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Biofilm-forming ability of *Staphylococcus aureus* strains isolated from human skin

Kyeho Shin [a,c], Yuna Yun [a], Sungwon Yi [a], Hyun Gee Lee [a], Jun-Cheol Cho [a,b], Kyung-Do Suh [b], Jooyoung Lee [c], Jiyong Park [c,*]

[a] Amore-Pacific Co. R&D Center 314-1, Bora-dong, Giheung-gu, Yongin-si, Gyeonggi-Do 446-729, Republic of Korea
[b] Division of Chemical Engineering, College of Engineering, Hanyang University, Seoul 133-791, Republic of Korea
[c] Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea

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ABSTRACT

Background: *Staphylococcus aureus* produces various toxins and enzymes, and its presence can exacerbate skin conditions. Previous studies have shown that *S. aureus* is involved in skin deterioration, even in normal tissue. Biofilm strains show much greater resistance to antimicrobial agents and therefore require a much higher concentration of biocide than planktonic counterparts.
Objective: As such, alternative strategies and more effective therapeutic agents against biofilm-producing *S. aureus* in skin are of great interest. Therefore, we turned our attention to differences in 50 clinical biofilm strains isolated from human facial skin.
Methods: Based on *S. aureus* density on facial skin, we divided donors into two groups: relatively low density (LSG) and high density (HSG). In general, strong biofilm-forming strains were detected in the HSG donors. Two strains from each of the groups were submitted to gene microarray analysis to investigate expression differences and confirmed by RT-PCR.
Results: In total, 111 of 7775 genes were differentially expressed between low (SA2 and SA7) vs. high (SA10 and SA33) biofilm-forming clinical strains. These genes include already well-known as biofilm formation related genes like *icaABCD* and *lrgAB*, and newly identified genes (*sdrC*, *sspBCP*) by RT-PCR. Comparison of gene expression differences between the two groups available at NCBI Gene Expression Omnibus accession number GSE44268.
Conclusion: Our results suggest that *S. aureus* density in the skin is closely related to biofilm-forming ability, and we have identified several potential target genes that may be involved in regulating biofilm formation in situ.

1. Introduction

Staphylococcus aureus is an aerophilic, Gram-positive bacterium that leads to skin and soft tissue infections, including atopic dermatitis [1,2]. However, the influence of *S. aureus* on normal skin remains unclear. We previously investigated correlations between *S. aureus* presence on normal skin and physiological cutaneous conditions, such as hydration, sebum secretion, pH and transepidermal water loss [3]. That study showed that *S. aureus* colonization correlates with the deterioration of normal healthy skin, in addition to its effects on damaged skin.

Biofilms are commonly associated with many diseases of human and can form on virtually any surface [4]. Moreover, they are linked to many persistent and chronic bacterial infections [5]. Biofilm formation begins with the attachment of planktonic microorganisms to a surface [6], and if they are not immediately removed, they fix themselves to the surface via cell adhesion structures and are highly resistant to antibiotics. They may present problems in many industrial and medical areas, such as contamination of drinking or cooling water, oil recovery, food processing, paper manufacturing and medical implants. Biofilm has been studied for a wide range of scientific disciplines including biomedicine, water engineering and evolutionary biology [7].

The formation of a mature biofilm involves a regulatory cascade that controls temporal and spatial gene expression [8]. To

Abbreviations: BF, biofilm; cfu, colony-forming units; CV, crystal violet; Glc, glucose; HSG, high density of *S. aureus* detected group; LSG, low density of *S. aureus* detected group; ORF, open reading frame; TSB, tryptic soy broth.

* Corresponding author.
E-mail address: foodpro@yonsei.ac.kr (J. Park).

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understand this process, comparative studies have been performed by systematic screening for overexpressed bacterial genes or open reading frames (ORF) that may affect biofilm development. An ardent search for factors involved in biofilm development has revealed the existence of a group of surface proteins with sequence similarities to the *S. aureus* biofilm-associated protein [9]. Studies by Davies and Geesey [10] examined *algC* expression, a gene required for alginate biosynthesis, using techniques that permit the concurrent monitoring of attachment and *algC* gene expression within individual cells throughout the process of biofilm development in *Pseudomonas aeruginosa*. Moreover, it was demonstrated that the expression of a substantial fraction of the *Escherichia coli* genome (approximately 38%) is altered during growth in a biofilm vs. planktonic mode [11]. Using microtiter plates, gene expression in planktonic- vs. biofilm-grown cells was examined in 885 strains carrying random Mu dX transposon insertions. These findings have provided new insights into biofilm development and physiology. In this study, we expand what is known about biofilm regulation by investigating the correlation between *S. aureus* density on facial skin and biofilm-forming ability, and we seek to identify biofilm formation-related genes by gene expression analysis using microarrays.

2. Materials and methods

2.1. Biofilm formation assay

Bacterial strains and culture condition. In total, 50 *S. aureus* strains isolated from left cheek, right cheek and forehead of 13 volunteers which have normal healthy skins confirmed by dermatological scientists were investigated. Forehead was chosen since it is typical oily part of face and cheeks represented dry skins [12]. Density of *S. aureus* in skin was measured using selected agar plate (30 mm × 30 mm), easy stamp (Hanil comed, Korea). We stamped the easy stamp on the forehead and cheeks then counted the positive red colony after 48 hours incubation at 35 °C. The subjects, *S. aureus* detected less than 4 colonies in an agar plate, are classified as LSG and more than 4 colonies detected subjects are named HSG. 17 strains were isolated from 8 low *S. aureus* detected group (LSG) volunteers, and 33 strains were isolated from 5 high *S. aureus* detected group (HSG) subjects. Even *S. aureus* isolates detected from same subjects, the strains and biofilm formation activity were not perfectly same.

The identification of all clinical *S. aureus* strains was confirmed by the automated Vitek32 microbiological identification system (Biomerieux, Inc., Marcy l'Etoile, France). Standard *S. aureus* ATCC 6538 was used as the reference strain. Stock cultures were maintained in 25% glycerol at −70 °C. All strains were cultured in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA) for 24 h at 32 °C, and the final cell concentration was approximately 10^9 colony-forming units (cfu)/mL after incubation.

2.2. Biofilm formation on polystyrene

Biofilm-forming ability was measured by determining adhesion to polystyrene microtiter plates. A slight modification of previously described methods was used [13,14]. Briefly, the wells of flat-bottomed microtiter plates (MICROTEST™ Tissue Culture Plate, 96-well, Becton Dickinson, Franklin Lakes, NJ, USA) were filled with 100 μL of TSB, with/without 1% glucose and 1% NaCl. A dilution of approximately 10^7 cfu/mL of an overnight bacterial culture (1:100 in TSB, 100 μL TSB) was added to each well. Negative control wells contained TSB only. After 48 h incubation at 32 °C, the medium was removed, and each well was washed twice with 100 μL of sterile water and then stained with 100 μL of 0.1% (w/v) crystal violet (CV) (Sigma, St. Louis, MO, USA) for 10 min. After the staining step, the wells were washed twice with 200 μL of sterile distilled water to remove excess dye. The stained adherent cells were dispersed with 100 μL of absolute ethanol. Biofilm formation was measured as the absorbance at 590 nm (OD_{590}) with a SpectraMax 190/ spectrophotometer (Molecular Devices, LLC., USA). All strains were tested in three independent experiments. We regarded 'biofilm-forming strains' are the strains which forming biofilm higher than 50% compared to *S. aureus* ATCC 6538.

2.3. Data analysis

The results were averaged after correcting for medium background. Biofilm formation of each strain was calculated from the optical density as follows:

$$\text{Biofilm formation}\,(\%) = \frac{\text{Test strain OD}_{590} - \text{negative control OD}_{590}}{\text{Positive control OD}_{590} - \text{negative control OD}_{590}} \times 100$$

The Minitab 16 software (Minitab Inc., PA, USA) was used to calculate the statistical significance of adding glucose and/or NaCl by a two-proportion test. A p-value of <0.05 was considered statistically significant.

2.4. Microarray analysis

Isolates from the 50 clinical isolates, two from LSG (strain number 2 and 7) and two from HSG donors (strain number 10 and 33) were selected. The four isolates were cultured in TSB with 1% glucose for 24 h at 32 °C to a concentration of about 10^9 cfu/mL and then the bacteria from 1 mL of each culture were harvested by centrifugation. Samples from these pellets were used for microarray analysis.

2.5. *GeneChip* S. aureus *genome array*

We monitored relative mRNA abundance in the four clinical *S. aureus* isolates using the GeneChip *S. aureus* Genome Array (GeneChip Expression, Affymetrix, Inc., USA) to study the patterns of gene expression. The arrays contain probe sets corresponding to more than 3300 *S. aureus* ORFs. Additionally, they contain probes to detect RNA of either forward or reverse orientation from more than 4800 intergenic regions. In total, 107 were used as a control to verify the test results. A total of 432 probes on the microarrays overlapped between ORFs and intergenic regions. Two samples from each strains were used for the microarray and two independent experiments were performed.

2.6. Statistical analyses

Statistical analyses were performed using the Affymetrix Command Console, R affy-package, and Expression Console 1.1 DAVID software (Affymetrix, Inc., USA) and were conducted by unpaired and two-tailed t-tests. A p-value of <0.05 was considered statistically significant.

2.7. Quantitative real-time PCR analysis

Total RNA was extracted by using Qiagen RNeasy mini kit (Qiagen Inc., USA) as manufacturer's instructions. RNA concentration was determined spectrophotometrically (NanoDrop spectrophotometer, Thermo Scientific, Fremont, USA).

cDNA was synthesized using the High Capacity Reverse Transcription Kit (Applied Biosystems, USA) and quantification was performed using the Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, USA). qPCR was conducted using the 7500