Rapid Diffusion of Fluorescent Tracers into Staphylococcus epidermidis Biofilms Visualized by Time Lapse Microscopy

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The transient diffusion of fluorescent tracers into biofilm cell clusters of Staphylococcus epidermidis was visualized by time lapse confocal scanning laser microscopy. Rhodamine B diffused into the center of cell clusters that were 200 to 600 μm in diameter within a few minutes. The apparent effective diffusion coefficient calculated from these data averaged $3.7 \times 10^{-5}$ cm$^2$ s$^{-1}$ or 11% of the value in pure water. Fluorescein diffused into biofilm more rapidly, with a diffusion coefficient that averaged $1.6 \times 10^{-4}$ cm$^2$ s$^{-1}$, or 32% of the value in water. This study provides direct, visual confirmation that solutes the size of many antibiotics and biocides can diffuse rapidly into biofilms.

MATERIALS AND METHODS

Bacteria and media. Staphylococcus epidermidis strain RP62A (ATCC 35984) was grown on tryptic soy broth (TSB) at 37°C. Full strength TSB was used to grow shake flask cultures that provided the inoculum for biofilm experiments. Biofilms were grown on 1/10 strength TSB. Autoclaved 1/10 strength TSB was used for cultivation of microbial cell clusters, noninvasively and in real time. This was accomplished by using fluorescent dyes that were imaged by confocal scanning laser microscopy. Quantitative image analysis was performed to extract numerical values of the effective diffusion coefficient in the biofilm.

The fluorescence intensity at the center of a cell cluster was measured as a function of time. The time series typically ran for 5 to 10 min. Images were analyzed in MetaMorph software (Universal Imaging Corporation, Downington, Pa.). From these experiments, a time series was initiated in which an image was collected every 5 s. At the same time, the flow of phosphate-buffered saline through the capillary was changed to buffer containing 5 mg of rhodamine B (Eastman Organic Chemicals, Rochester, N. Y.) per liter or 50 mg of disodium fluorescein (Sigma Aldrich, Milwaukee, Wis.) per liter. The time series typically ran for 5 to 10 min. Images were analyzed in MetaMorph software (Universal Imaging Corporation, Downington, Pa.).

Some experiments were performed in which a biofilm was exposed to both rhodamine B and fluorescein. A time series was initiated in which an image was collected every 5 s. At the same time, the flow of phosphate-buffered saline through the capillary was changed to buffer containing 5 mg of rhodamine B (Eastman Organic Chemicals, Rochester, N. Y.) per liter or 50 mg of disodium fluorescein (Sigma Aldrich, Milwaukee, Wis.) per liter. The time series typically ran for 5 to 10 min. Images were analyzed in MetaMorph software (Universal Imaging Corporation, Downington, Pa.).

Estimation of diffusion coefficient. The fluorescence intensity at the center of a biofilm cell cluster was extracted at each time point by using the MetaMorph software. The resulting intensity versus time data were exported to a spreadsheet. The solution to the diffusion equation in spherical coordinates (1) was fitted to the experimental data by adjusting the values of two parameters, the steady-state staining intensity and the effective diffusion coefficient, to minimize the sum of squares. The mean radius of the cell cluster was estimated from the transmission image of the cell cluster.

RESULTS

S. epidermidis formed extensive, heterogeneous biofilms in glass capillary tubes after 24 h of continuous development at 37°C (Fig. 1A). Thick biofilm formed in the corners of the flow cell and also occasionally as large clusters in the middle of the tube walls (Fig. 1B). These isolated, rounded clusters were
used for diffusion measurements. There were significant areas of the tube that had little or no biofilm accumulation.

The diffusion of rhodamine into the interior of cell clusters was imaged (Fig. 2). The dye first stained the periphery of a cell cluster and then progressively moved inward toward the center of the cluster (Fig. 2). A movie of this sequence can be viewed at http://www.erc.montana.edu/Res-Lib99-SW/Movies/Database/MD_DisplayScript.asp. The centers of cell clusters were the last regions to take up the stain. Image analysis of the intensity of red fluorescence at the center of the cell cluster allowed the diffusive penetration of the dye to be quantified (Fig. 3). Typical S-shaped curves were measured in these experiments. Image analysis was also applied to describe the radial profiles of staining intensity at different time points (Fig. 4).

We attempted to use a higher-magnification objective to address the question of whether there could be localized regions in the cluster interior where some of the cells were not stained by rhodamine B. While individual cells at the periphery of cell clusters could be resolved, it was not possible to obtain clear images of individual cells in the interior of cell clusters. This is probably due to the high cell density and relative opacity of these biofilms. Though clear pictures are lacking, these high-magnification explorations turned up no evidence for pockets of cells that were not accessed by the stain. Images at higher magnification suggested tight mosaics of cells, all of which were stained by the dye.

The time scale for diffusive penetration of rhodamine B into S. epidermidis cell clusters, defined as the time required to attain 90% of the equilibrium staining intensity at the center of the cell cluster, ranged from 1.1 to 6.7 min for cell clusters ranging in diameter from about 240 to 590 μm (Table 1). The effective diffusion coefficients derived from these data ranged...
from $2.0 \times 10^{-7}$ to $6.5 \times 10^{-7}$ cm$^2$ s$^{-1}$, or 6 to 18% of the diffusion coefficient in pure water (Table 1). The mean relative effective diffusion coefficient (the value in biofilm divided by the value in water) was 0.11 $\pm$ 0.05. The uncertainty indicated

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<th>$R$ (µm)</th>
<th>$T_{50}$ (s)</th>
<th>$T_{90}$ (s)</th>
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$^a$ $R$, cell cluster radius; $T_{50}$, time required to attain 50% of the equilibrium concentration at the cluster center; $T_{90}$, time required to attain 90% of the equilibrium concentration at the cluster center; $D_e$, effective diffusion coefficient; $D_e/D_{aq}$ relative effective diffusion coefficient. Mean $D_e$, $3.7 \times 10^{-7}$; mean $D_e/D_{aq}$ 0.11.

is the standard deviation. The diffusion coefficient of rhodamine B in water at 21.5°C, as calculated from the Wilke-Chang correlation, is $3.6 \times 10^{-6}$ cm$^2$ s$^{-1}$. There was no apparent trend of diffusion coefficient with cluster size.

The behavior exhibited by fluorescein in $S. epidermidis$ biofilm was different from that of rhodamine B. Rhodamine B fluorescence was brighter in biofilm than it was in the bulk fluid (Fig. 5), suggesting that this dye bound to the biofilm matrix and was concentrated there. In contrast, fluorescein did not stain the biofilm. Fluorescein fluorescence was strongest in the fluid outside the biofilm, while the biofilm itself remained dark even after prolonged incubation with the dye (Fig. 5). This suggested that fluorescein did not bind to the biofilm but was repelled by the biofilm matrix. Fluorescein fluorescence may also be quenched by the extracellular polymeric matrix of the biofilm. In some biofilm cell clusters, hollow centers were

$FIG. 3$. Rhodamine B staining intensity at the center of a $S. epidermidis$ biofilm cell cluster for the experiment dated 14 February 2003. Time zero corresponds to the first appearance of the dye in the flow cell. Open circles represent experimental data, and the line indicates the fitted diffusion equation in spherical coordinates.

$TABLE 1$. Summary of measured effective diffusion coefficients of rhodamine B in $S. epidermidis$ biofilm

$FIG. 4$. Profiles of rhodamine B staining intensity along radial transects through a $S. epidermidis$ biofilm cell cluster for the experiment dated 14 February 2003. Zero on the $x$ axis corresponds to the center of the cell clusters. Each curve is labeled with a time, in seconds, where time zero corresponds to the first appearance of the dye in the flow cell. Data were smoothed by the Lowess function to obtain the curves shown.

$FIG. 5$. A hollow $S. epidermidis$ biofilm cell cluster stained with rhodamine B (red) and negatively stained by fluorescein (green).
noted. These hollow regions were evident as zones that failed to stain with rhodamine B (Fig. 5) but did exhibit fluorescein fluorescence (Fig. 5). This observation shows that while fluorescein did not sorb to the biofilm matrix, it was able to permeate the matrix.

We note that the holes seen in some cell clusters are real; they have been confirmed by microscopic examination of cryosections, by transmission electron microscopy, and by magnetic resonance microscopy (data not shown).

In several cell clusters with such hollow centers, the diffusive penetration of fluorescein was measured by time lapse microscopy by using the same protocols used for rhodamine B. The time required to attain 90% of the equilibrium staining intensity at the center of the cell cluster ranged from 1.4 to 2.2 min for cell clusters ranging in diameter from about 360 to 590 μm (Table 2). The effective diffusion coefficients derived from these data ranged from 1.0 × 10⁻⁶ to 2.1 × 10⁻⁶ cm² s⁻¹, or 20 to 43% of the diffusion coefficient in pure water (Table 2). The mean relative effective diffusion coefficient for fluorescein was 0.32 ± 0.10, where the uncertainty indicated is the standard deviation. The diffusion coefficient of fluorescein in water at 21.5°C, as calculated from the Wilke-Chang correlation, is 4.9 × 10⁻⁶ cm² s⁻¹.

The relative diffusion coefficient of fluorescein was statistically significantly greater than that for rhodamine B by a factor of 2.9 (P = 0.006 by two-sided t test). In three experiments in which the diffusion of rhodamine B and fluorescein was measured in the same cell cluster, the diffusion coefficient for fluorescein averaged 3.0 times that for rhodamine (Fig. 6). In these three experiments, the relative effective diffusion coefficient for fluorescein averaged 2.4 times that for rhodamine B.

**DISCUSSION**

Confocal scanning laser microscopy has enabled the direct visualization of the diffusive penetration of fluorescent dyes into staphylococcal biofilm cell clusters, noninvasively and under continuous-flow conditions. Fluorescent tracers with molecular weights (MWs) of approximately 400 saturated the interior of cell clusters that were a few hundred micrometers in diameter within a few minutes. This qualitative result shows that the biofilm matrix does not exclude solutes of this size. By approximating cell clusters as hemispheres, it was possible to make quantitative determinations of effective diffusion coefficients in these biofilms.

The relative diffusion coefficients for rhodamine B (0.32) and fluorescein (0.11) in a staphylococcal biofilm measured in this investigation agree with reported relative effective diffusion coefficients for solutes of similar size in biofilms. The MW of rhodamine B is 442 and of fluorescein is 376. The mean relative effective diffusion coefficient for sucrose (MW, 342) in various biofilms is 0.19 (5). The relative diffusivity of the antibiotic ciprofloxacin (MW, 330) in *Pseudomonas aeruginosa* biofilms was reported to be 0.31 (8). The relative diffusion coefficient of chlorhexidine digluconate (MW, 898) in *Candida albicans* biofilms was approximately 0.2 (7). Tatevossian (9) reported a relative effective diffusion coefficient of inulin (MW, ~5,200) of 0.12 in dental plaque. Together these measurements suggest that solutes with MWs in the range of a few hundred to a few thousand diffuse in biofilms at 10 to 35% of the rate they do in pure water.

Our conclusion that antibiotic-sized solutes penetrate *S. epidermidis* biofilms is consistent with the few reports in which antibiotic penetration has been experimentally measured in biofilms formed by this microorganism (2, 3, 10, 11). The rapid penetration we observed is also in agreement with the work of Stone et al. (6), who used confocal scanning laser microscopy to demonstrate tetracycline permeation throughout *Escherichia coli* biofilms within 3 min.

The progressive pattern of inward diffusion (Fig. 2) was symmetric in every experiment. More specifically, there was no difference in the permeation of dyes on the upstream and downstream edges of a cell cluster. If convective flow within the cell cluster contributed to the transport of the tracer, then one would expect to see greater penetration of dyes on the upstream edge of the cluster than on the downstream edge. The fact that this was not observed confirms that convective transport inside the cell cluster was insignificant.
The slower diffusion of rhodamine B compared to fluorescein was likely due to adsorption of rhodamine B to the biofilm matrix. Sorption is predicted, on theoretical grounds, to retard the penetration of a solute diffusing in a heterogeneous medium (4). While rhodamine B clearly bound to the biofilm, there was no indication that fluorescein interacted with the biofilm matrix (Fig. 5).

The inability of antimicrobial agents to control microorganisms in biofilms is often attributed to the failure of these agents to penetrate the biofilm. This explanation is simple and intuitive and could apply to antimicrobial agents of diverse chemistries. But this hypothesis is probably incorrect. As the visual and quantitative data reported in this article demonstrate, there is no generic physical barrier to the permeation of solutes the size of most biocides and antibiotics into microbial biofilm. Antimicrobial agents likely do penetrate biofilms in most cases, except when subject to rapid neutralizing reactions in the biofilm (4, 5). Mechanisms of biofilm protection that derive from the biology of microorganisms in biofilms should be pursued.

ACKNOWLEDGMENT

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REFERENCES