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Substratum-Induced Morphological Changes in a Marine Bacterium and Their Relevance to Biofilm Structure

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The effects of surfaces on the physiology of bacteria adhering to surfaces or immobilized within biofilms are receiving more interest. A study of the effects of hydrophobic and hydrophilic substrata on the colonization behavior of a marine bacterium, SW5, revealed major differences in the morphology of SW5 on these surfaces. Using epifluorescence, scanning confocal laser, and on-line visualization (time-lapse video) microscopy, the organisms at hydrophobic surfaces were characterized by the formation of tightly packed biofilms, consisting of single and paired cells, whereas those at hydrophilic surfaces exhibited sparse colonization and the formation of chains more than 100 μm long, anchored at the surface by the terminal (colonizing) cell. The results are discussed in terms of the possible factors inducing the observed morphological differences and the significance of these differences in terms of biofilm structure and plasmid transfer when SW5 is the recipient organism.

Increasing attention is being paid to the effects of surfaces on microbial physiological properties (12, 21, 29), and reporter gene technology is being employed to investigate physiological changes in bacteria adhering to surfaces or immobilized within biofilms (9, 10, 13). Although bacteria are known to exhibit morphological variation in response to external environmental stimuli (27), few observations concerning the effects of surfaces on bacterial morphology have been reported, and as far as can be ascertained, no study has been undertaken to examine the effects of different substratum types on morphological responses in bacteria. Pertsovskaya et al. (26) reported the presence of fimbriae on adherent bacteria and their absence from nonattached cells. In this instance, the cell population may have been heterogeneous and only those cells expressing fimbriae were able to adhere to the surface examined. McCoy and Costerton (23), on the other hand, showed that a *Pseudomonas* sp. forming regular rod-shaped cells in liquid and in agar culture formed long filamentous cells in biofilms. Also, *Vibrio parahaemolyticus* cells grown on an agar surface show gross morphological differences relative to cells grown in liquid. In liquid, cells are short (about 1 μm long) and are motile by a single polar flagellum, whereas on agar, individual cells elongate (to several hundred micrometers) and become covered by hundreds of lateral flagella which cause swarming across the surface (5).

Previous work in our laboratory showed that the marine bacterium SW5 attached in different numbers to hydrophobic and hydrophilic substrata (4). This phenomenon may have affected the relative efficiency of gene transfer from donor bacteria into SW5 on these substrata. However, no investigation of the nature of the SW5 biofilm structures occurring on different substrata was carried out. The aim of the present study was to determine the effects of hydrophobic and hydrophilic substrata on the colonization behavior of SW5. The study revealed major differences in the morphology of SW5 on these substrata and has important consequences in terms of the structure of biofilms formed by the bacterium.

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MATERIALS AND METHODS

Bacterial strain and culture conditions. The nonmotile, hydrophobic (4), gram-negative bacterium SW5 was isolated by T. Neu in May 1988 from surfboard wax after exposure to seawater at Wanda Beach, Cronulla, Australia, and cultured in a minimal, artificial seawater medium (MMM) (25), supplemented with 20 mM glutamic acid (MMMglt) as the sole carbon and energy source. The average cell size of the organism grown in liquid culture (2.4 [standard deviation (SD), 3.8] by 1.3 [SD, 0.2] μm) was measured by using Magellan, a computerized interactive morphometry system developed by Paul Halasz.

Flow chambers and substrata. The laminar flow slide chambers employed were similar to those described by Korber et al. (15) and were sterilized by autoclaving. Colonization of the substratum was initiated by inoculating sterile laminar flow chambers with log-phase cells grown in MMMglt liquid culture at 30°C on a gyratory shaker at 120 rpm. The A_{600}s of cultures were determined and adjusted immediately prior to inoculation to 0.1 by dilution in MMMglt. Slide chambers were inoculated with a single 1.0-ml pulse of SW5 upstream from the chamber under static conditions, and flow was resumed 1 h after the addition of the inoculum. The bulk laminar flow rate was maintained at 2 cm s^{-1} throughout the experiments by using a peristaltic pump (Ismatec, Zürich, Switzerland). All experiments were carried out at room temperature (23 to 26°C).

The substrata mounted in the flow cells were acid-washed glass coverslips (64 by 22 mm; hydrophilic surface; mean water contact angle, spreading) or coverslips coated with dimethyldichlorosilane in 1,1,1-trichloroethane (Coatasil; Ajax Chemicals, Sydney, Australia) (hydrophobic surface; mean water contact angle, 84.4° [SD, 3.5; n = 12]). Preliminary experiments were also conducted in 20-ml flow reactors as described

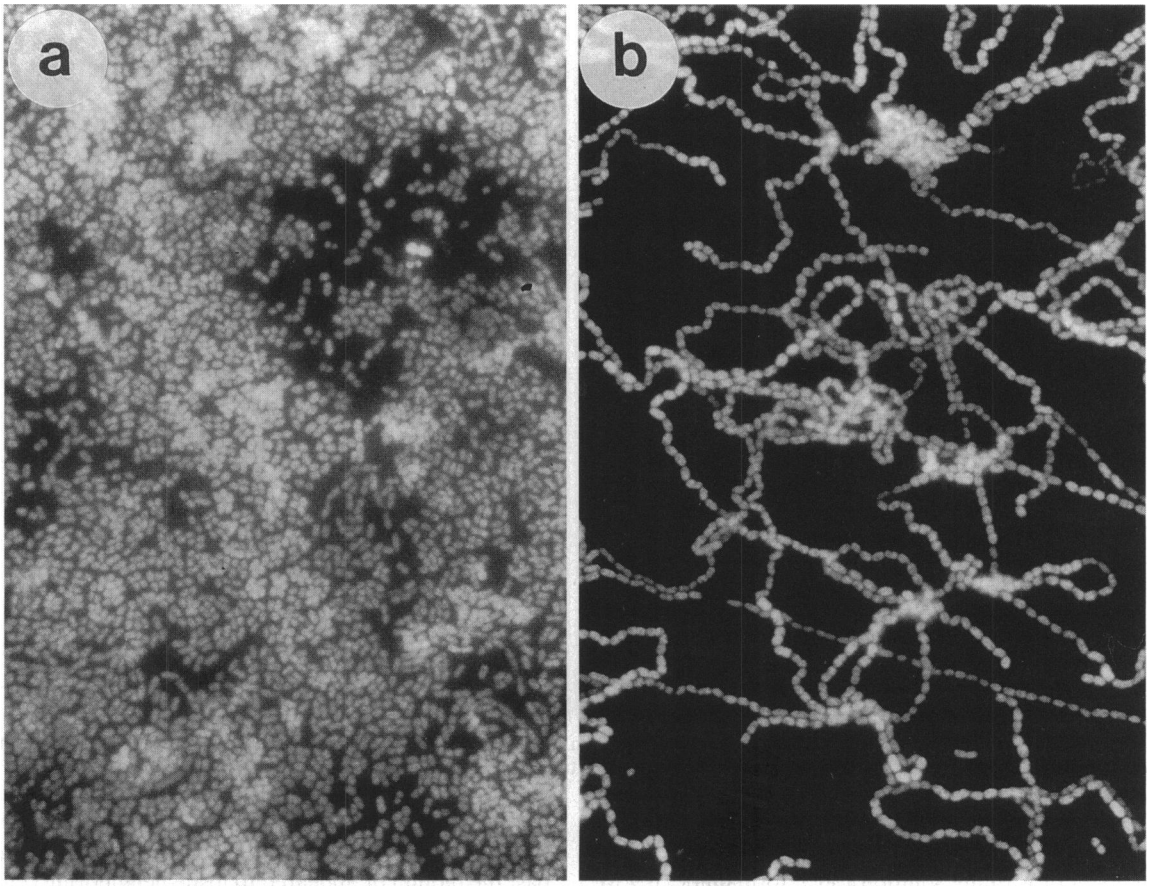

FIG. 1. Epifluorescence photomicrograph of a biofilm grown for 72 h on a hydrophobic (a) or hydrophilic (b) surface in a 20-ml flow reactor and hybridized with the eubacterial probe labeled with fluorescein isothiocyanate.

by Angles et al. (4) except that the substrata were coverslips, not glass beads.

Microscopy. For on-line visualization, the laminar flow slide chambers were mounted on the stage of a Zeiss Axioskop microscope (Carl Zeiss, Oberkochen, Germany) fitted with differential interference contrast optics. Continuous nutrient flow was maintained through the chambers during observation. Video recordings were made by using a Panasonic WV-BP500 CCTV camera (Matsushita Electric Industrial Co.) fitted to the Axioskop and connected to a Panasonic SVHS time-lapse video recorder (model AG-6720A).

Epifluorescence microscopy of cells hybridized with a fluorescently labeled oligonucleotide probe was done with the Axioskop fitted with an HBO 50-W mercury lamp and a 100×, 1.3-numerical-aperture oil immersion lens. The fluorescein isothiocyanate-labeled probe was visualized by using filter set 10 (Carl Zeiss). Photographs were taken with Ektachrome P1600 film (Eastman Kodak, Rochester, N.Y.).

Scanning confocal laser microscope (SCLM) images were obtained by using an Olympus GB200 instrument (Olympus Optical Co. Ltd., Tokyo, Japan) fitted with a piezoelectric *z* stage with a reproducibility of ±0.2 μm. The microscope was equipped with a 60×, 1.4-numerical-aperture oil immersion lens. An argon laser with emission line at 488 nm was used as the excitation source for the rhodamine B fluorophore. For examination by SCLM, biofilms were grown in flow chambers for 72 h. Positive staining of SW5 cells was achieved by fluorescence inclusion of rhodamine B (BBL Laboratory, Poole, England) (0.7 g of rhodamine B liter^{-1} in 0.15 M phosphate-buffered saline [PBS]–0.07 M Tris-HCl [pH 8]). The fluorophore (1 ml) was injected upstream of the flow chamber and retained in the chamber by sealing both inlet and outlet lines. A 1:200 dilution of the fluorophore achieved effective penetration of the biofilm, allowing *xy* and *xz* optical sectioning by SCLM. Images of serial optical sectioning of the biofilm were collected at 0.6-μm intervals, and the microVoxel 2.2 three-dimensional (3-D) image analysis program (32-bit version; Indec Systems, Inc., Capitola, Calif.) was used to construct the 3-D surface rendering of the biofilms and measure distances between cells and biofilm depth. The distances between cells were determined by measuring the mean spatial distance between the center of an attached cell and the center of its nearest neighbor. Biofilm depth was determined by measuring the distance above the attachment surface along transects in *xz* images.

Hybridization of biofilms. Fixation and hybridization of SW5 biofilms on coverslips were similar to the method described by Amann et al. (3). Pieces of coverslips (approximately 1 cm^2) from the 20-ml flow reactors were rinsed in PBS (pH 7.2) and fixed overnight in 4% paraformaldehyde at 4°C. Fixative was removed by three washes in PBS for 5 min each, with a short rinse in 0.1% nonionic detergent (Nonidet P-40; Sigma). Biofilms were dehydrated in a series of 50, 80, and 100% ethanol for 3 min each. Hybridization using the eubacterial 338 probe (2) labeled with fluorescein isothiocyanate was carried out overnight at 30°C as described by Amann et al. (1)

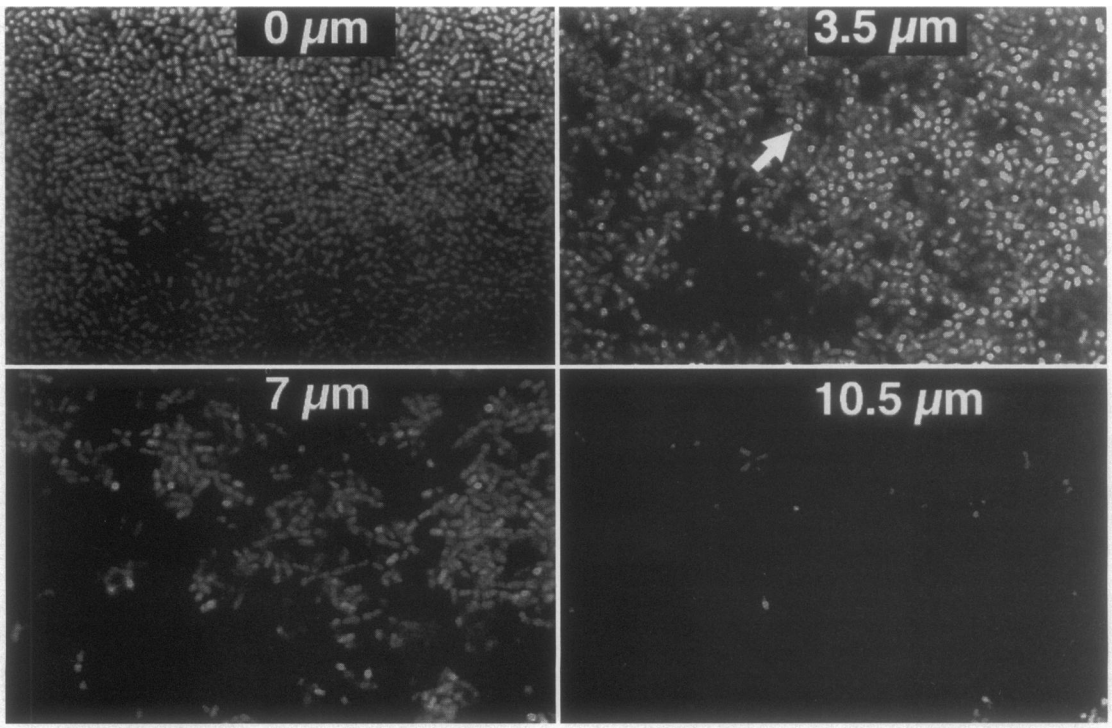

FIG. 2. Horizontal optical thin sections (*xy*) of a biofilm grown for 72 h on a hydrophobic surface and stained in situ. The distances of the optical sections from the substratum are indicated. Arrow, example of a cell attached in a vertical position. Magnification, ×1000.

and Stahl and Amann (28). Coverslips were washed three times with 40 μl of washing solution (30% formamide, 0.9 M NaCl, 0.1% sodium dodecyl sulfate, 100 mM Tris [pH 7.2]) at room temperature and then three times for 45 min each time at 30°C. The final rinse was in distilled water before air drying. The hybridized biofilms were then mounted in Citifluor (Citifluor UKC, Canterbury, United Kingdom) and visualized by epifluorescence microscopy.

RESULTS

Initial experiments involved SW5 biofilm development on coverslips in the reactor described by Angles et al. (4). After 3, 4, and 6 days, epifluorescence microscopy of biofilms hybridized with the 338 probe revealed that the hydrophobic coverslips were covered with almost confluent, and even multilayered, biofilms (Fig. 1a) whereas the biofilms on hydrophilic coverslips consisted of chains containing hundreds of cells (Fig. 1b). This differed from the nature of cells grown to log phase in liquid culture, which appeared only as single or paired cells. In view of these differences in the cell morphology of SW5 on the two substrata, a more detailed examination of biofilm formation on hydrophobic and hydrophilic substrata was attempted by using both SCLM and on-line time-lapse video microscopy.

Biofilm formation on a hydrophobic surface. Observed by time-lapse video microscopy, SW5 developed microcolonies originating from single or paired cells attaching in a face-to-face orientation at the surface and showing a packing maneuver similar to that described by Lawrence and Caldwell (16) and McLean and Nickel (24). As cell density increased and the biofilm started to become multilayered, cells were seen attaching or moving into a vertical position (Fig. 2). On cell division, daughter cells were either lost to the aqueous phase flowing past the biofilm or appeared to jostle neighboring cells aside in order to gain access to the substratum. There was no distinct alignment in relation to the flow.

Rhodamine labeling and SCLM orthogonal sectioning in the *x*, *y*, and *z* planes of the biofilm allowed a 3-D volumetric reconstruction of the biofilm (Fig. 3a). This reconstruction and the horizontal (*xy*) optical sectioning (Fig. 2) showed a biofilm structure that was tightly packed at the substratum and becoming increasingly diffuse near the outer edge, i.e., closest to the flow. Sagittal (*xz*) sectioning, i.e., vertical sectioning from the substratum towards the flow (Fig. 4a), confirmed both the tightly packed nature of the cells attached at the substratum and the structure of the biofilm. The mean spatial distribution between attached cells on the hydrophobic surface was 2.1 μm (SD, 0.9; $n = 45$). Biofilm depth was 8.5 μm (SD, 3.6; $n = 16$).

Biofilm formation on a hydrophilic surface. Time-lapse video microscopy revealed that chains, estimated as being over 100 μm long, had developed within the first 12 h. These chains streamed with the flow, sometimes moving out of the field of view (i.e., detaching), although the majority of chains must have been anchored to the surface, possibly by a single terminal cell or by only a few cells. Within 16 h the chains had formed a loosely structured biofilm, and by 22 h the chains had become entangled, resulting in blocked flow channels within the biofilm.

SCLM horizontal sectioning (Fig. 5) and the 3-D volumetric reconstruction (Fig. 3b) revealed a low density of cells attached to the substratum (mean spatial distribution of cells on the hydrophilic surface, 37.7 μm [SD, 28.3; $n = 32$). The biofilm depth was 37.0 μm (SD, 7.1; $n = 11$). As the cell density increased, the chains formed an interwoven mat of cells that was located away from the substratum. It was clear that

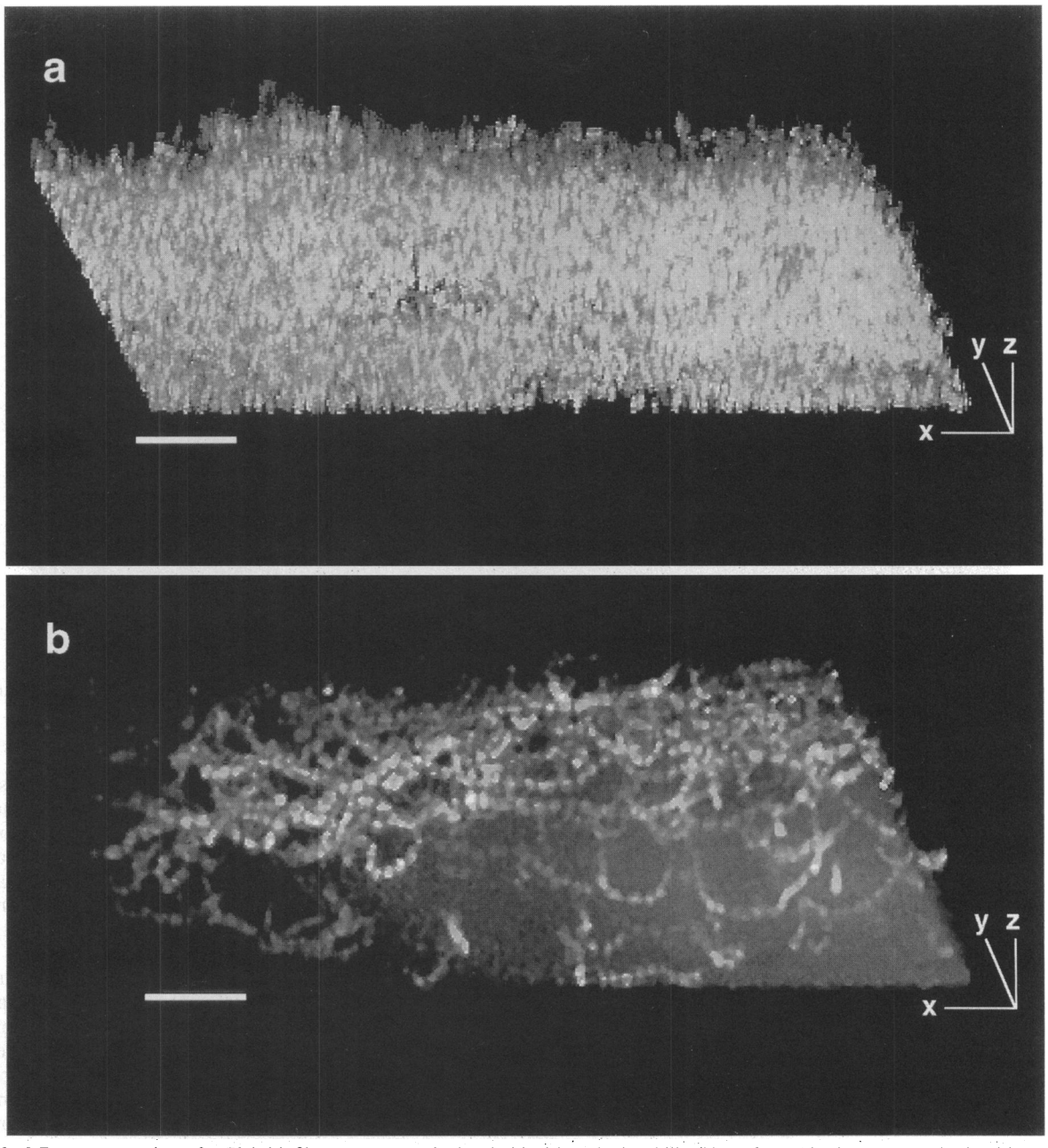

FIG. 3. 3-D reconstruction of a 72-h biofilm grown on a hydrophobic (a) or hydrophilic (b) surface. The image was obtained by combining horizontal optical sections taken at 0.6-μm intervals by using the microVoxel 3-D image analysis program. Background noise was introduced at the lower right of the hydrophilic-surface image to define the substratum. Bars, 10 μm.

each chain, often composed of more than 100 cells, was attached to the substratum by means of the single terminal cell. Sagittal sectioning confirmed this observation (Fig. 4b) and revealed the open structure of the filamentous biofilm.

Alternating substrata. In order to emphasize the controlling element of the substratum on cell morphology and biofilm structure, biofilms were developed on each of the substrata and then used to seed the alternative substratum downstream in the flow system. Biofilm development was monitored by on-line time-lapse video microscopy. Some single and paired SW5 cells which had sloughed off the hydrophobic substratum were observed developing chains on the hydrophilic surface, while others formed clumps, often entrapped within the mesh of chains, but during observation never formed chains themselves. Cells that did not form chains demonstrated a random sliding movement. These cells appeared to have some affinity to or be attached, but not immobilized, at the surface, in a process possibly resembling a form of reversible adhesion (22). Chains transferred from a hydrophilic surface to a hydrophobic surface rapidly attached to the substratum along the entire chain length. A confluent biofilm typical of a hydrophobic substratum then developed, with no further evidence of chain formation.

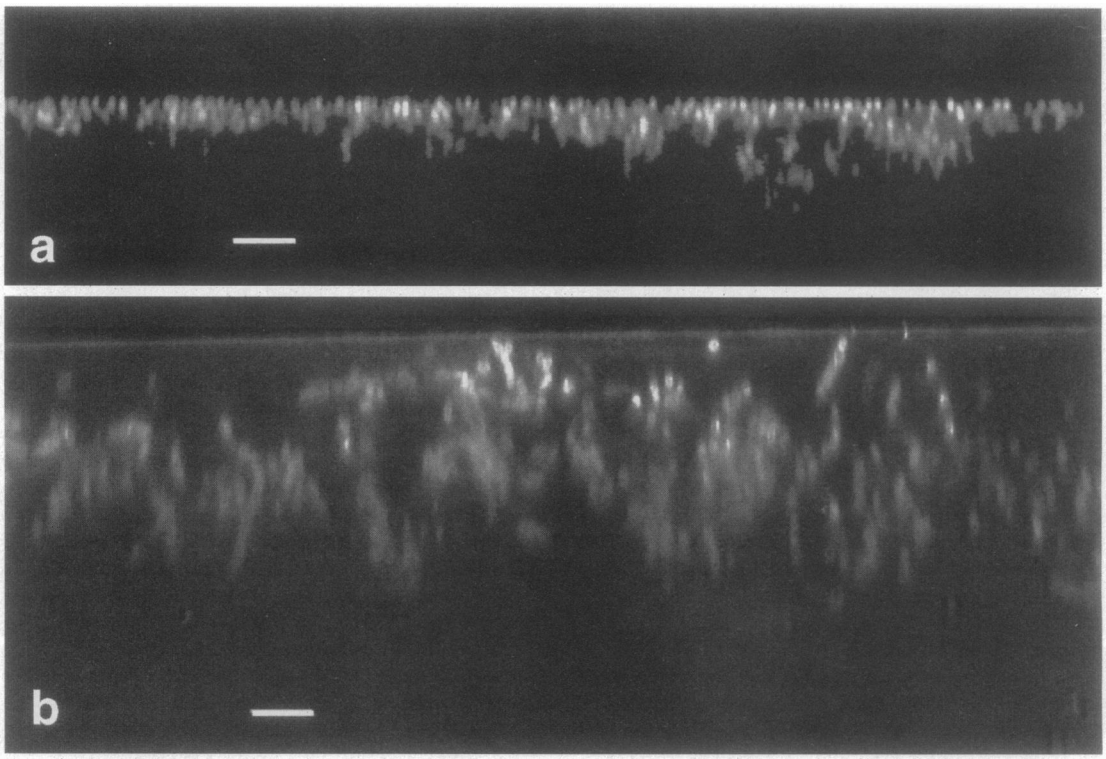

FIG. 4. Vertical (sagittal) sections (*xz*) showing the arrangement of a 72-h biofilm on a hydrophobic surface (a) and a hydrophilic surface (b). The surface of the substratum is at the top of each image. Bars, 10 μm.

DISCUSSION

Previous studies on surface colonization and biofilm formation by bacteria have paid scant attention to the effects of immobilization on the physiology and/or morphology of the bacteria. Reported changes at surfaces include altered expression of the *laf* (lateral flagellum) gene in, as well as the morphology of, *V. parahaemolyticus* grown on semisolid media (5), the *algC* and *algA* (alginate production) genes on solid substrata (10, 13), ultrastructural changes in various species including *Escherichia coli* and *Staphylococcus aureus* grown on semisolid media and on filters (18), the *gtf* (glucosyltransferase) operon in *Streptococcus mutans* cells attached to solid hydroxyapatite beads (14), and undefined genes, as detected by reporter gene activity, on solid, but not semisolid, substrata (9). In the present study, we consistently observed differences in both the distributions and the morphologies of SW5 cells adhering to and growing at hydrophobic and hydrophilic substrata. The organisms at hydrophobic surfaces were characterized by the formation of tightly packed biofilms consisting of single and paired cells, whereas those at hydrophilic surfaces exhibited sparse colonization of the surface and the formation of chains in excess of 100 μm, anchored at the surface by the terminal (colonizing) cell. The controlling influence of the substrata on biofilm development was reinforced by alternating the substrata colonized by the organism. It is notable that cells released from a biofilm formed on hydrophobic surfaces encountered a limited number of adhesion sites on the hydrophilic substrata and then formed chains whereas the chains released from hydrophilic surfaces colonized the hydrophobic substrata to form a tightly packed biofilm.

The mechanism(s) involved in the induction of chain formation in SW5 at hydrophilic surfaces is not clear at this time. Further investigations are in progress to examine alterations to the ultrastructure of SW5 exposed to hydrophilic substrata. It is interesting that any changes related to the regulation of cell separation induced in the initial colonizing cell continue to be expressed in all progeny cells, thus creating the long chains.

The recent application of SCLM to the examination of biofilms has changed our image of biofilm structure from one of a relatively continuous polymer matrix with microorganisms distributed uniformly throughout (7) to one in which discrete columns of microorganisms embedded in polymer are separated by water-filled voids (6, 17, 30). Given the observations of McCoy and Costerton (23) of continuous filament formation by a *Pseudomonas* sp. in biofilms and our present report of chain formation in SW5 at hydrophilic surfaces, caution needs to be exercised in making sweeping generalizations concerning the structure of all biofilms. It may be that there are many types of biofilm structures, depending on both the organism and the nature of the substratum. The recognition of such complex biofilm structures has profound implications on our understanding of mass transport of gases, substrates, and metabolic by-products into and out of the biofilms (11). Further in situ, on-line, nondestructive SCLM examination of biofilms originating from diverse sources should provide information on the general applicability of the current paradigm of biofilm structure.

The different biofilm structures on hydrophobic and hydrophilic substrata may provide an explanation for the observed differences in the frequency of gene transfer from a donor organism to a preformed biofilm of SW5 cells (4). Although SW5 appears to colonize hydrophobic surfaces more effectively than hydrophilic ones, the contiguity of the cells making up the chains and their length on the latter surface may, for example, trap more of the donor cells in the tangled filaments, allowing greater frequencies of gene transfer.

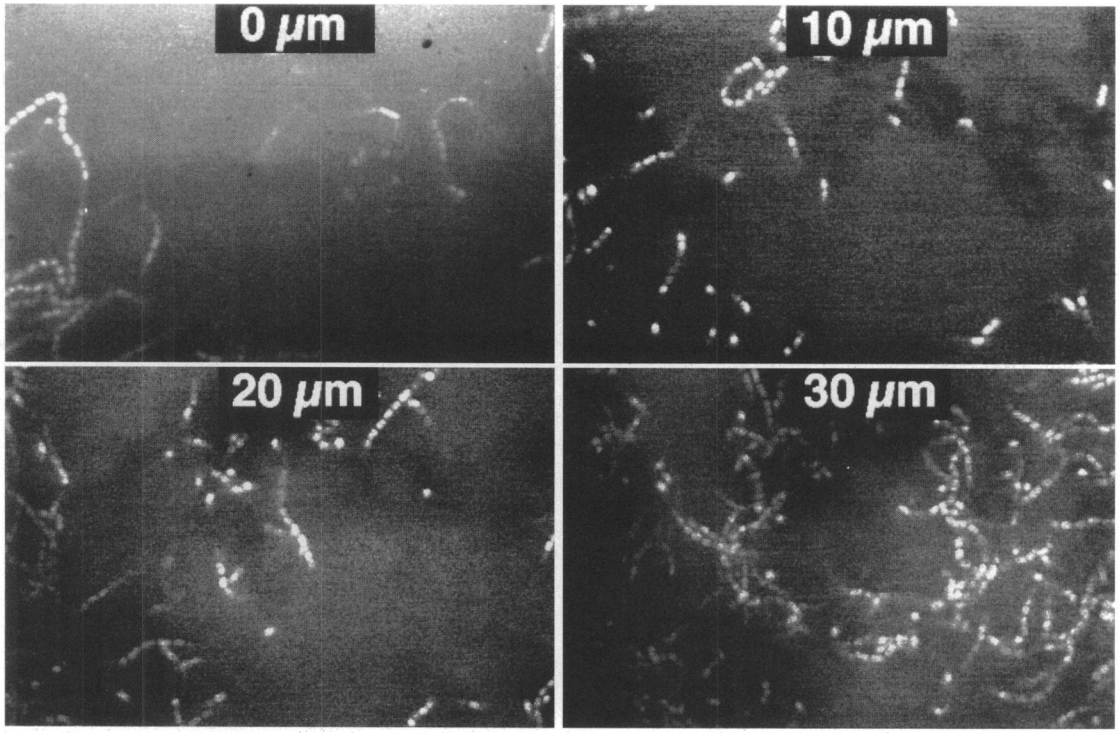

FIG. 5. Horizontal optical thin sections (*xy*) of a biofilm grown for 72 h on a hydrophilic surface and stained in situ. The distances of the optical sections from the substratum are indicated. Magnification, ×1000.

The broad applicability of our findings to biofilm formation and structure in general is not known at this stage. The results of this study emphasize once more the fact that many microorganisms do not behave in the same way in an immobilized state as in an aqueous phase. Since most microorganisms in nature spend most of their existence immobilized at surfaces or migrating between the immobilized and freely suspended states (8, 19, 20), it is imperative that more attention be paid to the physiological characteristics of these organisms in this immobilized condition.

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