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A dual fluorescence technique for visualization of *Staphylococcus epidermidis* biofilm using scanning confocal laser microscopy

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A new dual fluorescence technique is described which, when combined with scanning confocal laser microscopy (SCLM), can be used to visualize the components of biofilm produced by *Staphylococcus epidermidis*. Chemostat cultures of RP62A (a well-characterized slime-producing strain of *S. epidermidis*) were used to produce mature biofilm on polyvinylchloride (PVC) disks immobilized in a modified Robbins device using a 'seed' and 'feed' model system. Serial horizontal and vertical optical thin sections, as well as three-dimensional computer reconstructions, were obtained on *in situ* biofilm using the dual fluorescence procedure. Bacteria were visualized by green autofluorescence excited at 488 nm with an Argon laser. Cell-associated and exocellular matrix material (slime) was visualized by red fluorescence excited at 568 nm with a Krypton laser after interaction of the biofilm with Texas Red-labeled wheat germ agglutinin which is a slime-specific lectin marker. Structural analysis revealed that the cocci grew in slime-embedded cell clusters forming distinct conical-shaped microcolonies. Interspersed open channels served to connect the bulk liquid with the deepest layers of the mature, hydrated biofilm which increased overall surface area and likely facilitated the exchange of nutrients and waste products throughout the biofilm. The combined dual fluorescence technique and SCLM is potentially useful as a specific noninvasive tool for studying the effect of antimicrobial agents on the process of biofilm formation and for the characterization of the architecture of *S. epidermidis* biofilm formed *in vivo* and *in vitro* on medical grade virgin or modified inert polymer surfaces.

Keywords: *Staphylococcus epidermidis*; biofilm; laser scanning confocal microscopy; slime; lectin marker

Introduction

In humans, *Staphylococcus epidermidis* is the most common cause of infections associated with the use of medical devices and prosthetic implants [9]. This association with medical grade biomaterials has been explained, in part, by the capacity of the organism to form biofilms in which they colonize inert surfaces and grow embedded in an exocellular matrix material (slime) which they produce [2,5,22]. Lawrence *et al* [17] define 'biofilm' as 'an open system of cells, exopolymeric material, and extracellular spaces' and 'biofilm architecture' as the spatial arrangement of these three components. Biofilm architecture has been difficult to determine because images obtained by light or fluorescence microscopy are degraded by out-of-focus flare from focal planes above or below objects of interest [3]. This problem has been overcome by use of scanning confocal laser microscopy (SCLM) which allows horizontal and vertical optical thin sectioning of hydrated biofilms [3], including those that are formed *in vivo* [12]. SCLM imaging requires the use of fluorescent markers to differentiate the cells, matrix, or spaces of a biofilm. Fluorescein has been used as a positive stain for bacteria [29], as well as a nonspecific negative stain for biofilm formed by Gram-negative bacilli; in the latter, bacilli, which exclude the dye, appear black against a fluorescent background of matrix and spaces [4,17,18,29]. Wolfaardt *et al* [29] used the dye Nile Red, which binds to hydrophobic groups, to stain capsular material surrounding rod-like cells in biofilm formed by a complex degradative consortium of bacteria isolated from soil [28]. Fluorescein-labeled UEA gorse lectin, specific for L-Fucα-1,2Gal and (GlcNAcβ-1,4)$_2$ [10], was used to visualize the exopolymer matrix of *Hyphomicrobium* spp in a mixed marine biofilm [referred to in reference 3]. Size-fractionated fluor-conjugated dextrans have been used to determine the presence and penetration of vertical and horizontal channels (spaces) within biofilms formed by Gram-negative bacilli [3,4], as well as the coefficients of diffusion of these inert particles [18].

In the present study, a dual fluorescence technique combined with SCLM was developed to characterize the previously undescribed architecture of hydrated biofilm formed *in situ* by a well-characterized slime-producing strain of *S. epidermidis*. Two components of the biofilm were visualized: 1) cells, utilizing natural autofluorescence, and 2) slime matrix material, utilizing a specific Texas Red-labeled lectin probe [24]. The results were compared with images of the dehydrated biofilms obtained by conventional scanning (SEM) and transmission electron microscopy (TEM), which have been widely used to visualize *S. epidermidis* biofilm.

Materials and methods

Media and reagents

The following media and chemical reagents were used: FMC, an enriched chemically-defined medium [20], pH

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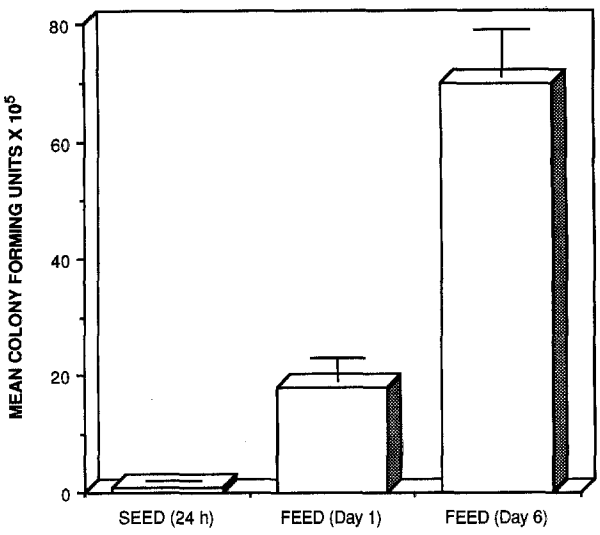

Figure 1 Results of colonization of PVC disks by *S. epidermidis* RP62A. The surfaces of the disks were exposed to a free-flow of the bacterial suspension for 24 h to seed the surfaces, followed by a free-flow of medium for 1 and 6 days in a combined chemostat-modified Robbins device model system. The data is expressed as mean colony forming units. Thin bars represent the standard error of the means of six determinations.

7.2; trypticase soy agar (TSA, Difco Laboratories, Detroit, MI, USA); phosphate-buffered saline (PBS), pH 7.2; FTA haemagglutination buffer (BBL, Cockeysville, MD, USA); Tween 20 and Tween 80 (Fisher Scientific Co, Pittsburgh, PA, USA); Count-Off (NEN Research Products, Boston, MA, USA); cacodylic acid (sodium salt) (Ernest F Fullam, Schenectady, NY, USA); glutaraldehyde (Eastman Kodak, Rochester, NY, USA); OsO_4 (Electron Microscopy Sciences, Ft Washington, PA, USA); uranyl acetate (EMS), the PolyBed 812 kit (Polysciences, Warrington, PA, USA); Texas Red-labeled *Triticum vulgaris* (wheat germ) agglutinin and wheat germ agglutinin labeled with 15 nm gold colloidal particles (E-Y Laboratories, San Mateo, CA, USA); SlowFade antifade reagent in glycerol/PBS (Molecular Probes, Eugene, OR, USA); and Crystal/Mount aqueous mounting medium (Biomeda Corp, Foster City, CA, USA). The remaining reagents were purchased from Sigma Chemical Co (St Louis, MO, USA); acid hydrolysate of casein (amicase, from bovine milk); $CaCl_2$; $MnCl_2$; sodium veronal (barbital); sodium acetate; and lead citrate.

Bacteria

A slime-producing strain of *S. epidermidis*, RP62A [6] (American Type Culture Collection no. 35984) was kindly supplied by Gordon Christensen (Harry S Truman Memorial Veterans' Hospital, Columbia, MO, USA).

Biomaterials

Injection-molded, medical-grade polyvinylchloride (PVC) test polymer disks (7.8 mm diameter × 2 mm thick) were supplied by Duane Horton (Mallinckrodt Medical, St Louis, MO, USA). Disks were cleaned with 0.1% Count-Off in dH_2O, washed exhaustively in dH_2O, drained and air-dried. Disks were placed in a modified Robbins flow-through device (MRD) [21] (University of Exeter, Department of Biological Sciences, Hatherly Laboratories, Exeter, UK) which is designed to hold 25 samples in a fixed position. This design prevents gravitational settling out of bacterial cells on the disks' surfaces because the surface of each disk is flush with the *top* of a trough that runs the length of the device. The device, with disks, was sterilized by ethylene oxide, and degassed for 7 days before use.

'Seed and feed' model for biofilm formation

A 750-ml volume of sterile FMC was pumped into the culture vessel of a Bioflo bench top chemostat (model C30, New Brunswick Scientific Co, Edison, NJ, USA); the medium was aerated at 0.2 L min^{-1} at 37°C and agitated at 200 rpm for 24 h to confirm sterility. A 12-ml vol of FMC was inoculated with RP62A, incubated at 37°C for 18 h, and used to inoculate the culture vessel. Fresh medium was then pumped in and the culture suspension was simultaneously pumped out at a rate of 30 ml h^{-1} until the culture reached a concentration of 2×10^9 CFU ml^{-1}, T_D of 14 h. At this point, the culture suspension was pumped, at a constant rate of 30 ml h^{-1}, through the MRD which was placed in an incubator at 37°C. The surfaces of polymer disks were 'seeded' by a continuous flow of bacteria through the device for 24 h; the MRD was switched to a culture vessel containing medium only, and seeded disks were exposed to a continuous flow of the medium for an additional 6 days.

Measurement of cell and slime components in in situ biofilm

Disks were removed from the MRD, drained, agitated for 5 s in sterile PBS, drained, and agitated in two fresh changes of PBS to remove unattached bacteria. Each disk was removed from the stub using a sterile needle, placed in a 10-ml vol of PBS that contained 0.1% Tween-80 and sonicated in a Branson 1200 ultrasonic cleaner waterbath (Branson Ultrasonics Corp, Danbury, CT, USA) for 45 min with intermittent vigorous vortexing to detach adherent bacteria [1] and to break up clumps. Ten-fold serial dilutions of each suspension were plated on TSA using the agar-overlay technique, incubated at 37°C for 18 h and the mean number of CFU determined. The arithmetic means, standard deviations, and standard errors of the means were determined for each set of quantitative data using the StatView 512+ computer package (BrainPower, Calabasas, CA, USA).

The percentage of slime matrix material was determined as follows: biofilm was harvested from 25 disks by scraping; the pooled material was suspended in PBS that contained 0.1% Tween-80 at a concentration of 10% (wet weight/volume); the suspension was homogenized in a Sorval Omni-Mixer at maximum speed for 10 min followed by centrifugation at 1500 × *g* for 20 min. Emulsified slime matrix material was present in the supernatant fluid. The percentage of slime was calculated by dividing the wet weight of the slime component by the wet weight of the total biofilm (cells + slime matrix). Determinations were based on three separate experiments.

Scanning electron microscopy

After removal from the MRD, disks were dipped 3× in PBS, taking care not to disturb the *in situ* biofilm, placed in vials, and fixed overnight in 2% glutaraldehyde in 0.2 M

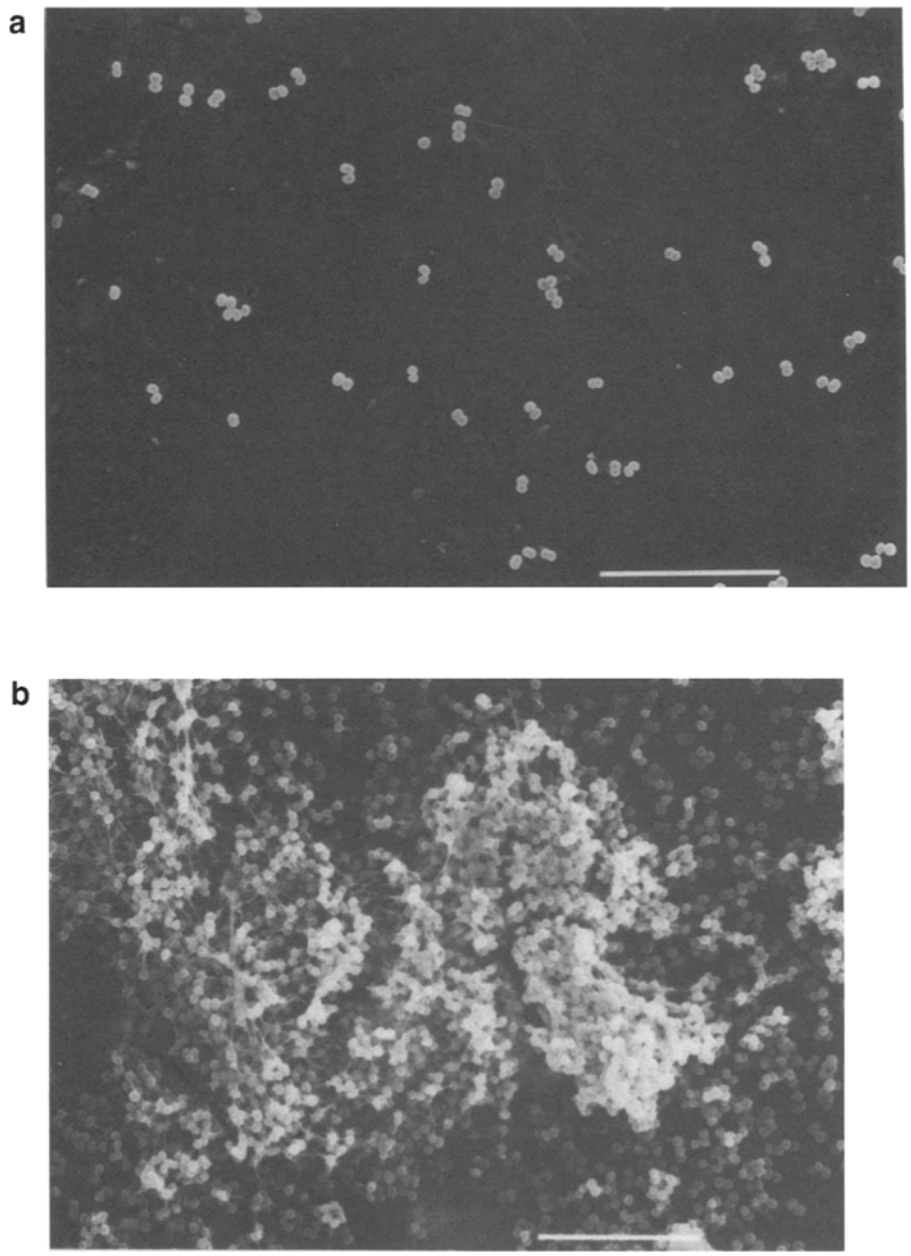

Figure 2 Scanning electron micrographs of the surface of PVC disks seeded with *S. epidermidis* RP62A (a) and then exposed to a continuous flow of sterile medium for an additional 6 days (b). The cocci were evenly distributed on the disk surface after 24 h (a), and appeared to form compact multilayered microcolonies with strands of amorphous exocellular matrix material (slime) by day 6 (b). Bar = 10 μm.

cacodylate-HCl buffer at 4°C. Disks were rinsed with 0.1 M PO_4 buffer, osmicated in 1% veronal-acetate buffered osmium [14] for 30 min, rinsed in buffered veronal-acetate buffer, dehydrated for 30 min each in 50%, 75%, and 95% *t*-butanol, and rinsed 3× in 100% *t*-butanol [15]. Disks, in 100% *t*-butanol, were put on ice to freeze, desiccated 2–3 h until completely evaporated, mounted, sputter-coated with gold-palladium and examined with a JEOL 840A scanning electron microscope (JEOL Ltd, Tokyo, Japan).

Lectin molecular probe

Disks were gently overlayed 3× with 1 ml of PBS, care being taken not to disturb the biofilm, prefixed overnight in 2% glutaraldehyde in 0.2 M cacodylate-HCl buffer at 4°C, overlayed 3× with 1 ml of 0.2 M cacodylate-HCl buffer (1 min each), and covered with a 1-ml vol of CT buffer (0.5% casein, 0.05% Tween 20, 0.1 mM Ca^{++}, and 0.1 mM Mn^{++} in PBS, pH 7.4) for 60 min at ambient temperature to quench free aldehyde groups and to minimize non-specific binding. Specimens were gently overlayed 3× with 1 ml of PBS, aspirated, and covered with 1 ml of CT buffer containing either 3 μg of gold-labeled WGA or 300 μg of Texas Red-labeled WGA. Specimens were maintained at 4°C for 18 h and subsequently overlayed 3× with 1 ml of PBS to remove unbound lectin. Specimens reacted with the gold-labeled WGA were postfixed with 2% glutaraldehyde in 0.2 M cacodylate-HCl buffer, dehydrated, and

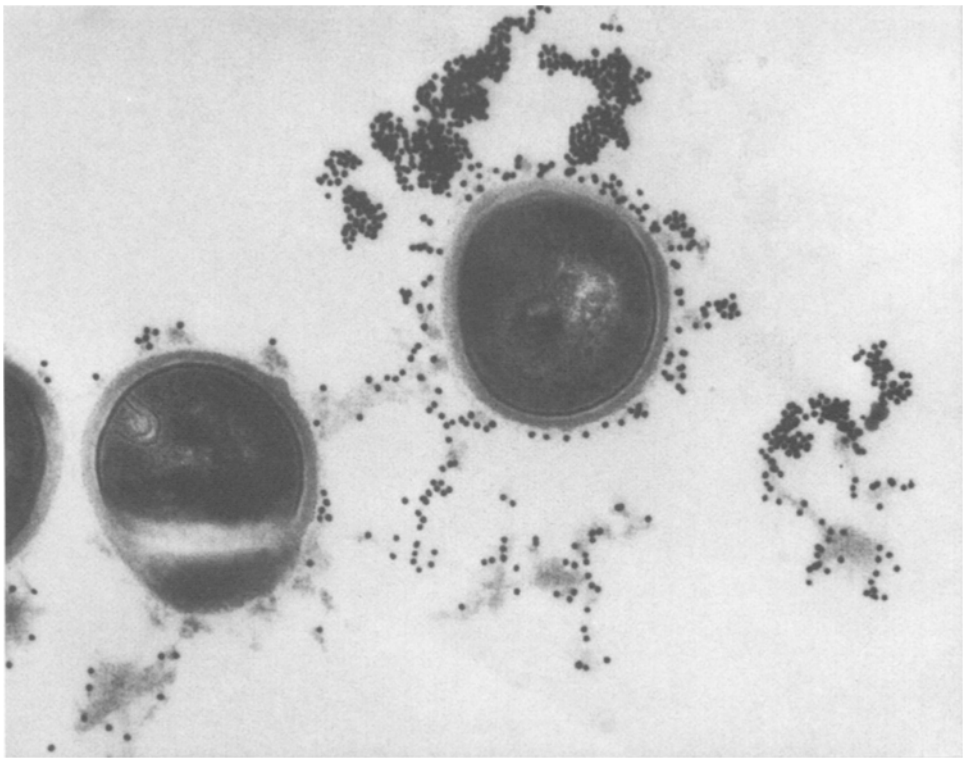

Figure 3 Transmission electron micrograph of *S. epidermidis* biofilm formed *in situ* on the surface of a PVC disk and reacted with gold-labeled WGA. Sections were stained with uranyl acetate-lead citrate. The presence of slime material was detected by bound WGA (15 nm gold particles), which is a slime-specific marker. Slime was detected in multiple, discrete areas of the cell surface and in the exocellular matrix material between cells. Original magnification = 34000.

embedded as described previously [24] for examination by transmission electron microscopy. After rinsing, specimens reacted with Texas Red-labeled WGA were overlaid with 1 ml of PBS. The disks were then drained, and one drop of SlowFade reagent was added to the biofilm surface for 1 min. The surface was drained, and an SIP cover glass (22 × 22 mm, No. 0 (0.08–0.13 mm) thickness; Baxter Healthcare Corp, Scientific Products Division, McGaw Park, IL, USA) was mounted onto the bacterial biofilm surface of the disk using Crystal/Mount aqueous mounting medium; likewise, the bottom surface of the disk was mounted to a glass slide, and the mounting fluid was allowed to set overnight at ambient temperature. Mounted specimens were stored at 4°C, in the dark, until examined by confocal microscopy.

Scanning confocal laser microscopy (SCLM) and 3-D rendering

Specimens were examined using an INSIGHT PLUS line-scanning confocal microscope (Meridian Instruments, Okemos, MI, USA) [27] with the scan unit mounted on a Nikon Diaphot equipped with a 100× oil immersion objective (n.a. 1.40). The autofluorescence of the cocci was excited by the 488-nm line from an Argon laser, and Texas Red was excited by the 568-nm line from a Krypton laser. The autofluorescence and Texas Red emissions were captured with a cooled charge-coupled device through 530/30-nm bandpass and 605/nm longpass filters, respectively. Multiple planes parallel to the disk surface of the samples (the *xy*-plane) were scanned at user-selectable increments in the *z*-axis. Three-dimensional (3-D) renderings were generated using the Simulated Fluorescence Process (SFP) algorithm [13,25], available in the system software. With this algorithm, a series of optical sections can be combined into one image, artificially illuminated at an adjustable angle, and projected against a reflective background plane. The characteristic shadows cast by the data assist in the visualization of three dimensions. The software permits generating SFP images from any viewing angle, thereby allowing precise localization of a fluorescent label in a scanned volume. In addition to *xy*-scans, planes perpendicular to the disk surface (the *xz*-plane) were collected by SCLM. All SFP images and *xz*-scans were automatically corrected for the mismatch in refractive index between the immersion oil and the mounting medium [26].

Results and discussion

PVC disks were exposed to a continuous flow of *S. epidermidis* for 24 h. After the disks were seeded with bacteria (adhesion phase), they were perfused with sterile medium for a period of 6 days (biofilm formation phase). This seed and feed approach allowed biofilm to develop and mature while preventing the problem of continuous 'reseeding' of the disks. Figure 1 demonstrates the number of viable staphylococci associated with the PVC disks after seeding for 24 h; the mean number of CFU increased 18-fold on day 1 of feeding and reached a maximum 70-fold increase by Day 6. Examination of *in situ* specimens by SEM showed that single, pairs, and small clusters of cocci were evenly

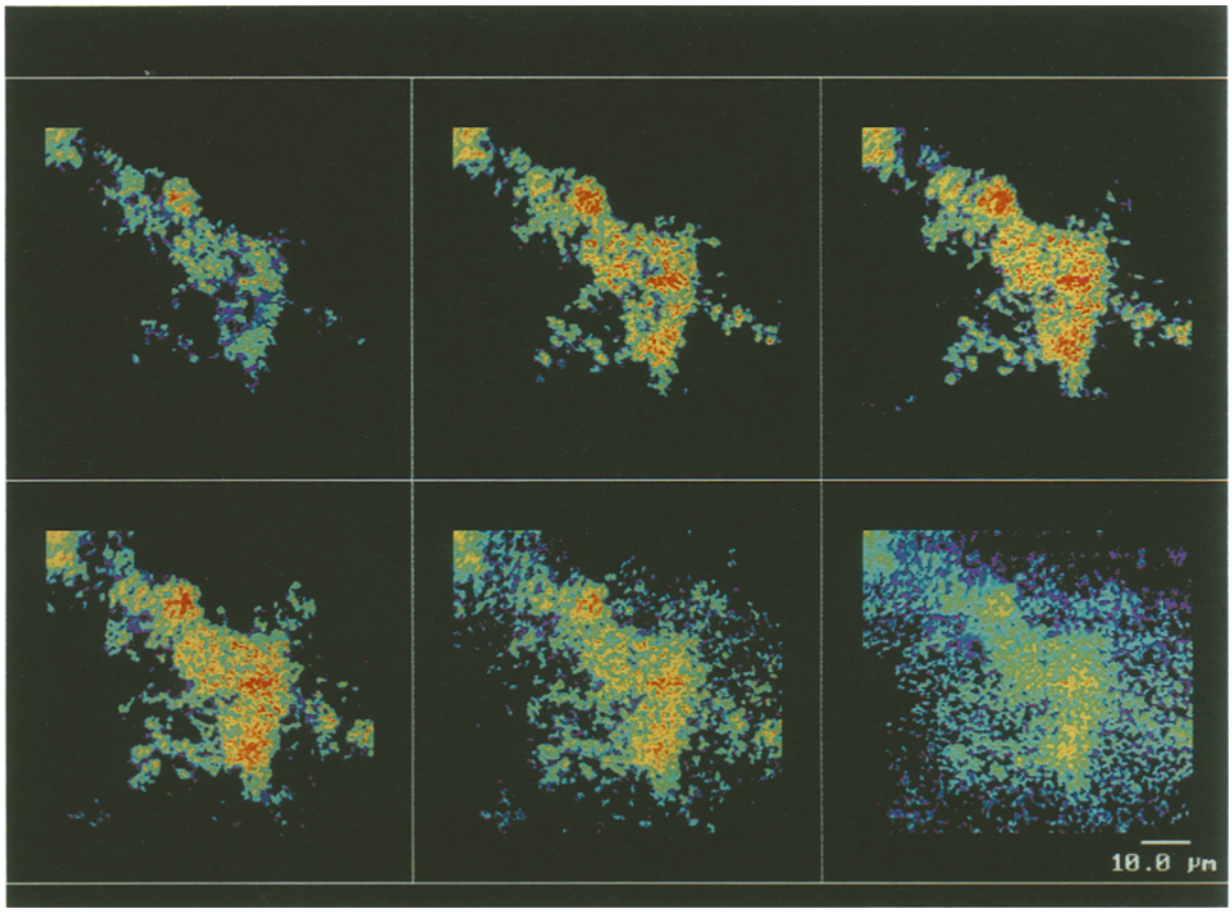

Figure 4 Optical sections (*xy* plane), obtained with an INSIGHT PLUS laser scanning confocal microscope using a 100× oil immersion objective. Sections depict the distribution of autofluorescent *S. epidermidis*, excited at 488 nm, in a hydrated biofilm (day 6), formed *in situ*, on the surface of a PVC disk. Data were collected at 0.5-μm intervals in the *z*-axis (0.133 μm *xy* pixel size). Selected sections of the series are shown at 1-μm intervals, beginning near the top of the biofilm (top left) and ending near the surface of the disk (bottom right). The images are displayed in a spectral palette, with low fluorescence intensity levels of cells on or near the surface of the disk indicated in purple and blue, intermediate levels in green, and high fluorescence intensity levels of cells furthermost away from the surface of the disk in yellow and red.

distributed over the disk surface after seeding for 24 h (Figure 2a). At the end of day 6, the cocci had formed compact multi-layered microcolonies (Figure 2b); individual cocci, within the microcolony, are clearly visible, and slime matrix material appears as 'strands' between the cocci. The localization of slime matrix material produced by Day 6 was determined by the modification of a previous 'sandwich' technique [24] in which we demonstrated that WGA could be used as a marker of slime because it binds specifically to *N*-acetyl glucosamine which is one of the chemical components of slime. In the present study gold-labeled WGA was reacted directly with *in situ* biofilm which was subsequently examined by TEM; representative results are shown in Figure 3. Exocellular slime appeared as 'strands' between cells and in the surrounding environment; slime was also associated with the cell surface.

Both SEM and TEM techniques required that biofilm specimens be dehydrated, a process known to significantly reduce the total volume of exocellular matrix material [23] and lead to collapse of the matrix, compression of the cells, and distortion of the architecture. To eliminate this problem, a combination of SCLM and a dual fluorescence technique was developed to study the hydrated biofilm. This approach was based on: 1) the fortuitous observation that the cocci autofluoresced at 488 nm excitation, and 2) the development of a procedure for the direct binding of Texas Red-labeled WGA to the slime matrix which produced red fluorescence at 568 nm excitation. The dual fluorescence approach gave clear results because of this wide separation of excitation wavelengths. Specifically, SCLM was used to examine the hydrated *in situ* biofilm after feeding with sterile medium for 6 days; multiple Z-series of fluorescent scans were done on areas of the biofilm which ranged in thickness from 5 μm to 20 μm. Figure 4 is representative of a series of optical sections obtained at 0.5-μm intervals in the *z*-axis and displayed at 1.0 μm intervals. Autofluorescent cells were present in concave microcolonies formed from irregular columns of cell clusters. This cell distribution pattern was different from those patterns reported by Lawrence *et al* [17] for hydrated biofilms produced by *Pseudomonas fluorescens*, *P. aeruginosa*, and *Vibrio parahaemolyticus*. The highest concentrations of pseudomonads were observed, by SCLM, at the interface of the biofilm with the substrate; the vibrios maintained a higher cell density near the outer regions of the biofilm with a more disseminated foundation of underlying cells. In addition, they

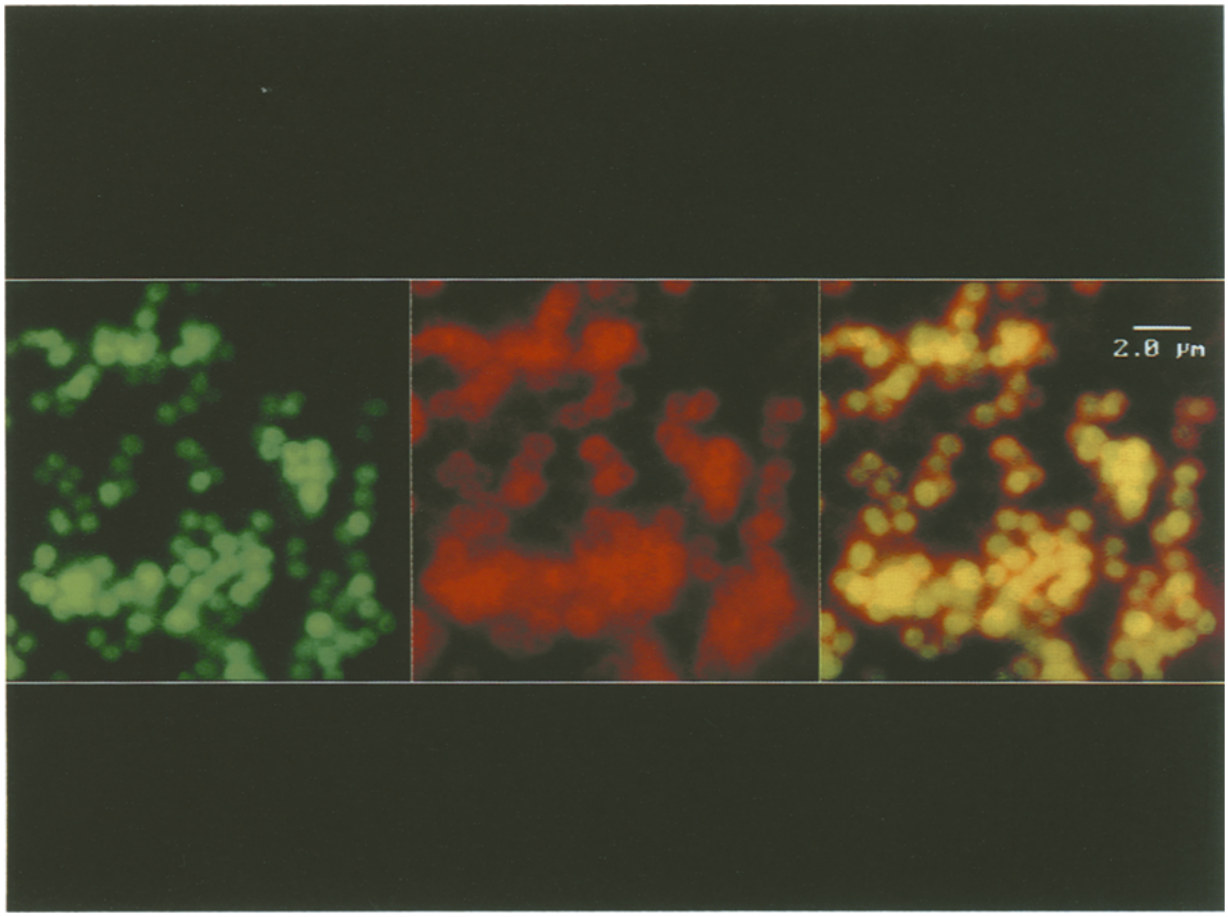

Figure 5 Spatial arrangement of components comprising the 6-day-old *S. epidermidis* biofilm. The green autofluorescent cells (left) appeared loosely arranged and interspersed with spaces. The *in situ* biofilm was reacted with Texas Red-labeled WGA to detect slime. The fully hydrated slime matrix material, which fluoresced red at 568 nm excitation, formed intense haloes surrounding individual cells and filled in much of the space between cells (center). A dual color image of the biofilm (right) shows the cells (yellow to green) embedded in slime matrix (red) with interspersed spaces. All three photographs show the same field.

found that mature biofilms showed no evidence of distinct microcolonies, indicating redistribution of motile cells within the biofilm during development. Motility contributes to the overall nature and morphology of biofilms formed by flagellated rods which grow as single cells [3,16,17]. In contrast, the distinct structural pattern exhibited by *S. epidermidis* may be explained, in part, by the fact that staphylococci divide in more than one plane to form clusters of cells (rather than single cells), and by the fact that these cells are not motile.

Examination of individual horizontal optical sections of the staphylococcal biofilm (Figure 5) showed a loose (rather than compact) spatial arrangement of the autofluorescing cocci. In contrast to the strands of slime seen by SEM and TEM (using dehydrated specimens), Figure 5 demonstrates that hydrated slime actually forms dense haloes around the cells and fills up much of the spaces separating individual cells and aggregates of cells. The slime component was found to comprise a mean of 51.5% (range of 48% to 56.6%) of mature (day 6) biofilm. A dual color image of autofluorescence of cells and Texas Red fluorescence of slime is also shown in Figure 5; this image demonstrates all three components of biofilm—cocci, exocellular matrix, and spaces. These spaces form part of a network of channels [19] that communicate between the surface of the disk and the outer fluid phase; they are best seen in Figure 6 which is an *xz* (cross-sectional)-view of a convex microcolony with fluorescing slime. In a recent review, Costerton *et al* [8] discussed the importance of similar channels observed in *P. aeruginosa* biofilm. They describe the channel network as a primitive 'circulatory system' designed for high access delivery of nutrients throughout the biofilm, as well as for removal of metabolic waste products. There are several possible explanations for the fact that *S. epidermidis* (growing in a biofilm) is resistant to antimicrobial agents even though, presumably, these agents would also have access to the channels. Costerton *et al* [8] have suggested that matrix material acts like a diffusion barrier (or ion-exchange column) to protect the matrix-encased cells from antimicrobial agents. In addition, the slow growth rate of bacteria embedded in a biofilm would affect their susceptibility to treatment with antibiotics (see recent review by Gilbert and Allison [11]) as would the fact that a high percentage of *S. epidermidis* strains are resistant to a wide-range of antibiotics.

Figure 7 shows a rotational series of 3-D reconstructions of the *in situ* hydrated biofilm. The multilayered cell aggregates, visible by SEM (Figure 2), were completely buried

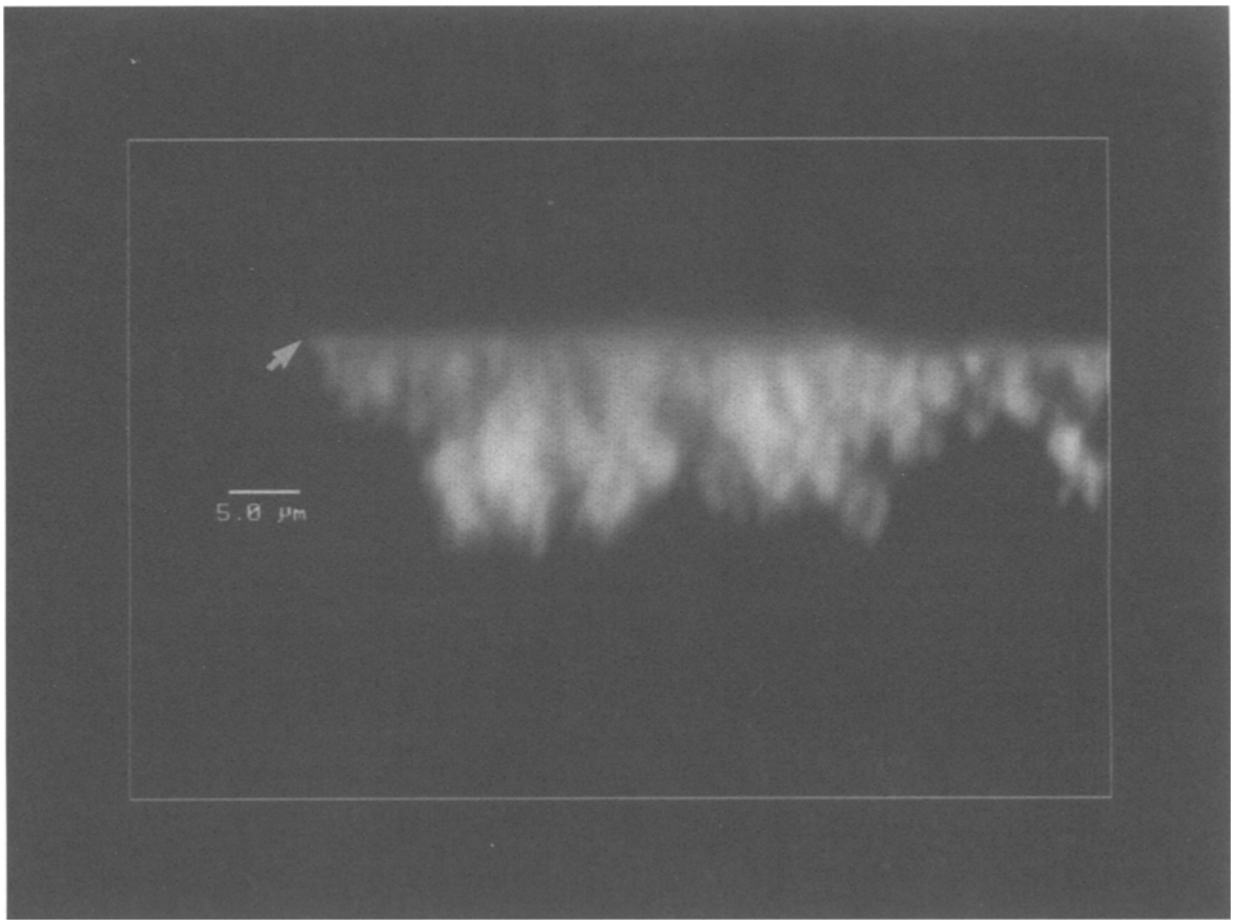

Figure 6 A cross-sectional view (*xz*-scan) of the *S. epidermidis* biofilm on the surface of a PVC disk; the specimen was reacted *in situ* with Texas Red-labeled WGA to detect slime. The arrow designates the interface between the surface of the polymer disk and the base of the biofilm. In this scan, only the exocellular slime matrix material is fluorescent. The topography of the surface is rough because of the difference in vertical growth of slime-embedded cell clusters within the microcolony. The intensity of fluorescence is decreased in the less-dense regions of the matrix. The entire biofilm is interspersed with open channels that connect the surface of the biofilm with the deep layers on the disk surface.

in slime matrix material and gave rise to a rough topography. These results are reminiscent of a recent environmental study by Wolfaardt *et al* [29] in which SCLM was used to demonstrate the architecture of diclofop-grown complex consortia biofilms. These biofilms also had a rough, conical-shaped morphology because most of the cells (cocci and rods) were concentrated in clusters, and vertical biofilm development originated from these clusters; this biofilm structure appeared to optimize the degradative activity of the bacteria grown in the presence of the herbicide and other chlorinated ring compounds. In the present study, *S. epidermidis* was not subjected to comparable environmental pressures; however, it is possible that production of exocellular slime matrix material exerted a physical pressure on the cells which enhanced vertical growth in clusters and contributed to the rough topography seen in Figure 7. Overall, the architecture of *S. epidermidis* biofilm, with its engineered channels, likely contributes to the well-documented difficulty in eradication of biomaterial-associated infections by enhancing the survival and persistence of the staphylococci and by protecting them from both the activity of antimicrobial agents and the host's immune system [7].

Acknowledgements

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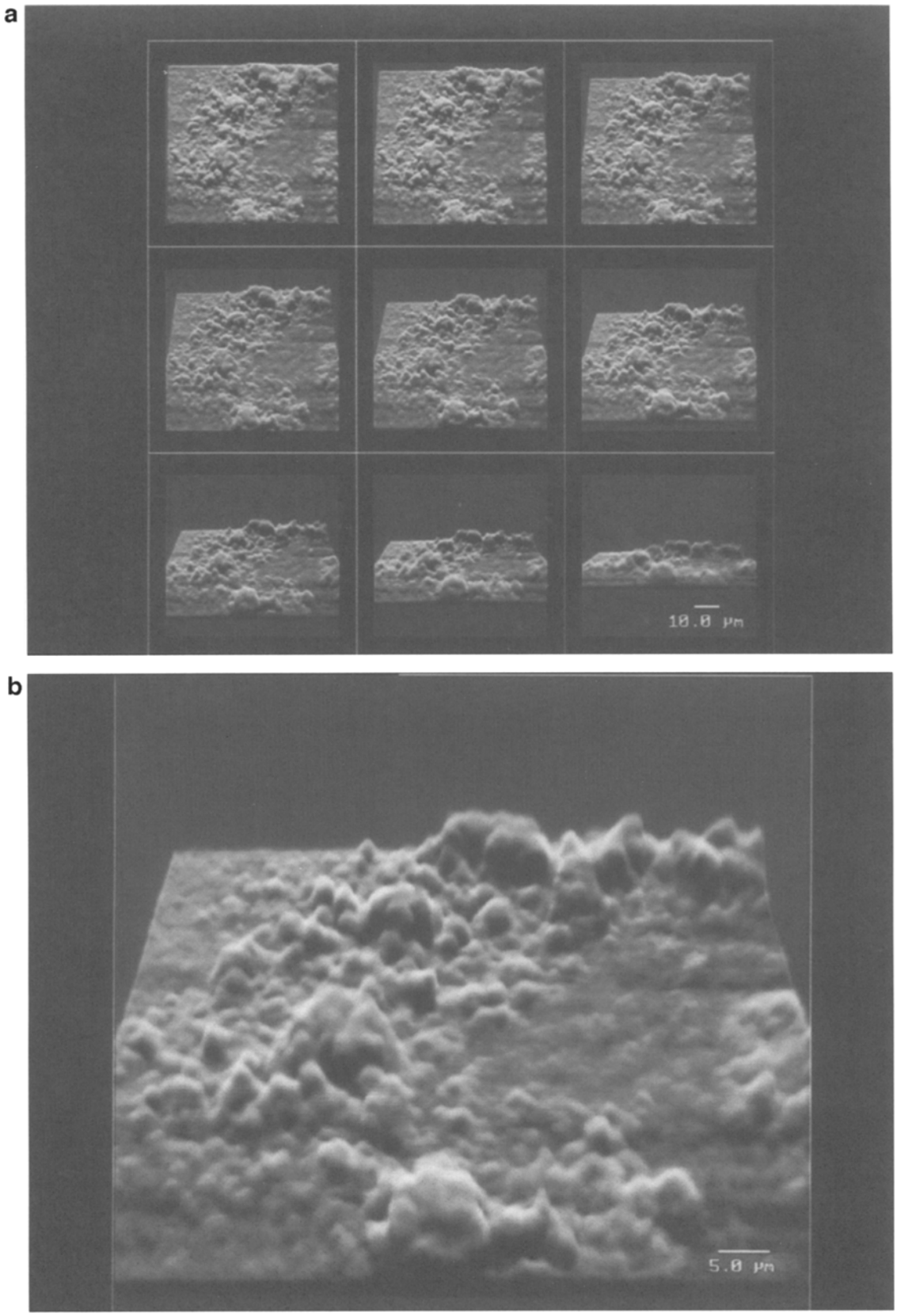

Figure 7 A rotational series of 3-D renderings of the hydrated *S. epidermidis* biofilm, on the surface of a PVC disk, reacted *in situ* with Texas Red-labeled WGA. Twenty-six optical sections were collected with the INSIGHT PLUS laser scanning confocal microscope at 0.5-μm increments in the *z*-axis, and reconstructed with the Simulated Fluorescence Process (SFP) algorithm at various viewing angles, as described in Materials and Methods. The matrix shows a rotational series of −10° steps in elevation (a), starting at 90° elevation (upper left) and ending at 10° elevation (bottom right), and a magnified view of the SFP reconstruction at 40° elevation (b). The reconstructions demonstrate that the disk surface is coated with slime matrix material; the uneven conical growth of cell clusters in the microcolonies embedded in slime contribute to the rough topography of the biofilm.

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