

Original Article

Biofilm Deficiency in Polysaccharide Intercellular Adhesin-Negative Variants of *Staphylococcus epidermidis* Selected by Subminimal Inhibitory Concentrations of Gentamicin

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SUMMARY: *Staphylococcus epidermidis* is a cause of orthopedic device-related infection, and to treat such infection, biofilms should be controlled. Polysaccharide intercellular adhesin (PIA) is associated with the biofilm-forming ability of staphylococcal strains. PIA in biofilm-positive staphylococcal strains can be detected by the Congo red agar (CRA) method. In this study, we used the CRA method to examine the effects of subminimal inhibitory concentrations (sub-MICs) of 11 antibacterial agents on PIA production by *S. epidermidis*. We found that the PIA-negative variants were selected only by sub-MICs of gentamicin (GM). This PIA-negative phenotype was maintained over several generations in the absence of GM. Such selection occurred in six of eight clinical isolates, as well as in the biofilm-positive control strain. No such selection occurred with aminoglycoside antibiotics except for GM. Most of the PIA-negative variants that were selected by GM showed a markedly lower biofilm-forming ability on stainless steel washers than their untreated parent strains. In conclusion, variants with lower biofilm-forming ability may be selected by a sub-MIC of GM. Investigation of the reason why variants with reduced biofilm-forming ability can be selected in the presence of sub-MICs of GM may contribute to strategies against biofilm-related infections.

INTRODUCTION

Orthopedists want to ensure the eradication of postoperative infections. Recently, biofilms produced by bacteria have attracted attention as a cause of chronic and intractable device-related infections (1). It has been suggested that the bacteria in biofilms are 100- to 1,000-fold more resistant to antibacterial agents than planktonic bacteria (2). It has also been suggested that the biofilm-forming ability of some staphylococcal strains is dependent in part on polysaccharide intercellular adhesin (PIA). Various reports have been published on the effects of subminimal inhibitory concentrations (sub-MICs) of antibacterial agents on biofilm formation and PIA production; however, the results are still controversial.

Here, we investigated whether sub-MICs of 11 antibacterial agents affect PIA production, and whether altered PIA production reflects biofilm-forming ability using a biofilm-positive control strain and eight clinical isolates.

MATERIALS AND METHODS

Bacterial strains and chemicals: The eight clinical isolates of *Staphylococcus epidermidis* used in this study

were obtained from infected sites in patients with bone and joint infections at Nagasaki University Hospital. *S. epidermidis* American Type Culture Collection (ATCC) 35984 (RP62A), which is a well-known biofilm producer (3), was used as a positive control for biofilm formation. These strains were stored in beads (MicroBank™; Pro-Lab Diagnostics, Richmond Hill, Canada) at -80°C. Congo red (1% Congo red solution) was purchased from Muto Pure Chemicals (Tokyo, Japan). Mueller-Hinton broth (MHB) and trypticase soy broth (TSB) were obtained from Becton-Dickinson and Company (Sparks, Md., USA). Agar was purchased from Ina Food Industry (Nagano, Japan). D-(+)-glucose was obtained from Wako Pure Chemical Industries (Osaka, Japan). Crystal violet (CV, 1% Pyoktanin Blue Solution) was purchased from Kanto Chemical (Tokyo, Japan). Phosphate-buffered saline (PBS) and blood agar were purchased from Gibco (Gaithersburg, Md., USA) and Eiken (Tochigi, Japan), respectively.

Antibacterial agents used in this study and determination of minimal inhibitory concentrations (MICs) of gentamicin (GM): The antibacterial agent-containing discs (BBL Sensi-Disc) used in this study were purchased from Becton-Dickinson Company, and the amount of each agent per disc was as follows: GM, 120 µg; amikacin (AMK), 30 µg; arbekacin (ABK), 30 µg; vancomycin (VCM), 30 µg; tetracycline (TC), 30 µg; chloramphenicol (CP), 30 µg; cefazolin (CEZ) 30 µg; fosfomicin (FOM) 50 µg; minocycline (MINO) 30 µg; rifampin (RFP), 5 µg; and clarithromycin (CAM), 15 µg.

MICs for suspended bacteria were measured by the microdilution method (4).

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Assessment of PIA production using the Congo red agar (CRA) method: PIA production was assessed using the CRA method as previously described (5). Briefly, colonies on blood agar were inoculated into 10 mL of TSB and incubated at 37°C for approximately 3 h ($OD_{600} = 0.2$). This culture was vortexed and then diluted 10-fold with PBS. Approximately 0.1 mL of the diluted suspension was dropped onto CRA plates (0.08% Congo red, 2.1% MHB, 0.5% glucose, and 1.7% agar), the plates were briefly air-dried, and then the antibacterial discs were put in place. The plates were incubated at 37°C for 48 h and then incubated at room temperature for 24 h. PIA production was determined by macroscopic observation since PIA-negative variants form red colonies, whereas PIA-positive variants form black colonies (5). PIA-negative variants were used in the subsequent experiments.

Assessment of biofilm formation: The biofilm-forming ability of the PIA-negative variants was compared with that of their cognate parent strains using a previously described method (6). Briefly, colonies were inoculated in antibacterial agent-free TSB and incubated at 37°C for approximately 3 h ($OD_{600} = 0.2$). Sterilized stainless steel washers (UW-0303-05; 6.0 mm diameter and 0.5 mm thickness, SUS304 quality containing 18% chrome and 8% nickel; Wilco, Tokyo, Japan) were im-

mersed in these cultures for 10 min to allow bacteria to adhere. Subsequently, the bacteria adhering to the washers were incubated in fresh TSB at 37°C for 24 h with the adhered surface down in order to facilitate biofilm formation.

The washer to which the biofilms adhered was washed gently with PBS to remove the planktonic bacteria, and then dried with a drier for approximately 5 min to fix the bacteria to the washer. The biofilm adhering to the washer was stained with CV for 2 min, and then washed twice with PBS to remove the excess stain.

Eight areas ($660 \mu\text{m} \times 480 \mu\text{m}$) of each washer were arbitrarily selected and full-color pictures were obtained using a digital optical microscope (VHX-100; Keyence, Osaka, Japan). The biofilm coverage ratio (BCR) was calculated as the ratio of the CV-stained area to total area of the washer. The color photograph was then converted to a grayscale TIFF image using Photoshop Elements 6 (Adobe Systems, San Jose, Calif., USA), and the BCR was measured with Scion Imaging software (Scion, Frederick, Md., USA). The data are presented as percentages and the mean \pm standard deviation (SD) of four replicates. The data were analyzed using Student's *t* test.

Assessment of revertants from serial passage of PIA-negative variants: PIA-negative variants underwent seri-

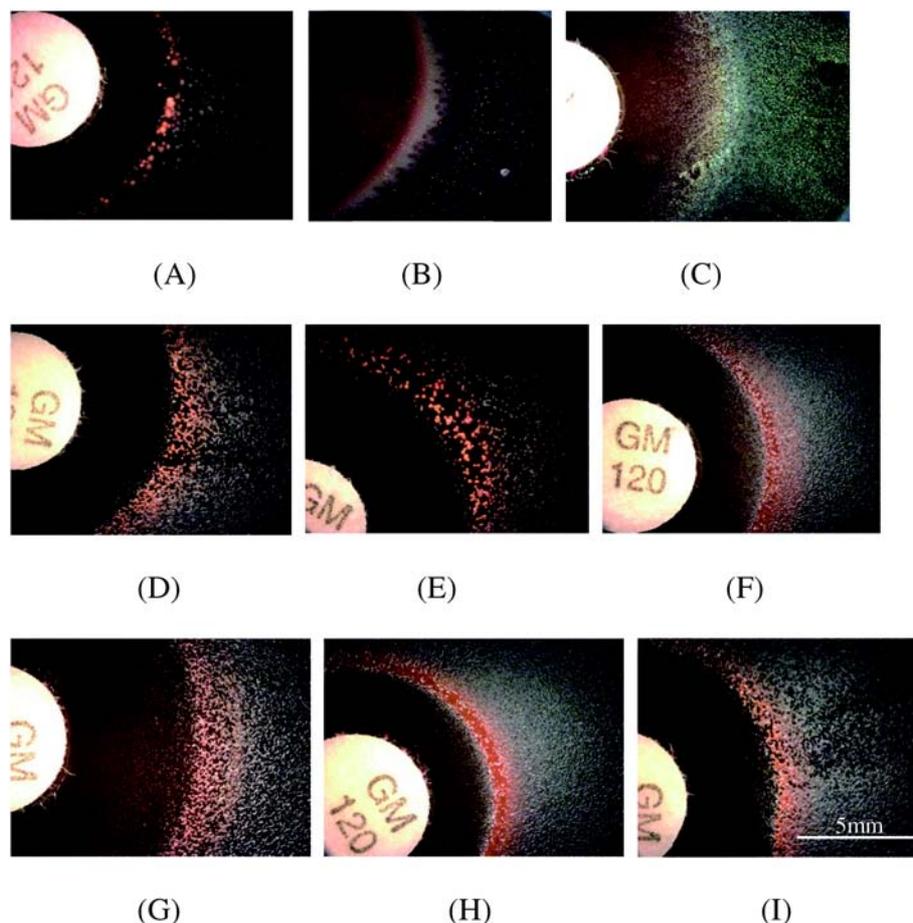


Fig. 1. The effects of subminimal inhibitory concentrations (sub-MICs) of gentamicin (GM) on polysaccharide intercellular adhesin (PIA) production were assessed using Congo red agar (CRA) method. (A) ATCC 35984, (B) strain no. 1, (C) strain no. 2, (D) strain no. 3, (E) strain no. 4, (F) strain no. 5, (G) strain no. 6, (H) strain no. 7, (I) strain no. 8. PIA-negative variants grow as red colony on CRA. PIA-negative variants appeared around inhibitory zones for all strains, with two exceptions (B and C).

al passage every 24 h in antibacterial-free TSB until PIA-positive prototypes (revertants) appeared (for a maximum of 2 weeks). The CRA method described above was also used to determine reversion. Revertants were identified when more than approximately 10% of the colonies that appeared were PIA-positive.

Sodium chloride (NaCl) (maximum, 3%) has been reported to promote reversion of some variants (7). To assess the effect of NaCl, serial passages were performed using antibacterial-free TSB with 0.5% NaCl until reversion occurred (for a maximum of 2 days).

RESULTS

PIA-negative variants appear at sub-MICs of GM:

The MICs of GM against clinical strain No. 1, 2, 3, 4, 5, 6, 7, and 8 and strain ATCC35984 were ≤ 0.5 , ≤ 0.5 ,

≥ 32 , ≥ 32 , ≥ 32 , ≥ 32 , 16, ≥ 32 , and $8 \mu\text{g}/\text{mL}$, respectively. PIA-negative variants appeared around the GM inhibitory zone of nearly all strains, with two exceptions (Fig. 1). In contrast, PIA-negative variants did not appear around the inhibitory zone of the other antibacterial agents used in this study (data not shown).

This PIA-negative phenotype was maintained in the absence of GM over several generations.

PIA-negative variants have impaired biofilm-forming ability compared with their cognate parent strains: The macroscopic images of CV-stained biofilms formed on stainless steel washers showed that the PIA-negative variant of ATCC35984 (Fig. 2B) had impaired biofilm-forming ability compared with the parent strain (Fig. 2A). The PIA-negative variants of the clinical isolates also had macroscopically impaired biofilm-forming ability compared with the parent strains. The BCRs of

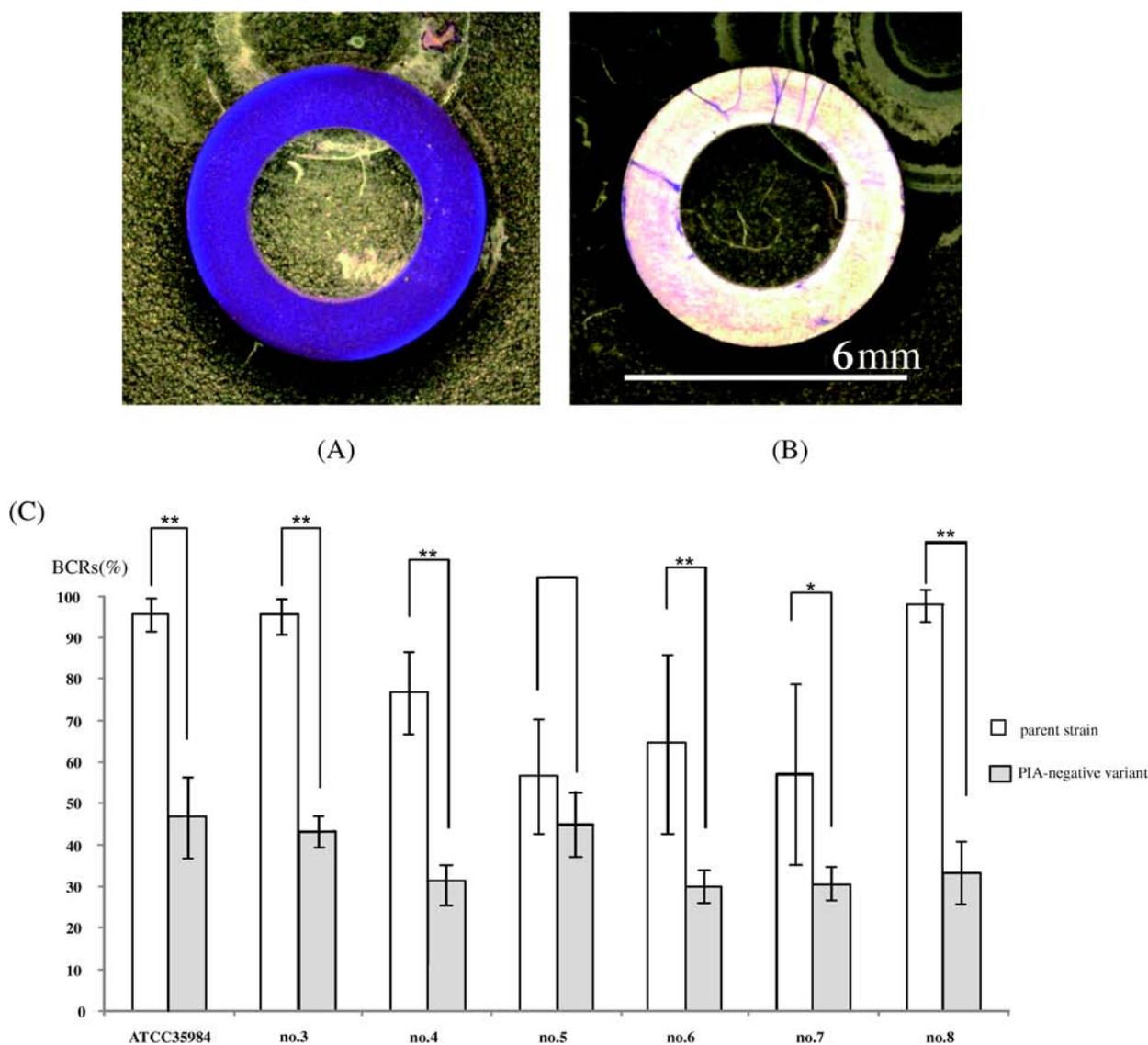


Fig. 2. Assessment of biofilm-forming ability of PIA-negative variants compared with that of the cognate parent strains using crystal violet (CV) staining of biofilms formed on stainless steel washers. The macroscopic images of the CV-stained biofilms formed on stainless steel washers showed that the PIA-negative variant (B) impaired the biofilm-forming ability compared with the parent strain of ATCC35984 (A). The biofilm coverage ratios (BCRs) were compared between the PIA-negative variants and the cognate parent strains derived from the PIA-positive control strain (ATCC35984) and clinical strains in which sub-MICs of GM selected PIA-negative variants (C). * $P < 0.05$ compared with parent strains. ** $P < 0.01$ compared with parent strains.

the PIA-negative variants, except for No. 5, were significantly lower than those of their cognate parent strains, and the BCR of strain No. 5 also tended to be lower than that of the parent strain ($P = 0.056$) (Fig. 2C).

PIA-negative variants reverted to the PIA-positive phenotype after serial passages; however, reversion was not promoted by NaCl: PIA-negative variants reverted to the PIA-positive phenotype after four or more passages. ATCC35984 reverted after 10 passages, No. 4, 5, 6, 7, and 8 reverted after 12 or 13 passages, and No. 3 reverted after four passages. NaCl did not promote the reversion of any strain tested in our study.

DISCUSSION

Although Rachid et al. previously reported that sub-MICs of GM had no effect on the expression of the *ica* genes that encode enzymes involved in PIA production (8), we found that PIA-negative variants were selected by sub-MICs of GM. It is unlikely that the PIA-negative variants are produced due to the action of Congo red at the concentration employed in the CRA method (9). Although the method used in our study is different from that used in their study, we confirmed that PIA-negative variants were selected by GM in the absence Congo red (data not shown). Therefore, we could not clarify the discrepancy between these studies; however, it was not dependent on the combination of GM and Congo red that we used.

The mechanism by which PIA-negative variants are selected by sub-MICs of GM is not fully understood; however, in a previous report by Ziebuhr et al., three possibilities were suggested. They classified naturally occurring PIA-negative variants into three groups. In the first group, the PIA-negative variants revert to the PIA-positive phenotype after only two serial passages or with the addition of NaCl, and expression of the *ica* operon is inhibited (7). In the second group, 9 to 12 serial passages are required for PIA-negative to PIA-positive reversion (7); this group includes phase variants proven to be caused by rearrangement of the *ica* operon (10,11). In the third group, reversion never occurs, and the *ica* operon is either completely or partially deleted (7). According to their classification, our variants might belong to the second group; however, gene arrangement was not confirmed, and further investigation is required.

The reason why PIA-negative variants were not obtained from two of the clinical isolates is unclear. However, the sensitivities of these strains to GM were higher (MICs $\leq 0.5 \mu\text{g/mL}$) than those of other strains. Therefore, for the two clinical isolates from which no PIA variant was selected, the antibacterial effect of GM may have been dominant to the PIA-negative variant-selecting effect.

We also showed that most PIA-negative variants had significantly impaired biofilm-forming ability. Accordingly, our results suggest that the impaired biofilm-forming ability of these variants may be due in part to the inhibition of PIA production.

Because inhibition of protein synthesis is the antibiotic mechanism of action for GM, we initially considered that inhibition of protein synthesis resulted in the selec-

tion of PIA-negative variants of the first group. It has also been reported that a protein synthesis inhibitor, CP, inhibited biofilm synthesis (12). However, our study showed that PIA-negative variants were not selected by CP or other aminoglycosides except GM. Therefore, the impaired biofilm-forming ability of our PIA-negative variants might be independent of protein synthesis inhibition. In addition, biofilm production is dependent on not only PIA production due to *ica* gene expression but also *agr* genes and other factors (13). Furthermore, *ica*-independent biofilm-forming strains have recently been reported (14). These complicated mechanisms might also contribute to the variation in biofilm-forming ability among our PIA-negative variants.

There was no significant difference between the BCRs of the parent strain and the PIA-negative variant of No. 5. We employed the BCR method because our laboratory is familiar with this method, and it is relatively reliable and reproducible (15). However, this method does not reflect the three-dimensional mass of biofilms, and thus it might have underestimated significant differences in biofilm-forming ability between the parent strain and the PIA-negative variant of isolate No. 5.

In conclusion, we reported that PIA-negative variants selected by sub-MICs of GM markedly impaired biofilm-forming ability. Our finding is clinically intriguing because biofilm formation frequently causes intractable infections. The mechanism underlying biofilm formation should be determined in order to provide effective anti-biofilm strategies against intractable infections.

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Conflict of interest None to declare.

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