Transmission of Monospecies and Dual-Species Biofilms from Smooth to Nanopillared Surfaces

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ABSTRACT The transmission of bacteria in biofilms from donor to receiver surfaces precedes the formation of biofilms in many applications. Biofilm transmission is different from bacterial adhesion, because it involves biofilm compression in between two surfaces, followed by a separation force leading to the detachment of the biofilm from the donor surface and subsequent adhesion to the receiver surface. Therewith, the transmission depends on a balance between donor and receiver surface properties and the cohesiveness of the biofilm itself. Here, we compare bacterial transmission from biofilms of an extracellular-polymeric-substance (EPS)-producing and a non-EPS-producing staphylococcal strain and a dual-species oral biofilm from smooth silicon (Si) donor surfaces to smooth and nanopillared Si receiver surfaces. Biofilms were fully covering the donor surface before transmission. However, after transmission, the biofilms only partly covered the donor and receiver surfaces regardless of nanopillaring, indicating bacterial transmission through adhesive failure at the interface between biofilms and donor surfaces as well as through cohesive failure in the biofilms. The numbers of bacteria per unit volume in EPS-producing staphylococcal biofilms before transmission were 2-fold smaller than in biofilms of the non-EPS-producing strain and of dual species. This difference increased after transmission in the biofilm left behind on the donor surfaces due to an increased bacterial density for the non-EPS-producing strain and a dual-species biofilm. This suggests that biofilms of the non-EPS-producing strain and dual species remained compressed after transmission, while biofilms of the EPS-producing strain were induced to produce more EPS during transmission and relaxed toward their initial state after transmission due to the viscoelasticity conferred to the biofilm by its EPS.

IMPORTANCE Bacterial transmission from biofilm-covered surfaces to surfaces is mechanistically different from bacterial adhesion to surfaces and involves detachment from the donor and adhesion to the receiver surfaces under pressure. Bacterial transmission occurs, for instance, in food processing or packaging, in household situations, or between surfaces in hospitals. Patients admitted to a hospital room previously occupied by a patient with antibiotic-resistant pathogens are at elevated infection risk by the same pathogens through transmission. Nanopillared receiver surfaces did not collect less biofilm from a smooth donor than a smooth receiver, likely because the pressure applied during transmission negated the smaller contact area between bacteria and nanopillared surfaces, generally held responsible for reduced adhesion. Biofilm left behind on smooth donor surfaces of a non-extracellular-polymeric-substance (EPS)-producing strain and dual species had undergone different structural changes than an EPS-producing strain, which is important for their possible further treatment by antimicrobials or disinfectants.

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The first step in biofilm formation is traditionally depicted as a transport step (1). In many applications where biofilms occur, bacteria are transported to a substratum material from a flowing fluid or air, examples being biofilm formation in marine environments (2), wastewater plants (3), and drinking water systems (4), or in the oral cavity where bacteria suspended in saliva adhere to oral hard and soft tissues but also to toothbrush filaments (5, 6). Such convective-diffusional mass transport systems are amply employed to study bacterial adhesion and biofilm formation (7). However, mass transport through bacterial transmission from one surface to another is a grossly neglected way of bacterial mass transport but arguably of equal or even bigger importance than convective-diffusional mass transport. Bacterial transmission in food processing or packaging can yield severe health problems (8). In daily life, bacteria are transmitted from kitchen sponges to kitchen appliances, from public soap dispensers to hands, and so on (9), which can also easily result in disease. UV irradiation of toothbrushes has been advocated to prevent the transmission from a bacterially contaminated brush to the oral cavity during the next brushing (10). Contact lens-induced corneal keratitis is initiated by bacterial transmission from the lens case to the contact lens and from the contact lens to the cornea (11). Patients admitted to a hospital room previously occupied by a patient with methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococcus (VRE), or *Acinetobacter baumannii* have an elevated risk of becoming infected by the same pathogens through transmission (12). Catheter-related infections are induced by bacterial transmission from the skin of a patient and from health care workers to the catheter (13), leading to increased mortality, morbidity, and hospital costs (14).

Bacterial transmission from a biofilm-covered surface to a clean surface is an entirely different process than that for bacterial adhesion to surfaces, as it involves detachment from a donor surface and adhesion to a receiver surface under an applied pressure and for a particular duration of time. Bacterial transmission is determined by the force exerted by the receiving surface on biofilm organisms, relative to the adhesion force of bacteria to the donating surface or the cohesion forces between bacteria in the biofilm, as shown, for instance, for bacterial transmission from storage cases to contact lenses (15, 16).

Recently, there is a rising interest in various forms of engineered surfaces for use in hospitals and nursing homes, where the risks of nosocomial infections and epidemic spreads are high (17). Micro- or nanostructured surfaces were shown to reduce the adhesion of bacteria on hydrogels and titanium oxide surfaces (18, 19). Depending on the bacterial strain, this reduction was found to be more effective on well-arranged patterns than on randomly organized nanostructures (20). Yet, like with almost every surface assumed to be “nonadhesive,” a low number of bacteria always adhere to any surface due to the multitude of adhesion mechanisms bacteria have at their disposal, and even low numbers of bacteria tend to grow out into a biofilm with all possible negative consequences, especially in health care settings. Micro- and nanostructured surfaces are generally considered not to be an exception (21). However, recent studies have suggested that regardless of whether or not they reduce bacterial adhesion numbers, nanostructured surfaces may yield entirely different and potentially more important effects on adhering bacteria. Multiple nanoscale contacts between a nanostructured surface and a bacterium have been suggested to cause bacterial cell death (22), while bacteria adhering in submonolayer numbers and involved in the transmission between surfaces showed pressure-induced production of extracellular polymeric substances (EPS) when contacting nano-sized pillars and 2-fold-higher bacterial cell death after transmission (23). These properties make nanostructured surfaces attractive for use in applications in which bacterial transmission and its consequences are to be prevented.

FIG 1 SEM micrographs of lithographically prepared nanopillars on Si surfaces with different interpillar distances of 200 nm (A), 400 nm (B), and 800 nm (C). Scale bars, 500 nm.

Bacterial transmission from a surface with submonolayer bacterial coverage, i.e., the initial adhesion of a single bacterium in the absence of growth into multiple layers, can only occur through adhesive failure of the bond between an adhering bacterium and the donor surface and subsequent adhesion to the receiver surface. However, in the case of the presence of a biofilm on the donor surface, transmission can occur through adhesive failure and/or cohesive failure in the biofilm. Considering the importance of bacterial transmission and the promises of nanostructured surfaces voiced throughout the literature for the control of biofilm formation (21), the aim of this study was to compare the transmission of staphylococcal biofilms of EPS- and non-EPS-producing strains and a dual-species oral biofilm (consisting of a rod- and coccus-shaped bacterium) from smooth to nanopillared silicon (Si) surfaces with different pillar-to-pillar distances under a constant pressure. Biofilm thicknesses on both donor and receiver surfaces were determined using optical coherence tomography (OCT), while also the numbers of bacteria adhering on the surfaces were determined after the dispersal of the biofilms from the donor and receiving surfaces. Biofilms were visualized using confocal laser scanning microscopy (CLSM) after LIVE/DEAD staining, while the contact between individual bacteria and nanopillared surfaces was imaged using scanning electron microscopy (SEM). The novelty of this paper is that we studied the transmission of bacteria in their biofilm mode of growth, which is highly different from the transmission of initially adhering single bacteria as done in a previous study by Hizal et al. (23).

RESULTS

The transmission of staphylococcal biofilms of EPS- and non-EPS-producing strains and a dual-species oral biofilm was studied from a smooth donor to smooth and nanopillared Si receiver surfaces with different pillar-to-pillar distances (Fig. 1) under a constant pressure. Both EPS-producing *Staphylococcus epidermidis* ATCC 35984 and non-EPS-producing *S. epidermidis* 252 grew sizeable biofilms with thicknesses on smooth Si donor surfaces of 72 ± 32 and 56 ± 19 μm (differences not significant), respectively. The dual-species biofilm of *Actinomyces naeslundii* T14V-J1 (rod shaped) and *Streptococcus oralis* J22 (coccus shaped) showed 2-fold-thicker biofilms of 120 ± 20 μm , as determined using OCT. Transmission successively exerted compressive and tensile forces on the biofilm. Transmission from a smooth donor to a smooth Si receiver left approximately 25% of the initial biofilm thickness on the donor, transmitting approximately 15% to the receiver and therewith creating a percentage “thickness loss” of 60%, regardless of the strain involved (Fig. 2A to C). In the absence of a demonstrable major loss of bacteria during the experiment (see below), the sum of the biofilm thicknesses on the donor and receiver should add up to 100%, on the basis of which the loss of thickness can be interpreted as due to biofilm compression. The percent thickness of biofilms of the EPS-producing staphylococcal strain transmitted to nano-

FIG 2 (A, B, C) The percent thicknesses of biofilms after transmission from a smooth donor to smooth and nanopillared receiver surfaces with respect to the initial biofilm thickness on the donor surface before transmission, together with the percent thicknesses lost during transmission. (D, E, F) The total numbers of bacteria after transmission from a smooth donor to smooth and nanopillared receiver surfaces with respect to the initial numbers of bacteria on the donor surface before transmission. (G, H, I) Similar to panels D, E, and F but for the surface coverage by biofilm. Panels A, D, and G refer to EPS-producing *S. epidermidis* ATCC 35984, panels B, E, and H are for non-EPS-producing *S. epidermidis* 252, and panels C, F, and I refer to the dual-species oral biofilms composed of *A. naeslundii* T14V-J1 and *S. oralis* J22. Error bars denote standard deviations over triplicate experiments with separately grown biofilms and different sample surfaces. Markers indicate significant differences ($P < 0.05$) between indicated groups.

pillared receiver surfaces was larger than that in the transmission to a smooth receiver surface (Fig. 2A), whereas in contrast, transmission of the non-EPS-producing staphylococcal strain and of bacteria from the dual-species biofilms yielded similar thicknesses on smooth or nanopillared receiver surfaces (Fig. 2B). Biofilms composed of the non-EPS-producing staphylococcal strain and dual species had larger percentage biofilm thicknesses transmitted to nanostructured surfaces with pillar-to-pillar distances of 200 nm than to those with other pillar-to-pillar distances (Fig. 2B and C).

The initial biofilms on smooth Si surfaces before transmission contained approximately 10^9 bacteria/cm², as obtained after biofilm dispersal and microscopic enumeration, of which around 10% were transmitted to a smooth receiver (Fig. 2D to F). Higher numbers of bacteria of the EPS-producing staphylococcal strain were transmitted to nanopillared surfaces than the non-EPS-producing staphylococcus and the dual-species biofilm (Fig. 2D to F). During transmission, only a minor number of bacteria (CFU) were “lost” (0.28 log units, on average, across different receiver surfaces and strains).

Finally, an analysis of OCT images indicated that 100% of the smooth donor surfaces were covered with biofilm before transmission, while after transmission, donor surfaces remained to be covered for approximately 25% to 50% by biofilm for the staphylococcal strains regardless of the pillar-to-pillar distance of the receiver surfaces. For the dual-species biofilm, the surface coverage was highest on the smooth donor surface after transmission and decreased with increasing pillar-to-pillar distances (Fig. 2I). Surface coverage of the receiver surfaces after transmission ranged from 10% to 50% (Fig. 2E and F). Biofilm surface coverage after transmission to the 200-nm pillar-to-pillar distance receiver surface was highest and was significantly ($P < 0.05$) more than that to a smooth receiver surface for the non-EPS-producing staphylococcal strain, whereas

FIG 3 Bacterial cell densities in biofilms before and after transmission from a smooth donor surface to smooth and nanopillared receiver surfaces. (A) Densities of EPS-producing *S. epidermidis* ATCC 35984 biofilms. (B) Densities of non-EPS-producing *S. epidermidis* 252 biofilms. (C) Densities of the dual-species *A. naeslundii* T14V-J1 and *S. oralis* J22 biofilms. Error bars denote standard deviations from triplicate experiments with separately grown biofilms and different sample surfaces. Markers indicate significant differences ($P < 0.05$) between indicated groups.

for the EPS-producing staphylococcal strain, coverage on the receiver was significantly higher for both the 400- and 800-nm pillar structures. For the dual-species oral biofilm, there was no significant difference in surface coverage after transmission on the receiver surface between smooth and nanopillared surfaces (Fig. 2I).

The combination of the biofilm thicknesses and the numbers of bacteria in the different biofilms enables the calculation of the bacterial density in the biofilms, expressed as the number of bacteria per μm^3 of biofilm. Before transmission, bacterial densities in biofilms of the EPS-producing strain (Fig. 3A) were 2-fold smaller than in biofilms of the non-EPS-producing staphylococcal strain (Fig. 3B) and in the dual-species biofilm (Fig. 3C). This difference in bacterial density increased with respect to the biofilms left behind on the donor surfaces after transmission regardless of the

pillar-to-pillar distance, mostly due to an increase in bacterial density for the non-EPS-producing staphylococcal strain, while the density for the EPS-producing strain remained low. For the dual-species biofilm, an increase of bacterial density after transmission was shown with increasing pillar-to-pillar distances. However, in all biofilms on the receiver surfaces, regardless of strains and species, the density differences were negligible and without statistical significance.

CLSM images of staphylococcal biofilms on donor surfaces showed the clear presence of EPS patches in biofilms of *S. epidermidis* ATCC 35984 (Fig. 4A) that were absent in biofilms of *S. epidermidis* 252 and in the dual-species ones (Fig. 4B and C). In biofilms of *S. epidermidis* ATCC 35984 left behind on the smooth donor after transmission, EPS appeared organized in filamentous structures (Fig. 4A). After transmission, the EPS distributed in a fine-dotted structure on the smooth receiver but had a much more pronounced and bigger-dotted structure on the nanopillared receiver (Fig. 4A). There are no noteworthy differences in the appearance of biofilms of non-EPS-producing *S. epidermidis* 252 biofilms after transmission, neither on the donor nor on the receiver surfaces, regardless of the type of nanopillaring (Fig. 4B).

SEM images of the *S. epidermidis* ATCC 35984 biofilm on the nanopillared receiver surfaces showed pressure-induced EPS production in the neighborhood of the transmitted staphylococci (Fig. 5A). At a higher magnification (Fig. 5A inset), EPS was also visible relatively far away from the transmitted staphylococci, likely indicating direct EPS transmission from the biofilm on the donor. No EPS was seen in the transmission in *S. epidermidis* 252 (Fig. 5B and inset) and the dual-species biofilm (data not shown).

DISCUSSION

Biofilms with incomplete surface coverage were found on both donor and receiver surfaces after biofilm transmission for EPS- and non-EPS-producing staphylococcal strains and a dual-species oral biofilm composed of *A. naeslundii* and *S. oralis*, indicating that adhesive failure at the interface between biofilms and the donor surfaces occurred as well as cohesive failure in the biofilms. Importantly, both biofilms are highly different and relevant in widely various applications, such as in hospital environments (staphylococci) and oral health care (streptococci and actinomyces). The transmission of the biofilm to the nanopillared surfaces was surprising, as under convective-diffusion conditions in the absence of compression forces, staphylococcal adhesion forces on the nanopillared surfaces are much smaller as a result of a minimal contact area than adhesion forces to smooth surfaces (23) or, for that matter, between bacteria (24, 25). Adhesion forces to both surfaces are likely strengthened by the compression phase of the transmission process negating the effects of minimal contact area, putting them on par with the cohesive forces in the biofilm. This would also explain why the percent thickness data (Fig. 2A) show little significant differences among the nanopillared surfaces with different pillar-to-pillar distances: under the condition of equal adhesion and cohesion forces, it is impossible to predict at which position in a biofilm failure resulting in transmission will occur.

Before transmission, the bacterial density in the biofilms was around 0.1 bacterium per μm^3 for the EPS-producing staphylococcal strain and 0.2 bacterium per μm^3 for a non-EPS-producing staphylococcal strain and the dual-species biofilm (Fig. 3). This confirms that most of the volume, especially in biofilms of EPS-producing strains, is occupied by EPS or water-filled channels and voids but not by microbes (26, 27). Transmission is a succession of biofilm compression and elongation during the separation phase. Compression irreversibly increases the density of the non-EPS-producing staphylococcal strain on all receiver surfaces, regardless of nanopillaring, but not of the EPS-producing strain that maintains a low density. The densities of the dual species increased by a factor of three due to the inefficient packing of the rods and cocci in the uncompressed biofilm. This suggests that biofilms of the non-EPS-producing strain and dual-species biofilms remained compressed after transmission without noticeable strain relaxation, while biofilms of the EPS-producing strain relaxed toward their initial state after transmission due to the short viscoelastic relaxation times conferred to the

FIG 4 CLSM images of biofilms of EPS-producing, non-EPS-producing staphylococci, and dual species *A. naeslundii* and *S. oralis* before and after transmission from a smooth donor to a smooth or nanopillared (400-nm pillar distance) receiver. (A) *S. epidermidis* ATCC 35984. (B) *S. epidermidis* 252. (C) *A. naeslundii* T14V-J1 and *S. oralis* J22. EPS appears as blue fluorescent regions. Scale bars, 50 μm .

FIG 5 Scanning electron micrographs of staphylococci in direct contact with a nanopillared receiver surface. (A) EPS-producing *S. epidermidis* ATCC 35984. (B) Non-EPS-producing *S. epidermidis* 252. Note that for better visibility, bacteria and EPS patches have been artificially colored green and blue, respectively. Scale bars, 500 nm.

biofilm by the EPS (28). Also, the EPS-producing *S. epidermidis* strain is induced to produce more EPS during the compression phase of transmission, as shown by the electron micrographs of the interface between staphylococci and a nanopillared surface (Fig. 5A), which reduces the bacterial density (see Fig. 3). Pressure-induced EPS production has also been observed previously in the transmission of bacterial sub-monolayers involving nanostructured surfaces (23) and may perhaps be considered a step toward cell death as a result of multiple nanoscale high-pressure contact points (29). Accordingly, EPS was distributed differently after transmission in the biofilm on the smooth donor than before transmission. After transmission, elongated EPS-rich filaments were seen, which speculatively developed during transmission and the separation of the donor and receiver. During separation, the viscoelastic properties of the EPS will enable the formation of elongated filamentous structures that try to keep the donor and receiver surfaces together until final separation. After separation, they collapse as filamentous structures on top of the biofilm. EPS-rich filaments were not seen on smooth receiver surfaces but only on fine-structured EPS dots. On nanopillared receiver surfaces, much larger EPS dots were seen, presumably produced under the influence of high local pressures arising from the nanopillared surfaces (Fig. 4).

In summary, bacterial transmission in EPS-producing and non-EPS-producing staphylococcal biofilms and in a dual-species oral biofilm on smooth Si donor surfaces was not significantly different to smooth than to nanopillared Si receiver surfaces. After transmission, biofilms were found on both donor and receiver surfaces, including empty patches on the donor surfaces, suggesting that transmission occurred in both strains through adhesive failure at the interface between the biofilm and the smooth Si surface and through cohesive failure in the biofilm. Bacterial densities in biofilms of the non-EPS-producing strain and in dual-species biofilms increased after transmission, while there was indication of pressure-induced EPS production on nanopillared receiver surfaces and a rearrangement of EPS over the surfaces of biofilms left behind on donor surfaces for the EPS-producing strain. Thus, biofilms left behind on smooth donor surfaces of a non-EPS-producing strain and of a dual-species biofilm underwent different structural changes than the biofilm of an EPS-producing strain, which is important for their possible further treatment by antimicrobial or disinfectants. In compressed biofilms with more closely packed bacteria and accordingly higher bacterial densities, the penetration of antimicrobials into the biofilm will be more difficult than in biofilms with a more open structure and lower density.

MATERIALS AND METHODS

Fabrication of Si nanopillared surfaces. The preparation of the Si nanopillared surfaces was described in detail elsewhere (23). Briefly, polished 4-inch (10-cm) Si wafers were degreased in acetone and deionized water and dried by N_2 . Next, a negative-tone photoresist (NR-250P; Futurrex, Inc., Franklin, NJ, USA) layer was spin coated over the Si wafers and baked at 150°C for 1 min. The samples were

exposed to linearly polarized light (325 nm) using a He-Cd laser (Kimmon, Tokyo, Japan) in a Lloyd-mirror laser interference lithography system with two degrees of freedom (23, 30) regulated to create pores with diameters of 100, 150, and 370 nm at intercore distances of 200, 400, and 800 nm, respectively, and were baked for 1 min at 100°C. The exposed photoresist layers were subsequently developed with resist developer, RD 6 (Futurrex Inc., Franklin, NJ, USA), diluted (3:1 by volume) in deionized water for different time periods (15 to 25 s) to achieve the desired pore diameters, followed by rinsing with deionized water. Finally, e-beam evaporation (PVD75; Kurt J. Lesker, Jefferson Hills, PA, USA) was employed to deposit a chrome metal layer of a uniform thickness of around 50 nm through the nanopatterned photoresist layer (pore pattern) onto the Si wafer at a deposition rate of $0.2 \text{ nm} \cdot \text{s}^{-1}$, and the photoresist layer was removed in piranha solution (a mixture of H_2SO_4 [98%] and H_2O_2 [95%] with a volume ratio of 3:1). To achieve pillar structures, Si surfaces were etched in the deep reactive ion etching with O_2 and SF_6 in a cryogenic mode at -100°C (Plasmlab 100; Oxford Instruments, Abington, UK). Finally, the chrome masks were removed in chrome etchant (MicroChemicals Inc., Ulm, Germany) at 30°C for 1 min, followed by water rinsing and N_2 drying. The specimens were imaged by SEM (Quanta FEG 450; FEI, Hillsboro, Oregon, USA) to check the surface topographies and uniformity of the nanostructures over the sample area. Finally, 1-cm^2 samples were cut from the fabricated Si nanopillared wafers (Fig. 1).

Biofilm transmission assays were carried out using smooth Si surfaces as donors and nanopillared Si surfaces as receiver surfaces. Prior to the assays, all samples were cleaned by sonication for 5 min in 2% RBS35 (Sigma-Aldrich, St. Louis, MO, USA), followed by another 5 min in sterile demineralized water. Samples with nanopillared surfaces were individually sonicated in separate containers to avoid any damage during sonication. Subsequently, the samples were rinsed with sterile demineralized water and dried using N_2 . Finally, the samples were cleaned for 10 min using air plasma at 12 kPa (Diener ATTO, Ebhausen, Germany) to remove any residues and achieve hydrophilicity. The samples were stored in sterile petri dishes until use.

Bacterial strains and growth condition. EPS-producing *S. epidermidis* ATCC 35984 (31) and non-EPS-producing *S. epidermidis* 252 were originally isolated from a patient with catheter-associated sepsis and from stool (32), respectively. The *A. naeslundii* T14V-J1 and *S. oralis* J22 strains were both isolated from the human oral cavity (33). Both staphylococcal strains and *S. oralis* J22 were grown aerobically and *A. naeslundii* T14V-J1 was grown anaerobically on blood agar plates from frozen stocks for 24 h at 37°C . One single colony was used to make a preculture in 10 ml of tryptone soya broth (TSB; Oxoid, Basingstoke, England) supplemented with 0.25% D-(+)-glucose ($\text{C}_6\text{H}_{12}\text{O}_6$) (Merck, Darmstadt, Germany) and 0.5% NaCl (Merck) for both staphylococcal strains, in Todd-Hewitt broth (Oxoid) for *S. oralis* and in Schaedler broth supplemented with hemin at $0.01 \text{ g} \cdot \text{liter}^{-1}$ for *A. naeslundii* and cultured for 24 h at 37°C under the appropriate conditions. This preculture was used to inoculate a second culture of 200 ml, which was grown for 16 h at 37°C . The number of bacteria in the final culture was adjusted to 1×10^9 bacteria per ml for staphylococci and *S. oralis* and 1×10^8 per ml for *A. naeslundii*, as measured using a Bürker-Türk counting chamber.

Biofilm formation. Smooth Si donor samples were placed on the bottoms of petri dishes filled with 15 ml bacterial suspension and left for bacterial adhesion at 37°C . After 1 h, the staphylococcal suspension was carefully removed and replaced with 15 ml of fresh TSB supplemented medium. Subsequently, a biofilm was grown for 48 h at 37°C , refreshing TSB after 24 h. For dual-species biofilm formation, the *A. naeslundii* suspension was first added to the smooth Si donor sample and left for 1 h, and after washing, the *S. oralis* suspension was added and left for 1 h. After washing, brain heart infusion broth (Oxoid) supplemented with 0.1% yeast medium was added and the biofilm was grown aerobically for 48 h at 37°C . The growth medium was refreshed after 24 h.

Prior to further OCT analysis and total bacterial counts, the growth medium was carefully removed, and donor surfaces with biofilm were moved into a new petri dish with 10 ml phosphate-buffered saline (PBS; 10 mM potassium phosphate, 0.15 M NaCl, pH 6.8) and analyzed with OCT under PBS (see "Biofilm analysis with OCT," below).

Biofilm transmission assay. After OCT analysis of biofilms on the smooth donor surface, the PBS was removed carefully and a clean nanopillared receiver surface, with a plastic weight attached on the back side, was placed on top of the biofilm-covered donor surface, resulting in a pressure of 0.7 kPa. This pressure was chosen as it falls in the same range as the pressure of holding a cup of coffee or using a door handle (34). After 5 min of contact, the surfaces were rapidly separated ($<1 \text{ s}$) from each other by holding the donor surface with a tweezer and simultaneously lifting the receiver surface perpendicularly from the donor. Five minutes was chosen as a contact time, as it is relevant in many applications involving transmission and yielded more reproducible data than longer or shorter contact times. Both donor and receiver surfaces were placed into a new petri dish filled with 10 ml PBS for OCT analysis. All experiments were carried out in triplicates for the smooth and each (200, 400, and 800 nm) of the nanopillared surfaces using newly grown staphylococcal biofilms.

Biofilm analysis with OCT. The thicknesses of biofilms on donor and receiver surfaces were assessed with an OCT Ganymede II (Thorlabs Ganymede, Newton, NJ, USA), while immersed in 10 ml PBS. Biofilms on the smooth donor surfaces were assessed before and directly after transmission and, on nanopillared receiver surfaces, directly after the transmission. Biofilms were imaged by taking a three-dimensional (3D) scan of the biofilm over the entire 1-cm^2 surface area of a sample, recording at least three scans for each biofilm. Subsequently, OCT images were processed using the Otsu method to define the top border of the biofilm. The substratum, which is normally much whiter than the biofilm, was recognized and the elevated whiteness was used to set the biofilm bottom. The average height of a biofilm over three scans was calculated over the center 0.5-cm^2 area of the biofilm to exclude possible irregularities toward the edges of the sample. The biofilm thickness after

transmission on donor and receiver surfaces was also expressed as a percentage of the initial biofilm thickness on the donor surface before transmission.

A top-view photograph was taken together with the 3D scan and used to calculate the percentages of the donor and receiver surfaces after transmission visibly covered with biofilm. Coverage was determined by color threshold analysis using FIJI software and expressed as percent surface coverage with respect to the initial coverage by biofilm on the donor surfaces before transmission, which was always 100%.

Total number of bacteria in biofilms. The total numbers of bacteria in biofilms on the surfaces both before and after transmission were assessed by dispersing the biofilms with a sterile 5-mm interdental brush (Albert Heijn, Zaandam, The Netherlands) and suspending them in 5 ml PBS. Furthermore, the sample, brush, and bacterial suspension were transferred to a sterile tube and sonicated for 1 min to disperse staphylococci remaining on the brush or sample and to break bacterial aggregates. Subsequently, the numbers of bacteria were counted in a Bürker-Türk counting chamber. Note that separate biofilms were grown on smooth Si surfaces, without performing transmission, to assess the number of bacteria in the initial biofilm before transmission. The numbers of bacteria enumerated were expressed as log values per cm² and as a percentage of the initial number of bacteria on the donor. A combination of the total number of bacteria in a biofilm with its thickness and surface coverage per cm² yielded the bacterial density (number of bacteria per unit volume) in a biofilm.

Confocal laser scanning microscopy. Biofilms were also visualized using CLSM. To this end, biofilm-covered samples were immersed in LIVE/DEAD stain (BacLight; Molecular Probes, Leiden, The Netherlands) containing SYTO9 (3.34 mM) and propidium iodide (20 mM) for 30 min and kept in the dark at room temperature. This staining enabled the assessment of live (green fluorescent) and dead (red fluorescent) bacteria in a biofilm. After washing with PBS, biofilm-covered samples were immersed in calcofluor white (50 mM Fluorescent Brightener 28; Sigma-Aldrich, St. Louis, MO, USA) for 30 min, a polysaccharide staining agent used to visualize EPS (35). Next, biofilm-covered samples, immersed in PBS, were imaged by CLSM (Leica TCS-SP2; Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) at $\times 40$ magnification, with laser excitation at 488 nm and 351 nm for LIVE/DEAD stain and Fluorescent Brightener 28, respectively. The images were stacked, optimized, and analyzed using FIJI software.

SEM imaging of staphylococcal biofilms. For SEM imaging, biofilm-covered samples were fixed in a fixation buffer (1% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer) for 2 h at room temperature, after which the surfaces were washed 5 times with 0.1 M cacodylate buffer, followed by 1 h of incubation at room temperature in 0.1 M cacodylate buffer supplemented with 1% OsO₄. Subsequently, the samples were washed 5 times with demineralized water and dehydrated with 30%, 50%, and 70% ethanol for 15 min each and three times with 100% ethanol for 30 min at 4°C. Finally, the samples were exposed to ethanol (100%) and tetramethylsilane (1:1 by volume) for 10 min, followed by a 15-min exposure to tetramethylsilane and air drying. Samples were sputter coated with 2- to 3-nm-thick electrically conducting metal (Au/Pd) to prevent the charging of the nonconducting section on the surface (i.e., bacteria) and analyzed by SEM, using 5, 10, and 20 kV accelerating voltages under high vacuum. The images of the biofilms were recorded both before and after transmission.

Statistics. Data were assessed for normality using a Shapiro-Wilk test ($P < 0.05$). Subsequently, the differences between multiple groups were assessed using an analysis of variance (ANOVA), after the equality of variances was tested using Levene's test ($P > 0.05$), and a Bonferroni *post hoc* test was performed to identify the differences between groups. Differences between two groups were assessed using an independent *t* test ($P < 0.05$). Statistics were performed using SPSS 23 (IBM Corp., Armonk, NY, USA).

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