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Chapter 10

Staphylococcal Biofilms: Pathogenicity, Mechanism and Regulation of Biofilm Formation by Quorum-Sensing System and Antibiotic Resistance Mechanisms of Biofilm-Embedded Microorganisms

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Additional information is available at the end of the chapter

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Abstract

Staphylococcal infections are reported to cause very important problems in hospitalized and immunocompressed patients worldwide due to their tough and irresponsive treatment by antibiotics. Biofilm-embedded bacteria that gain resistance to immune defense and antibiotics by antibiotic degrading enzymes, efflux pumps, and certain gene products of which expression are changed by the quorum sensing cause chronic and recurrent infections such as indwelling device–associated infections. Biofilm-embedded sessile community has heterogeneous cells that have wide range of different responds to each antimicrobials. *Staphylococcus epidermidis* (*S. epidermidis*) and *Staphylococcus aureus* (*S. aureus*) that are mostly known pathogenic strains can induce gene expression of biofilm that has an important role in the pathogenesis of staphylococcal infections and causes bacterial attachment and colonization on biotic such as tissues or abiotic surfaces such as prosthetic surfaces that may act as a substrate for microbial adhesion when microorganisms exposed to stress conditions. This expressed and matured biofilm causes bacterial spread to whole body, consequently, spread of infection in to whole body. It is hard to treat biofilm infections, and new agents are being researched to prevent formation and dissemination of biofilm. Defining the virulence and the role of biofilm of *S. epidermidis* and *S. aureus* in chronic and recurrent infections such as indwelling device–associated infections, the mechanism and the global regulation of biofilm production by quorum-sensing system, inactivation of biofilm formation, and the resistance patterns of biofilm-embedded microorganism against antimicrobials are important.

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Keywords: staphylococcal biofilm, mechanism and regulation of biofilm formation, quorum-sensing system, antimicrobial resistance of biofilm, *Staphylococcus aureus*, *Staphylococcus epidermidis*, pathogenicity

1. Introduction

Staphylococcus epidermidis (S. epidermidis) and *Staphylococcus aureus* (S. aureus) are the most common causes of indwelling device–associated infections, and nosocomial and community acquired infections can produce biofilm as a virulence factor [1]. The biofilm infections such as *S. epidermidis* and *S. aureus* infections are important problems in hospitalized and immunocompressed patients worldwide due to their tough and irresponsive treatment by antibiotics. Biofilm-producing bacteria resist to immune defense, antibiotics, and many antimicrobial agents. Biofilm-embedded bacteria gain antibiotic resistance by antibiotic-degrading enzymes, efflux pumps, and certain gene products of which expression are changed by the quorum sensing [2, 3]. Biofilm-embedded sessile community has heterogeneous cells that have wide range of different responds to each antimicrobials [2]. So, every antibiotic has a different effect against different metabolically active cells that are present in the different layers of biofilm and persister cells that are evolved to survive in biofilm. It is hard to treat biofilm infections that are generally recurrent infections and of which treatments are tough and irresponsive [3].

Staphylococci that construct the human skin flora can contaminate indwelling devices. By this way, they are inserted to human by contaminated indwelling devices. When microorganisms exposed to stress conditions, gene expression of biofilm is induced as a stress response. The biofilm that is a slime-like glycocalyx causes bacteria to survive in the stress conditions. Staphylococci adhere, colonize, and infect biotic surfaces such as tissue or abiotic surfaces such as prosthetic surfaces that may act as a substrate for microbial adhesion and causes bacterial spread to whole body by forming biofilm that is a slime-like glycocalyx [1, 4, 5]. The virulence and the role of biofilm of *S. epidermidis* and *S. aureus* in chronic and recurrent infections such as indwelling device–associated infections, the mechanism, and the global regulation of biofilm production by quorum-sensing system, especially *agr*-quorum-sensing system, inactivation of biofilm formation, and the resistance patterns of biofilm-embedded microorganism against antimicrobials are discussed in this chapter.

2. The biofilm, virulence, and Staphylococcus

2.1. The pathogenesis of Staphylococcus biofilm

The biofilm has an important role in the pathogenesis of staphylococcal infections. The biofilm causes bacteria to survive in the stress conditions such as UV damage, metal toxicity, anaero-

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bic conditions, acid exposure, salinity, pH gradients, desiccation, bacteriophages, and amoebae and to resist antibiotics, antimicrobials, and host immune defense [5–8]. The main pathogen of implant infections is staphylococci that cause 80% of all prosthetic infections [9]. The biofilm of bacteria causes chronic infections such as indwelling device–related infections, chronic wound infections, chronic urinary tract infections (UTI), cystic fibrosis pneumonia, chronic otitis media (OM), chronic rhinosinusitis, periodontitis, and recurrent tonsillitis [10]. The biofilm infections are the main important problems in hospitalized and immunocompressed patients in worldwide due to their tough and irresponsive treatment by antibiotics. In biofilm, bacteria are not distrupted completely by antibiotics even high doses of antibiotics used *in vivo* [3, 11, 12]. Infected device can expose the patient to a higher risk of mortality. Orthopedic surgery and trauma indwelling device-related infections that make treatment difficult by antibiotics [13] cause removal of implant out of the body to eradicate biofilm and overcome biofilm-related infections [14] and may cause functional loss of the infected limb [15, 16].

2.2. Staphylococcal biofilms as a virulence factor

The biofilm that anchored to abiotic or biotic surfaces is a slime-like glycocalyx in which sessile community of microorganisms embedded. This extracellular polymeric substance that is constituted by matrix of polysaccharide, teichoic acids, extracellular DNA (eDNA), and staphylococcal proteins is produced by biofilm producing microorganisms [4, 17, 18]. Polysaccharide intracellular adhesin (PIA) is a specific polysaccharide in glycocalyx composed of β-1,6–linked N-acetylglucosamine residues (80–85%) and non-N-acetylated D-glucosaminyl residues that are an anionic fraction and contain phosphate and ester-linked succinate (15–20%) [18]. Although PIA is a main mechanism of biofilm formation in *S. aureus* and *S. epidermidis*, surface proteins are the other alternative mechanism of biofilm formation. Extracellular matrix has large water-filled channels, accumulates antibiotic-degrading enzymes such as β-lactamases [19], and plays a role in the adaptive resistance mechanisms due to eDNA constituent [20] (**Figure 3**).

2.3. Mechanisms of biofilm formation

Bacterial biofilm formation is a complex and multifactorial process. The biofilm formation process consists of adherence/adhesion/attachment, aggregation/maturation/accumulation, and detachment/dispersal phase. The last step is the dispersal of mature biofilm-embedded bacteria out of the biofilm [21] (**Figure 1**).

2.3.1. Attachment (adhesion or adherence) phase

When conditions favor biofilm formation, biofilm formation that begins with the adherence of the bacteria to a surface that act as a substrate for microbial adhesion continues with the aggregation formed by cell–cell adhesion [22] (**Figure 1**).

Staphylococcal adherence to an abiotic surface of indwelling prosthetic device depends on physico-chemical structure of medical device and surface components of Staphylococci such as wall teichoic acid (WTA) [23], lipoteichoic acid (LTA) [23], accumulation-associated

protein (Aap) [24], autolysins AtlA [25] and AtlE [26]. The staphylococcal adherence to a biotic surfaces such as host cells and plasma protein-coated prosthetic surface is mediated by cell wall-anchored (CWA) proteins such as the fibrinogen-binding protein SdrG/Fbe of *S. epidermidis* and fibrinogen-/fibronectin-binding proteins FnBPA and FnBPB and clumping factors A and B of *S. aureus* [27].

Several microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) that are able to bind to human matrix proteins such as fibronectin and fibrinogen and colonize are expressed in *S. epidermidis* and *S. aureus* at the first step [28]. Adherence of bacteria to an extracellular matrix component, fibronectin, fibrinogen, and plasma clot is mediated by expressed surface adhesins such as Bap coded by bap gene [29], surface protein G (SasG) [22], fibronectin-binding proteins (FnbA and FnbB) of *S. aureus* [30], and the fibrinogen-binding protein SdrG/Fbe of *S. epidermidis* [27]. Adherence of *S. aureus* to collagenous tissues and cartilage is mediated by collagen-binding protein, Cna. Some antibodies can block bacterial attachment to these tissues by blocking Cna. Adherence of *S. aureus* to fibrinogen in the presence of fibronectin is mediated by clumping factor A and B (ClfA, ClfB) that are effective in foreign body and wound infections. Also, plasma-sensitive surface protein (Pls) participates in the attachment to fibrinogen and fibronectin. Protein A that is present in cell wall and encoded by *spa* gene in *S. aureus* impair opsonization and phagocytosis by binding to Fc domain of immunoglobulin G (IgG) in the wrong orientation. Endovascular diseases are emerged by *S. aureus* as a result of the binding of protein A to von Willebrand factor in damaged endothelium [31].

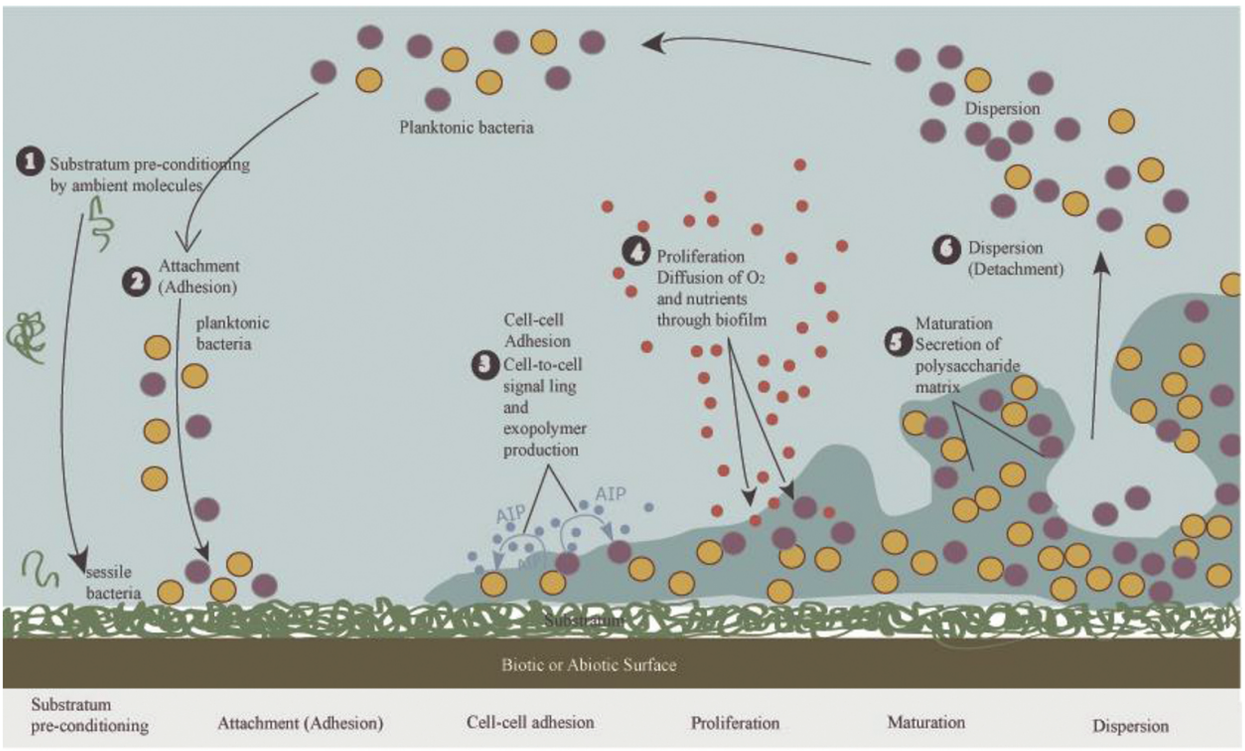


Figure 1. The stages of biofilm formation.

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2.3.2. Accumulation (aggregation or maturation) phase

After adherence of staphylococcus to biotic and abiotic surfaces, exopolysaccharide (EPS) such as PIA or PNAG that are produced by *ica* operon (*ica*-dependent form) starts to be produced, extracellular matrix (ECM) is constructed by PIA/PNAG, extracellular DNA (eDNA), and surface proteins [cell wall-anchored (CWA) proteins] in *ica*-independent form, and bacterial colonies become mature [2, 27]. The cell wall-anchored (CWA) proteins not only provide bacterial adherence but also provide intercellular adhesion, biofilm accumulation, and maturation [27]. Aggregation that is mediated by the synthesis of either polysaccharide intercellular adhesion/poly-N-acetylglucosamine (PIA/PNAG) [30, 32] is formed in cell clusters till multi-layer-structured biofilms formed. Several staphylococcal surface proteins that mediate primary attachment of bacteria such as clumping factors A and B, fibrinogen-/fibronectin-binding proteins FnbA and FnbB of *S. aureus* or the fibrinogen-binding protein SdrG/Fbe of *S. epidermidis* that are cell wall-anchored proteins (CWA) also promote intercellular adhesion and construct the aggregation of bacteria in *ica*-independent biofilm formation rather than PIA [33] (**Figure 1**).

In the initial cell-surface interaction of motile bacteria, adherence of motile cell to surface is facilitated by flagella of motile cell. After adherence motile species that undergo cellular differentiation in biofilm lose their motility by paralyzing their flagella and become nonmotile [34]. Klausen et al. [35] revealed that wild-type strain and isogenic flagellar mutant of *Pseudomonas aeroginosa* both forms biofilms which have structural differences.

2.3.3. The detachment (or dispersal) phase

In the detachment stage, sessile cells turn into planktonic state that can spread and colonize other surfaces and form biofilm on these infected regions [2] (**Figure 1**). Detachment of microorganisms from biofilm can be caused by bacteria themselves, such as enzymatic degradation of the biofilm matrix such as dissolution of adhesins by proteases, nucleases, and a group of small amphiphilic α-helical peptides, known as phenol-soluble modulins (PSMs) functioning as surfactants [27], and quorum sensing or by external forces such as fluid shear forces, corrosion, and human intervention [36] (**Figure 2**). During detachment of motile microorganism rather than staphylococcus, cells express genes that are for motility such as transcription of pilus and ribosomal proteins and are almost seen in planktonic cells [37].

2.4. Types of biofilm formation

2.4.1. PIA-dependent biofilm formation

Positively charged PIA provides intercellular attachment via binding to bacteria of which surface is negatively charged [27]. All *S. aureus* strains contain *icaADBC* gene of which product is PIA constructs biofilm formation [31]. *Ica* locus have been identified in many staphylococcus species like *S. aureus* and *S. epidermidis* but except *S. haemolyticus* and *S. saphrophyticus* [9]. *ica* is regulated by stress conditions, such as anaerobic conditions, extreme temperature, osmolarity, ethanol, and antibiotics. icaA, icaD, icaC, and icaB are the genes of *ica*ADBC locus.

icaA and icaD contribute to exopolysaccharide synthesis and encode N-acetylglucosaminyl transferase as a transmembrane enzyme to synthesize poly-N-acetylglucosamine polymer. While poly-N-acetylglucosamine polymer is translocated to cell surface of bacteria by *ica*D gene, the polymer is fixed to the outer surface of bacteria by deacylation of poly-N-acetylglucosamine polymer by the product of *ica*B gene [9]. Regulator gene *icaR* that is located upstream of the *icaADBC* operon encodes a transcriptional repressor in both *S. epidermidis* and *S. aureus* and *icaADBC* genes are upregulated in response to anaerobic growth such as inside of biofilm. Under anaerobic conditions, PIA is induced by SrrAB (the staphylococcal respiratory response regulator) that binds to upstream of the *icaADBC* operon. Insertion sequence (IS256) can regulate *ica* by reversible inactivation in *S. epidermidis* and some strains of *S. aureus*. TcaR (transcriptional regulator of the teicoplanin-associated locus) and IcaR are repressors of ica operon transcription and repress PIA expression. While deletion of *icaR* gene increases *ica* gene expression, PIA production, deletion of *tcaR* gene had no effect against *ica* gene, PIA production. Transcription of IcaR is repressed by Rbf that is a protein regulator of biofilm formation and leads expression of *ica* gene, PIA production, whereas transcription of IcaR is induced by Spx that is a global regulator of stress response genes and regulates biofilm formation negatively [18].

2.4.2. PIA-independent biofilm formation

Biofilms not only can be constructed by *ica* gene of which product is PIA, but also constructed by *ica*-independent (PIA-independent) form. Biofilm is generated not only by PIA that is a main component of biofilm production but also by a number of proteins. When *icaADBC* is deleted, PIA is not produced but the biofilm formation so, virulence is not affected. In this case, biofilm formation can be constructed rather than PIA. In the catheter infection, biofilm formation of clinical isolates of *S. aureus* of which *ica* cluster is mutated is not reduced [18]. Fitzpatrick et al. revealed that biofilm formation of the *icaADBC* operon-deleted MRSA mutants was not affected, whereas biofilm formation of the *icaADBC* operon-deleted MSSA mutants was impaired. This study showed that ica-independent biofilm formation is strain specific [38].

PIA-independent biofilms were constructed by accumulation-associated proteins (Aap) of *S. epidermidis*, biofilm-associated protein (Bap) that is a surface protein of *S. epidermidis* and *S. aureus* and Bap-related proteins of *S. aureus* [18]. Other surface proteins that involve in the PIA-independent biofilm formation are SasG, SasC, protein A, fibronectin-binding proteins FnBPA and FnBPB, cell wall-anchored (CWA) proteins including clumping factors A and B, autolysins AtlA and AtlE or wall teichoic acid (WTA), the fibrinogen-binding protein SdrG/Fbe, lipoteichoic acids (LTA) of *S. aureus* and the fibrinogen-binding protein SdrG/Fbe of *S. epidermidis* [27].

Scientists determined that medical MRSA isolates produce protein-dependent biofilm such as FnBP- and Aap-dependent biofilms in animal models that have indwelling device–associated infection. O'Neill et al. [30] and McCourt et al. [39] revealed that biofilms of certain isolates of HA-MRSA from CC8 and CC22 and CA-MRSA from USA300 lineage (CC8) were FnBPs-dependent.

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Autolysin Atl that is a wall-anchored protein of *S. aureus* and causes initial attachment of *S. aureus* to surfaces can be cleaved into amidase and glucosaminidase that cause cell lysis, eDNA release, and cell accumulation. Then, biofilm maturation of FnBP-dependent biofilm phenotype is constructed by FnBPs [25].

In biofilm production of *S. aureus*, cell-cell interactions are facilitated by α-toxin that is a haemolytic toxin. Nevertheless, the mechanism of integral role of α-toxin has not been known clearly. β-toxin that is a sphingomyelinase and causes hemolysis and lyse lymphocytes plays a stimulative role in the biofilm production of *S. aureus* by covalently cross-linking to itself in the occurrence of DNA in matrix of staphylococcal biofilms [40].

S. aureus biofilms can be stabilized by amyloid fibrils that are formed by aggregated PSM on the surface of bacteria and aggregated signal peptide AgrD [41].

2.5. The global regulation of biofilm formation

2.5.1. The regulation of Staphylococcal biofilm by agr-quorum-sensing system

Biofilm production is provided by the equilibrium between the productions of amyloid fibrils and phenol soluble modulins (PSMs) that are extracellular polymeric substances and their catabolism by enzymes such as nucleases and proteases that are expressed by agr-QS regulator system that use two-component system signal transduction system (TCS). The control of planktonic and sessile bacteria and the biofilm expression is regulated by coordinated mechanisms [41] (**Figure 2**).

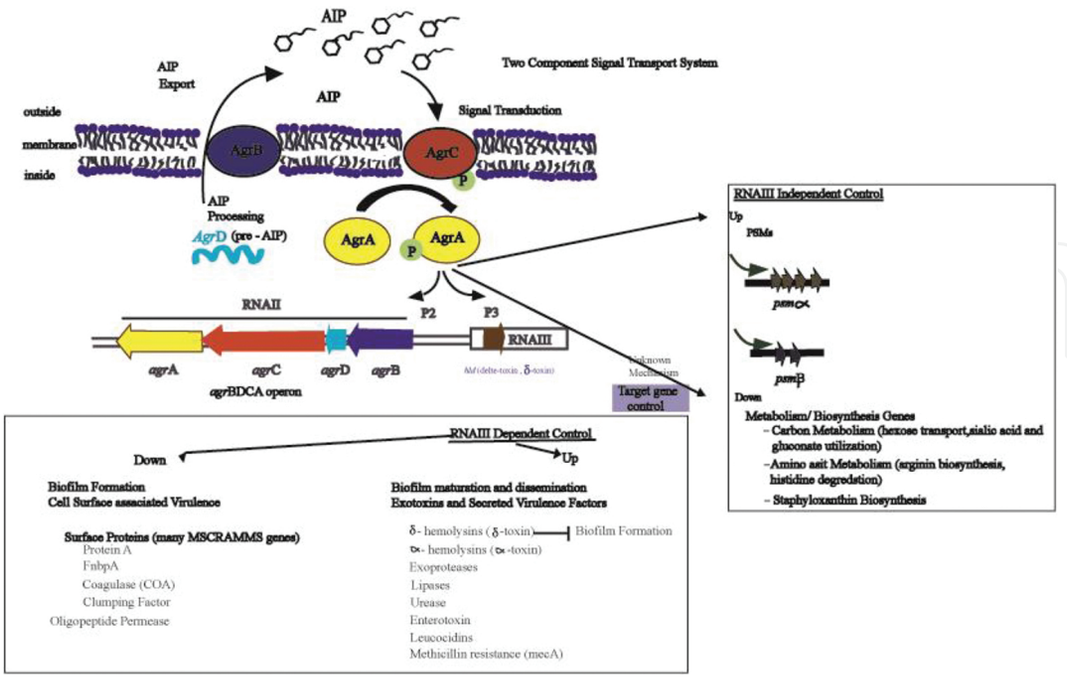


Figure 2. The regulation of biofilm formation by agr-quorum-sensing system.

The biofilm formation of staphylococci is fully expressed *in vivo*, whereas the biofilm formation of staphylococci is not fully expressed all the time *in vitro* unless nutrient supplementations are added to growth media and is provided. Increased amount of biofilm formation due to fully expression occurs in stress conditions such as starvation, thermal stress, heat shock, salt, certain antibiotics, iron limitation, subinhibitory concentrations of ethanol, accumulations of metabolites, oxidative stress, low pH, and changes in osmolarity *in vitro*. Bacteria sense stimuli from the environment and bacterial density and then respond to stimuli by upregulating expression of biofilm formation, virulence factors production such as toxins, etc. [9].

Staphylococcus use quorum-sensing systems (QS) for intercellular communication and biofilm formation. Accessory gene regulator (Agr) system regulates cell density-dependent gene expression using two-component signal transduction system [42]. Agr and LuxS systems that are required for autoinducer peptide (AIP) production as a pheromone are quorum-sensing systems in staphylococci [43]. Bacteria sense pheromones as stimuli that are released by the density of bacteria belonging to the same group and express biofilm formation [9]. AIP production starts in exponential phase of bacterial growth [44]. There are four proteins that are sensor histidine protein kinase AgrC, DNA-binding response regulator AgrA, AgrD that is a prepheromone, and AgrB that exports and modifies AgrD, present in this system. The signal is transported to bacteria by binding of AIP to AgrC. When AIP binds to AgrC, DNA-binding regulator AgrA is activated by His-dependent phosphorylation of AgrC [42]. By the binding of activated DNA-binding regulator AgrA to P2 and P3 promoters in *agr* operon (*agr*BDCA), RNAII and RNAIII are transcripted, respectively [44]. The *agr*BDCA operon codes RNAII transcript that encodes AgrB, D, C, A from *agrB*, *D*, C, *A* genes as a components of agr system, and RNAIII transcript that include *hld* gene encodes the δ-hemolysin (termed δ-toxin or δ-PSM) [42]. RNAIII regulates the expression of agr-governed virulence factors such as CWA proteins as a surface proteins and exotoxins at transcriptional and translational level. Independently of RNAIII (RNAIII independent control), AgrA also directly regulates the expression of α-PSMs and β-PSMs by binding to their promoters in *psm* operon in *S. aureus* and involves in the downregulation of genes contribute carbohydrate and amino acid metabolism [44] (**Figure 2**).

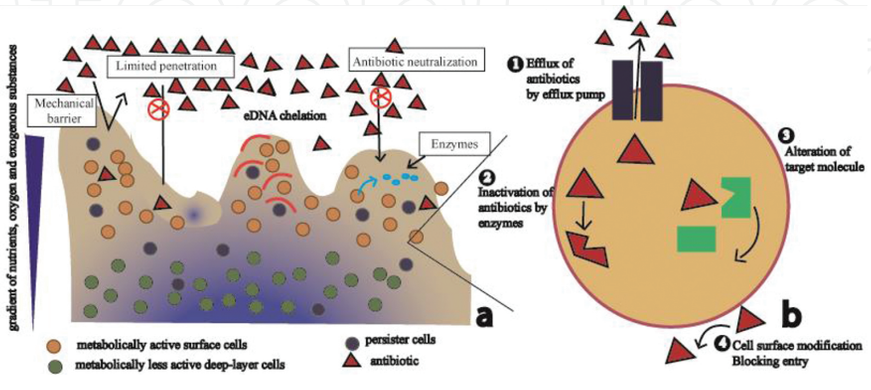


Figure 3. The biofilm-embedded bacteria. (a) The heterogeneous sessile community of biofilm. (b) Antibiotic resistance mechanisms of biofilm-embedded bacteria.

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The regulation mechanisms of RNAIII for target genes can be at transcriptional and translational level, and its regulation can be direct or indirect. Fourteen stem-loop and two long helices construct structure of RNAIII. Each domain regulates the expression of each target gene. Translation of α-hemolysin (*hla*) upregulated by hairpin loop H2 and H3. In contrast to this, the repression of early expressed virulence genes of *S. aureus* such as coagulase, protein A, and the repressor of toxins (Rot) is comprised by hairpin H13, H14, and H7 of RNAIII. Hairpins such as H7, H13, and H14 that are complementary to Shine-Dalgarno sequences (SD) of target mRNA act as an antisense RNA and inhibit initiation of translation and cause RNAaseIII-mediated degradation of target mRNA [45] (**Figure 4**).

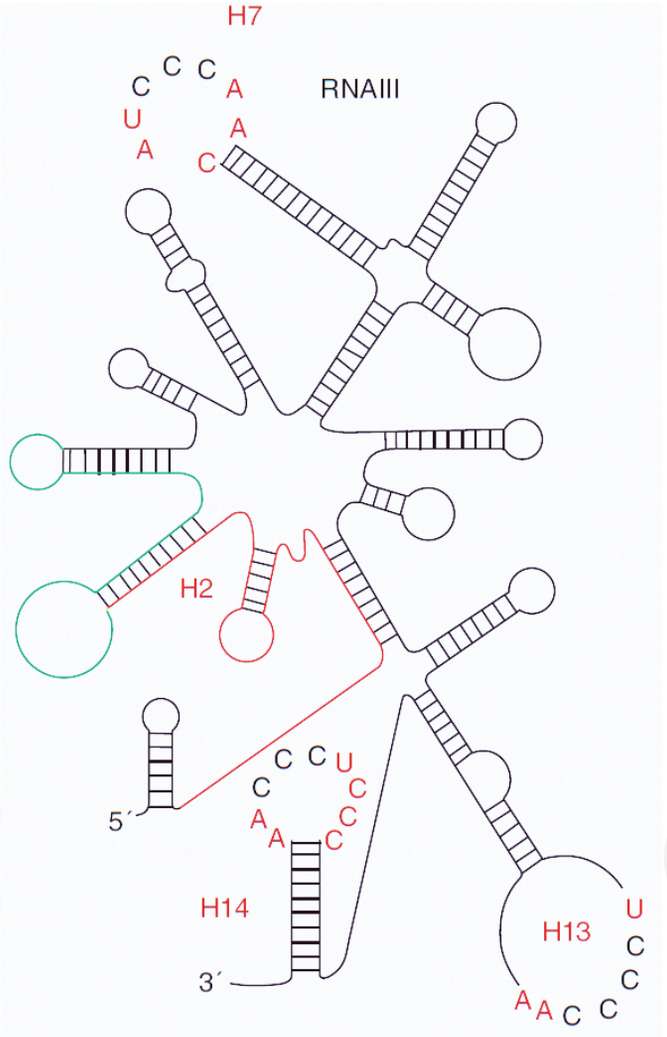


Figure 4. The structure of RNAIII [44]

Staphylococcal virulence factors are expressed with accessory gene regulator (agr) system in response to cell density [9]. During the beginning of the biofilm-related staphylococcal infection, adhesion factors (surface proteins) such as MSCRAMMs are upregulated. After initial attachment and colonization had been happened, during early stationary growth phase

of bacteria, toxins and other acute virulence factors such as degradative exoenzymes (such as δ-hemolysin, lipases and proteases that disperse bacteria) are upregulated and non-aggressive colonization surface proteins such as MSCRAMMs are downregulated by *agr*-QS regulator system [1, 46]. Adherence is reduced by downregulated genes of CWA, due to surface proteins are no longer needed after colonization, by the way initial biofilm formation is decreased indirectly [5]. Expression of staphylococcal toxins such as enterotoxin B, toxic shock syndrome toxin-1, exfoliative toxins, fibrinolysin, α, β, γ, and δ hemolysins, other phenol-soluble modulins (PSMs), leucocidin, capsular polysaccharide (type 5 and 8), serine protease, and DNase is increased (upregulated), and expression of surface proteins and biofilm formation is decreased (downregulated) by *agr* of *S. aureus* and *S. epidermidis* [9, 44]. Infection is dispersed to other surfaces by the detachment of biofilm that is caused by the upregulation of the expression of PSMs that have an important role in acute infection [1]. In chronic biofilm-associated infection of *S. aureus* high amount of QS or *psm* gene mutants are present, by the way, mutants favor compact biofilm development and biofilm/infection cannot be dispersed to other surfaces [46, 47].

The production of PIA/PNAG, PIA/PNAG-degrading enzymes, and matrix components of staphylococcal biofilm is not regulated by QS [44, 46].

Phenol-soluble modulins (PSMs) are surfactant-like staphylococcal peptides and are controlled by *agr* locus function in biofilm maturation, biofilm structuring/destructuring, dispersal, and dissemination by distruption of non-covalent interactions between biofilm matrix molecules. PSMs have a role in the pathogenesis of *S. aureus* and *S. epidermidis* biofilm-associated infections [9, 21, 46]. In contrast to soluble PSMs, PSMs that are aggregated form amyloid fibrils that contribute to stability of the biofilm [27, 41]. *S. aureus* and *S. epidermidis* catheter-related infections can be controlled by PSM surfactant-mediated QS control of biofilms for biofilm maturation and dissemination [48, 49]. The biofilm maturation is not only caused by PSM surfactants but also enzymatic degradation of biofilm matrix components by proteases and nucleases [46]. But Beenken et al. [50] revealed that nuclease did not disperse *S. aureus in vitro*. Hochbaum et al. [51] revealed that D-amino acids trigger biofilm dispersal of *S. aureus*.

Agr (AIPs) of each strain belongs to different *agr* classes of which biofilm-forming capacities and syndromes are different. Four main classes of AIPs (Agr) are present in *S. aureus* and *S. epidermidis*. *S. aureus* strains of which *agr* classes are *agr* II and *agr* III are high and medium biofilm formers due to having defective and inactive *agr*, respectively. Non-defective and active *agr* is present in *agr* I and *agr* IV strains that are weak biofilm producers [52]. *agr* IV *S. aureus* strains are more associated with exfoliative syndromes. *agr* I *S. aureus* strains are isolated from endocarditis and superficial infections. *agr* II and *agr* III *S. aureus* strains are isolated from endocarditis and nasal colonization, respectively [53]. Mortality due to *agr* II–caused infections is higher than *agr* I–caused infections [54]. The prevalences of *agr* I type among the *S. epidermidis* clinical isolates and *S. epidermidis* localized in skin flora are approximately 89% and 52%, respectively [55]. The sequences of AIPs that belong to *agr* I, II, III, and IV classes in *S. aureus* and *S. epidermidis* are YSTCDFTM, GVNACSSLF, YINCDFLL, YSTCYFTM, YNPCASYL, DSVCASYF, YNPCSNYL, YNPCANYL, respectively [55, 56].

http://dx.doi.org/10.5772/62943

To control biofilm-associated staphylococcal infections, production of virulence factors and antibiotic resistance, QS can be disrupted by inhibition of signal production, degrading signals, and suppressing synthase and receptors [9].

2.5.2. The regulation of Staphylococcal biofilm by other than Agr

2.5.2.1. sarA

Two-component regulator gene locus encoded by arlRS is regulated by *agr* and *sarA* loci. *sar*A and *agr* have opposite functions in staphylococcal global regulation. When enough quorum population is present, at the beginning of attachment phase *sar*A is upregulated. During the initial stages, SarA enhances expression of PIA, adhesions, and EPS, by the way, induces attachment and early biofilm formation. SarA also represses nuclease and protease synthesis. After attachment of bacteria, agr system works and virulence factors that cause dispersal, nucleases and proteases and PSMs are produced [18].

2.5.2.2. sigB

The *sigB* operon of which product is σ^B in *S. aureus* upregulates ica transcription, and the factors for early stages of biofilm formation including FnbpA, clumbing factor, and coagulase and downregulates factors that are efficient in dispersal and in passing to planktonic state such as β-hemolysin, enterotoxin B, serine protease (SplA), cysteine protease (SplB), the metallo-protease Aur, staphopain, and leukotoxin D [18].

2.5.2.3. ArIRS

The biofilm formation of *S. epidermidis* [57] and *S. aureus* [58] can be also regulated by ArIRS that uses TCS. The biofilm formation of *S. epidermidis* is regulated by ArIRS in ica-dependent manner, whereas in *S. aureus*, this is ica-independent manner [59]. ArlRS also plays a role in the modulation of bacterial autolysis, as a result of eDNA release that participates in biofilm matrix [9].

2.5.2.4. lytSR

LytSR operon that is the other TCS of *S. aureus* plays a role in the activity of murein hydrolase that is an autolysin and distrupt structural components of the bacterial cell wall, consequently, autolysis. *Lrg*/*cid* operon that is a target of this system regulates lysis of cell during biofilm formation [60]. The regulator LytR that is effected by stimuli bound LytS sensor histidine kinase protein activates transcription of genes under its control. The regulator LytR upregulates the expression of *lrgA* and *lrgB* genes [61]. Encoded LrgA by *lrgA* is an antiholin and inhibits the extracellular activity of murein hydrolases, whereas *cidA* gene encodes holin protein that effects the activity of murein hydrolase, consequently, cell lysis and release of eDNA that participate in biofilm matrix [9].

2.5.3. Inactivation of ica by sequences

2.5.3.1. IS256

Although *S. epidermidis* strains are *ica* positive, they cannot produce biofilm due to IS256 insertion sequence that is inserted within the *ica* operon. Ziebuhr et al.[62] revealed that if bacterial genomic DNA contained IS256, IS256 was not seen within *ica* locus. They also revealed that although *S. epidermidis* strains that caused indwelling device–associated infection was *ica* positive and the insertion of IS256 is not seen within *ica* locus, strains did not produce biofilm ("off switch") [62]. These results showed that IS256 is not a natural occurring global regulator mechanism of biofilm production. The similar results were gained for *S. aureus*. IS256 that was inserted within *ica*C gene of *S. aureus* strain prevented biofilm formation by inactivating *ica*C gene [63].

2.5.3.2. Tetranucleotide tandem repeat

*ica*C inactivation caused by the expansion or contraction of tetranucleotide tandem repeat inhibits PIA/PNAG formation in *S. aureus* [64]. The reading frame of *ica*C is shifted by tetranucleotide tandem repeat ("ttta"), and this contributes premature stop of IcaC protein, consequently, inhibited PIA/PNAG production ("off switch"). Mutated *ica*C is preferred for the indwelling device-associated infections due to off switching of PIA/PNAG production.

2.6. Treatment of biofilm

To provide protection against *S. aureus* and *S. epidermidis* biofilm-associated infections vaccine that causes production of antibodies against PNAG and PSM peptides can be used. Researchers had revealed that mutant *S. aureus* of which icaB is over-expressed and produces high amount of surface associated PNAG was more opsonized by antibodies and undergoes to phagocytosis. But immune response is ineffective antibodies produced against PIA/PNAG of vaccine bind secreted PIA/PNAG of bacteria rather than surface-associated PIA/PNAG of bacteria [65]. Conjugate vaccine that contains *S. aureus* PNAG and clumping factor A can accelerate immune response [66]. Bacterial dispersal from indwelling medical devices can be prevented by antibodies against PSM peptides [48]. Brady et al. [67] had treated chronic osteomyelitis with a combination of antibiotic and quadrivalent vaccine that contains four antigens, which are glucosaminidase, an ABC transporter lipoprotein, a conserved hypothetical protein, and a conserved lipoprotein. By this way, Brady et al. [67] had reduced biofilm formation of *S. aureus* on infected tibias.

Kaplan et al. [68] and Whitchurch et al. [69] concluded that DNase I in human serum can degrade eDNA in biofilm matrix, by the way bacterial biofilms are degreased.

Nitric oxide (NO) that is a product of anaerobic respiration can cause dispersal of microorganism from mature biofilm by stimulation of c-di-GMP phosphodiesterases activity [70]. c-di-GMP biosynthesis inhibitors can be an alternative treatment for preventing biofilm formation and mature biofilm dispersal. The combinations of dispersin B (EPS-degrading

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enzymes) and disinfectants such as triclosan with antibiotics that are used in the treatment of wound and skin infections provides synergistic removal of biofilms [71].

3. The mechanisms of antibiotic resistance in biofilm-embedded microorganism

Biofilm-embedded bacteria are more resistant to antimicrobial agents than planktonic bacteria. It is difficult to eradicate biofilm, and this causes serious clinical problem [72].

Antibiotic resistance (tolerance) that is caused by biofilm and permit bacteria to survive is a physiological state by which mutational changes not caused [73]. Impermeability of peptidoglycan by efflux pumps, antibiotic-degrading enzymes, the charge of polymers [73], and certain gene products that are produced in biofilms [3] are the other antibiotic resistance mechanisms of bacteria rather than the biofilm [3]. Biofilm can gain higher antibiotic tolerance by antibiotic degrading enzymes such as beta-lactamases, efflux pumps, and certain gene products of which expression are changed by the quorum sensing as a stress response [3, 74]. Biofilms resist to beta-lactam antibiotics by beta-lactamases. Beta-lactamases that are produced by bacteria play a key factor in the biofilm caused resistance to beta-lactam antibiotics [3].

3.1. The heterogeneous sessile community and the physiology of biofilm

Biofilm-embedded sessile community has heterogeneous cells that are in the different growth states. Bacterial growth rate is reduced by stress conditions such as nutrient and oxygen limitation at the lower parts of the biofilm, and low metabolic activity. Low metabolically active cells (slow growing cells) are seen at the deeper parts of the biofilm, whereas high metabolically active cells (rapid growing cells) are seen at the surfaces of the biofilm. These heterogeneous cells that consist of low and high metabolically active cells have wide range of different responds to each antimicrobial. Antibiotic penetration through the biofilm is reduced by reduced bacterial growth rate. The biofilm-related resistance mechanisms such as oxygen limitation and low metabolic activity, reduced antibiotic penetration through the biofilm, and gaining genetic adaptations such as increased changes in the genes of the DNA repair systems play a key factor in the biofilm tolerance to antibiotics [3]. But some antibiotics such as colistin are just effective against slow-growing cells seen at the deeper parts of the biofilm not against rapid growing cells that acquired adaptive resistance by upregulation of the LPS-modification (arn) operon [75]. Persister cell population that is present in the biofilms of *S. epidermidis* can withstand to inhibitory concentrations of antibiotics [76] (**Figure 3**).

3.2. Nutrient limitation

Some researchers demonstrated that nutrient limitation-related antibiotic resistance is not due to the reduced growth rate of microorganism, but rather to the activation of regulated stress responses. Nutrient limitation-related antibiotic resistance is controlled by complex regulato-

ry pathways [77]. During starvation, the activation of the stringent response participates in antibiotic resistance such as fluoroquinolone resistance in *E. coli* biofilms [23]. Also, some researchers demonstrated that certain efflux pumps in *P. aeruginosa* are upregulated in the low-oxygen conditions [78] (**Figure 3**).

3.3. Biofilm matrix

Usually, the decreased antibiotic penetration through the biofilm is caused by antibiotics that may bind to the structural contents of biofilm matrix [3] rather than reduced diffusion of antibiotics through the biofilm matrix [10] (**Figure 3**).

3.4. Agr expression

Antibiotic susceptibility of biofilm-embedded bacteria decreases according to the planktonic state. The virulence of *agr* defective strains is lesser than the wild type. Expression of *agr* that imposes a fitness cost on *S. aureus* effects drug resistance of staphylococcal biofilm. It has been revealed that RNAIII production (provides fitness cost of bacteria) of *agr*-positive bacteria is induced by subletal doses of ciprofloxacin, mupirocin, and rifampin [79]. The adaptability of *S. aureus* to antibiotics involves the *agr* locus. *S. aureus* resists to drugs by adapting to antibiotics with *agr* locus. Ciprofloxacin, mupirocin, and rifampin are more effective against *agr*-defective bacteria. These antibiotics just must be used in *agr*-deficient mutants or *agr*-negative *S. aureus* when designing antimicrobial chemotherapy. *agr*-defective strains are isolated frequently in hospital-acquired *S. aureus* (HA-*S. aureus*) infections. Due to broad antibiotic usage in hospitals, the prevalence of agr-defective strains among hospital-acquired *S. aureus* infections is high and ranges between 15% and 60% [80].

Agr expression of biofilm producer staphylococcus has also been associated with the drug resistance of some antibiotics. It has been also revealed that the effect of rifampin against *agr*-defective *S. aureus* mutants was increased, whereas the effect of oxacilline unchanged [79]. *agr* negative or *agr* dysfunction strains have a fitness advantage over *agr* positive strains in the presence of some antibiotics such as vancomycin. Vancomycin susceptibility is reduced in VISA (vancomycin-intermediate *S. aureus*) due to the thickening of cell wall that is the result of the combination of cell wall biosynthesis activation and decreased autolytic activity. *agr* mutations have been correlated with the rise of VISA. *agr* defects that reduce autolysis decrease susceptibility of vancomycin of VISA [81].

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