁻³⁰

Urease Activity Investigation

Christensen Urea agar was used to determine the urease activities of all strains.³¹ The 20 µl supernatant of the 1:100 diluted samples were taken grown on urea agar after being produced on Brain heart medium. The tubes were evaluated after 24 h incubation at 37 °C. Urea hydrolysis in this medium was shown by a change in color from the pale yellow of the fresh medium to intense red-violet color.

CONCLUSION

SPSS Ver 10.0 was used for statistical analysis. Spearman's correlation coefficient was used to evaluate the relationship between biofilm production and enzyme activities of the working clinical isolates. The statistical error margin was accepted as 5%.

S. aureus strains were isolated from various clinical specimens (eye, urine, nasal swab, etc.). Numbers and percentages of biofilm positive and negative specimens were shown on Congo red agar medium (Table 1).

Table 1. Of the 175 S. aureus strains used in the study, 87 wound (49.7%), 25 blood culture (14.3%), 35 tracheal aspirate (20.0%), 10 sputum (5.7%), 8 catheter 4.6%) and 10 (5.7%) were isolated from various clinical specimens (eye, urine, nasal swab, etc.). Numbers and percentages of biofilm positive and negative specimens were shown on Congo red agar medium

For example, where it is isolated	n	Biofilm			
		Negative		Positive	
	n	(%)	n	(%)	
Wound	87	34 (39.1)	53 (60.9)		
Blood	25	17 (68.0)	8 (32.0)		
Tracheal aspirate	35	31 (88.6)	4 (11.4)		
Sputum	10	7 (70.0)	3 (30.0)		
Catheter	8	7 (87.5)	1 (12.5)		
Other	10	5 (50.0)	5 (50.0)		
Total	175	101 (57.7)	74 (42.3)		

The Extracellular Enzyme Production among the Isolates

The relationship between biofilm production and lipase, protease and urease activities of all isolates was investigated.³²

Lipolytic activity of S. aureus strains was showed on Tween 20 agar (Figure 2). Of the clinical isolates, 146 (83.4%) showed lipase activity whereas 29 (16.6%) did not have lipase activity. Lipase (+) was detected in 68 (91.9%) and Lipase (-) was detected in 6 (8.1%) in 74 samples of biofilm positive in Congo red agar medium. In biofilm negative 101 samples, 78 (77.2%) were lipase (+) while 23 (22.8%) were lipase (-) (Table 2).

Table 2. Relationship between biofilm formation and lipase activity. There was a good correlation between lipase activity and biofilm production of the isolates (r=0.195, p=0.10).

	Biofilm		
	Positive	Negative	Total
Lipase	n(%)	n(%)	n(%)
Positive	68 (91.9)	78 (77.2)	104 (59.4)
Negative	6 (8.1)	23 (22.8)	71 (40.6)
Total	74	101	175

Of the 175 clinical isolates, 104 (59.4%) showed proteolytic activity whereas 71 (40.6%) showed no proteolytic activity. Protease (+) was detected in 44 (59.5%) and protease (-) was detected in 30 (40.5%) of the 74 samples in which biofilm was positive in Congo red agar medium. Protein (+) was found in 60 (59.4%) and protease (-) was detected in 41 (40.6%) of the biofilm negative 101 samples (Table 3).

Table 3. Relationship between biofilm formation and protease activity.

	Biofilm		
	Positive	Negative	Total
Protease	n(%)	n(%)	n(%)
Positive	44 (59.5)	60 (59.4)	104 (59.4)
Negative	30 (40.5)	41 (40.6)	71 (40.6)
Total	74	101	175

There was no correlation between biofilm production and proteolytic activity of the isolates (r= 0.001, p = 0.99).

144 (82.3%) of the clinical isolates showed urease activity, while 31 (17.7%) had no urease activity. Urease (+) was found in 63 (85.1%) and urease (-) was detected in 11 (14.9%) in 74 samples of biofilm positive in Congo red agar medium. Urease (+) was found in 81 (80.2%) and urease (-) was detected in 20 (19.8%) of the biofilm negative 101 samples (Table 4).

Table 4. Relationship between biofilm formation and urease activity. There was no relationship between biofilm production and urease activity of the isolates ($r=0.06$, $p=0.4$).

	Biofilm		
	Positive	Negative	Total
Urease	n(%)	n(%)	n(%)
Positive	63 (85.1)	81 (80.2)	144 (82.3)
Negative	11 (14.9)	20 (19.8)	31 (17.7)
Total	74	101	175

DISCUSSION

Clinical impact of bacterial biofilm. The study of bacteria residing in biofilms as an interactive community has recently gained a great deal of interest, in part, because a number of human diseases are involved in biofilms. Several mechanisms have been proposed to explain why pathogens in biofilms are more virulent than their planktonic counterparts.⁸ First, pathogens in biofilms can initiate an infection through the seeding or dispersal of biofilm clumps, which contain large numbers of cells. Second, among the phenotypically heterogeneous pathogens within a biofilm, certain virulent phenotypes might survive and spread within the biofilm matrix. Finally, the closely related cells within biofilms might initiate quorum-sensing networks, which regulate virulence gene expressions.⁹ Taken together, the dense aggregated virulent organisms within the context of biofilms might be a major contributor to the pathogenesis of bacterial biofilm-related infections.^{10,11}

The success of *S. aureus* as a pathogen and its ability to cause such a wide range of infections are the result of its extensive virulence factors. Biofilm formation occurs through a series of steps, which begins, with the initial attachment of planktonic bacteria to a solid surface that is present at the air-water/liquid interface. This step is followed by subsequent proliferation and accumulation of the cells in small multilayer cell clusters known as microcolonies. The microcolonies then further proliferate to form giant assemblages of cells enmeshed in an extracellular matrix, which covers entire surfaces and protects its inhabitants from detrimental effects of all sorts.⁴⁻⁶ A mature well-established biofilm is not a static structure, rather it is highly dynamic in nature, where old cells are constantly being dispersed and new members being recruited for this surface-associated community to expand, at all times. The composition of the extracellular matrix is very difficult to ascertain and variable among different bacterial species and even within the same species under different environmental conditions.³ Despite this fact, exopolysaccharides are an essential component of virtually all biofilm structures, providing the necessary matrix in which the bacterial cells are initially embedded.⁷ Bacterial cells have to protect themselves from a pH that is too low.¹³ Bacterial cells have assumed that the urease activity determined contributes to the persistence of the bacterial cells in the biofilm by counteracting the low pH values caused by the production of lactic acid, acetic acid, and formic acid. Beenken et al. have also reported up-regulation of the urease operon in 7-day-old biofilms.⁵ According

to Resch et al. urease activity might be an important factor for keeping the biofilm alive.¹⁵ Since excess ammonia would be toxic for the bacterial cells, they should have some mechanism of resistance against this chemical and should have enzymes or other mechanisms to detoxify this compound. Bacterial proteases secreted into an infected host may exhibit a wide range of pathogenic potentials.¹⁵ Bacterial protease is reported as a pathogenic factor.²² *S. aureus* has two enzymes, metalloprotease and serine protease.²⁶⁻³⁰ the distribution of both enzymes varies greatly between strains. Researchers report that the protease activity is a pathogenic factor.^{26,28} Urease activity is said to be important in the maintenance of biofilm formation. The bacterial cells in the biofilm protect themselves by up-regulation of the urease genes from the pH decreasing by the production of lactic acid, formic acid, which is the result of metabolism.^{15,16,19} Coagulase-negative staphylococci were demonstrated to present lipase and protease activities more often than coagulase-positive staphylococci. *Staphylococcus aureus* produces some of the industrially important extracellular enzymes. Lipase is the most abundant enzyme produced by these bacteria. The hypnotic activity of staphylococci was originally observed.¹⁰ The hypnotic activity is now known to be the result of the enzymes lipase and esterase that act against water-soluble, water-insoluble glycerol esters and on water-soluble Tween polyoxyethylene esters. Therefore, staphylococci split a variety of lipid substrates with lipase acting on fat-soluble glycerides and with esterase acting on water-soluble esters.⁹ It is known that staphylococci tend to remain in lipid secretions in the cutaneous habitat of the host organism.¹⁰ Lipids are found ubiquitously on the surface of human skin and are largely composed of sebum-derived triacylglycerides.¹¹ When the natural host defense is weakened the opportunistic pathogens invade the host. One of these microorganisms is *Staphylococcus epidermidis* known as the human cutaneous commensal that lives on the skin of its host which is also able to become an opportunistic pathogen.^{21,33,34} It is thought that during the infection process, two secreted lipases support the colonization and growth of the bacteria by the cleavage of the triacylglycerols derived from the sebum of the skin.^{21,35,36} The clinical studies have proven that *Staphylococcus aureus* than those isolated from the more superficial ones suggesting that lipase activity might be important for nutrition or dissemination of the bacteria.^{36,37} The strongest hint that was ever found out about the correlation between lipase activity and pathogenicity of staphylococci is the detection of anti-lipase IgG antibodies in patients with the *Staphylococcus aureus* infections, which showed the pathogenic potential of the extracellular lipase.^{38,39} Staphylococcal lipase is encoded by *geh* which stands for glycerol ester hydrolyze that was identified from *Staphylococcus epidermidis* strain in the studies aiming to identify extracellular colonization factors important for the persistence of cutaneous bacteria on the skin.^{15,35,37} The lipase activity was significantly higher in strains isolated from deep or subcutaneous infections, i.e, septicemia, pyomyositis, osteomyelitis, aerobic and anaerobic furunculosis, than in strains from superficial infections, i.e. impetigo, or from the nasal mucosa.⁴⁰

In our study, we found no correlation between biofilm formation and urease and protease activities, bacterial pathogenicity factors, and found a correlation between lipase and biofilm production. Although there are no previous studies in this area.³² These results suggest that the formation of lipase and biofilm, which play a role in settlement, may function together in pathogenic strains.

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