J Infect Chemother 21 (2015) 55–61

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Original article

Effect of incubation atmosphere on the production and composition of staphylococcal biofilms

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ARTICLE INFO

Article history:
Received 13 August 2014
Received in revised form
19 September 2014
Accepted 2 October 2014
Available online 31 October 2014

Keywords:
Biofilm composition
Staphylococcus aureus
Staphylococcus epidermidis
Poly-β (1,6)-*N*-acetyl-$_D$-glucosamine
Extracellular DNA
Microbial surface components recognizing adhesive matrix molecules

ABSTRACT

Staphylococcus aureus and *Staphylococcus epidermidis* are pathogenic bacteria that often cause invasive infections in humans. In this study, we characterized the composition and growth characteristics of staphylococcal biofilms under various incubation atmospheres. We assessed the effect of incubation atmosphere (aerobic, 5% CO_2, anaerobic, and microaerobic) on the biofilm production capabilities of *S. aureus* strains isolated from healthy volunteers and from patients with catheter-related bloodstream infection. In addition, the composition of *S. aureus* and *S. epidermidis* biofilms was determined by assessment of biofilm degradation after treatment with DNase I, proteinase K, and dispersin B. The strains obtained from healthy volunteers and patients showed similar biofilm formation capabilities. Biofilms of *S. aureus* were rich in proteins when developed under ambient atmospheric conditions, 5% CO_2, and microaerobic condition, whereas *S. epidermidis* biofilms contained large amounts of poly-β (1, 6)-*N*-acetyl-$_D$-glucosamine when developed under ambient atmospheric conditions and microaerobic condition. The biofilm-producing capability of *S. epidermidis* was considerably higher than that of *S. aureus* under aerobic condition. Staphylococcal isolates obtained from healthy individuals and patients with catheter-related infections have similar biofilm-forming capabilities. Under microaerobic conditions, *S. aureus* and *S. epidermidis* form protein-rich and poly-β (1, 6)-N-acetyl-D-glucosamine-rich biofilms, respectively. These components may play an important role in the development of biofilms inside the body and may be the target molecules to prevent catheter-related infections caused by these organisms.

1. Introduction

Patients with indwelling venous catheters for supplementary administration of fluids, electrolytes, nutrients, or antimicrobial agents have increased risk of catheter-related bloodstream infection (CRBSI), which can sometimes be fatal. *Staphylococcus epidermidis* and *Staphylococcus aureus* are frequently found on the human epidermis and in the nasal cavity of healthy individuals [1] and are the major causative agents of CRBSI and other nosocomial infections. Bacterial biofilms cannot be readily eliminated by host immune defense mechanisms (leukocytes, antibodies, and complement system) [2,3]. These biofilms also have increased resistance to antimicrobial agents [4,5].

Biofilm formation begins with the attachment of bacteria to surfaces of indwelling medical devices. Bacteria multiply and form complex biofilm structures. Previous research has shown that staphylococcal biofilms are comprised of DNA, protein, and polysaccharides [6–8]. Furthermore, the DNA component of the biofilms may be a byproduct of cell lysis, and it is suggested that genes such as *cidR*, *cidA*, *lrgA* and *lrgB* may play a role in this lysis [9–11]. Proteins in staphylococcal biofilms include accumulation-associated protein, biofilm-associated protein, and fibronectin-

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http://dx.doi.org/10.1016/j.jiac.2014.10.001
1341-321X/

binding protein, and collectively, the biofilm-related proteins are referred to as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [6,7,12–14]. Previous studies have indicated that many MSCRAMMs are regulated by the accessory gene regulator during quorum sensing [15–17].

Polysaccharides constitute a major part of staphylococcal biofilms and are comprised of teichoic acids and poly-β (1, 6)-*N*-acetyl-D-glucosamine (PNAG) [18–22]. Studies have suggested that synthesis of PNAG is regulated by the *icaADBC* operon that encodes polysaccharide-synthesizing enzymes [19–21]. More than 60% of *S. aureus* strains have the *ica* operon [23–25]. Compared to the wild-type strains, *icaA*-deficient mutants exhibited decreased pathogenesis *in vivo* [26]. Therefore, biofilm-associated PNAGs may contribute to biofilm pathogenicity [8]. In *Staphylococcus* spp., genes involved in the production of these biofilm components are regulated by the sigma factor σ^B [27–30]; therefore, the amount of biofilm produced varies in response to environmental stressors such as D-glucose [31], salt concentration [32], ethanol [32], and high or low pH [23].

In this study, we quantitatively analyzed biofilm formation capabilities of isolates obtained from CRBSI patients and healthy volunteers to determine whether *S. aureus* strains causing CRBSI are more efficient at biofilm production than the colonizing strains. Furthermore, we analyzed the influence of gas composition on biofilm production and the biofilm composition by using three substrate-specific enzymes: DNase I, proteinase K, and dispersin B.

2. Materials and methods

2.1. Strains and culture conditions

In total, seven methicillin-sensitive *S. aureus* (MSSA) strains (No. 1, 5, 13, 14, 16, 18, and 25), five methicillin-resistant *S. aureus* (MRSA) strains (No. 3, 12, 20, 23, and 26), two methicillin-sensitive *S. epidermidis* (MSSE) strains (No. 6 and 9), 10 methicillin-resistant *S. epidermidis* (MRSE) strains (No. 2, 8, 9, 10, 11, 15, 17, 21, 22, and 24), one *Staphylococcus hominis* strain, and one *Staphylococcus haemolyticus* strain were isolated from blood samples obtained from patients diagnosed with CRBSI [33] at Nagoya University Hospital between September 2007 and January 2009. The epidemiological analysis, antibiogram data, and the sample collection location (ward) and day indicated that these strains were not related to each other. All 12 *S. aureus* strains and 12 *S. epidermidis* strains isolated in this study were used for the analysis. Additionally, 20 *S. aureus* isolates, including 10 MSSA and 10 MRSA strains, were randomly selected from our departmental library of bacterial strains, which includes 225 strains of *S. aureus* isolated from nasal swabs of healthy volunteers that were recruited in the period between 2004 and 2005 in Japan. Informed consent was obtained from all recruited volunteers. This study was approved by the Ethics Committee of Nagoya University (Approval Number 2012-0191).

Strains were pre-cultured in 5 mL of tryptic soy broth (TSB) medium (Becton Dickinson Co., Winnipeg, Canada) supplemented with 0.5% (w/v) D-glucose (Wako Pure Chemical Industries, Osaka, Japan) and 3% (w/v) NaCl (Wako). Next, each overnight bacterial culture was diluted 200-fold by using fresh TSB supplemented with 0.5% D-glucose and 3% NaCl. Diluted bacterial cultures were added to a 96-well microtiter polystyrene plate (180 μL/well) and incubated for 24 h at 37 °C. A CO_2 incubator (MCO-175, Sanyo Electric Co., Ltd., Tokyo, Japan) was used for bacterial culturing by using ambient air or ambient air supplemented with 5% CO_2 (abbreviated as 5% CO_2). Bacterial biofilm culture under microaeration (O_2: 6%–12%, CO_2: 5%–8%) and anaeration (>16% CO_2) was performed using an AnaeroPack-microaeration pack and an AnaeroPack-anaerobic pack (Mitsubishi Gas Chemical Company, Tokyo, Japan), respectively.

2.2. Biofilm formation

Amount of biofilm produced was measured as previously described [34–36]. Briefly, after the bacterial culture was cultivated for 24 h, the culture supernatant was aspirated and discarded, and the adherent biofilm was gently washed thrice with sterile deionized water. The wells were dried for 10 min at room temperature. Biofilm in each well was then stained with 200 μL of 0.25% safranin solution with 0.5% ethanol for 15 min. The wells were washed with sterile water three times and dried. Absorbance of biofilms (nine spots/well) was measured at 492 nm by using a plate reader (Powerscan4, DS Pharma Biochemical, Osaka, Japan), and the average absorbance value of nine spots was obtained from five wells for each strain (45 spots/strain) in a single experiment. The amount of biofilm produced by each strain was calculated by determining the average of three absorbance values obtained by three independent experiments.

2.3. Analysis of biofilm composition

Enzymes used in this study were as follows: DNase I (Sigma–Aldrich, St. Louis, MO; 100 μg/mL), proteinase K (Sigma–Aldrich; 100 μg/mL), and dispersin B (Kane Biotech, Winnipeg, Canada; 40 μg/mL). The reaction buffer consisted of 20 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 1 mM $CaCl_2$ and was used for all digestion assays. Biofilms formed by 24-h incubation under the aforementioned conditions were washed once with sterile water, following which enzyme solution (100 μL) was added to each well for 90 min at 37 °C. After digestion, the biofilm was washed once with sterile water, stained with safranin, and washed again with sterile water, as described above. Buffer alone (without enzyme) was added to control sample wells containing biofilms, and these wells were incubated for the same time, along with the enzyme-added wells. To assess whether biofilms were digested by each enzyme, results were evaluated by three independent experiments. Statistical analyses were performed to analyze the differences between two groups.

2.4. Detection of icaA and icaD by PCR

Of the 10 primers used for the detection of *icaA* and *icaD* genes, the sequences of four primers have been previously reported [37]. Sequences of the remaining six primers that were designed for this study are shown in Table 1. *Ex Taq* (TaKaRa Shiga, Japan) was used for performing PCR. PCR conditions employed were as following: initial incubation for 3 min at 96 °C; followed by 38 cycles of 94 °C for 30 s, 56 °C or 52 °C for 30 s, and 72 °C for 45 s; and final elongation for 5 min at 72 °C on a T3000 Thermocycler (Biometra, Germany).

Table 1
DNA sequences of the primers.

Primer	Sequence (5′ → 3′)	Reference
icaA_forward_primer_1	TCTCTTGCAGGAGCAATCAA	[37]
icaA _reverse_primer_2	TCAGGCACTAACATCCAGCA	[37]
icaD_forward_primer_1	ATGGTCAAGCCCAGACAGAG	[37]
icaD_reverse_primer_2	CGTGTTTTCAACATTTAATGCAA	[37]
icaA_a-l_f	TGGGATACYGAYATGATTAC	This study
icaA_a-l_r	TTACGTYTTAATGCTTTTGGAAA	This study
icaD_e-cR	TCATRCTTCACGACCTTTC	This study