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Confocal laser microscopic observation of glycocalyx production by *Staphylococcus aureus* in vitro

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Abstract

We used a scanning confocal laser microscope to study the effects of various agents on sugar production by *Staphylococcus aureus* in vitro. *S. aureus* cells attached to coverslips in Pl–TSB (plasma:tryptic soy broth = 1:1) were stained with fluorescein isothiocyanate-conjugated concanavalin A (FITC-conA) and were more strongly stained over time. We considered that the materials that stained positive for FITC-conA consistent with *S. aureus* cells were sugars, probably glycocalyx, produced by the *S. aureus* cells. Since the cells in the stationary growth phase alone were strongly stained with FITC-conA, all *S. aureus* cells attached to the coverslips in Pl–TSB were considered to be in this phase (low growth rate). The positive staining for FITC-conA was markedly reduced when fibrin was not formed in Pl–TSB with plasmin and sucrose, and was also markedly reduced when the fibrin in Pl–TSB was destroyed with plasmin. In conclusion, the results of the present study indicate that the existence of fibrin is essential for glycocalyx production and biofilm formation of *S. aureus* cells to aid in the attachment of *S. aureus* cells in vitro, because *S. aureus* cells attached on coverslips and fibrin alone produce glycocalyx. Of the antimicrobial agents tested, sulfadiazine silver most strongly inhibited the production of FITC-conA-positive materials by *S. aureus* cells at a sub-MIC concentration. Plasmin, sucrose, and sulfadiazine silver may be useful topical applications for use on clinical dermatology for the prevention and the treatment of staphylococcal biofilms. We consider that this simple method is very useful for the detection of *S. aureus* glycocalyx on dermatology field.

Keywords: Confocal laser microscope; *Staphylococcus aureus*; Glycocalyx; Concanavalin A

1. Introduction

Bacteria that adhere to implanted medical devices or damaged tissues can become the cause of persistent infection. These bacteria encase themselves in a hydrated matrix of polysaccharides and proteins, forming a slimy layer known

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as a biofilm [1,2]. Direct microscopic examination of colonized surfaces shows the dense aggregates of bacteria held together by diffuse extracellular polymers [2]. Exopolysaccharides are produced not only by mucoid strains, but also by all tested nonmucoid strains of *Staphylococcus aureus* [3]. Various terms such as glycocalyx, slime, or exopolysaccharide have been used to designate the biofilm produced by bacteria [4]. While the relationship between glycocalyx, slime, and exopolysaccharides is unclear [4], the term glycocalyx is general and also refer to bacterial slime and exopolysaccharides [5]. Products of the intercellular adhesion (ica) operon in *S. aureus* synthesize a linear β-1,6-linked glucosaminylglycan. This extracellular polysaccharide mediates bacterial cell–cell adhesion and is required for biofilm formation, which is thought to increase the virulence of *S. aureus* in associated with prosthetic biomedical implants [6,7]. *S. aureus* forms a fibrin-rich biofilm in the presence of plasma which is highly resistant to the immune system and conventional chemotherapy [8]. *S. aureus* can grow in a broth containing 70% sucrose, but plasma coagulation by *S. aureus* is inhibited in such conditions, which create high osmotic pressure [9]. The real structure of the bacterial glycocalyx can only be determined using a confocal laser scanning microscope (CLSM) and after the glycocalyx has collapsed during dehydration to produce clectron-dense accretions [10]. Concanavalin A (conA) is using as a marker for detecting slime on staphylococcal cells and its mechanism is due to the reaction of conA and mannose [11]. However, there are no reports of glycocalyx detection associated with *S. aureus* using CLSM. In the present study, we analyzed glycocalyx production by *S. aureus* cells in vitro and the influence of various agents on its production using CLSM for the purpose of clinical use on dermatology.

2. Materials and methods

2.1. Bacterial strains

We employed four strains of *S. aureus* cells: the protein A-deficient mutant, designate the C7 strain, isolated from the Cowan 1 strain [12,13]; the N strain (mec A gene positive, coagulase type IV and enterotoxin A producer) isolated from furunculosis; the AD strain (coagulase type VII producer) isolated from infected skin lesions associated with atopic dermatitis; and the 209 P strain. The C7 strain was used for the study of the anti-*S. aureus* antibody. The N and AD strains were used for the study of sugar production and the influence of various antimicrobial agents on its production, because they were the strains isolated from skin lesions. The 209P strain was used for the study of sugar production and the influence of agents other than antimicrobial agents on its production, because it was the only strain that coagulated plasma after incubation for 6 h among the four strains. The MICs for the N and AD strains using oxacillin (MPIPC; Sigma, St. Lois, MO) were 32 and 0.06 μg/ml, respectively.

2.2. Laser microscope

Images were obtained with an LSM 510 confocal microscope (Carl Zeiss Co, Ltd, Germany) in conjunction with an Axioplan 2 MOT microscope (Carl Zeiss). The photomicroscope was equipped with a $100\times$, 1.3-numerical aperture (NA) oil immersion, phase-contrast lens. When used in conjunction with confocal laser technology, the high-NA lens has the potential to produce images with sub-200-nm horizontal resolution in the xy axis and with reduced defocused information from xz materials. The scanning control and the image processor were housed in an IBM PC/AT-compatible desktop computer. An argon laser with maximum emission lines at 488 nm was used as the excitation source for the fluorescent probes. All measurements were made through the following absorbing filter set: 585 nm$<$ in the case where safranine was used; 505–530 nm in the case where fluorescein isothiocyanate-conjugated concanavalin A (FITC-ConA) and FITC conjugated immunoglobulin (Ig) were used.

2.3. Bacterial suspension for inoculation

The *S. aureus* strains were grown on Mueller–

Hinton (MH; Difco, Detroit) agar plates at 37 °C overnight. The bacterial cells were suspended in 0.01 M phosphate buffered saline (PBS; pH 7.4) and harvested by centrifugation at 6000 × *g* for 10 min at 4 °C. They were then resuspended in PBS and centrifuged, as described above. The process was repeated three times. The washed bacteria were resuspended in tissue culture dishes (35 × 10 mm^2 style; Becton Dickinson, New Jersey) and polypropylene microcentrifuge tubes (1 ml; Iuchi BioSystems, Tokyo) containing 10^8 cfu/ml and were used in the following experiments.

2.4. Observation of the C7 strain

Cell suspensions of the C7 strain (the protein A-deficient strain) were inoculated into 2 ml of tryptic soy broth (TSB; Nissui Pharmaceutical Co., Tokyo) on 1.0 cm^2 coverslips (Sumitomo Bakelite, Tokyo). Because the C7 strain could not attach on coverslips in plasma after incubation for 3 h, we used TSB in this experiment. After incubation for 3 h at 37 °C, the coverslips were washed three times by PBS for 5 min at room temperature and immersed in 0.1% safranine (pH 7.4; Wako Pure Chemicals Co., Kyoto) for 5 min. After washing by PBS, the coverslips were immersed in 1% bovine serum albumin/PBS for 30 min, and then immersed in × 40 anti-*S. aureus* antibody (mouse IgM: Cosmo Bio Co., Tokyo) for 60 min. After washing by PBS, the coverslips were immersed in × 100 FITC-labelled anti-mouse IgM antibody (rat IgG: Cosmo Bio) for 60 min. After washing by PBS, the coverslips were embedded using prolong™ antifade kit (Molecular Probes, Leiden, The Netherlands) and were observed using a laser microscope.

2.5. Observation of glycocalyx production of the 209P strain, N strain, and AD strain

Cell suspensions of the 209P strain, N strain, and AD strain were inoculated into 2 ml of Pl–TSB (rabbit plasma (Denka Seiken, Tokyo):TSB = 1:1) on 1.0 cm^2 coverslips. After incubation for 3 and 6 h at 37 °C, the coverslips were washed by PBS and immersed in 0.1% safranine for 5 min. After washing by PBS, the coverslips were immersed in FITC-ConA (50 μg/ml) for 5 min at room temperature. After washing by PBS, the coverslips were embedded and were observed using a laser microscope.

2.6. Effect of a sub-MIC concentration of various antimicrobial agents against glycocalyx production

Cell suspensions of the N strain and the AD strain were inoculated separately into 2 ml of Pl–TSB with 1/4 MIC each of cefdinir (CFDN), imipenem (IPM), fosfomycin (FOM), roxithromycin (RXM), minocycline (MINO), ofloxacin (OFLX), and sulfadiazine silver (SD–Ag) on 1.0 cm^2 coverslips. After incubation for 6 h at 37 °C, the coverslips were immersed in safranine and FITC-conA, and were observed as described above.

2.7. Effect of plasmin and sucrose against glycocalyx production

Cell suspensions of the 209P strain cells were inoculated separately into 2 ml of Pl–TSB with 1 Sigma unit (≈ 3 WHO units)/ml of plasmin (fibrinolysin; Sigma) and 70% sucrose (Kowa, Tokyo) on 1.0 cm^2 coverslips. After incubation for 6 h at 37 °C, the coverslips were immersed in safranine and FITC-conA, and were observed as described above. The use of 1 Sigma unit/ml of plasmin did not influence the growth rate of the 209P strain in TSB after incubation for 18 h at 37 °C.

2.8. Observation of cells in the exponential growth phase and the stationary growth phase, and the floating cells

Cell suspensions of the 209P strain cells were inoculated into 2 ml of TSB. After incubation for 3 and 24 h at 37 °C, cell suspensions washed by PBS were inoculated into 2 ml of Pl–TSB on 1.0 cm^2 coverslips. After incubation for 3 h at 37 °C, the coverslips were immersed in safranine and FITC-conA, and were observed as described above. We considered that bacterial

cells at 3 and 24 h pre-incubation in TSB were in the exponential growth phase and the stationary growth phase, respectively.

Cell suspensions of the 209P strain cells were also inoculated into 2 ml of Pl–TSB without coverslips. After incubation for 3 h at 37 °C, bacterial cells were immersed in safranine and FITC-conA, and were observed as described above. We considered that bacterial cells after 3 h incubation in Pl–TSB without coverslips were the floating cells.

2.9. Effect of plasmin and amylase against fibrin clot

Cell suspensions of the 209P strain cells were inoculated into 2 ml of Pl–TSB on 1.0 cm^2 coverslips. After incubation for 6 h at 37 °C, plasma coagulation was observed around the coverslips. The coverslips with fibrin clots were immersed separately in 2 ml of Pl–TSB with or without 1 Sigma unit/ml of plasmin for 18 h at 37 °C. The coverslips with fibrin clots were also immersed in 2 ml of PBS with or without 0.1% amylase (Sanko, Tokyo) for 1 h at 37 °C. The coverslips were immersed in safranine and FITC-conA, and were observed as described above.

3. Results

The staining patterns of safranine were completely consistent with those of FITC-labelled anti-mouse IgM antibody (or anti-*S. aureus* antibody) in the C7 strain.

Table 1 shows the staining patterns of FITC-conA in three *S. aureus* strains on coverslips after incubation for 3 and 6 h. Positive staining of FITC-conA was consistent with microspheres stained positive with safranine and increased strongly over time. Fig. 1 shows the single- and double-staining patterns of safranine and FITC-conA in the N strain on coverslips in Pl–TSB after inoculation for 3 h. Less than 50% of the cells were strongly positive with FITC-conA and more than 50% were weakly positive with FITC-conA. Plasma coagulation was observed around the coverslips in the case of the 209P strain cells alone after incubation for 6 h, but was not observed in Pl–TSB with plasmin and sucrose. Fig. 2 shows the double-staining patterns of safranine and FITC-conA in the 209P strain on coverslips in Pl–TSB after inoculation for 6 h. More than 50% of the cells were strongly positive with FITC-conA and less than 50% were weakly positive with FITC-conA. The fibril-like structures were ar-

Table 1
Staining pattern of FITC-conA in three *S. aureus* cells on coverslips

Medium for inoculation	After inoculation	Organisms
		N strain, AD strain and 209P strain on coverslips
Pl–TSB (plasma:TSB = 1:1)	3 h	3
	6 h	4 (coagulation and fibril-like structures were observed with the 209P strain)
		N strain and AD strain on coverslips
Pl–TSB with CFDN, IPM, FOM, RXM, MINO, OFLX at 1/4 MIC	6 h	2
Pl–TSB with SD–Ag at 1/4 MIC	6 h	0
		209 P strain on a coverslip
Pl–TSB with 1 Sigma unit/ml of plasmin, 70% sucrose	6 h	1 (coagulation and fibril-like structures were not observed)

Organisms were directly inoculated into Pl–TSB with coverslips from the agar plates. Valuations of staining patterns of FITC-conA. 0: negative in all cells; 1: weakly positive in less than 50% of cells and negative in more than 50% of cells; 2: weakly positive in all cells; 3: strongly positive in less than 50% of cells and weakly positive in more than 50% of cells; 4: strongly positive in more than 50% of cells and weakly positive in less than 50% of cells.

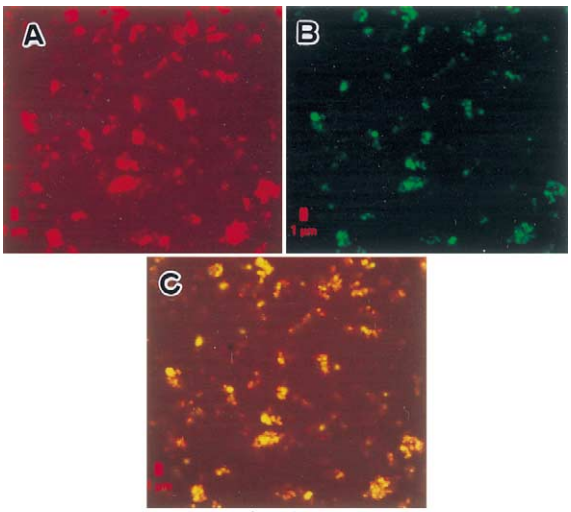

Fig. 1

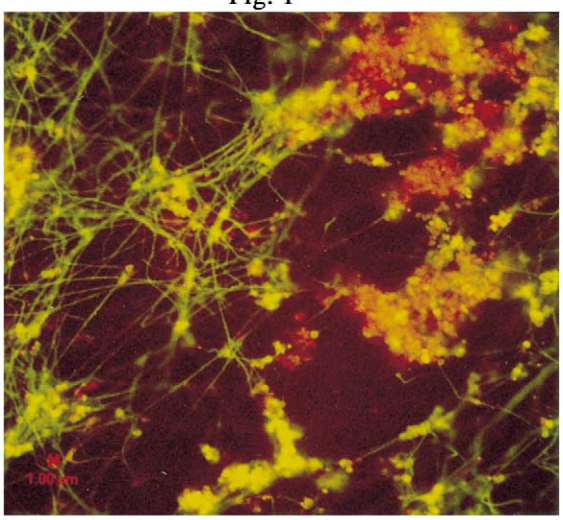

Fig. 2

Fig. 1. The staining patterns of safranine and FITC-conA in the N strain on coverslips in Pl–TSB after incubation for 3 h at 37 °C. (A) The staining patterns of safranine. The N strain cells were stained with safranine. (B) The staining patterns of FITC-conA. Less than 50% of cells were strongly positive with FITC-conA and more than 50% were weakly positive with FITC-conA. (C) The double-staining patterns of safranine and FITC-conA. The N strain cells were stained with both safranine and FITC-conA.

Fig. 2. The double-staining patterns of safranine and FITC-conA in the 209P strain on coverslips in Pl–TSB after incubation for 6 h at 37 °C. The 209P strain cells were stained with both safranine and FITC-conA. More than 50% of cells were strongly positive with FITC-conA and less than 50% were weakly positive with FITC-conA. The fibril-like structures were arranged like a spider's thread and the 209P strain cells were observed to have grown around the fibril-like structures. The staining patterns of FITC-conA of *S. aureus* cells were most strongly decreased in Pl–TSB with SD–Ag at 1/4 MIC.

Table 2 shows the staining patterns of FITC-conA in the 209P strain cells in the exponential growth phase and the stationary growth phase, and the floating cells after incubation for 3 h. All cells in the stationary growth phase alone were stained strongly positive with FITC-conA. The cells in the exponential growth phase and the floating cells attached to a coverslip in Pl–TSB did not show the positive staining of FITC-conA in this incubation hours (3 h).

Table 3 shows the staining patterns of FITC-conA in the 209P strain cells on coverslips with fibrin clots in Pl–TSB with or without plasmin after incubation for 18 h. Also shown are the patterns in PBS with or without amylase after incubation for 1 h. The staining patterns of FITC-conA in Pl–TSB with plasmin and in PBS with amylase were reduced in comparison with those in control without any of them.

4. Discussion

Bacterial biofilm formation is major concern in association with indwelling medical devices, since the biofilm provides bacteria with considerable resistance to host defenses and antimicrobial agents [7]. Susceptibility tests with in vitro biofilm models have shown the survival of bacterial biofilms after treatment with antibiotics at concentrations hundreds or even a thousand times the MIC of bacteria measured in a suspension culture [2].

Because the staining patterns of safranine were completely consistent with those of FITC-labelled anti-mouse IgM antibody (or anti-*S. aureus* anti-

Fig. 2. (*Continued*)
were observed to have grown around the fibril-like structures. The colors of microcolonies stained with safranine alone, safranine and FITC-conA (weakly positive), and safranine and FITC-conA (strongly positive) were red, orange, and yellow, respectively.

Table 2
Staining pattern of FITC-conA in the 209P strain cells

Medium for inoculation	After inoculation	Organisms		
		On coverslip		In Pl–TSB
Culture conditions		Exponential growth phase[a]	Stationary growth phase[b]	Floating cells[c]
Pl–TSB	3 h	0	5	0

[a] Organisms were inoculated into Pl–TSB with coverslips after pre-incubation in TSB for 3 h.
[b] Organisms were inoculated into Pl–TSB with coverslips after pre-incubation in TSB for 24 h.
[c] Organisms were directly inoculated into Pl–TSB without coverslips from the agar plates.

body), the *S. aureus* cells were considered to be stained with safranine. Asynchronous populations contain organisms in all phase of growth. Growth rate differences have been indicated as a possible cause of susceptibility change and the hydrophobicity of the cell surface [14]. Pöhlmann-Dietze et al. reported that *S. aureus* Newmann cells from the exponential growth (2.5 h) phase were more adherent to human endothelial cells than bacteria harvested in the stationary growth phase (14 h) [15]. Because cells in the stationary growth phase alone were strongly stained with FITC-conA (Table 2), all *S. aureus* cells attached on coverslips were considered to be in the stationary growth phase (low growth rate).

Because lectins are plant proteins with high affinity for specific sugar residues, they are used as probes to detect these residues. For example, conA is known to specifically bind to α-mannose (2-epimer of D-glucose) [16]. *S. aureus* glycocalyx is an extracellular polyanionic biofilm containing sugars, and other components [5]. Baselga et al. reported that more than 80% of *S. aureus* bacterial cells produce exopolysaccharides after in vivo growth and that the production decreases to less than 10% when bacteria are grown in regular laboratory media [3]. Takahasi et al., using gas–liquid chromatography, determined that sugars in glycocalyx produced by methicillin-resistant *S. aureus* (MRSA) consist of mannose and glucose [17]. Amylases are a group of hydrolases that split complex carbohydrates constituted of α-D-glucose units linked through the carbon atoms 1 and 4 located on adjacent glucose residues [18]. Buxton et al., using scanning electron microscopy (SEM), reported that *S. aureus* glycocalyx tends to form globe-shaped microspheres consistent with *S. aureus* cells [5]. The appearance of *S. aureus* glycocalyx as visualized by SEM [5] was consistent with the FITC-conA positive materials observed in the present study. On the basis of the binding-character of conA to α-mannose and the reduction in positive staining of FITC-conA after treatment with amylase, we concluded that the materials stained positive for FITC-conA consistent with *S. aureus* cells were sugars, probably glycocalyx, which were produced by the *S. aureus* cells. We thought that

Table 3
Staining pattern of FITC-conA in the 209P strain cells on coverslips with fibrin clots

Medium for inoculation	After inoculation	Organisms on coverslips
Pl–TSB with 1 Sigma unit/ml of plasmin	18 h	1 (coagulation and fibril-like structures were not observed)
Pl–TSB alone (control)	18 h	4 (coagulation and fibril-like structures were observed)
PBS with 0.1% amylase	1 h	1 (fibril-like structures were observed)
PBS alone (control)	1 h	4 (fibril-like structures were observed)

Organisms attached on coverslips with fibrin clots after incubation for 6 h were directly inoculated into Pl–TSB and PBS. Valuations of staining patterns of FITC-conA. 0: negative in all cells; 1: weakly positive in less than 50% of cells and negative in more than 50% of cells; 4: strongly positive in more than 50% of cells and weakly positive in less than 50% of cells; 5: strongly positive in all cells.

reaction of conA and mannose was monitored as a marker for detecting *S. aureus* glycocalyx in this study. We previously reported that SD–Ag killed *S. aureus* cells in fibrin clots [9]. In the present study, we found that, of the antimicrobial agents tested at the sub-MIC levels, SD–Ag most strongly inhibited the FITC-conA-positive materials produced by *S. aureus* cells.

Fibrin is split by plasmin, a serine protease that hydrolyzes peptide bonds in the triple-stranded connector rod regions [19]. The 209P strain cells that were adhered to the coverslips in presence of plasma were often associated with the fibril-like structures (Fig. 2). The fibril-like structures around the 209P strain shown in Fig. 2 were suggested to be fibrin on the basis of the staining patterns of fibrin formed by the culture–supernatant of the 209P strain acquired using by sterile syringe filter (data not shown), the appearance of the fibrin in SEM [20], and the destruction observed in the presenc of plasmin (Table 3).

The presence of a fibrin-rich biofilm is a well-known factor responsible for prolonging *S. aureus* infections. The biofilm of *S. aureus* is reinforced with fibrin fibers, making it more resistant to physical effects than are other bacterial biofilms [8]. We previously reported that the attachment of *S. aureus* cells to coverslips, the conversion of fibrinogen to fibrin, and the production of glycocalyx by *S. aureus* cells are minimum requirements for the production of an biofilm on coverslips [21]. If plasma coagulation does not occur in the presence of plasmin and sucrose (Table 1), the formation of fibrin-rich biofilms by *S. aureus* will probably be inhibited. The positive staining for FITC-conA was markedly reduced when fibrin was not formed in Pl–TSB with plasmin and sucrose (Table 1), and was also markedly reduced when fibrin in Pl–TSB was destroyed with plasmin (Table 3).

In conclusion, the results of the present study indicate that the existence of fibrin is essential for glycocalyx production and biofilm formation of *S. aureus* cells to aid in the attachment of *S. aureus* cells in vitro, because *S. aureus* cells attached on coverslips and fibrin alone produce glycocalyx. Plasmin, sucrose, and SD–Ag may be useful topical applications for use on clinical dermatology for the prevention and the treatment of staphylococcal biofilm. Now we are detecting *S. aureus* glycocalyx on various infected skin lesions such as impetigo contagiosa and atopic dermatitis in this method (data not shown). So, we consider that this simple method is very useful for the detection of *S. aureus* glycocalyx on dermatology.

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