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# Biofilm Formation and Cell Surface Properties of *Staphylococcus aureus* Isolates from Various Sources

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**Abstract** This study investigated biofilm formation, cell surface hydrophobicity, colony spreading, and slime production for 112 *Staphylococcus aureus* strains isolated from various sources (leaf vegetables, pea leaf, perilla leaf, *Kim-bab*, person, and animal). When biofilm formation was classified by origin, *S. aureus* isolated from animal origin showed a significantly higher level of biofilm formation than others ($p \leq 0.05$). When *S. aureus* groups with different levels of biofilm formation (very strong, strong, moderate, and weak) were evaluated for the correlation with cell surface properties, there was a positive correlation between biofilm formation and hydrophobicity ($r$=0.926). Biofilm formation and colony spreading on tryptic soy broth (without dextrose) also showed positive correlation ($r$=0.863). In contrast, biofilm formation and slime production were negatively correlated ($r$= −0.973). Based on these results, the biofilm forming ability of *S. aureus* differs depending on their origin and might be affected by cell surface properties such as cell surface hydrophobicity.

**Keywords:** *Staphylococcus aureus*, biofilm, cell surface properties, hydrophobicity, correlation

## Introduction

*Staphylococcus aureus* is a non-flagellated, gram-positive pathogen that causes numerous diseases, including suppurative wound infections, meningitis, and sepsis (1). *Staphylococcus* spp., especially *S. aureus* is considered as one of the world's leading causes of foodborne diseases (2). *S. aureus* can survive under strong stress conditions (e.g. dryness, high pressure, and high salt concentration) for long periods of time (3). Previous study reported that foodborne illness by *S. aureus* can be caused by cross-contamination under these high stress conditions (4).

Recently, biofilm formation has received considerable attention from the food industry. Biofilm was recognized as highly ordered cellular structures of bacteria formed through irreversible attachment, proliferation, and production of an extracellular polymeric substance (EPS) on solid surfaces (5). EPS is composed of polysaccharides, proteins, extracellular DNA (eDNA) and phospholipids. EPS facilitates the adhesion of microorganisms to surfaces, and protects them from antimicrobial agents. Therefore, when bacterium forms the biofilm, they become highly resistant to heat, desiccation, acidic conditions, high salt concentrations, antibiotics and other food preservatives (6). Many studies have demonstrated that gram-positive bacteria are capable of adhering to and forming biofilm on food-contact surfaces such as polystyrene, glass, stainless steel (7). Among gram-positive bacteria, *Listeria monocytogenes*, *S. aureus*, and *Bacillus cereus* are able to easily form biofilms in food processing environments; in particular, *S. aureus* has shown a remarkable ability to attach to various surfaces (8).

Biofilm formation of *S. aureus* is influenced by a number of factors including cell surface hydrophobicity, colony spreading, and slime production. Cell surface hydrophobicity is generally associated with bacterial adhesiveness and varies from organism to organism and from strain to strain (9). Cell surface charge and hydrophobicity play an important role in initiating microbial adhesion (10). Previous study has shown that there is a positive correlation between bacterial cell hydrophobicity and their attachment to hydrophobic surfaces (11). Biofilm formation was identified to be more effective on polypropylene surfaces than on hydrophilic surfaces such as stainless steel (7). Therefore, it is important to consider the choosing materials for equipment, conveyer belts, food storage tanks, and food processing pipelines in food processing facilities. Although *S. aureus* does not have flagella, recent studies have reported that *S. aureus* can rapidly spread on soft agar surfaces and the phenomenon named as "colony spreading" (1). Also, previous studies reported that colony spreading was related to biofilm formation. For example, *Mycobacterium* spp. spread on the surface of a growth medium by sliding motility and biofilm formation has been correlated with colony spreading as motility in some mycobacteria (12). However, whether colony spreading is a mechanism involved in regulating biofilm formation and virulence in *S. aureus* is still unknown. The capacity for a

pathogen to form a biofilm may also correlate with its ability to produce slime, a substance primarily composed of polysaccharide intercellular adhesin (PIA) (13). Christensen *et al.* (14) found that specific strains of coagulase-negative staphylococci (CNS) coated the walls of culture tubes with a viscid film of bacteria or "slime". Previous studies aimed at identifying slime-producing bacterial strains were based on the ability of bacterial isolates to adhere to and grow on plastic surfaces, forming detectable biofilms (15). While the correlation between slime production and biofilm forming properties of bacteria remains unclear, slime detection using Congo red agar (CRA) is commonly used as a method to assess *S. aureus* isolates for their ability to generate biofilms (16).

As previously mentioned, there are many properties implicated in biofilm formation. But, a clear correlation between the cell surface properties of *S. aureus* and its biofilm forming ability has not been established. Therefore, this study determined the biofilm forming ability of 112 *S. aureus* isolated from various sources. Moreover, correlation between the cell surface properties (cell hydrophobicity, colony spreading, and slime production) and biofilm formation were evaluated.

## Materials and Methods

**Bacterial strains and growth conditions** One hundred and twelve *S. aureus* strains were obtained from various origins with different sources (Table 1). Stock cultures were stored at −80°C in 0.7 mL of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) supplemented with 0.3 mL of 50% glycerol. All the cultures were maintained on tryptic soy agar (TSA; Difco Laboratories, Detroit, MI, USA) slants at 4°C and subcultured monthly. *S. aureus* was incubated for 24 h at 37°C in TSB before experiments.

**Biofilm formation assay** A biofilm formation assay using the crystal violet staining method was performed (16). Individual wells of sterile 96-well polystyrene microtiter plates were filled with 90 μL of TSB and each well was inoculated with 10 μL of 112 *S. aureus* strains, respectively. Biofilms were permitted to form on the surfaces of the plate. Negative control wells containing TSB alone were included in each assay. *S. aureus* strains were incubated at 37°C for 24 h. The medium in the wells was discarded and the wells were subsequently rinsed three times with distilled water (200 μL/well). After air-drying for 30 min, the wells were stained with 50 μL of 0.5% crystal violet for 1 min. Excess cells were removed by washing three times with distilled water (200 μL/well). The optical density (OD) of each well was measured at 595 nm using a spectrophotometer (Spectronic 20 Genesys; Spectronic Instruments, Rochester, NY, USA).

**Bacterial hydrophobicity** The bacterial adhesion to hydrocarbons (BATH) assay was performed as described by Goulter *et al.* (17). The 112 *S. aureus* strains in the stationary phase were incubated for 24 h at 37°C, collected by centrifugation (10,000×*g* for 10 min), and resuspended in phosphate buffered saline (PBS, pH 7.2). The $OD_{595\,nm}$ of the suspension was adjusted with PBS to 1.0±0.2. The absorbance of 50 μL of each bacterial cell suspension (Ac) was measured using a spectrophotometer (Specronic 20 Genesys). Bacterial cell suspensions (90 μL) were overlaid with 30 μL of the hydrocarbon *n*-nonane, and were then added to 45 μL of the 4 M ammonium sulfate. The suspensions were vortexed for 5 min. The 96-well plate was allowed to stand at room temperature for 30 min. After the incubation, 50 μL of the lower aqueous layer was removed using a pipette, and the $OD_{595\ nm}$ was measured (Ab). The ratio of the absorbance of the experimental samples (Ab) to that of the control suspension (Ac) was used to calculate the percentage of cells bound to hydrocarbon using the following equation: cell surface hydrophobicity (%)=(Ac−Ab)/Ac×100

**Colony spreading assay** The colony spreading assay was performed

**Table 1.** Origin and source of the 112 *Staphylococcus aureus* strains used in this study

| Strains | No. | Origin | Source[1)] |
|---|---|---|---|
| R1, R2, R4, R5, R9, R10, R11 | 7 | Leaf vegetable | Rural Development Administration |
| H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, H17, H18, H19, H20, H21, H22, H23 | 23 | Pea leaf | Rural Development Administration |
| D1, D3, D4, D5, D6, D7, D8, D9, D10, D12, D13, D14, D15, D16, D17, D18, D19, D20, D22, D23, D24, D25, D26, D28, D29, D30, | 26 | Perilla leaf | Rural Development Administration |
| K13, K14, K15, K17, K18, K19, K20, K21, K23, K24, K25, K26 | 12 | *Kim-bab* | Kyungwon University |
| S198, SS25 | 40 | Person | Rural Development Administration |
| K1, K3, K4, K5, K7, K8, K9, K10, K11, K12 | | | Kyungwon University |
| C1, C2, C5, C6, C7 | | | Asan Medical Center |
| C30, C32, C34, C35, C36, C37 | | | Korea University Guro Hospital |
| C64, C65, C66, C68, C69, C80A, C71 | | | Kyeongbuk University |
| C76, C78, C79, C80B, C88 | | | Korean National Institute of Health |
| C93, C96, C98, C99, C100 | | | Korean Network of Antimicrobial Resistance |
| C50, C51, C52, C53 | 4 | Animal | Chonbuk National University |

[1)]*S. aureus* strains were obtained from the described source.

as described by Kaito and Sekimizu (1). Soft agar (0.24%, w/v) was added to TSB and TSB without dextrose, then autoclaved at 121°C for 15 min. Sterile medium (20 mL) was poured into a petri dish (80 mm diameter), and the plates were dried for 20 min in a safety cabinet. Bacterial cultures (2 μL) were spotted onto the center of the plates and dried for 10 min in a safety cabinet. The plates were covered and incubated at 37°C for 8 h. Morphology was obtained using a digital camera and morphological analysis was performed by measuring the diameter of colonies.

**Slime-producing ability onto Congo red agar (CRA)** Slime production by each strain was analyzed by cultivating the strains on Congo red agar (CRA; Sigma-Aldrich, St. Louis, MO, USA) (18). CRA plates (0.8 g/L CRA and 36 g/L saccharose to TSA) were incubated for 24 h at 37°C. Slime-producing strains form black colonies on CRA, whereas non-producing strains develop red colonies. As previously described (19), the strains were categorized as follows: very black, black, and almost black colonies with a rough, dry, and crystalline consistency were considered normal slime-producing strains, whereas smooth very red, red, and Bordeaux-colored colonies were classified as non-slime-producing strains. And, the slime-producing ability of each strain was quantified by assigning a point that corresponded to the colony color. Points were assigned as follows: very red (−3), red (−2), Bordeaux (−1), almost black (1), black (2), and very black (3).

**Statistical analysis** All experiments were performed in triplicate with duplicated sets. Results for all experiments were presented as mean±standard deviation (SD). Data were analyzed using the ANOVA procedure with Duncan's multiple comparison tests of SAS to determine the significant differences ($p \leq 0.05$).

## Results and Discussion

**Biofilm formation in *S. aureus* isolated from various origins** The biofilm forming ability of 112 *S. aureus* strains isolated from various origins (leaf vegetable, pea leaf, perilla leaf, *Kim-bab*, person, and animal) was summarized in Table 2. *S. aureus* strains were divided into four groups based on their ability to form biofilms as described by Stepanoviæ *et al.* (20): weak ($OD_{595\ nm} \leq 0.3$), moderate ($0.3 < OD_{595\ nm} \leq 0.6$), strong ($0.6 < OD_{595\ nm} \leq 0.9$), and very strong ($OD_{595\ nm} > 0.9$). The majority of *S. aureus* strains (93 of 112) were categorized as strong or moderate in their ability to form biofilms. When biofilm formation was classified by origin (Fig. 1), *S. aureus* isolated from animal origin showed a significantly higher biofilm forming ability than strains from other origins ($p \leq 0.05$). These results suggest that the ability of *S. aureus* to produce biofilm is different depending on their origin. Previous studies have also reported the effect of pathogen origin on biofilm formation. Cha *et al.* (21) evaluated the biofilm formation of several methicillin-resistant *S. aureus* (MRSA) strains. The biofilm forming capacity of MRSA was significantly different depending on the site of isolation ($p$=0.001). Similarly, biofilm formation by *L. monocytogenes* isolates of lineage I was significantly greater than that by isolates of lineage II (22). Among various serotypes (1/2a, 1/2b, 3a, 4a, and 4b) of *L. monocytogenes*, serotype 1/2a strain produced significantly higher levels of biofilm than the others (23). Mohamed *et al*. (24) identified that biofilm formation by *Enterococcus faecalis* isolated from patients with endocarditis was significantly greater than that of strains isolated from other sources (non endocarditis). These data suggest that biofilm forming ability might differ depending on the origin or serotype of the strain. Here, *S. aureus* isolated from animal origin showed significantly higher levels

**Table 2.** *Staphylococcus aureus* strains grouped according to their biofilm forming ability

| Biofilm-forming ability | Origin | | | | | | Total number ($n$=112) |
|---|---|---|---|---|---|---|---|
| | Leaf vegetable ($n$=7) | Pea leaf ($n$=23) | Perilla leaf ($n$=26) | Kim-bab ($n$=12) | Person ($n$=40) | Animal ($n$=4) | |
| Very strong ($OD_{595\ nm}$>0.9) | R5 | H1 | - | - | C7, C30 C68, C79 | C52, C51 C53, C50 | 10 |
| Strong (0.6$OD_{595\ nm}$<0.9) | R1, R2, R9, R4, R10 | H2, H7, H10, H8, H20, H11, H9, H21, H12, H3, H4, H23, H17, H13, H6, H18 | D25, D9, D23, D10, D16, D12, D7 | K21, K14, K25 | C78, C32, C80A, C64, C2, C69, C1, C65, C76 | - | 40 |
| Moderate (0.3$OD_{595\ nm}$<0.6) | R11 | H14, H5, H22, H15, H16, H19 | D22, D24, D18, D19, D15, D13, D20, D14, D17, D30, D28 | K23, K19, K13, K24, K15, K17, K26, K20, K18 | K12, C66, C99, C88, K11, K10, S198, C96, C6, C37, K9, K7, C36, C80, C93, C71, SS25, K5, C5, K8, K4, C98, C34, K3, C100, K1 | - | 53 |
| Weak ($OD_{595\ nm}$<0.3) | - | - | D5, D6, D1, D4, D26, D3, D29, D8 | - | C35 | - | 9 |

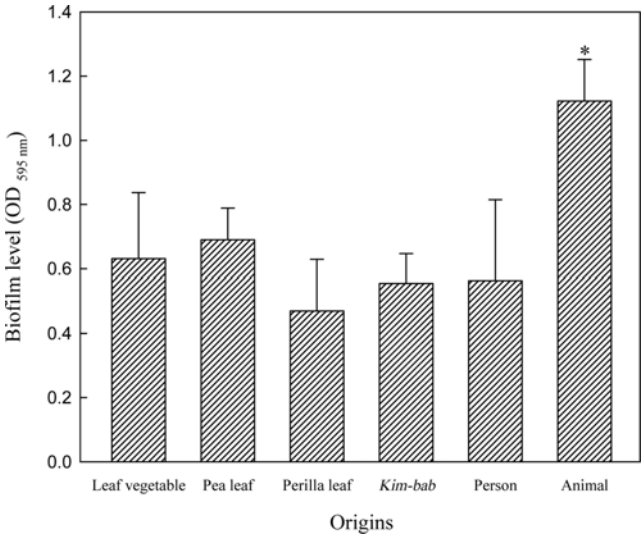


**Fig. 1.** The levels of biofilm formation in *Staphylococcus aureus* depending on their origins (leaf vegetable, pea leaf, perilla leaf, *kim-bab*, person, and animal). The results are expressed means±standard deviations obtained from triplicates. symbol (*) indicated significantly higher level of biofilm formation than other isolates from origins ($p \leq 0.05$).

of biofilm than *S. aureus* from other origins. But, only four strains of *S. aureus* from animal origin were evaluated in this study. Therefore, it would be prudent to examine additional strains of animal origin in order to confirm the effect of origin on biofilm forming ability.

**Cell surface properties of *S. aureus* and correlation between biofilm formation and cell surface properties** Cell surface properties including bacterial cell surface hydrophobicity, colony spreading, and slime production were analyzed for the 112 *S. aureus* strains (Table 3). Cell surface hydrophobicity values were expressed as the BATH%. The BATH% of the very strong biofilm forming group was 28.86± 13.11, 27.00±11.08% for the strong group, 20.59±7.70% for the moderate group, and 15.46±7.57% for the weak group. Cell surface hydrophobicity was increased according to levels of biofilm formation. Colony spreading assays indicated that *S. aureus* has the ability to spread on soft agar. When *S. aureus* was spotted onto soft agar, morphology of colony spreading was irregular (data not shown). Some colonies were spread out a lot, but other colonies were not. For colonies spreading on soft agar supplemented with dextrose, colony diameters were 0.89±0.50, 1.88±2.11, 1.22±1.17, and 0.67± 0.29 cm for the very strong, strong, moderate, and weak biofilm forming groups, respectively (Table 3). On soft agar without dextrose, colony diameters were 2.81±2.30, 2.47±2.47, 2.20±2.30, and 0.77± 1.11 cm for the very strong, strong, moderate, and weak groups, respectively. Therefore, the extent of colony spreading on soft agar without dextrose correlated with the biofilm forming ability of the group. In contrast, there was no correlation between colony spreading and biofilm forming ability when bacteria were spotted on soft agar supplemented with dextrose. Slime production of the *S. aureus* was tested using the CRA method. Color of the colonies was assessed and a corresponding numerical value representing the slime-producing ability of that strain was assigned (see Materials and Methods for details; Fig. 2). Negative values were assigned to red non-slime-producing colonies, whereas positive values were given to black slime-producing colonies (18,19). Slime production was 1.90± 1.80, 2.08±1.53, 2.34±0.94, and 2.44±0.89 (arbitrary units) for the very strong, strong, moderate, and weak biofilm forming groups, respectively (Table 3). Therefore, slime production was negatively correlated with biofilm forming ability. The cell surface properties evaluated in this study (cell surface hydrophobicity, colony spreading, and slime production) showed clear correlations with biofilm forming ability. There were, however, no significant differences in cell surface properties amongst strains within a single group ($p>0.05$).

Groups by biofilm forming ability (very strong, strong, moderate, and weak) were evaluated for the correlation with cell surface properties (cell surface hydrophobicity, colony spreading, and slime production) of each *S. aureus* strain (Fig. 3). The study found a positive correlation between biofilm forming ability, measured using crystal violet staining, and cell surface hydrophobicity, measured using the BATH assay ($r=0.926$) (Fig. 3A). Furthermore, a positive correlation was found between biofilm formation and colony spreading on soft agar without dextrose ($r=0.863$) (Fig. 3C). In contrast, supplementing the soft agar with dextrose abolished this correlation ($r=0.136$) (Fig. 3B). Lastly, there was a negative correlation between biofilm formation and slime production ($r= -0.973$) (Fig. 3D). However, although there was correlation between averages values in groups showing different biofilm forming ability and other cell surface properties, there was no significant difference of cells surface properties including hydrophobicity, colony spreading, and slime production among groups ($p>0.05$).

According to the report previously published, the biofilm forming ability of a particular bacterial strain is affected by its cell surface

**Table 3.** Cell surface hydrophobicity, colony spreading, and slime production in *Staphylococcus aureus* strains grouped by biofilm forming ability

| Biofilm-forming ability | Hydrophobicity (%) | Colony spreading (with dextrose) (cm) | Colony spreading (without dextrose) (cm) | Slime production |
|---|---|---|---|---|
| Very Strong ($OD_{595\ nm}$>0.9) ($n$=10) | 28.86±13.11[1)] | 0.89±0.50 | 2.81±2.30 | 1.90±1.80 |
| Strong (0.6$OD_{595\ nm}$<0.9) ($n$=40) | 27.00±11.08 | 1.88±2.11 | 2.47±2.47 | 2.08±1.53 |
| Moderate (0.3$OD_{595\ nm}$<0.6) ($n$=53) | 20.59±7.70 | 1.22±1.17 | 2.20±2.30 | 2.34±0.94 |
| Weak ($OD_{595\ nm}$<0.3) ($n$=9) | 15.46±7.57 | 0.67±0.29 | 0.77±1.11 | 2.44±0.89 |

[1)]Data is represented as mean±standard deviation of *S. aureus* strains grouped by biofilm forming ability.

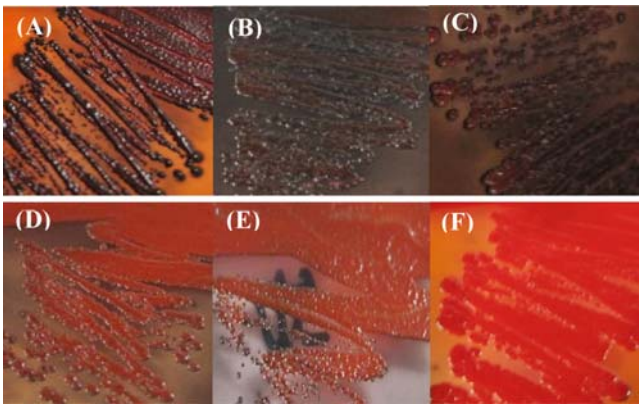


**Fig. 2.** Slime production of *Staphylococcus aureus* on Congo red agar (CRA). Slime ranges in color from black to red. Black colonies and red colonies represent slime-positive and slime-negative colonies, respectively. (A) very black, (B) black, (C) almost black, (D) bordeaux, (E) red, and (F) very red colonies were assigned numerical values of 3, 2, 1, -1, -2, and -3, respectively.

properties (25). Pasmore *et al*. (26) demonstrated that a higher surface hydrophobicity in *Pseudomonas aeruginosa* correlated well with a greater incidence of biofilm formation on the surface of ultrafiltration membrane surfaces found in water processing systems. Similarly, the attachment strength of *Salmonella* sp., *Escherichia coli*, and *L. monocytogenes* to cantaloupe rind was strongly correlated with their cell surface hydrophobicity (27). Together, this implicates cell surface hydrophobicity is correlated with strong surface attachment and the initiation of biofilm formation. On the other hand, other studies have shown a negative correlation or failed to find a correlation between the hydrophobicity of microbial strains and biofilm formation. Auger *et al*. (28) demonstrated that the hydrophobicity of *B. cereus* was not positively correlated with biofilm formation in polyvinyl chloride (PVC) microtiter plates. In addition, 12 *Streptococcus thermophilus* strains formed biofilm in dairy processing plants, but the attachment of those strains to stainless steel did not correlate with hydrophobicity (29). From these results, the correlation of bacteria between hydrophobicity and biofilm formation could be different depending on types of bacteria or experimental conditions.

Biofilm forming ability was associated with the extent of colony spreading. Diameter of colony spreading on soft agar was a commonly used measure for bacterial motility (1). O'Toole and Kolter (30) showed that cell motility (colony spreading) was necessary for microcolony formation and, therefore, required for normal biofilm development. Furthermore, bacterial motility has been related to persistence in the environment (31). In this study, the colony spreading and biofilm formation of *S. aureus* were affected by at least one environmental factor, dextrose. Ueda *et al.* (32) reported that colony spreading of *S. aureus* was promoted by the addition of glucose to soft agar plates. However, present studies show no clear tendency or enough data between colony spreading and biofilm formation under environmental conditions. Tojo *et al*. (33) also demonstrated that slime production was the primary factor affecting

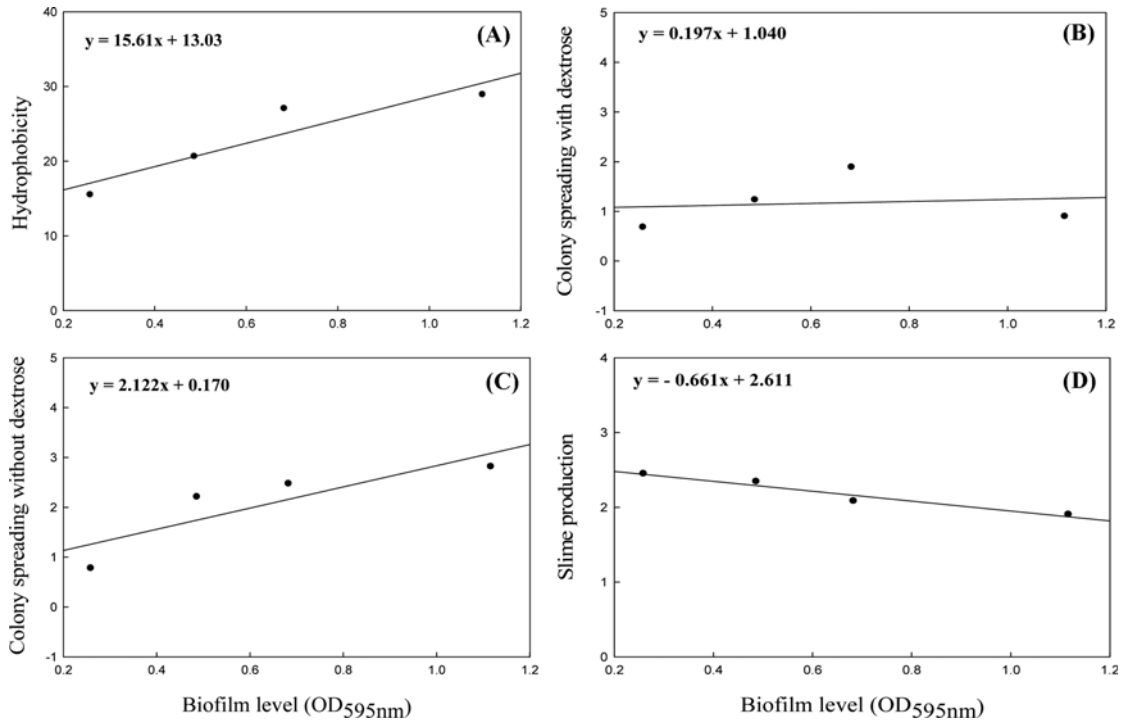


**Fig. 3.** Correlation between the biofilm forming ability of *Staphylococcus aureus* and cell surface properties (cell surface hydrophobicity, colony spreading in the presence and absence of dextrose, and slime production). (A) cell surface hydrophobicity ($r$=0.926), (B) colony spreading on soft agar in the presence of dextrose ($r$=0.136), (C) colony spreading on soft agar in the absence of dextrose ($r$=0.863), and (D) slime production on CRA ($r$= –0.973).

the ability of a pathogen to adhere to artificial materials. In slime-producing bacteria, genes required for adhesion and biofilm-associated proteins were expressed (34). In this study, the result showed a negative correlation between biofilm formation and slime production. However, Oliveria *et al*. (35) showed that colony phenotype in the CRA assay correlated with and biofilm formation in *S. aureus* according to the kappa coefficient test ($\kappa$=0.259). Therefore, relationship between slime production and biofilm formation is not yet known. It is clear, however, that the color change of slime in the CRA assay only occurs toward the stage of biofilm formation.

The results of this study indicate the biofilm formation ability is different depending on bacterial origins. Also, correlation between biofilm formation and cell surface properties (cell surface hydrophobicity, colony spreading, and slime production) in *S. aureus* strains was confirmed. Although cell surface properties play a clear role in regulating biofilm formation, the relationship between these properties and biofilm forming ability are poorly understood. And, to control biofilm formation by *S. aureus* in the food industry, mechanism of biofilm formation related with cell surface properties of pathogens under environmental factors needs to be studied.

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**Disclosure** The authors declare no conflict of interest.

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