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Gene Transfer Efficiency in Gonococcal Biofilms: Role of Biofilm Age, Architecture, and Pilin Antigenic Variation

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ABSTRACT

Extracellular DNA is an important structural component of many bacterial biofilms. It is unknown, however, to which extent external DNA is used to transfer genes by means of transformation. Here, we quantified the acquisition of multidrug resistance and visualized its spread under selective and nonselective conditions in biofilms formed by *Neisseria gonorrhoeae*. The density and architecture of the biofilms were controlled by microstructuring the substratum for bacterial adhesion. Horizontal transfer of antibiotic resistance genes between cocultured strains, each carrying a single resistance, occurred efficiently in early biofilms. The efficiency of gene transfer was higher in early biofilms than between planktonic cells. It was strongly reduced after 24 h and independent of biofilm density. Pilin antigenic variation caused a high fraction of nonpiliated bacteria but was not responsible for the reduced gene transfer at later stages. When selective pressure was applied to dense biofilms using antibiotics at their MIC, the double-resistant bacteria did not show a significant growth advantage. In loosely connected biofilms, the spreading of double-resistant clones was prominent. We conclude that multidrug resistance readily develops in early gonococcal biofilms through horizontal gene transfer. However, selection and spreading of the multiresistant clones are heavily suppressed in dense biofilms.

IMPORTANCE

Biofilms are considered ideal reaction chambers for horizontal gene transfer and development of multidrug resistances. The rate at which genes are exchanged within biofilms is unknown. Here, we quantified the acquisition of double-drug resistance by gene transfer between gonococci with single resistances. At early biofilm stages, the transfer efficiency was higher than for planktonic cells but then decreased with biofilm age. The surface topography affected the architecture of the biofilm. While the efficiency of gene transfer was independent of the architecture, spreading of double-resistant bacteria under selective conditions was strongly enhanced in loose biofilms. We propose that while biofilms help generating multiresistant strains, selection takes place mostly after dispersal from the biofilm.

In biofilms, bacteria are embedded in an extracellular matrix. The matrix consists mainly of polysaccharides, proteins, nucleic acids, and lipids (1). It has been demonstrated for many species that extracellular DNA (eDNA) is an important structural element and involved in adhesion. eDNA increases tolerance against cationic antibiotics and antimicrobial peptides (2, 3). Furthermore, it has been proposed that eDNA facilitates horizontal gene transfer between cells in the biofilm, but experimental proof for this hypothesis is lacking (1).

Recently, biofilm formation by the human pathogen *Neisseria gonorrhoeae* (gonococci) has been demonstrated. There is evidence that biofilms form *in vivo* and that they are associated with long-period asymptomatic carriage of *N. gonorrhoeae* in women (4). An essential component of gonococcal biofilms is extracellular DNA (5). In particular, microcolonies are stabilized by eDNA under oxygen depletion (6). Treatment with DNase destroys biofilms. The thermonuclease NucA determines the thickness of the biofilm (5), potentially controlling its remodeling. Gonococci can use a type IV secretion system for secreting DNA, and the latter is effective for transformation in liquid culture (7). Secreted single-stranded DNA (ssDNA) is important for early biofilm formation, but double-stranded DNA (dsDNA) dominates in gonococcal late biofilms (8). In the closely related species *Neisseria meningitidis*, release of eDNA was mediated by lytic transglycosylase and cytoplasmic *N*-acetylmuramyl L-alanine amidase in early biofilms and by phospholipase A-dependent autolysis in late biofilms (9).

It is tempting to speculate that eDNA is used for genetic exchange by transformation. Transformation is the import and inheritable integration of eDNA from the environment (10). Many bacterial species are naturally competent for transformation. They assemble highly efficient machines for DNA import (11). The DNA binding step requires type IV pili (T4P). Once bound, the DNA is imported into the cell against considerable external force (12, 13). The final step of transformation is the integration of the newly acquired DNA into the chromosome by homologous recombination. Transformation is therefore dependent on RecA. The probability of integration is determined by the degree of homology between the imported DNA and the chromosomal DNA (14).

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N. gonorrhoeae is competent for transformation throughout all growth phases (15), and it imports DNA at a high rate. It can import ~40 kbp DNA within several minutes and store the DNA in the periplasm (16). However, *N. gonorrhoeae* can rapidly lose competence for transformation by switching off type IV pili through antigenic variation (17, 18). T4P antigenic variation is mediated by a gene conversion system that transfers parts of the silent loci into the expressed locus of the major pilin subunit *pilE* by homologous recombination (19). Thus, antigenic variation depends on RecA (20). Four or five silent loci carry about 18 silent copies of *pilE* that lack a promoter and ribosome binding site. During antigenic variation, a portion of a silent copy is recombined into the *pilE* locus while the silent copy remains unchanged (19). Some of the silent copies carry stop codons, and thus, recombination into the expressed locus produces a nonpiliated (and thus noncompetent) variant (18, 20).

To our knowledge, few quantitative data on the efficiency of transformation in biofilms are available. For *Acinetobacter* sp. (21, 22) and *Streptococcus mutans* (23) the efficiency of transformation was measured after supplying the biofilms with an excess of transforming DNA. For both species, the transformation frequencies were highest in early biofilms. Other nonquantitative studies demonstrated gene transfer by transformation between different species in oral biofilms (24, 25).

In this work, we designed an approach that allowed us to directly visualize bacteria that acquired multidrug resistance through gene transfer by transformation within a gonococcal biofilm. We found that gene transfer was efficient in early biofilms. Pilin antigenic variation was responsible for loss of piliation but did not explain the reduction of gene transfer in late biofilms. Using micropatterning techniques, we controlled the biofilm architecture and showed that spreading of multidrug resistance occurred from the biofilm surface and was strongly suppressed in dense biofilms.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *N. gonorrhoeae* (see Table S1 in the supplemental material) was grown overnight at 37°C and 5% CO_2 on agar plates containing gonococcal base agar, consisting of 10 g/liter Bacto agar (BD Biosciences, Bedford, MA, USA), 5 g/liter NaCl (Roth, Darmstadt, Germany), 4 g/liter K_2HPO_4 (Roth), 1 g/liter KH_2PO_4 (Roth), 15 g/liter Bacto Proteose peptone no. 3 (BD), and 0.5 g/liter soluble starch (Sigma-Aldrich, St. Louis, MO), and the following supplements: 1 g/liter D-glucose (Roth), 0.1 g/liter L-glutamine (Roth), 0.289 g/liter L-cysteine-HCl · H_2O (Roth), 1 mg/liter thiamine pyrophosphate (Sigma-Aldrich), 0.2 mg/liter $Fe(NO_3)_3$ (Sigma-Aldrich), 0.03 mg/liter thiamine HCl (Roth), 0.13 mg/liter 4-aminobenzoic acid (Sigma-Aldrich), 2.5 mg/liter β-NAD (Roth), and 0.1 mg/liter vitamin B_{12} (Sigma-Aldrich). Before each experiment, gonococcal colonies were resuspended in GC medium.

Flow chamber biofilms. Biofilms were cultivated at 37°C in ibidi μ-Slides $I^{0.8}$ Luer. For experiments on the silicone surface, a bottomless sticky-Slide $I^{0.8}$ Luer was used. Silicon masters with stripes of 10-μm width and 1-μm depth were used to introduce patterns into poly(dimethylsiloxane) (PDMS [silicone]). PDMS surfaces were prepared by curing Sylgard 184 (Dow Corning) at 60°C overnight and transferred onto microscope cover slides. Coverslips (25 by 75 mm) were covered with a flat or patterned silicone (PDMS) and mounted to the self-adhesive underside of the flow cell.

For biofilm growth in a continuous-flow chamber, GC medium was diluted 1:10 in phosphate-buffered saline (PBS), pH 7.4, and 1% IsoVitaleX, 1 mM $MgCl_2$, 5 mM sodium bicarbonate, and 100 μM sodium nitrite were added. Bacteria from overnight plates of each strain were resuspended in GC-PBS to an optical density at 600 nm (OD_{600}) of 0.1, and 200 μl of each culture was inoculated into biofilm chambers and left for 1 h at 37°C to allow attachment to the glass surface. After 1 h, the flow was resumed and pumped through the flow cells at a flow rate of 3 ml/h for each channel (0.2 mm/s) by using a peristaltic pump (model 205U; Watson Marlow, Falmouth, United Kingdom).

Determination of the fraction of transformants. Biofilms were removed from the chamber and suspended in GC medium and diluted 1:10 in phosphate-buffered saline. To disrupt cell aggregates, biofilms were dispersed by pipetting, sonicating for 30 s at 20% amplitude with an ultrasonic processor (UP100H; Hielscher), and vortexing for 10 s at high speed. Cell separation was confirmed by light microscopy. Resuspended biofilm cells were serially diluted 10-fold, and 50 μl of each dilution was plated onto nonselective GC agar plates and on GC agar plates containing 2.5 μg/ml erythromycin and 100 μg/ml spectinomycin. Plates were incubated for 2 to 3 days at 37°C, 5% CO_2. Transformants were verified by dual expression of green fluorescent protein (GFP) and mCherry, confirmed by fluorescence microscopy (TE2000; Nikon). The transformation efficiency was defined as the number of resistant colonies normalized by the number of colonies on nonselective plates.

RESULTS

Strategy for quantification and direct visualization of multidrug resistance acquired by gene transfer. Throughout the paper, we will distinguish between transformation and gene transfer. Transformation refers to the process of DNA uptake and integration into the genome. Gene transfer denotes the transfer of DNA from one strain to another strain. As a consequence, the rate of gene transfer is dependent on the rate of transformation and additionally on the rates at which eDNA is generated and transported through the biofilm.

For directly visualizing the acquisition of multidrug resistance, we generated two gonococcal strains, namely, strain 1 (*igA1*::*gfp ermC*) and strain 2 (*lctP mcherry aadA aspC*) (Fig. 1). *ermC* encodes a 23S RNA methylase and confers resistance against erythromycin. *ermC* together with *gfp* was inserted into the *igA1* locus. *aadA* encodes a spectinomycin aminoglycoside-3′ adenyltransferase and confers resistance against spectinomycin. *aadA* together with *mcherry* was inserted between *lctP* and *aspC*. The growth rates of the two strains were comparable (see Fig. S1 in the supplemental material), and the fluorescent proteins were still stably expressed after passaging over the time course of 3 days (see Fig. S2 and the text in the supplemental material).

For most of our biofilm experiments, the two strains were mixed at a ratio of 1:1. Chromosomal DNA from strain 1 entering the extracellular space became a substrate for transformation of strain 2 and vice versa. Since the genes expressing the fluorescent protein and the antibiotic resistance were flanked by regions that were homologous to wild-type (wt) chromosomal DNA (*igA1* and *lctP aspC*, respectively), both the resistance gene and the fluorescence gene were integrated in one piece by homologous recombination. We verified that transformants growing on selective plates with both erythromycin and spectinomycin at their MIC were fluorescent both in the green (GFP) and red (mCherry) channel. The transformation rates for strain 1 with genomic DNA (gDNA) from strain 2 and vice versa for planktonic cells were comparable and on the order of the following: $a_{\text{planktonic}} \approx 10^{-4}$ cell^{-1} h^{-1} ≈ 10^{-4} cell^{-1} generation^{-1} at the saturating DNA concentration (see Fig. S3 in the supplemental material). In the presence of antibiotics, the transformation rates were lower by a factor of ~2.

For characterizing the fraction of transformants, bacteria were plated in the presence of erythromycin and spectinomycin at con-

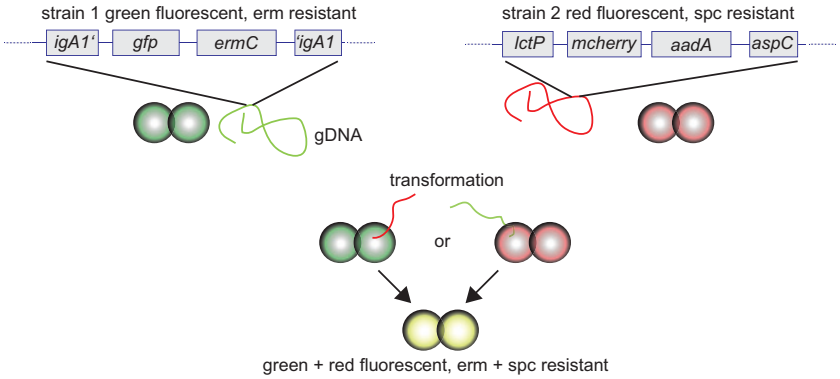

FIG 1 Detection scheme for acquisition of multidrug resistance by gene transfer. (a) Gonococcal strain 1 contains a *gfp* and *ermC* insertion within the *igA1* gene. (b) Strain 2 contains *mcherry* and *aadA* insertions between *lctP* and *aspC*. By transformation with complementary chromosomal DNA, an *igA1::gfp ermC lctP mcherry aadA aspC* strain is generated. erm, erythromycin; spc, spectinomycin.

centrations exceeding the MICs of each antibiotic. For direct visualization of transformants, we used confocal microscopy.

Gene transfer by means of transformation is efficient in early gonococcal biofilms. First, we compared the efficiency of gene transfer between planktonic cells and cells within the biofilm. Strains 1 and 2 were inoculated at a ratio of 1:1. Planktonic cells were grown in a shaking flask for 5 h. For biofilm formation, we inoculated cells into a flow chamber. The bottom of the flow cell was covered with flat silicone (PDMS). Microcolonies were motile and frequently fused to form large colonies (see Fig. S4 in the supplemental material). After 24 h and 48 h of growth, red and green bacteria were still mostly mixed. After various periods of time, gonococci were removed and plated with erythromycin and spectinomycin at concentrations exceeding the MICs of each antibiotic. The fraction of double-resistant cells was obtained by normalizing the number of colonies to the number of recovered cells. Already after 5 h, a significant fraction of transformants ($\sim 0.5 \times 10^{-4}$) was detected in the biofilm (Fig. 2). The fraction increased by a factor of ~4 after 22 h. When cells were grown planktonically in a shaking flask for 5 h, the fraction of transformants was lower by a factor of ~2 than in biofilms. We expect the gonococci to form biofilms even in a shaking flask after 5 h, and therefore we did not attempt to observe transformation under planktonic growth for longer periods of time. After 22 h, the fraction of double-resistant bacteria did not increase significantly, suggesting a strong decrease of gene transfer in late biofilms.

As a negative control, we used a $recA_{ind}$ strain in which *recA* was

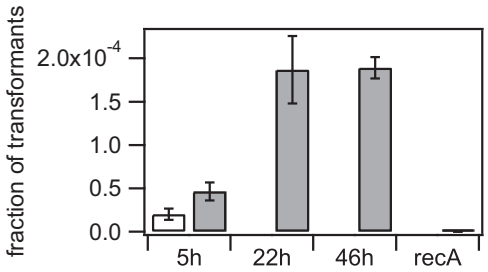

FIG 2 Gene transfer efficiency throughout biofilm development. Fraction of transformants (wt) as a function of time in a developing biofilm. *recA*, *recA*-inducible strain in the absence of induction. White, planktonic growth; gray, biofilm. $n \geq 3$ for each condition; error bars indicate standard deviations.

under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter. Since we did not add IPTG and *recA* is essential for transformation, the strain was not transformable. No double-resistant bacteria could be detected after 24 h of biofilm growth (Fig. 2).

For gene transfer to occur, extracellular DNA (eDNA) must be available. We visualized the distribution of eDNA at different stages of biofilm development of strain 1 (green) by staining the biofilm with propidium iodide (Fig. 3a and b). Propidium iodide is a DNA stain that does not penetrate intact cell membranes and will therefore stain eDNA and the DNA in dead cells that is bound to become eDNA. Already after 3 h, eDNA was clearly visible. In late biofilms, eDNA was present throughout the biofilm and accumulated at the center of the microcolonies.

In addition, we assessed the effect of DNase treatment. To characterize the biofilm architecture, we measured the biofilm mass (volume per area), which is also a quantitative measure for the density of the biofilm. Moreover, we determined the roughness coefficient as follows: $R = 1/N \sum_i |D_i - \langle D \rangle| / \langle D \rangle$, where D_i is the local biofilm thickness, $\langle D \rangle$ is the average thickness, and N is the number of pixels. ComStat was used to derive these values from confocal stacks (26). We confirmed that the biomass was considerably reduced when the biofilm was grown under continuous DNase treatment (see Fig. S5a in the supplemental material). The roughness of the biofilm increased (see Fig. S5b in the supplemental material). Gene transfer was not detectable after 5 h of continuous treatment with DNase (Fig. 3c). When the biofilm was grown for 5 h without DNase and subsequently DNase was added for an additional 17 h, no increase in the fraction of transformants was observed between 5 h and 22 h. These experiments confirm that eDNA was essential for gene transfer.

In summary, we have shown that gene transfer by means of transformation is very efficient in early gonococcal biofilms. In late biofilms, the efficiency decreased strongly.

Loss of T4P through pilin antigenic variation is not responsible for the decrease of gene transfer probability in late biofilms. Since we observed that the probability for gene transfer was higher in early biofilms than in late biofilms, we investigated whether loss of transformability was caused by pilin antigenic variation. Antigenic variation causes loss of T4P at a high rate (18), thus generating nontransformable bacteria. We performed the biofilm gene transfer assay in an antigenic-variation-deficient

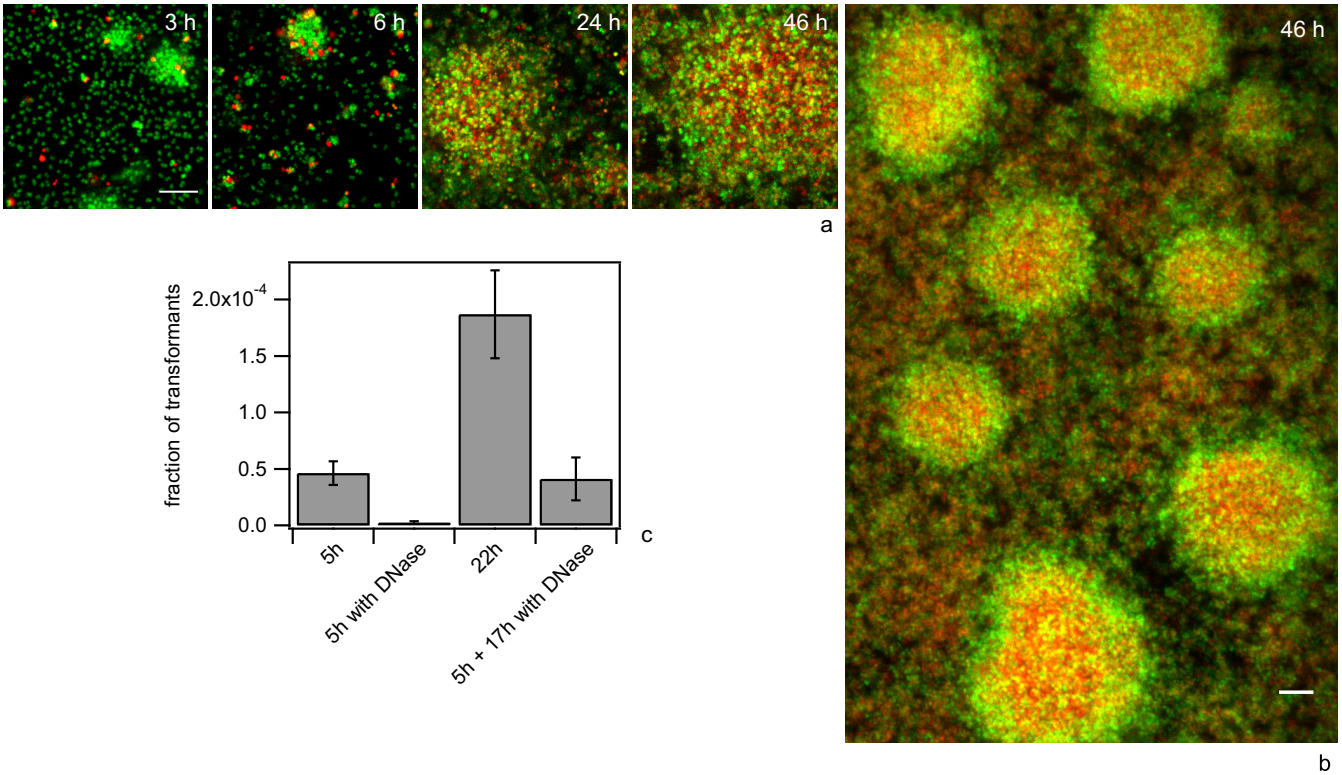

FIG 3 Distribution of free DNA in gonococcal biofilms and DNase treatment. (a) Typical confocal plane after 3 h, 6 h, 24 h, and 46 h of growth on flat surface after staining with propidium iodide. (b) Average projection of typical confocal stack after 46 h of growth. Green, bacteria; red, extracellular DNA. Scale bars, 10 μm. (c) Fraction of transformants under continuous treatment with DNase between 0 and 5 h and between 5 and 22 h compared to untreated biofilm. DNase was applied at a concentration of 10 U/ml. $n \geq 3$ for each condition; error bars indicate standard deviations.

(*avd*) background, in which the antigenic variation of the major subunit of the type IV pilus was suppressed (27). To verify changes due to antigenic variation between the inoculum and the final bacteria in a 46-h biofilm, we sequenced the variable regions of the pilin gene *pilE*. In strain 1, large stretches of the highly variable regions were exchanged (see Fig. S6 in the supplemental material). The modified stretches showed the sequence of silent pilins as expected for antigenic variation. The *avd* strain showed only single amino acid substitutions after 46 h. Thus, sequencing confirmed that pilin antigenic variation occurred within the biofilm and that it was suppressed in the *avd* strain. The loss of pili as a consequence of pilin antigenic variation was most relevant for gene transfer. The morphology of gonococcal colonies grown on agar plates indicates whether or not the bacteria generate T4P (27). We characterized piliation after 5 h, 22 h, and 46 h of growth in the biofilm by plating gonococci and evaluating colony morphology. The fraction of bacteria with a nonpiliated phenotype was ~50% after 46 h (Fig. 4a). The probability of finding nonpiliated gonococci was severely suppressed in the *avd* strain, indicating that the major cause for loss of T4P was pilin antigenic variation. Nevertheless, the fraction of transformants was not significantly larger for antigenic-variation-deficient bacteria. Between 22 h and 46 h

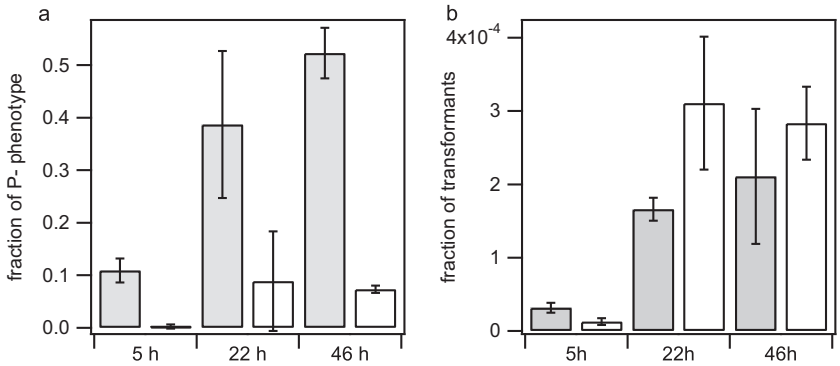

FIG 4 Effect of pilin antigenic variation on the gene transfer probability. (a) Fraction of bacteria with a nonpiliated phenotype. (b) Fraction of transformants (wt) as a function of time in a developing biofilm. Gray, wt; white, pilin antigenic-variation-deficient *avd* strain. $n \geq 3$ for each condition; error bars indicate standard deviations.

of biofilm growth, no further increase of the fraction of transformants was observed (Fig. 4b), indicating that the strong reduction of gene transfer in the late biofilm was not primarily due to pilin antigenic variation.

Surface topography governs the architecture of gonococcal biofilms. We were interested in finding out how the architecture of biofilms affected the efficiency of gene transfer in gonococcal biofilms. We had previously shown that structured surfaces affect the motility and microcolony formation of gonococci (28). Grooves with a height of 1 μm are sufficient to trap motile gonococci (29). To test whether the architecture of biofilms could be governed by surface microstructuring, the bottom of the flow cell was covered either with flat silicone (PDMS) or with patterned silicone. Gonococci were motile at the surface and formed mixed red and green microcolonies within several hours. We found that the biofilm mass was considerably lower for biofilms grown on structured surfaces (Fig. 5a and b). After 48 h, the architecture of the biofilm was very different in appearance when bacteria were grown on the structured surface and on the flat surface; in particular, biofilms grown on patterned surfaces were considerably rougher (Fig. 5c). Whereas the layer had grown dense on the flat surface, individual microcolonies were still prominent on the structured surface.

We conclude that surface structuring governs the architecture of gonococcal biofilms, in particular its density and roughness.

The gene transfer efficiency is independent of biofilm architecture. Next, we addressed the question of whether the architecture of the biofilm and in particular its density and roughness affected the probability of gene transfer. Using microstructured surfaces, the architecture of the biofilm was strongly affected (Fig. 5). We found that the fraction of transformants was independent of biofilm architecture, i.e., the gene transfer efficiency did not depend on whether gonococci were grown on flat or structured surfaces (Fig. 6).

Spreading of multiresistant transformants under selective conditions depends on biofilm architecture. Double-resistant gonococci are expected to have a fitness advantage under selective conditions. Therefore, we investigated how transformants spread in the presence of erythromycin and spectinomycin. To start with, strains 1 and 2 were inoculated at equal ratios and grown for 24 h in the absence of antibiotics. Subsequently, antibiotics were continuously supplied at a concentration of 100 μg/ml for spectinomycin and 2.5 μg/ml for erythromycin for 24 h. The concentrations of the antibiotics were chosen such that the sensitive strains did not grow and the cell density showed a decrease after 6 h of growth in liquid culture. Using time lapse confocal microscopy, we observed that the structure of the biofilm became destabilized with frequent rearrangements of microcolonies. The biomass did not decrease during drug exposure and even showed a slight increase ($P \leq 0.05$) on flat surfaces between (i) 24 h without selection and (ii) 24 h without selection plus 24 h with selection (Fig. 7a, b, and e). The roughness was comparable between selective and nonselective conditions (Fig. 7f).

Under selective conditions, dual-color transformants were observed by confocal microscopy (Fig. 7a and b). The transformants formed mostly patches, suggesting that they were the offspring of a single double-resistant bacterium. On flat surfaces, the patches were rare and most contained only a few bacteria. These patches were preferentially located at the surface of the biofilm (Fig. 7c). At locations where the biofilm was not intact, larger patches were observed (see Fig. S7a in the supplemental material). When using *recA*$_{ind}$ backgrounds without induction, we never observed dual-color patches (see Fig. S7b in the supplemental material). Furthermore, the fraction of transformants was not significantly increased under selective conditions (Fig. 7g), suggesting that application of antibiotics did not confer a selective advantage to dual-resistant gonococci in dense biofilms.

When grown on structured surfaces, large patches of transformants were observed frequently (Fig. 7b). These patches span from the substrate to the surface of the biofilm (Fig. 7d). In agreement with this observation, the fraction of double-resistant bacteria was strongly increased, by a factor of ~7, after 1 day of growth under selective conditions (Fig. 7g).

It was conceivable that selection was efficient in regions where gene expression was active, since erythromycin and spectinomycin affect translation. To test for the activity of gene expression, we generated strain P_{lac}*gfp* (K-Ng-330), in which *gfp* expression was inducible with IPTG. We found that induction was effective in early biofilms (see Fig. S8 in the supplemental material). When the biofilm was grown for 22 h without induction and subsequently IPTG was added for another period of 22 h, *gfp* was expressed (see Fig. S9 in the supplemental material). Efficient expression occurred only within patches at the surface of the biofilm, reminiscent of the pattern of double-resistant bacteria shown in Fig. 7. We note, however, that this correlation may be caused by other factors, including hindered penetration of IPTG into the biofilm or hindered folding of GFP under oxygen-limiting conditions within the biofilm.

We conclude that dense biofilms with an intact architecture inhibit spreading of multiresistance under selective conditions. When the structure is not intact and large surface areas are exposed, multiresistant clones generated by gene transfer spread rapidly.

DISCUSSION

Gene transfer is efficient in early gonococcal biofilms. We have demonstrated that gene transfer is efficient in gonococcal biofilms. At early stages, i.e., at the level of microcolonies, gene transfer is more efficient than in planktonic cells, potentially because the eDNA is trapped within the microcolony. In the following, we will estimate the rate of gene transfer in early biofilms. Via gene transfer between strain 1 and strain 2, four different strains can occur. In addition to the initial green and red strains, the dual-color green-red strain and the wt strain can emerge. Since transfer continually occurs, we expect an equilibrium between all four strains for very long time periods. In our experiments, however, the fraction of dual-color and wt strains is very low compared to that of strains 1 and 2. Therefore, we can perform a rough estimate of the gene transfer rate using the Luria-Delbrück theory. It predicts that for an exponentially growing population, the average number of transformants as a function of time behaves as follows: $<m(t)> = atN(t)$, where m is the number of transformants, a the rate of gene transfer, and N the total cell number (30). Assuming that the cells grow exponentially for up to 24 h of biofilm development, the fraction of transformants, $<m(t)>/N(t)$, is expected to increase linearly with time. Considering the data shown in Fig. 2, we find the following gene transfer rate at 5 h and at 24 h: $a_{\text{biofilm}} \approx 10^{-5}$ cell^{-1} h^{-1}. For planktonic cells, transformation of strain 1 with saturating concentrations of chromosomal

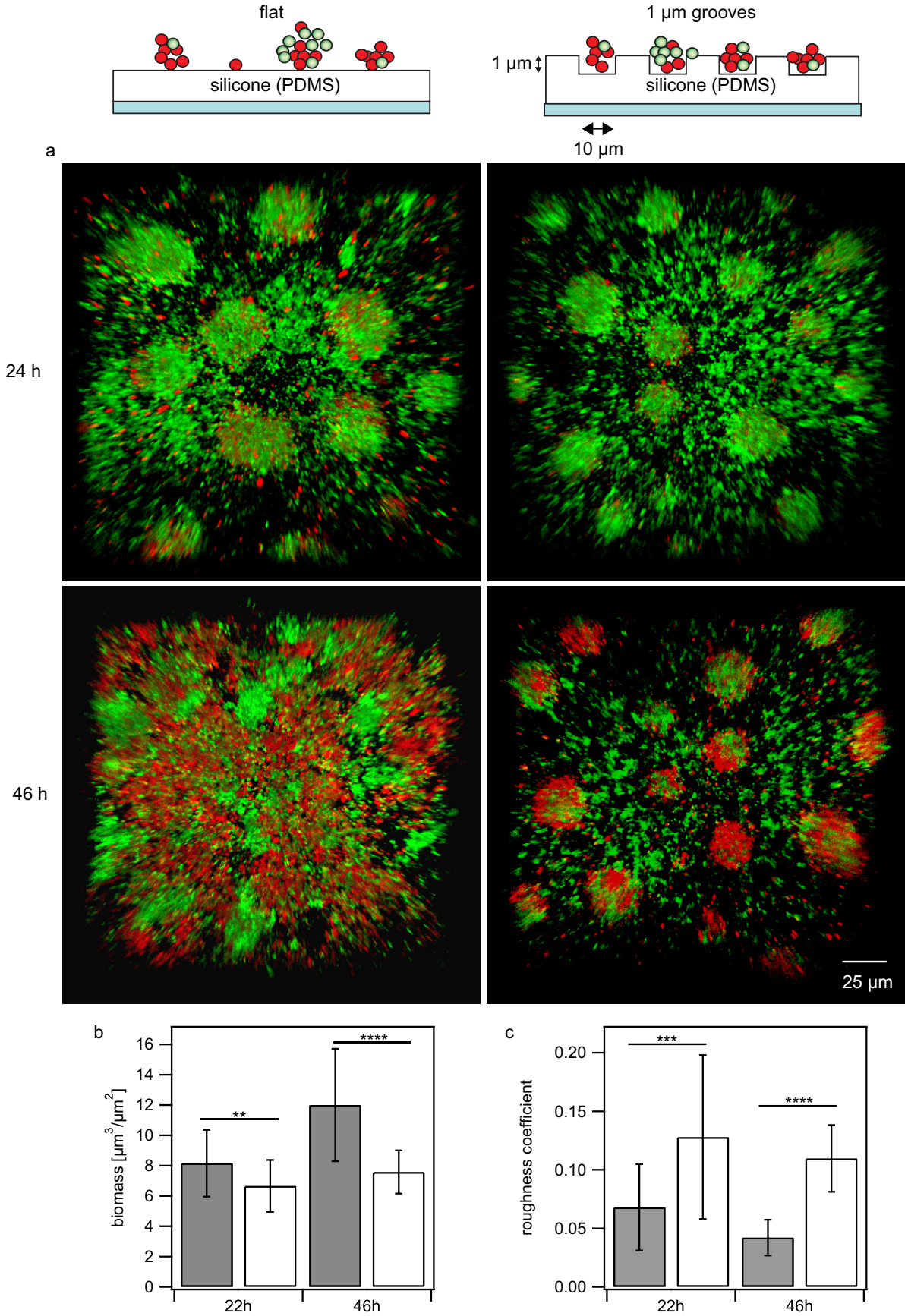

FIG 5 Biofilm architecture is influenced by surface structure. (a) Confocal volume plot near the glass coverslip after 22 h and 46 h of growth of strain 1 and strain 2 mixed at a 1:1 ratio on a flat surface (left column) and on 1-µm grooves (right column). (b) Biofilm mass; (c) roughness coefficient. Gray, flat PDMS; white, 1-µm-deep grooves (**, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$).

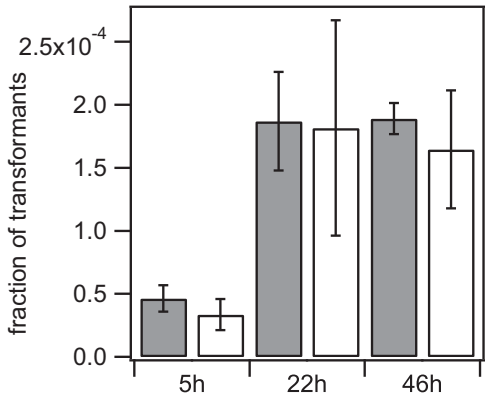

FIG 6 Gene transfer efficiency does not depend on biofilm roughness and density. Fraction of transformants as a function of time. Gray, flat surface; white, 1-μm grooves. $n \geq 3$ for each condition; error bars indicate standard deviations.

DNA from strain 2 and vice versa occurred at the following rate: $a_{\text{planktonic}} \approx 10^{-4}\ \text{cell}^{-1}\ \text{h}^{-1}$. We attribute the difference to the fact that within early biofilms eDNA is produced and transported too slowly to saturate the transformable bacteria with DNA, suggesting that production and transport of DNA are the rate-limiting steps in early biofilms.

Potential causes for decrease of gene transfer efficiency in late biofilms. Between 24 h and 48 h, the fraction of transformants did not increase in the absence of selection. One possible explanation for the reduced gene transfer rate was the loss of T4P through mutations. By far the most likely cause for loss of T4P is pilin antigenic variation (18). However, we found that an antigenic-variation-deficient strain showed no increase in the fraction of transformants between 24 h and 48 h. The total fraction was comparable to that of the wild type, indicating that loss of pili due to antigenic variation was not the major cause for the reduction of gene transfer in late biofilms. While the most likely cause of loss of piliation has been attributed to antigenic variation (18), phase variation of genes involved in pilus biogenesis might reduce the transformability within late biofilms. However, for the *avd* strain we found that more than 90% of the gonococci had piliated colony morphology after 24 h. Therefore, most likely other mechanisms inhibit gene transfer. Although gonococci are continuously supplied with fresh medium, oxygen might become limiting within biofilms, inhibiting transformation (20). Oxygen levels have a strong effect on type IV pilus dynamics (6, 31) and are therefore likely to affect the transformation rate. Another explanation for the reduction of gene transfer probability might be a reduced mobility of DNA. With increasing density of the biofilm matrix, the movement of DNA between bacteria is likely to be inhibited. In particular, it is unclear whether eDNA is free in solution or whether a large fraction of DNA is masked by extracellular vesicles (32). The transformation rates of *Acinetobacter* sp. and of *Streptococcus mutans* under exposure to external plasmid DNA were measured as a function of biofilm age (21, 23). For both species the transformation rate was highest in early biofilms, in agreement with our data. Furthermore, efficient conjugation was observed in biofilms. However, transconjugants were found predominantly at the surface of the biofilm, again suggesting that within the bulk of the biofilm, gene transfer was inhibited (22).

Multiresistant bacteria do not spread under selective conditions in intact biofilms. We observed that the fraction of dual-resistance gonococci did not increase when the 24-h-old biofilm was treated with erythromycin and spectinomycin for another 24 h on flat surfaces. On the other hand, on the structured surface with decreased biofilm mass, i.e., lower density, spreading was very efficient. The concentrations of the antibiotics (100 μg/ml spectinomycin, 2.5 μg/ml erythromycin) were chosen such that the sensitive strains did not grow, and the cell density showed a decrease after 6 h of growth in liquid culture. It has been argued that within biofilms bacteria are in the stationary state, which protects them from the action of bacteriostatic antibiotics (33). However, in our setup the biofilm mass on the flat surface increased between 24 h and 48 h even when antibiotics were applied, whereas no increase was observed on structured surfaces. Interestingly, the pattern of cells in which expression of *gfp* was induced in the late biofilm (see Fig. S9 in the supplemental material) was reminiscent of the pattern of dual-color bacteria in dense biofilms (Fig. 7). Patches of bacteria at the surface of the biofilm expressed *gfp*. Likewise, patches of double-resistant bacteria were observed at the surface. Since both erythromycin and spectinomycin affect translation, selection is likely to act mostly on cells that have a high rate of gene expression. Other explanations for reduced induction of P_{lac}*gfp* (K-Ng-330) are reduced diffusion of IPTG within the biofilm and reduction of oxygen, which is required for folding of GFP. Theoretical estimates together with experiments in *Pseudomonas aeruginosa* biofilms strongly suggest that IPTG can diffuse rapidly within the biofilm (34, 35). It is therefore tempting to speculate that reduced gene expression activity within dense biofilms affects tolerance to the antibiotics. Another mechanism could be sequestration of the antibiotics by the biofilm matrix (33). eDNA has been shown to increase the tolerance of biofilms against positively charged antibiotics, including aminoglycosides such as spectinomycin (2, 3). We propose that the dense biofilm matrix on the flat surface and reduction of gene expression activity prevent spreading of double-resistance bacteria by reducing the fitness advantage of the transformants.

Surface topography governs biofilm architecture. Gonococci adhere well on the patterned surface, but the biofilm mass is strongly reduced after 2 days and the biofilm is considerably more susceptible to antibiotic treatment. The size of the grooves was large enough for a single bacterium to adhere, and we have shown previously that gonococci are motile within these grooves (29). Recent reports show that decreased adherence of bacteria to micropatterned surfaces is a general phenomenon. *P. aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Escherichia coli* showed severely reduced biofilm mass when grown on Sharklet structures with dimensions similar to those of our structures (36). Marine biofilm volumes were strongly reduced on micropatterned surfaces with similar dimensions, and their composition and sensitivity to antibiotics were altered (37). Whereas decreased wetting of the surface by bacterial appendages and biofilm matrix may explain decreased biofilm formation on nanopatterned surfaces (38), the underlying mechanism that causes reduced biofilm mass on microstructured surfaces remains elusive. In fact, patterning can even increase the biofilm mass through flagellum-mediated attachment (39). In this study, we have used micropatterning as a tool to influence biofilm architecture without changing the genome of the gonococci.

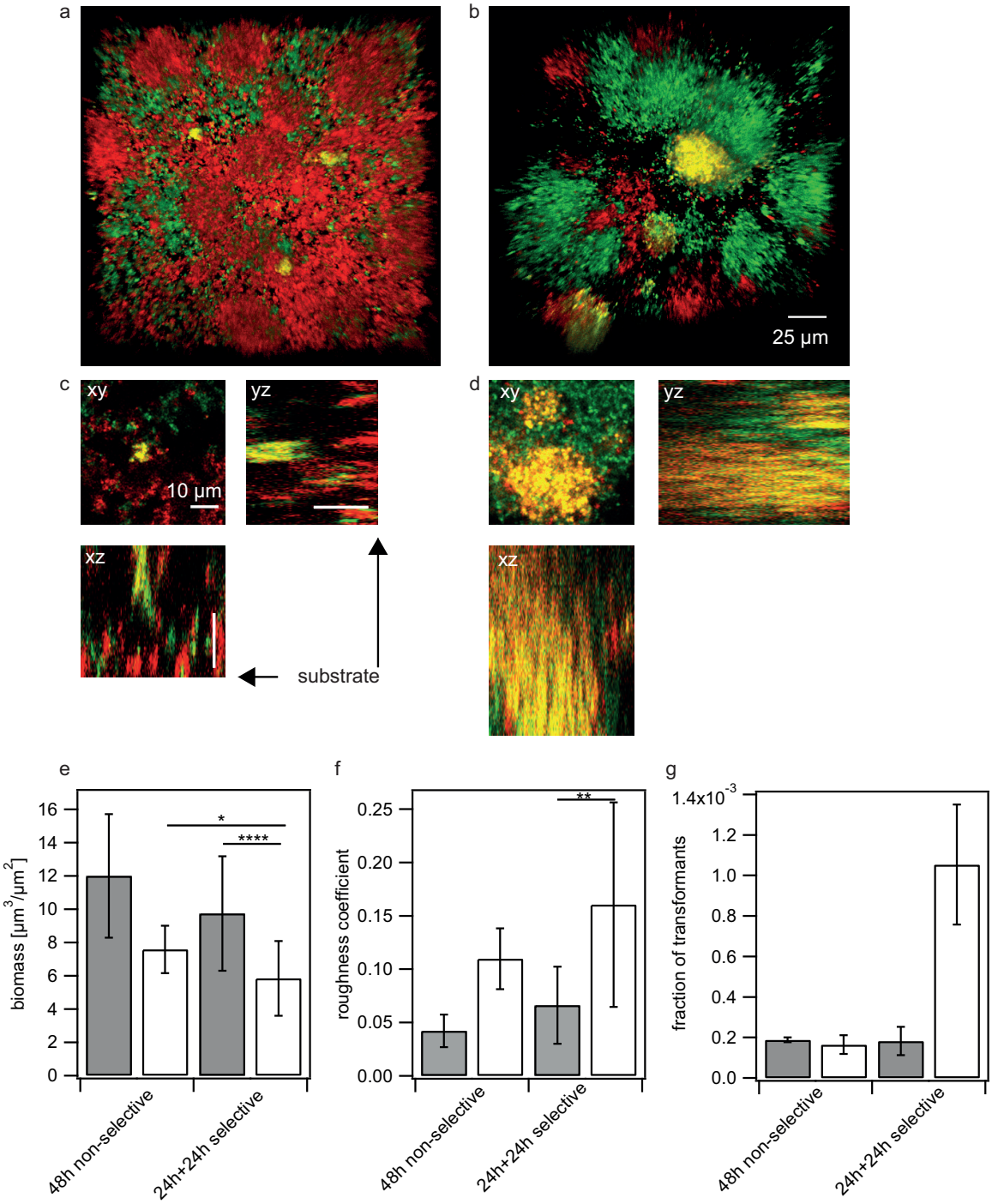

FIG 7 Spreading of multiresistant clones under selective pressure. Biofilms (strain 1 and strain 2 mixed at a 1:1 ratio) were grown without antibiotics for 24 h and subsequently treated with 2.5 μg/ml erythromycin and 100 μg/ml spectinomycin. Confocal volume plot on flat surface (a) and 1-μm grooves (b) and orthogonal views on flat surface (c) or 1-μm grooves (d). (e) Biomass; (f) roughness coefficient (*, $P < 0.05$; **, $P < 0.005$; ****, $P < 0.00005$); (g) fraction of transformants. $n \geq 3$ for each condition; error bars indicate standard deviations. Gray, flat; white, 1-μm grooves.

Conclusion. We have quantified the transfer of genes conferring antibiotic resistance between single-resistance bacteria within gonococcal biofilms of different age and the subsequent spreading of double-resistance gonococci. Gene transfer was efficient in early biofilms. It will be very interesting to assess the underlying reason for reduction of gene transfer rate in late biofilms by investigating the mechanisms of DNA transfer. Furthermore, host cells may affect the rate of gene transfer during infection. Most likely, the architecture of biofilms depends on the interaction of gonococci with the host cell surface. Moreover, differential regulation of type IV pilus-specific genes may influence the probability of transformation. Future experiments will address how the

rate of gene transfer is affected by the host cells. Unexpectedly, the rate of spreading under selective conditions was below the detection limit for intact biofilms, suggesting that the biofilm may act as a reservoir for multiresistant clones that are only selected for when they disperse from the biofilm.

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