Staphylococcus epidermidis Forms Floating Micro-colonies in Platelet Concentrates at the Early Stage of Contamination

Yasuo Motoyama,[a] Nobuyasu Yamaguchi,[b] Mami Matsumoto,[a] Tomoaki Ichijo,[b,c] Hideto Nagumo,[d] Noboru Kagami,[a] Yoshihiko Tani,[e] Masahiro Satake,[d] and Masao Nasu*,[b]

[a]Asahi Breweries, Ltd., 5–4–31 Minami-Aoyama, Minato-ku, Tokyo 107–0062, Japan, [b]Environmental Science and Microbiology, Graduate School of Pharmaceutical Sciences, Osaka University, 1–6 Yamada-oka, Suita, Osaka 565–0871, Japan, [c]Research Institute for Humanity and Nature, 457–4 Motoyama, Kamigamo, Kita-ku, Kyoto 603–8047, Japan, [d]Tokyo Metropolitan Red Cross Blood Center, 2–1–67 Tatsumi, Koto-ku, Tokyo 135–0053, Japan and [e]Osaka Red Cross Blood Center, 2–4–43 Morinomiya, Jyoto-ku, Osaka, Osaka 536–8505, Japan

(Received May 1, 2009; Accepted July 25, 2009; Published online July 31, 2009)

Staphylococcus epidermidis (*S. epidermidis*) often cause sepsis and related diseases by transfusion of contaminated platelet concentrates (PCs). The proliferation process of this bacterium in PCs has been unclear, thus, bio-imaging system was applied for analyzing the dynamics of *S. epidermidis* in PCs. *S. epidermidis* were spiked into PCs or Luria Bertani (LB) broth. These samples were collected at each sampling time during incubation (up to 7 days), and colony-forming-units were counted. Bacterial number and their size distribution in each sample were also determined with a new bio-imaging system. The morphological characters of *S. epidermidis* growing in the samples were observed precisely by scanning electron microscopy (SEM). The numbers of *S. epidermidis* were stable for 48 hr after the spiking as lag-phase, while the bio-imaging analysis also showed that aggregates proliferated during "lag-phase." The aggregates were also observed in LB media, however, their sizes were much smaller than those in PCs. SEM suggested that the aggregates were micro-colonies (MCs) of staphylococcal cells and cores of the MCs are composed with platelets (PLTs). Out results suggested that *S. epidermidis* formed floating MCs in PCs during "lag-phase." Therefore, the term of lag phase of *S. epidermidis* in PCs should be called as "pseudo-lag phase." The initial processes of forming MCs in PCs are thought to be an interaction between bacterial cells and PLTs. Floating MCs would be the source of biofilms on the inside of PC storage bags. New information obtained in this study would be useful for understanding the dynamics of growing bacteria in PCs.

Key words —— platelet concentrate, *Staphylococcus epidermidis*, micro-colony, proliferation process, bio-imaging, fluorescent vital staining

INTRODUCTION

Sepsis and related diseases caused by bacterially contaminated platelet concentrates (PCs) constitute a serious health risk,[1] with several numbers bacterially contaminated units being annually transfused in the U.S.A.[2–4] *Staphylococcus epidermidis* (*S. epidermidis*) is a Gram-positive, non-motile, facultative anaerobic coccus. This bacterial species is also part of the normal skin flora and it is most frequently isolated from contaminated blood products.[3] Because *S. epidermidis* grows very slowly in PCs[5,6] and form biofilms on the insides of PC storage bags after storage for three days, it can escape detection.[7] Presently, inactivation techniques[8,9] as well as rapid microbial detection methods[1] are required to ensure the safety of PCs. To develop these techniques, understanding of the proliferation process of this pathogenic bacterium in PCs is important.

Here, we analyzed the dynamics of *S. epidermidis* in PCs by plate counting and by using a novel bio-imaging system.

*To whom correspondence should be addressed: Environmental Science and Microbiology, Graduate School of Pharmaceutical Sciences, Osaka University, 1–6 Yamada-oka, Suita, Osaka 565–0871, Japan. Tel.: +81-6879-8170; Fax: +81-6879-8174; E-mail: nasu@phs.osaka-u.ac.jp

MATERIALS AND METHODS

Bacterial Strains and Samples —— *S. epidermidis* strains ATCC 12228 (biofilm-negative strain) and ATCC 35984 (biofilm-positive strain), respectively, were cultivated in Luria Bertani (LB) broth at 35°C for 16 hr, diluted in phosphate buffered saline (PBS[−]), and enumerated using a bio-imaging system, μFinder (Asahi Breweries, Tokyo, Japan) as described below. Storage bags (Terumo, Tokyo, Japan) containing 100 ml of fresh PC (apheresis; Platelet Concentrates Nisseki; Japanese Red Cross, Tokyo, Japan) or LB broth were spiked with cultured staphylococcal cells (1000 cells; counted by the μFinder) by using syringes and then flat-shaken at 22°C (temperature for PC storage) for up to 168 hr (7 days).

Monitoring of Staphylococcal Growth in PC or LB Broth —— Three ml of PCs or LB media in the bags were sampled by using syringes at 24, 48, 72, 96, 120, 144 and 168 hr (1, 2, 3, 4, 5, 6 and 7 days) after the spiking. Samples were spread onto Soybean Casein Digest (SCD) plates and incubated aerobically at 35°C for 72 hr. Formed colonies were counted macroscopically.

Both samples at each sampling time were also analyzed using the bio-imaging system, μFinder (Asahi Breweries), as described by Motoyama *et al.*[10] This bio-imaging system consists of an epifluorescence microscope equipped with an autofocusing unit, a Charge Coupled Device (CCD) camera and a XY-motorized stage, which were controlled by a personal computer. This system firstly scans the defined area of the surface of a membrane rapidly and automatically under blue light irradiation at lower resolution. During scanning, all detected fluorescently-stained cells were automatically discriminated from other particles or platelet debris based on their morphological characters and fluorescence intensities. Real-time results were displayed on the computer screen showing the number of discriminated spots and their positions on the scanned membrane, with each fluorescence image. Subsequently, each fluorescent cell, which was discriminated from other particles or platelet debris at lower resolution, was re-analyzed automatically at higher resolution using three different excitation wavelengths for accurate discrimination of bacterial cells from other particles.

Firstly, platelets (PLTs) and leucocytes were selectively lysed with trypsin and polyoxyethylene (23) lauryl ether (PC lysis reagent; Asahi Breweries). After filtration through by metalized filters (pore size, 0.4 μm; Asahi Breweries), bacterial cells remaining on the filters were stained with the esterase indicator, carboxyfluorescein diacetate[11–13] (CFDA; Invitrogen, Carlsbad, CA, U.S.A.) in GAN buffer (phosphate buffer for CFDA-staining of staphylococcal cells; Asahi Breweries) to detect viable *S. epidermidis*. The bio-imaging system automatically discriminated bacterial cells from other noise particles in the sample and enumerated them rapidly. The sizes of bacterial cells and aggregates in each sample were determined by this bio-imaging system.

These experiments were repeated several times.

Scanning Electron Microscopy (SEM) —— Cells in stationary phase were fixed in 4% (w/v) paraformaldehyde for 16 hr at 4°C and passed through polycarbonate (pore size, 0.2 μm) or polypropylene (pore size, 10 μm) filters (ADVANTEC, Tokyo, Japan) and washed with PBS. The filters were placed on a SEM pore holder (JEOL Datum, Tokyo, Japan) connected to a cooling holder (JEOL Datum) and incubated with 50 μl of *t*-butyl alcohol at room temperature for 10 min. The holder was covered with a cooling cap (JEOL Datum) and chilled with liquid nitrogen for 30 s. After the cooling cap was removed, the holder was immediately placed in the specimen chamber of the scanning electron microscope (JSM 5610L; JEOL Datum). The samples were then sputter-coated with evaporated gold for 2 min using Quick Coater (Sanyu Denshi, Tokyo, Japan) and observed by SEM.

RESULTS

CFU and Bio-imaging Analysis

Monitoring the growth of *S. epidermidis* in PCs showed that the CFU remained stable during a 48-hr lag phase after spiking, and the number of *S. epidermidis* reached 1×10^7 CFU/ml in PCs by 144-hr (Fig. 1A). Direct observation using the μFinder showed similar results (Fig. 1B). Monitoring the growth in LB media showed similar dynamics while the number of *S. epidermidis* reached $> 1 \times 10^8$ CFU/ml (Fig. 1C) or counts/ml (Fig. 1D; determined by the μFinder).

From direct observations using the μFinder, not only single bacterial cells but also various sizes of aggregates were observed in PC when spiked with *S. epidermidis*. Figure 2 shows representative images of aggregates in PCs spiked with ATCC35984

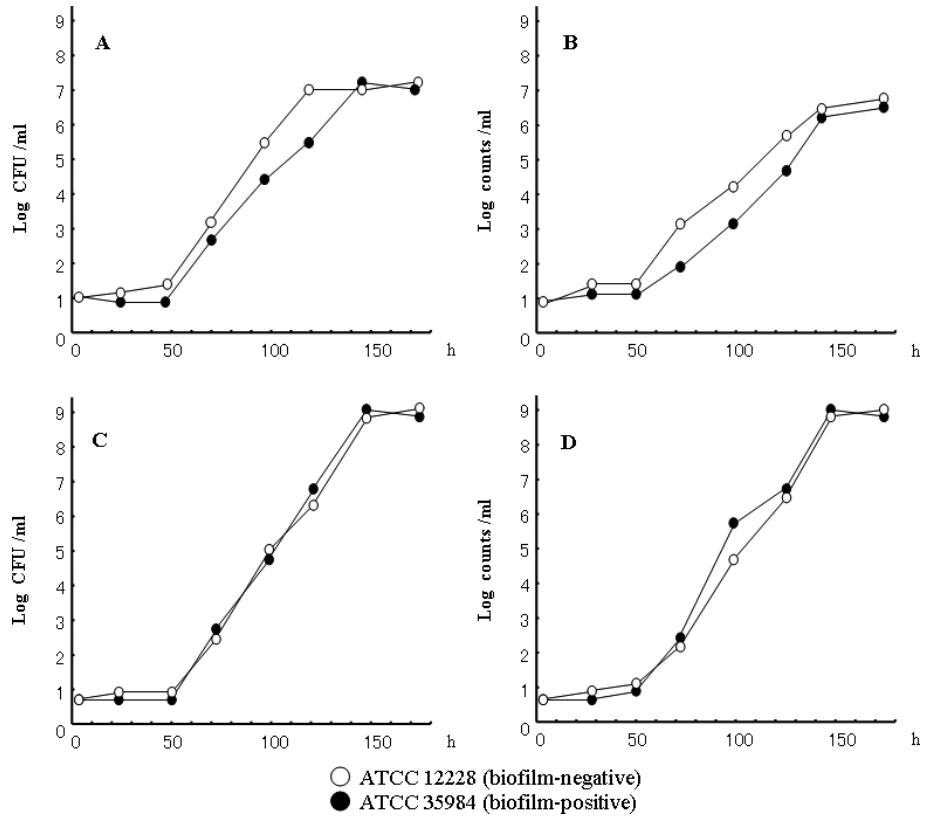

Fig. 1. Growth Curves of *S. epidermidis* in Platelet Concentrates (A and B) and LB Media (C and D)
A and C: Bacterial number determined as CFU by plate counting. B and D: Bacterial number determined by μFinder analysis.

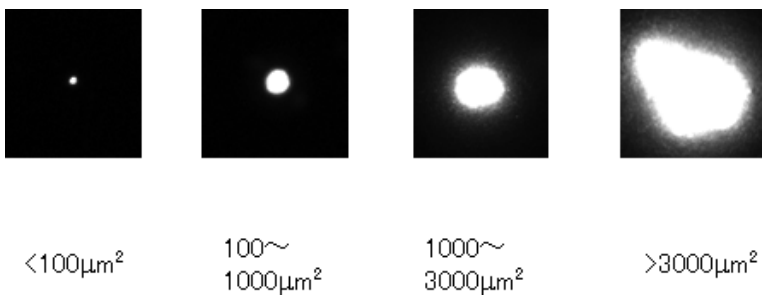

Fig. 2. Representative Images and Sizes of Growing Staphylococcal Micro-colonies (Strain ATCC 35984 Cultured in PCs) Detected by μFinder Analysis

strain, detected by the μFinder. Similar aggregates were observed when examining PCs contaminated with the ATCC 12228 strain. Figure 3 shows that the size distribution of the aggregates spiked with *S. epidermidis* strains; those distributions were completely different between in PCs and LB media. The sizes of the aggregates in PCs increased during the lag phase and finally reached $>3000\,\mu m^2$ at the end of the lag phase (48 hr; Fig. 3A and B), while aggregates in LB media were much smaller than those in PCs (less than $300\,\mu m^2$ as shown in Fig. 3C and D). The maximum sizes of aggregates in PCs and LB reached >10000 and $300\,\mu m^2$, respectively. The sizes of the aggregates, particularly these of ATCC12228, decreased after the end of the lag phase (Fig. 3A).

These experiments were repeated several times with different PCs, and similar results were obtained. The standard deviation of data was 1.0–27% (average 9.7%) in Fig. 1 and 3.0–25% (average 11%) in Fig. 3.

SEM Analysis

Figure 4 shows the SEM findings of aggregates growing in PCs (Fig. 4A, B and C) and LB (Fig. 4D). Staphylococcal cells formed aggregate in

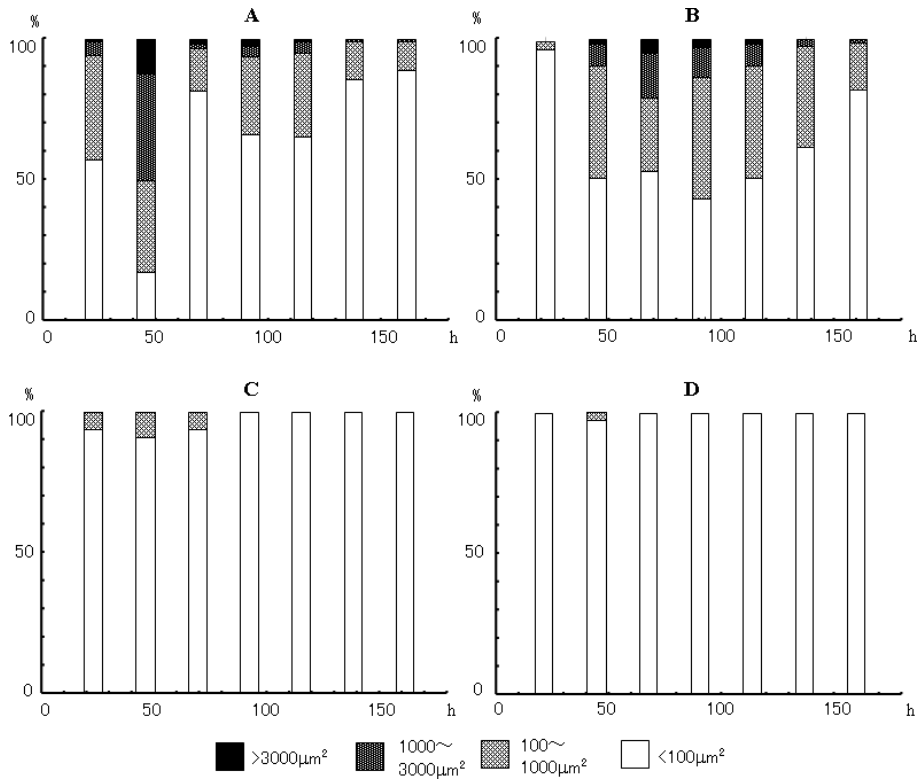

Fig. 3. Changes in Size Distribution of Staphylococcal Micro-colonies during Incubation in Platelet Concentrates (A and B) or LB Media (C and D)

A and C, ATCC 12228; B and D, ATCC 35984.

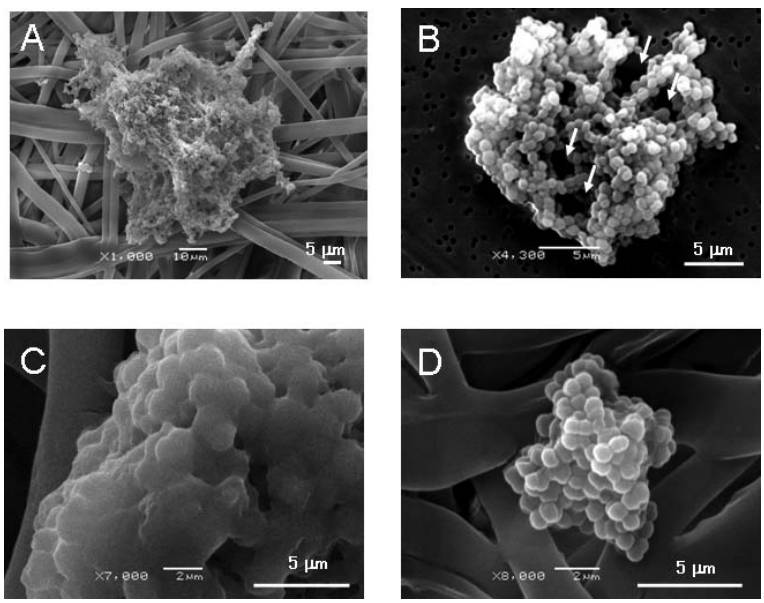

Fig. 4. SEM of Micro-colonies of *S. epidermidis*

A and B: Micro-colony of *S. epidermidis* ATCC 12228 (biofilm-negative strain) in platelet concentrates observed without (A) or after (B) selective lysis of platelets. C: Micro-colony of *S. epidermidis* ATCC 35984 (biofilm-positive strain) in platelet concentrates without selective platelet lysis. D: Micro-colony of *S. epidermidis* ATCC 35984 in LB medium.

PC as shown in Fig. 4A, while several gaps in the aggregate appeared after selective PLT lysis with enzyme and detergent (Fig. 4B, white arrow).

The surface of the aggregates formed by the biofilm-positive strain in PC seemed to be covered with more of a secretion product (Fig. 4C), than

either the biofilm-negative strain growing in PC (Fig. 4A) or the biofilm-positive strain growing in LB (Fig. 4D).

DISCUSSION

The growth kinetics of staphylococcal cells (Fig. 1) and the μFinder results (Fig. 3) showed that the sizes of the micro-colonies (MCs) in PCs increased and finally reached $> 3000\,\mu m^2$ at the end of the lag phase. Thereafter, MCs became degraded (Fig. 3A and B) and result in increased numbers of bacteria (Fig. 1A and B). That is, over time reduction of larger aggregates may be a consequence of the increasing number of growing bacterial cells.

Large aggregates were observed in PC after the spiking of staphylococcal cells (Fig. 2, Fig. 3A and 3B), while only few or no aggregates were observed in PC without the bacterial inoculation (data not shown). Furthermore, the counts of the aggregates obtained by the μFinder analysis were almost as same as CFU (Fig. 1A and 1B). Those results indicate that the aggregates were not noise particles but bacterial MCs. SEM analysis strongly supported this fact (Fig. 4). Therefore, it was suggested that *S. epidermidis* proliferated by forming floating-MCs in PC suspension during "lag phase." From these results, we propose the proliferation process of *S. epidermidis* in PCs at early stage should be called as "pseudo-lag phase."

Furthermore, SEM analysis showed that several gaps in the aggregates appeared after the selective PLT lysis (Fig. 4B). This might mean that PLTs were cores of staphylococcal MCs in PCs. In fact, pathogenic bacteria do interact with PLTs, which can result in PLT activation and subsequent aggregation.[14] Therefore, the initial process of MC formation might be assisted by these mechanisms.

Greco *et al*. showed that *S. epidermidis* form biofilms on the insides of PC storage bags at 3–5 days after inoculation,[7,15] while dynamics of this bacterium in short storage term (within 3 days) was not precisely determined. We confirmed that this bacterium formed floating MCs in PC suspension, which reached to the maximum sizes at 1–3 days after inoculation. From these observations, it was strongly suggested that floating MCs would be the source of the biofilms on PC storage bags.

In addition, the secretion products of the biofilm-positive strain (ATCC 35984) were particularly obvious in PC (Fig. 4C), rather than the biofilm-negative strain (ATCC12228) (Fig. 4A). Some strains of *S. epidermidis* produce mucus that encases the cells and aids in their adherence to and accumulation on smooth surfaces of medical devices.[16] Greco *et al*. indicated that both biofilm-positive and -negative strains of this bacterium form biofilms on the insides of PC storage bags.[7] Our results also indicated that both biofilm-positive and -negative strains formed aggregates not in LB media but in PCs. Therefore, it was speculated that floating MCs play an important role in forming biofilms on PC storage bags, rather than by producing secretions. Biofilms are resistant to disinfectants due to having slime layers.[15] Therefore, further studies on the growth kinetics of floating MC-forming bacteria in PCs should be important to improve transfusion medicines.

Acknowledgements This study was in part supported by the JSPS Grant-in Aid for Scientific Research (C) (19590118) and the JSPS Grant-in Aid for Scientific Research (A) (21256002). Y. M. and N. Y. contributed equally to this work.

REFERENCES

1) Schmidt, M., Karakassopoulos, A., Burkhart, J., Deitenbeck, R., Asmus, J., Muller, T. H., Weinauer, F., Seifried, E. and Walther-Wenke, G. (2007) Comparison of three bacterial detection methods under routine conditions. *Vox Sang.*, **92**, 15–21.
2) Goldman, M. (2004) Bacterial contamination of platelet concentrates: where are we today? *Vox Sang*., **87**, 90s–92s.
3) Brecher, M. E. and Hay, S. N. (2005) Bacterial contamination of blood components. *Clin. Microbiol. Rev*., **18**, 195–204.
4) Eder, A. F., Kennedy, J. M., Dy, B. A., Notari, E. P., Weiss, J. W., Fang, C. T., Wagner, S., Dodd, R. Y., Benjamin, R. J. and the American Red Cross Regional Blood Centers (2007) Bacterial screening of apheresis platelets and the residual risk of septic transfusion reactions: the American Red Cross experience (2004–2006). *Transfusion*, **47**, 1134–1142.
5) Stormer, M., Cassens, U., Kleesiek, K. and Dreier, J. (2007) Detection of bacteria in platelet concentrates prepared from spiked single donations using cultural and molecular genetic methods. *Transfus. Med.*, **17**, 61–70.
6) Sugiura, S., Takahashi, I., Inoue, C., Takayanagi, M. and Kamiya, T. (2007) Evaluation of three bacte-

ria detection systems for contamination of apheresis platelets. *Japanese Journal of Transfusion and Cell Therapy*, **53**, 35–42.
7) Greco, C., Martincic, I., Gusinjac, A., Kalab, M., Yang, A.-F. and Ramirez-Arcos, S. (2007) *Staphylococcus epidermidis* forms biofilms under simulated platelet storage conditions. *Transfusion*, **47**, 1143–1153.
8) Klein, H. G., Anderson, D., Bernardi, M.-J., Cable, R., Carey, W., Hoch, J. S., Robitaille, N., Sivilotti, M. L. A. and Smaill, F. (2007) Pathogen inactivation: making decisions about new technologies: Report of a consensus conference. *Transfusion*, **47**, 2338–2347.
9) McCullough, J. (2007) Pathogen inactivation: a new paradigm for blood safety. *Transfusion*, **47**, 2180–2184.
10) Motoyama, Y., Yamaguchi, N., Matsumoto, M., Kagami, N., Tani, Y., Satake, M. and Nasu, M. (2008) Rapid and sensitive detection of viable bacteria in contaminated platelet concentrates using a newly developed bio-imaging system. *Transfusion*, **48**, 229–236.
11) Yamaguchi, N. and Nasu, M. (1997) Flow cytometric analysis of bacterial respiratory and enzymatic activity in the natural aquatic environment. *J. Appl. Microbiol.*, **83**, 43–52.
12) Yamaguchi, N., Baba, T., Nakagawa, S., Saito, A. and Nasu, M. (2007) Rapid monitoring of bacteria in dialysis fluids by fluorescent vital staining and micro-colony methods. *Nephrol. Dial. Transplant.*, **22**, 612–616.
13) Baba, T., Yamaguchi, N., Matsumoto, R. and Nasu, M. (2009) Bacterial population dynamics in a reverse-osmosis water purification system determined by fluorescent staining and PCR-denaturing gradient gel electrophoresis. *Microbes and Environments*, **24**, 163–167.
14) Fitzgerald, J. R., Foster, T. J. and Cox, D. (2006) The interaction of bacterial pathogens with platelets. *Nat. Rev. Microbiol.*, **4**, 445–457.
15) Donlan, R. M. and Costerton, W. (2002) Biofilms: survival mechanisms of clinically relevant microorganism. *Clin. Microbiol. Rev.*, **15**, 167–193.
16) Christensen, G. D., Simpson, W. A., Bisno, A. L. and Beachey, E. H. (1982) Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infect. Immun.*, **37**, 318–326.