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Bacterial Adhesion to Hydroxyl- and Methyl-Terminated Alkanethiol Self-Assembled Monolayers†

K. MARK WIENCEK AND MADILYN FLETCHER*

Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Maryland 21202

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The attachment of bacteria to solid surfaces is influenced by substratum chemistry, but to determine the mechanistic basis of this relationship, homogeneous, well-defined substrata are required. Self-assembled monolayers (SAMs) were constructed from alkanethiols to produce a range of substrata with different exposed functional groups, i.e., methyl and hydroxyl groups and a series of mixtures of the two. Percentages of hydroxyl groups in the SAMs and substratum wettability were measured by X-ray photoelectron spectroscopy and contact angles of water and hexadecane, respectively. SAMs exhibited various substratum compositions and wettabilities, ranging from hydrophilic, hydroxyl-terminated monolayers to hydrophobic, methyl-terminated monolayers. The kinetics of attachment of an estuarine bacterium to these surfaces in a laminar flow chamber were measured over periods of 120 min. The initial rate of net adhesion, the number of cells attached after 120 min, the percentage of attached cells that adsorbed or desorbed between successive measurements, and the residence times of attached cells were quantified by phase-contrast microscopy and digital image processing. The greatest numbers of attached cells occurred on hydrophobic surfaces, because (i) the initial rates of adhesion and the mean numbers of cells that attached after 120 min increased with the methyl content of the SAM and the contact angle of water and (ii) the percentage of cells that desorbed between successive measurements (ca. 2 min) decreased with increasing substratum hydrophobicity. With all surfaces, 60 to 80% of the cells that desorbed during the 120-min exposure period had residence times of less than 10 min, suggesting that establishment of firm adhesion occurred quickly on all of the test surfaces.

The consequences of bacterial adhesion to nonbiological surfaces can be beneficial or deleterious depending on the situation. Bioreactor and biofilter systems rely on the adhesion and growth of bacterial cells on support materials for effective operation (4). Also, bacterial biofilms can be utilized to promote the attachment of other organisms in the aquaculture of invertebrates (10). In marine and estuarine environments, irreversible attachment and the subsequent growth of bacteria on surfaces can affect the attachment of other organisms, often leading to the fouling of heat exchangers, ship hulls, and other synthetic surfaces (2, 18, 23). As a result, the study of bacterial adhesion and the effects of substratum surface properties has received considerable attention.

Although the forces involved in bacterial adhesion are not fully understood, it is evident from numerous studies of different systems that substratum wettability is an important factor in bacterial adhesion (9, 12, 13, 26), as well as in protein adsorption (33) and the attachment of eukaryotic cells (21, 39). Many studies of bacterial adhesion and substratum wettability have utilized different materials, e.g., glass, metals, and polymers, to represent substrata with different wettabilities (12, 38, 40). Other studies have used chemical treatments of materials such as polystyrene to create a series of substrata with different wettabilities (9, 35). However, chemical treatments often cause severe denaturation of the surface, leading to increased roughness and wetting hysteresis (27). Substratum roughness, created by manufacturing processes or chemical or physical treatments, has been shown to affect substratum wettability (17, 27) and bacterial adhesion (35, 38) but seldom is measured or accounted for in adhesion studies.

In addition to the problems associated with using different materials to study the effects of substratum wettability, the inter- and intrasample chemical and physical heterogeneity of the materials used by different investigators may contribute to some of the conflicting results reported in the literature (30, 38). Trace impurities in many of the polymers used in adhesion studies result in uncertainties concerning the types of functional groups present at the substratum surface and may contribute to the substratum heterogeneity. A rigorous study of the effects of substratum wettability on bacterial adhesion will require a model system that allows control of the type, amount, and configuration of functional groups at the substratum surface with minimal changes in the inter- and intrasample surface roughness.

Long-chain alkanethiols, $HS(CH_2)_nX$, adsorb from solution onto gold substrata and form oriented, well-ordered monolayers. The sulfur moieties of the alkanethiol bind to the gold, causing the molecules to pack tightly in an orientation nearly perpendicular to the surface of the gold film (32). For an alkanethiol of sufficient chain length ($n > 10$), the resulting single-component monolayer consists of an ordered, organic film with wetting properties that are determined by the terminal functional group, X (5). Several studies of the surface properties of self-assembled monolayers (SAMs) have focused on single- and mixed-component SAMs with methyl and hydroxyl functional groups (5, 14). Generally, results have indicated that hydroxyl-terminated SAMs are wetted by water and hexadecane with advancing contact angles (θ_a) of $<10°$, whereas methyl-terminated SAMs are hydrophobic [θ_a(water) $\approx 110°$] and oleophobic [θ_a(hexadecane) $\approx 45°$] (5). The wettability of mixed SAMs composed of two alkanethiols with equal chain lengths but with polar (hydroxyl, carboxyl, or ni-

* Corresponding author. Mailing address: Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 600 East Lombard St., Baltimore, MD 21202. Phone: (410) 783-4800.

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trile) and nonpolar (methyl) terminal functional groups increases as a function of the mole fraction (χ_p) of the polar component in the monolayers (8, 14).

In this study, we have used methyl- and hydroxyl-terminated SAMs to determine the effects of substratum composition and wettability on adhesion of an estuarine bacterial strain, MI-1A. The kinetics of adhesion, including quantification of the amount of cell adsorption and desorption and cell residence times, on single- and mixed-component alkanethiol SAMs were measured in a laminar flow environment under defined shear stress.

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MATERIALS AND METHODS

Bacterial strain and culture conditions. An estuarine bacterial strain, designated MI-1A, was isolated from the surface of a polycarbonate coupon in a heavily used harbor of the David Taylor Naval Research Laboratory, Annapolis, Md. The isolation medium was *Pseudomonas* Isolation Agar (Difco, Detroit, Mich.) supplemented with 1.0% (wt/vol in distilled water) Instant Ocean marine salts (IO) (Aquarium Systems, Mentor, Ohio). IO is a synthetic medium formulated to match the composition of seawater. MI-1A is a gram-negative, rod-shaped bacterium identified as a *Pseudomonas* sp. The strain is motile and produces a green, fluorescent pigment in broth cultures. Taxonomic analysis of the strain was conducted with the BIOLOG system (Biolog, Inc., Hayward, Calif.), which identifies microorganisms on the basis of utilization of different carbon sources. Analysis of the isolate yielded a high similarity index (SIM = 0.800) with *Pseudomonas putida* (type A1). In addition, the gel electrophoresis band patterns resulting from 5S rRNA and tRNA (15) also suggest that the isolate is a *Pseudomonas* sp. (data not shown).

For all adhesion experiments, cultures of MI-1A were grown with shaking (125 rpm) at 20°C in PYE medium (0.1% peptone, 0.07% yeast extract) with 1.0% IO. Cultures in early stationary phase were harvested by centrifugation, washed twice, and resuspended in 1.0% IO (pH 8.0) to an optical density of 0.280 to 0.370 (5.5×10^7 to 7.5×10^7 cells per ml). The density of cells in the suspension was determined from a standard calibration curve of optical density and acridine orange direct counts of cells cultured and harvested under similar conditions.

SAMs. SAMs composed of undecanethiol or mercaptoundecanol were constructed on gold-covered glass coverslips individually or as a mixture by methods based on those of Bain et al. (5). Mercaptoundecanol, a hydroxyl-terminated alkanethiol, was synthesized by an organic substitution reaction between 11-bromo-1-undecanol (98%; Aldrich Chemical Co., Milwaukee, Wis.) and thiol-acetic acid (96%; Aldrich) (6). The alkanethiol product was purified on a column of silica gel (70/230 mesh; Aldrich) by using 1:1 hexane-ethyl acetate as the eluant, and the purity of the compound was verified qualitatively by nuclear magnetic resonance. A methyl-terminated alkanethiol, undecanethiol, was used as received (92%; Phaltz and Bauer, Inc., Waterbury, Conn.).

To construct the SAMs, clean glass coverslips (22 by 40 mm, no. 1.5; Corning) were coated with ca. 5 nm of chromium (99.995%; Aldrich) followed by a thin (ca. 40-nm-thick) layer of gold metal (99.99%; Aldrich) by thermal evaporation (model DV-502 vacuum evaporator; Denton Vacuum, Cherry Hill, N.J.). The metals (11 to 14 mg of chromium chips and 25 cm of gold wire) were evaporated successively from separate tungsten baskets under a 5-μtorr (ca. 7×10^{-4} Pa) vacuum to prevent oxidation of the chromium films. The thickness of each metal film was calculated from the mass of the metal that was evaporated and from the geometry of the evaporation chamber but was not measured directly.

The monolayers were prepared by overnight immersion of the gold-coated coverslips in a 1 mM ethanol solution of the alkanethiol(s). After removal from the coating solution, the SAMs were rinsed three times with 100% ethanol, blown dry with argon, and used immediately for substratum analysis or adhesion experiments. The five test surfaces consisted of (i) single-component SAMs constructed from mercaptoundecanol or undecanethiol and (ii) a series of three mixed-component SAMs constructed from different mole fractions of the two alkanethiols in the coating solution. The series of mixed SAMs was constructed from solutions with χ_p^{sol} values of 0.80, 0.53, and 0.25, where χ_p^{sol} represents the mole fraction of the polar alkanethiol (mercaptoundecanol) in the coating solution.

The substratum composition of one set of SAMs was determined by X-ray photoelectron spectroscopy (XPS) as detailed by others (5, 6). Analysis of the intensity and shape of the peak resulting from O(1s) photoelectrons yields information about the amount of oxygen present at the SAM surface indicating terminal hydroxyl groups. All data were treated with a Shirley background subtraction routine and an 80% Gaussian–20% Lorentzian peak-fitting procedure before normalization of the O(1s) intensities to the mean oxygen intensity, O(1s)*. O(1s)* was detected from several different samples of single-component, mercaptoundecanol monolayers.

The wettability of each set of SAMs was determined from the advancing contact angles (θ_a) of water and hexadecane. The contact angles of water and hexadecane are indicators of the polar and dispersion components, respectively, of substratum surface free energy (14). Advancing contact angles of water (freshly deionized and distilled) or hexadecane (99%; Sigma; purified by passage through a column of neutral, grade I, activated alumina [Aldrich]) on the SAMs were measured with a mechanical goniometer (LPI, Liss, England) at room temperature. Droplets of water (5 μl) or hexadecane (3 μl) were applied to each SAM with a micropipettor or a Hamilton syringe, respectively, by lowering a droplet to the surface until contact was made and then raising the applicator (6). The mean contact angles on each sample were calculated from three drops (six angles) measured on different areas of the surface.

Analysis of adhesion kinetics. A polycarbonate, parallel-plate chamber (0.5 by 14 by 25 mm) similar to that designed by Owens et al. (28) was used for the in situ measurements of bacterial adhesion. The interior dimensions and geometry of the chamber included a gradual inlet and outlet to provide laminar flow (37). However, the exterior of the chamber was modified from the original design of Owens et al. (28) to accommodate the optics of the microscope (Axioplan; Zeiss, Thornwood, N.Y.); these modifications included the use of gaskets composed of Parafilm (American National Can, Greenwich, Conn.) to seal the chamber. The hydrodynamics of flow through the chamber, including calculation of the shear stress and Reynolds' number, have been described previously (28, 29). Laminar flow through the chamber was verified with dye tests.

The flow chamber was connected with sterile tubing downstream of an input flask placed in a 20°C water bath and upstream of a peristaltic, pulseless pump (Micropump; Cole-Parmer Instrument Co., Niles, Ill.) used to pull the cells in suspension through the chamber at a fixed flow rate of 2 ml/min (shear stress = 58 mN/m^2) in a once-through system. Before introduction of the cell suspension, the system was filled with sterile 1.0% IO, preventing exposure of attaching or attached cells to the air-water interface during the experiment (11, 31, 34).

Adhesion of MI-1A to single- and mixed-component SAMs was measured in the laminar flow chamber by phase-contrast microscopy and digital image processing (IBAS image processing system; Kontron Elektronik GmbH, Munich, Germany). During the 120-min experimental period, images of one field of view (ca. 4,500 μm^2) were captured with a charge-coupled device camera (Sony Corp., Park Ridge, N.J.) utilizing an image-averaging routine that effectively removed from further processing steps any cells that were not immobilized on the surface for at least 40 ms, in addition to improving the signal-to-noise ratio (25). With a macro we had written with the IBAS software, the averaged image was then subjected to several automated processing steps to obtain a binary image for the subsequent measurement of adhesion (20, 37). One of the processing steps, an object selection routine, was necessary to remove from subsequent counting routines any objects that were not attached cells, such as artifacts generated from the background. The routine discards objects that do not fall within the ranges of several size and shape parameters that are characteristic of cells of MI-1A.

The following four parameters that describe the kinetics of adhesion of MI-1A to single- and mixed-component SAMs were measured for four trials with each type of surface.

(i) The initial rate of net adhesion was calculated from a linear regression analysis of the net number of attached cells at ca. 2-min intervals during the first 30 min of exposure.

(ii) The percentage of cells that adsorbed or desorbed between each successive measurement was determined. The percent adsorption and desorption rates are quantitative indicators of the reversibility of adhesion and are related to the residence time of attached cells on the substratum surface. The rates were determined by subtractive analysis of successive images of the same microscopic field captured at 2-min intervals during the 2-h period of exposure. Percent adsorption is the percentage of cells attached in the current field that adsorbed since the previous measurement was made; percent desorption is the percentage of cells attached in the previous field that desorbed between measurements. Each image was superimposed over the previous image to identify which cells had adsorbed since the previous image was captured. If any part of an individual cell in an image did not overlap a cell on the previous image, the cell was counted as newly adsorbed. In the same manner, the percentage of cells that desorbed between successive measurements was determined by superimposition of the successive images. The amount of cell adsorption or desorption is expressed as a rate following division by the time interval between successive images, yielding the fraction of cells that adsorbed or desorbed per unit of time. These parameters of adhesion kinetics are identical to the desorption constant α described by Busscher et al. (11), but here they are expressed as percentages: percent adsorption rate = $[(\Delta n_{b12}/n_{b2}) \cdot (t_2 - t_1)] \cdot 100$, where Δn_{b12} is the number of cells that adsorbed between times t_1 and t_2 and n_{b2} is the number of attached cells at time t_2; and percent desorption rate = $[(\Delta n_{b12}/n_{b1}) \cdot (t_2 - t_1)] \cdot 100$, where Δn_{b12} is the number of cells that desorbed between times t_1 and t_2 and n_{b1} is the number of attached cells at time t_1. As described previously (24), these measurements may underestimate the amount of adsorption and desorption if cells desorb and other cells adhere to the same sites between measurements. However, the total area covered by attached cells was always less than 10% of the total area of substratum, reducing the likelihood that cells would attach to a site previously occupied by desorbed cells within the time interval (≈2 min) between measurements. Also, visual observations confirmed that this did not occur in the current study.

(iii) A separate subroutine was used to directly measure the residence time of attached cells for two trials with each of the five types of SAMs. The algorithm of the residence time program described by Meinders et al. (24) was used to construct an analogous subroutine with the analysis software of the IBAS. The residence time subroutine is similar to the adsorption and desorption measurement routine (described above) in that the current image is compared with a stored reference image to determine which cells have adsorbed or desorbed. However, instead of using the previous image as the reference image, the residence time subroutine compares the current image with separate compilation images, one each for adsorption and desorption. The compilation images are created during the experiment by assigning grey levels (1 to 255) to separate binary images of newly adsorbed or desorbed cells and adding each multiphase image to the appropriate compilation image (24). After the experiment was completed, a separate program was used to scan the compilation images and record the times of adsorption and desorption for cells that had attached during the 120-min exposure period. Although this subroutine can also yield the percentage of cells that adsorbed or desorbed per minute, the method has a greater tendency to underestimate the amount of adsorption and desorption. The residence time routine uses compilation images which record the adsorption and desorption of cells throughout the experiment, rather than using the previous image as a reference image. This increases the chance that cells will adsorb in an area previously occupied by a desorbed cell and will decrease the amount of adsorption and desorption measured by the subroutine.

For each experiment, the residence times of cells that adsorbed to the substratum during the exposure period were determined for two different populations: the population of cells that desorbed from the substratum (reversible adhesion) and the population of cells that did not desorb (irreversible adhesion). The residence times of the population that desorbed from the substratum were further analyzed for the distribution of residence times in increasing 10-min intervals as a percentage of the population.

(iv) Net attachment at the end of the experimental period also was determined. After the 2-hour exposure period, the flow chamber was rinsed for 10 min with sterile 1.0% IO at a flow rate of 2 ml/min to remove nonattached cells. Subsequently, a separate image analysis program was used to calculate the mean number of attached cells from several (5 to 10) fields of view.

Four trials were conducted for each of the five types of SAMs by using separate cultures of MI-1A. The Student *t* test was used to test the significances ($P < 0.05$) among the mean initial adhesion rates and the number of attached cells after 120 min.

RESULTS

Substratum composition and wettability of SAMs. The gold films supported on chromium-primed glass that were used for self-assembly of the alkanethiol monolayers were highly reflective but were sufficiently transparent to allow microscopic observation of attached cells. Analysis of the surface roughness of a limited number of the gold films by atomic force microscopy (Nanoscope III; Digital Instruments, Santa Barbara, Calif.) indicated root mean square roughness values of <3 nm.

The substratum compositions and wettabilities of single-component SAMs of mercaptoundecanol or undecanethiol were characteristic of well-ordered monolayers in that (i) the surface properties of the SAMs were determined by the terminal hydroxyl or methyl groups and (ii) mixed-component SAMs constructed from three different mole fractions of the hydroxyl-terminated alkanethiol in the coating solution, i.e., χ_p^{sol} values of 0.80, 0.53, and 0.25, exhibited substratum compositions and wettabilities that ranged between the properties of the single-component SAMs. XPS detected the amount of oxygen present in single- and mixed-component SAMs that contain mercaptoundecanol by the intensities of photoelectrons emitted from excited oxygen atoms within the terminal hydroxyl groups. A small amount of oxygen, probably due to adsorption of contaminants from the air during preparation of the sample for XPS, was detected in the single-component SAMs constructed from undecanethiol. The background was subtracted from the intensities of all the SAMs before normalization against the amount of oxygen detected in single-component SAMs of mercaptoundecanol. XPS of the mixed SAMs indicated that the proportion of hydroxyl groups in the mixed-component monolayers (%OH) increased with the mole fraction of the polar component in the coating solution, χ_p^{sol} (Fig. 1). The mixed SAMs constructed from solutions with χ_p^{sol} values of 0.80, 0.53, and 0.25 yielded surfaces with 83, 40, and 17% of the hydroxyl-terminated alkanethiol in the monolayers, respectively.

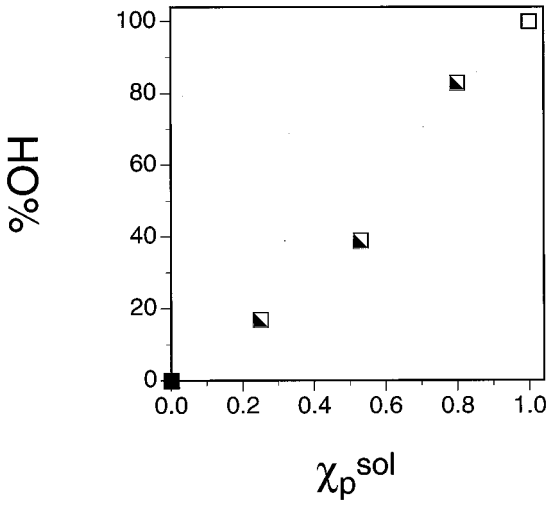

FIG. 1. Percentage of mercaptoundecanol (%OH) detected in single-component SAMs of mercaptoundecanol (□) and undecanethiol (■) and in mixed SAMs (◪) constructed from solutions with χ_p^{sol} values of 0.25, 0.53, and 0.80, where χ_p^{sol} is the mole fraction of the hydroxyl-terminated alkanethiol in the coating solution. Symbols represent the photoelectron intensities of oxygen from the SAMs, normalized to the mean intensity from two single-component SAMs of mercaptoundecanol.

The wettabilities of single-component monolayers constructed from mercaptoundecanol for water and hexadecane were indicative of very hydrophilic [θ_a(water) $\leq 10°$] and oleophilic [θ_a(hexadecane) $\leq 10°$] surfaces. In contrast, SAMs composed of undecanethiol were hydrophobic [θ_a(water) $\approx 105°$] and oleophobic [θ_a(hexadecane) $\approx 38°$]. The advancing contact angles of water on the mixed-component SAMs constructed from mercaptoundecanol and undecanethiol increased as the percentage of the hydroxyl-terminated alkanethiol in the monolayers decreased (Fig. 2). Hexadecane spread ($\theta_a \leq 10°$) on mixed SAMs composed of 83 and 40% OH. However, at lower percentages of hydroxyl groups (%OH $\leq$ 17), the advancing contact angles of hexadecane increased.

Initial rate of adhesion. The initial rate of adhesion of MI-1A to the test substrata was measured as the slope from a least-squares fit of 7 to 15 measurements of the number of attached cells during the first 30 min of exposure. Very few cells (one to five per field) attached to the single-component, mercaptoundecanol SAMs during the 120-min exposure period, including the first 30 min (Fig. 3). The initial rates of adhesion to single- and mixed-component SAMs which contained undecanethiol increased with substratum hydrophobicity [θ_a(water)] and, correspondingly, with the decreasing percentage of hydroxyl groups in the monolayers. However, because of the variation among different trials with the same surface, only the mean initial rates of MI-1A adhesion to undecanethiol and 83% OH mixed SAMs were significantly different ($P < 0.05$). After ca. 30 min, the rate of net adhesion to each of the four types of SAMs containing undecanethiol began to decrease. Stationary conditions (rate $\approx$ 0) were achieved only for the 83 and 40% OH mixed-component SAMs (data not shown).

Percent adsorption and desorption rates. The highest percentages of cells that adsorbed and desorbed per minute were observed with the single-component SAMs constructed from

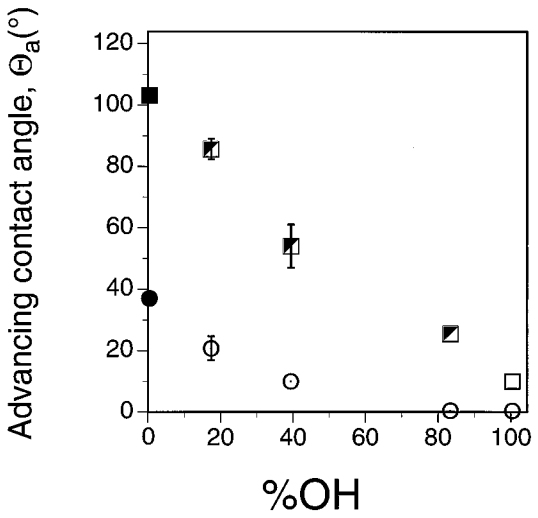

FIG. 2. Advancing contact angles of water (squares) and hexadecane (circles) on single (□, ○, ■, and ●)- and mixed (◩ and ⊙)-component SAMs of mercaptoundecanol (open symbols) and undecanethiol (closed symbols) as a function of the percentage of the polar alkanethiol in the monolayers, %OH. Symbols represent the mean contact angles of the test liquids calculated from results obtained with four samples of each type of SAM. Error bars represent the standard errors of the mean and generally are smaller than the symbols.

mercaptoundecanol (Fig. 4). However, because of the extremely small amount of adhesion of MI-1A on the single-component, mercaptoundecanol SAMs, the percent adsorption and desorption rates reflect the measurement of only a few cells (less than five) per field. Net adhesion to the test surfaces containing undecanethiol was much greater than that to mercaptoundecanol SAMs, providing a larger sample of the cell population from which to calculate the percent adsorption and desorption rates. With each of these test surfaces, the percent adsorption rate decreased dramatically during the first

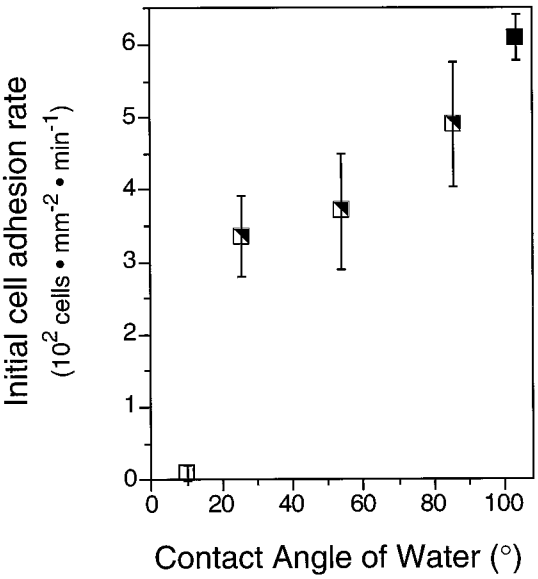

FIG. 3. Mean initial adhesion rates of MI-1A to single-component SAMs of mercaptoundecanol (□) and undecanethiol (■) and to mixed SAMs (◹) constructed from solutions with χ_p^{sol} values of 0.80, 0.53, and 0.25. Error bars represent the standard deviations of the mean calculated from several trials.

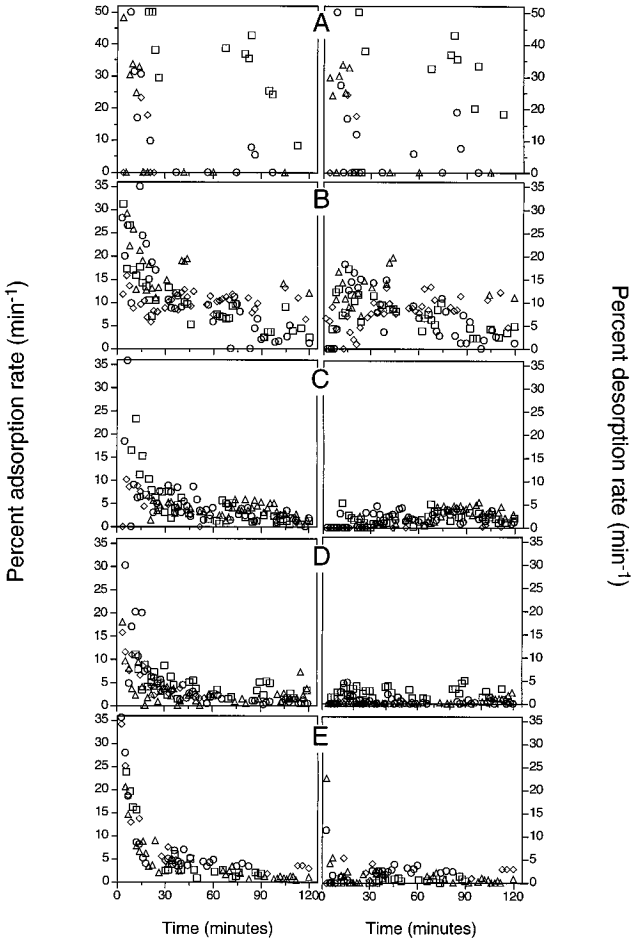

FIG. 4. Percent adsorption (left panels) and desorption (right panels) rates of MI-1A on single- and mixed-component SAMs constructed from mercaptoundecanol and undecanethiol. Different symbols represent four different trials with each type of surface. Substrata are single-component SAMs of mercaptoundecanol, %OH = 100 (A); mixed-component SAMs, %OH = 83 (B); mixed-component SAMs, %OH = 40 (C); mixed-component SAMs, %OH = 17 (D); and single-component SAMs of undecanethiol, %OH = 0 (E). %OH, percentage of mercaptoundecanol in the SAMs. Data collected at 2-min intervals are illustrated.

30 min of exposure. During this period, the percent adsorption rate was much greater than the desorption rate. After approximately 30 min, the percentage of cells that adsorbed per minute had decreased to the levels of desorption. Generally, bacterial adsorption and desorption on the mixed-component SAMs decreased with increasing substratum hydrophobicity. Among these substrata, the percentages of cell adsorption and desorption were highest on the 83% OH mixed SAMs. Adsorption and desorption were similar for the 40 and 17% OH and undecanethiol SAMs, although the rates measured for the 40% OH SAMs were slightly higher than those for the SAMs with lower compositions of undecanethiol. The percent desorption of MI-1A from 17% OH and undecanethiol SAMs during the 2-h period of exposure generally was low (<5%), indicating irreversible adhesion and a long residence time of most of the cells that attached to the substratum. There was less variability among the adsorption and desorption data from different trials than observed with the initial rates of net adhesion.

Residence times of cells. The residence times of individual

TABLE 1. Reversible adhesion of MI-1A on SAMs during the 120-min exposure period[a]

SAM (%OH)	No. of cells adsorbed	% Desorbed
83[b]	373	76
	185	76
40	379	53
	243	47
17	461	49
	252	11
0	856	50
	384	33

[a] The total number of cells that adsorbed and the percentage of the total population that desorbed were determined from analysis of the residence time compilation images (see Materials and Methods).

[b] %OH, percentage of mercaptoundecanol in the SAMs. Two trials were conducted with each type of SAM, the same as those used to compile Fig. 5.

cells that attached to the substrata during the 120-min exposure period were measured for two trials with the four types of SAMs containing undecanethiol. For each experiment, the data were separated according to two populations, the population of cells that desorbed at some time during the exposure period and the population of cells that were still attached at 120 min. Table 1 lists the total number of cells that adsorbed to the substrata during the exposure period and the percentage of the total that desorbed. Although there is considerable variation between two trials for the same type of SAM, the trend indicates that the total number of cells that contact the substratum is independent of substratum wettability. In contrast, the desorption of MI-1A appears to be affected by substratum wettability. The percentage of cells that desorbed during the exposure period decreased from 76% with the most hydrophilic SAM (83% OH) to 30 to 50% with the more hydrophobic SAMs. The histograms shown in Fig. 5 depict the distribution of residence times in increasing 10-min intervals as a percentage of the population that desorbed. In all cases, the highest percentage of the population of cells that desorbed from the substratum exhibited residence times of less than 10 min. Comparing the cells with residence times of less than 10 min among the four types of monolayers shows that the highest percentages were observed on the most hydrophilic SAMs (83% OH). There were few consistent differences in the percentages of cells with residence times of less than 10 min among the 40 and 17% OH and undecanethiol SAMs, although all were lower than the percentages observed for the 83% OH SAMs.

Mean number of attached cells after 120 min. The measurements described above were conducted over time on a single microscopic field of the substratum surface. The numbers of attached cells after 120 min were determined for several microscopic fields to discover the homogeneity of attachment on a larger area of the substratum and the degree to which the net adhesion measured on one field of view reflected the amount of attachment to other areas of the substratum. For each trial, the mean number of attached cells was within 10% of the number measured after 2 h on a single field of view. In addition, there was no significant increase or decrease in the numbers of attached cells during the 5 to 10 min required to measure several different fields, indicating that the rate of net adhesion was near zero following the 10-min period of rinsing with sterile IO. The results averaged from four trials with each type of SAM indicated that the mean number of attached cells after 120 min increased with the increasing contact angles of water (Fig. 6). The level of cell adhesion to the SAMs containing undecanethiol was significantly higher ($P < 0.05$) than that of adhesion to the single-component SAMs of mercaptoundecanol. However, among the 40 and 17% OH mixed SAMs and the single-component SAMs of undecanethiol, the mean numbers of attached cells were not significantly different. The level of variation in the number of attached cells after 2 h among different microscopic fields was extremely low (average standard deviation, <10 cells per field), indicating that all the single- and mixed-component SAMs exhibited homogeneous surfaces with respect to adhesion.

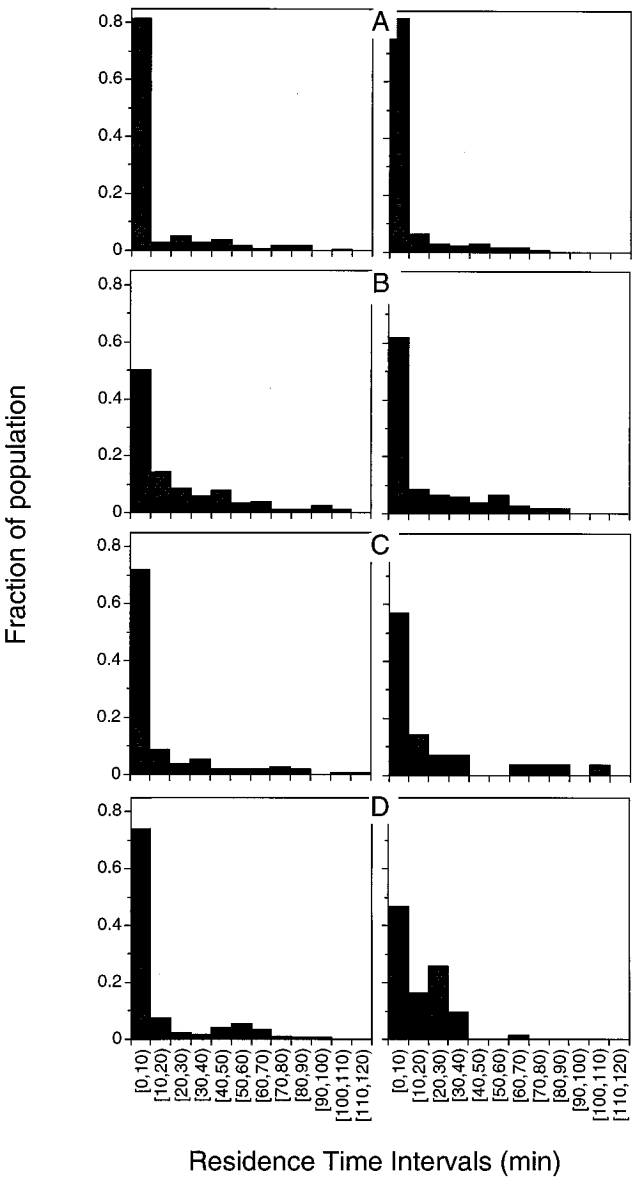

FIG. 5. Distribution of individual residence times for the population of cells of MI-1A that desorbed from the SAMs during the 120-min exposure period. The numbers of cells within increasing residence time intervals are expressed as the fraction of the total number of cells that desorbed. Two trials with each type of SAM are shown (left and right panels, respectively). Substrata are mixed-component SAMs, %OH = 83 (A); mixed-component SAMs, %OH = 40 (B); mixed-component SAMs, %OH = 17 (C); and single-component SAMs of undecanethiol, %OH = 0 (D). %OH, percentage of mercaptoundecanol in the SAMs. Single-component SAMs of mercaptoundecanol (%OH = 100) were not included because of the extremely small numbers of attached cells.

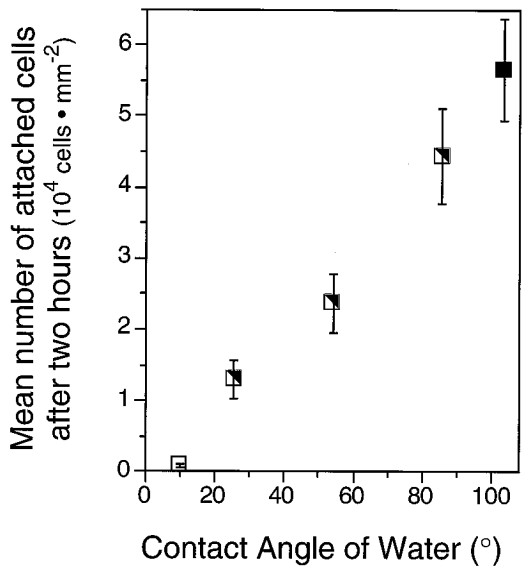

FIG. 6. Mean numbers of attached cells of MI-1A after 2 h of exposure. Substrata are single-component SAMs of mercaptoundecanol (□) and undecanethiol (■) and mixed SAMs (◥) constructed from solutions with χ_p^{sol} values of 0.80, 0.53, and 0.25. Each symbol represents the mean for several trials with a selected surface. Error bars represent the standard errors of the mean. For each experiment, the mean number of attached cells was calculated from 5 to 10 microscopic fields.

DISCUSSION

In most studies of substratum wettability and bacterial adhesion, different materials are used to represent test surfaces with a range of substratum wettabilities. The effects of other surface properties (e.g., charge, roughness, and chemical composition), which vary among materials, make it difficult, however, to distinguish the effects of substratum wettability on adhesion (30, 35, 38). Also, many materials used as test surfaces have unknown chemical compositions, which may differ among samples of the same material because of trace impurities. In contrast, SAMs can provide a series of well-ordered, smooth substrata with known compositions and wettabilities (5–7). To our knowledge, the use of SAMs in the study of substratum wettability and bacterial adhesion has not been reported previously. The goals of this study were to determine (i) the role of substratum wettability on adhesion of MI-1A by using hydroxyl- and methyl-terminated SAMs and (ii) the relative contributions of adsorption, desorption, and the residence times of attached cells to net adhesion.

The surface properties of the SAMs constructed in our laboratory were consistent with previous descriptions of these surfaces (5–7). The relative amount of the hydroxyl-terminated alkanethiol in the SAMs, as determined by XPS, increased as the mole fraction of mercaptoundecanol in the coating solution increased. This is in general agreement with results of previous studies of 11-carbon alkanethiols assembled onto 200-nm-thick gold films supported on silicon wafers (5, 6). The advancing contact angles of water and hexadecane on the single- and mixed-component SAMs also correspond well with previous results obtained with similar types of SAMs composed of mercaptoundecanol and undecanethiol (5, 7). The range of wettabilities exhibited by the single-component and mixed SAMs was comparable to the ranges observed in previous studies of bacterial adhesion and substratum wettability but had the advantage that variations in only two functional groups were used to control the surface properties. Previous studies employed different materials with numerous chemical differences as test surfaces (13, 38, 40). In addition, the roughness of the gold films that were used to support the SAMs was ca. 30-fold less than the roughness of polished polymers used in previous studies (35, 40).

The kinetics of adhesion to the SAMs are reflected in the net adhesion rates, the percentage of cells that adsorbed or desorbed per minute, the residence time of cells that desorbed during the 120-min experimental period, and the mean number of attached cells after 120 min, calculated from several fields. The rates of adhesion of MI-1A during the first 30 min to SAMs containing methyl groups were significantly higher than the rates of adhesion to single-component, hydroxyl-terminated SAMs. This suggests that the initial attractive interactions which result in an increase in the rate of adhesion during the first 30 min are similar among SAMs containing undecanethiol. After ca. 30 min, the rates of net adhesion to the SAMs containing methyl groups began to decrease as a result of several possible effects: the existence of a limited number of attachment sites, which are saturated during the early stages of exposure (11, 36); repulsion of bacteria that are approaching the surface by bacteria that are already attached (1, 36); and the conditioning of the substratum by substances released from the bacteria and adsorbed onto the substrata, which could begin to alter the surface properties of the SAMs after ca. 30 min (3, 13, 20).

The number of bacteria attached to any surface at a given time is the net result of two processes, i.e., adsorption and desorption. For decades, the model of nonspecific bacterial adhesion has been described as a two-stage process. Initial, reversible adsorption of cells at some distance from the substratum surface is followed by time-dependent metabolic activities (e.g., synthesis of adhesive polymers) or the removal of interfacial water, resulting in the cell moving closer to the substratum surface and adhering irreversibly (3, 11, 19, 22). Although several studies have attempted to determine the role of substratum wettability in the model of bacterial adhesion, many of those investigators measured only the net number of attached cells (13, 35). Also, exposure of attached cells to the air-water interface probably removed most of the reversibly attached cells from the substratum, resulting in the measurement only of the irreversibly attached population (11, 31). By directly measuring and quantifying the reversibility of adhesion through the percent desorption rate and the residence times of attached cells, we can determine more precisely how substratum wettability affects the mechanism of bacterial adhesion.

One of our goals in this study was to determine the relative effect of adsorption and desorption on the net adhesion of MI-1A. Both the adsorption and desorption rates of MI-1A decreased with increasing substratum hydrophobicity. The effect of surface hydrophobicity on adsorption rates suggests that the interfacial forces responsible for the initial binding of MI-1A are greater on hydrophilic than on hydrophobic SAMs. This conclusion is inconsistent, however, with the net adhesion (i.e., initial adhesion rate and mean number of attached cells after 2 h), which increased with substratum hydrophobicity. It appears that desorption, rather than adsorption, determines the net number of attached cells at any given time. The decrease in percent desorption rate on increasingly hydrophobic SAMs indicates a transition from reversible to irreversible adhesion. It has been suggested that interfacial water, which adsorbs to polar functional groups on the substratum surface through a hydrogen-bonding network, may act as a barrier that prevents initial irreversible adhesion (11, 16). The results from this study support those previous findings because the percent-

age of cells that adhered reversibly was directly related to the amount of polar functional groups in the SAMs.

The relative importance of desorption in the process of bacterial adhesion is also supported by the data collected from the residence time compilation images. The effect of substratum wettability on the total numbers of cells that adsorbed to the substrata was not consistent, but it appears to be relatively insignificant. Separation of the total numbers of cells that adsorbed to each type of SAM into two populations, i.e., the population that desorbed and the population that remained attached, indicated that substratum wettability does, however, affect the fate of cells after initial adsorption. Although the data exhibit considerable variability, the decrease in the percentage of cells that desorbed was greatest between the 83 and 40% OH mixed SAMs, a trend that also was observed with the adsorption and desorption rates (see above). The percentage of cells that desorbed from the more hydrophobic substrata was higher than expected on the basis of the low percent desorption rates from these SAMs. On these surfaces, we observed a small number of cells ($\leq$10% of the attached population) that adsorbed to the substratum and then exhibited slight flipping, rotating, or sliding maneuvers without desorbing. Such activities could cause the residence time image-processing routine to count these cells as desorbed. In contrast, the processing routine that was used to calculate the desorption rates compared the current image with the previous image and therefore did not detect these slight movements.

The transition from reversible to irreversible adhesion is an important component of the two-step model described above. While some studies have indicated a role of physiological responses by the organism in the process (20), other studies indicate that rapid aging of cell-substratum bonds is responsible for the development of irreversible adhesion (24, 26). The analysis of the residence time distribution done in this study suggests a rapid transition from reversible to irreversible adhesion. Data demonstrated that when bacteria desorbed they did so quickly, generally in $<$4 min. In addition, visual observation of the adhesion kinetics indicated that, especially with hydrophilic SAMs, many cells adsorbed and desorbed at time intervals of much less than 4 min (often in seconds). These intervals correspond well to the residence times reported in several previous works (19, 36). In contrast, a recent study of *Vibrio parahaemolyticus* indicated that the adhesion to glass was reversible for a period of 30 min before a transition to irreversible adhesion occurred (20). However, the residence time for individual cells was not measured. The rapid transition from reversible to irreversible adhesion shown here and in previous studies (19, 26, 36) suggests that, contrary to the accepted model (18, 23), physiological responses by the bacteria were not responsible for the development of irreversible cell adhesion. However, this does not discount the possibility that physiological responses such as exopolysaccharide production could be induced by irreversible adhesion (3).

Substratum wettability appears to have little effect on the distribution of residence times of cells that desorb from the substratum. Data suggest that the probability of cells remaining attached after initial immobilization on the substratum increases considerably after 4 min, regardless of substratum wettability. This agrees with a previous study in which little or no effect of substratum wettability on the time required for development of irreversible cell adhesion was reported (26). Analysis of both the residence times of attached cells and the percent adsorption and desorption rates suggests that substratum wettability appears to affect the numbers of cells that attach reversibly or irreversibly, rather than affecting the residence time required for development of irreversible adhesion.

Cell attachment to the SAMs was homogeneous within and among different microscopic fields, with no observable clustering of cells at specific areas of the substrata. Additionally, the compilation diagrams used to record the adsorption and desorption of cells throughout the 120-min exposure period did not reveal any clustering of cell images due to adsorption and desorption of cells at specific areas of the SAMs. These findings provide evidence in support of previous observations (7) that if the hydroxyl- and methyl-terminated alkanethiols phase-separate into macroscopic islands in the mixed SAMs, the islands are no larger than a few microns. If larger islands were formed in this study, they would have been revealed by the adhesion of several cells to specific areas of the substratum. Although the formation of islands smaller than ca. 1 μm^2 might result in the adhesion of single cells to specific sites of the substratum, we did not observe cells adhering selectively to sites previously occupied by attached cells. It has been suggested that chemical or physical substratum heterogeneity could result in sites that favor initial immobilization of bacterial cells (38). Microscopic islands that are too small to be detected by measurement of contact angles and XPS may provide microsites that favor adhesion of a single cell.

In conclusion, utilizing a simple system consisting of hydroxyl and methyl functional groups, this study has created a foundation for the measurement and analysis of bacterial adhesion using alkanethiol SAMs. Additional questions remain about which properties of the substratum actually affect adhesion. Adhesion of MI-1A increased both with decreasing amounts of mercaptoundecanol in the SAMs and with decreasing substratum wettability. As with previous studies of protein adsorption on SAMs (33), this study did not allow differentiation between the significance of specific functional groups and the more general property, wettability. Studies now under way will focus on the role of substratum composition compared with that of wettability in adhesion and on the role of substratum hydrophobicity (contact angles of water) compared with that of substratum oleophobicity (contact angles of hexadecane).

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REFERENCES

1. **Adamczyk, Z., B. Siwek, and M. Zembala.** 1991. Negative cooperativity in adsorption and adhesion of particles. Biofouling **4:**89–98.
2. **Alberte, R. S., S. Snyder, B. J. Zahuranec, and M. Whetstone.** 1992. Biofouling research needs for the United States navy: program history and goals. Biofouling **6:**91–95.
3. **Allison, D. G., and I. W. Sutherland.** 1987. The role of exopolysaccharides in adhesion of freshwater bacteria. J. Gen. Microbiol. **133:**1319–1327.
4. **Atkinson, B., and H. W. Fowler.** 1974. The significance of microbial film in fermenters, p. 223–277. *In* T. K. Ghose, A. Fiechter, and N. Blakebrough (ed.), Advances in biochemical engineering. Springer-Verlag KG, New York.
5. **Bain, C. D., J. Evall, and G. M. Whitesides.** 1989. Formation of monolayers by the coadsorption of thiols on gold: variation in the head group, tail group, and solvent. J. Am. Chem. Soc. **111:**7155–7164.
6. **Bain, C. D., E. B. Troughton, Y. Tao, J. Evall, G. M. Whitesides, and R. G. Nuzzo.** 1989. Formation of monolayer films by the spontaneous assembly of organic thiols from solution onto gold. J. Am. Chem. Soc. **111:**321–335.
7. **Bain, C. D., and G. M. Whitesides.** 1988. Formation of two-component surfaces by the spontaneous assembly of monolayers on gold from solutions

containing mixtures of organic thiols. J. Am. Chem. Soc. **110:**6560–6561.
8. **Bain, C. D., and G. M. Whitesides.** 1989. Formation of monolayers by the coadsorption of thiols on gold: variation in the length of the alkyl chain. J. Am. Chem. Soc. **111:**7164–7175.
9. **Bidle, K., H. H. Wickman, and M. Fletcher.** 1993. Attachment of a *Pseudomonas*-like bacterium and *Bacillus coagulans* to solid surfaces and adsorption of their S-layer proteins. J. Gen. Microbiol. **139:**1891–1897.
10. **Bonar, D. B., R. M. Weiner, and R. R. Colwell.** 1986. Microbial-invertebrate interactions and potential for biotechnology. Microb. Ecol. **12:**101–110.
11. **Busscher, H. J., J. Sjollema, and H. C. Van der Mei.** 1990. Relative importance of surface free energy as a measure of hydrophobicity in bacterial adhesion to solid surfaces, p. 335–359. *In* R. J. Doyle and M. Rosenberg (ed.), Microbial cell surface hydrophobicity. American Society for Microbiology, Washington, D.C.
12. **Dexter, S. C., J. D. Sullivan, J. Williams III, and S. W. Watson.** 1975. Influence of substrate wettability on the attachment of marine bacteria to various surfaces. Appl. Microbiol. **30:**298–308.
13. **Fletcher, M., and G. I. Loeb.** 1979. Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. Appl. Environ. Microbiol. **37:**67–72.
14. **Folkers, J. P., P. E. Laibinis, and G. M. Whitesides.** 1992. Self-assembled monolayers of alkanethiols on gold: comparisons of monolayers containing mixtures of short- and long-chain constituents with CH_3 and CH_2OH terminal groups. Langmuir **8:**1330–1341.
15. **Höfle, M. G.** 1992. Rapid genotyping of pseudomonads by using low-molecular-weight RNA profiles, p. 116–126. *In* E. Galli, S. Silver, and B. Witholt (ed.), *Pseudomonas*: molecular biology and biotechnology. American Society for Microbiology, Washington, D.C.
16. **Israelachvili, J.** 1992. Interfacial forces. J. Vacuum Sci. Technol. **10:**2961–2971.
17. **Israelachvili, J. N., and M. L. Gee.** 1989. Contact angles on chemically heterogeneous surfaces. Langmuir **5:**288–289.
18. **Lappin-Scott, H. M., and J. W. Costerton.** 1989. Bacterial biofilms and surface fouling. Biofouling **1:**323–342.
19. **Lawrence, J. R., P. J. Delaquis, D. R. Korber, and D. E. Caldwell.** 1987. Behavior of *Pseudomonas fluorescens* within the hydrodynamic boundary layers of surface microenvironments. Microb. Ecol. **14:**1–14.
20. **Lawrence, J. R., D. R. Korber, and D. E. Caldwell.** 1992. Behavioral analysis of *Vibrio parahaemolyticus* variants in high- and low-viscosity microenvironments by use of digital image processing. J. Bacteriol. **174:**5732–5739.
21. **López, G. P., M. W. Albers, S. L. Schreiber, R. Carroll, E. Peralta, and G. M. Whitesides.** 1993. Convenient methods for patterning the adhesion of mammalian cells to surfaces using self-assembled monolayers of alkanethiolates on gold. J. Am. Chem. Soc. **115:**5877–5878.
22. **Marshall, K. C., R. Stout, and R. Mitchell.** 1971. Mechanism of the initial events in the sorption of marine bacteria to surfaces. J. Gen. Microbiol. **68:**337–348.
23. **McFeters, G. A.** 1984. Biofilm development and its consequences, p. 109–124. *In* K. C. Marshall (ed.), Microbial adhesion and aggregation. Springer-Verlag KG, Berlin.
24. **Meinders, J. M., J. Noordmans, and H. J. Busscher.** 1992. Simultaneous monitoring of the adsorption and desorption of colloidal particles during deposition in a parallel plate chamber. J. Colloid Interface Sci. **152:**265–280.
25. **Meinders, J. M., H. C. Van der Mei, and H. J. Busscher.** 1992. *In situ* enumeration of bacterial adhesion in a parallel plate flow chamber—elimination of in focus flowing bacteria from the analysis. J. Microbiol. Methods **16:**119–124.
26. **Meinders, J. M., H. C. Van der Mei, and H. J. Busscher.** 1994. Physicochemical aspects of deposition of *Streptococcus thermophilus* B to hydrophobic and hydrophilic substrata in a parallel plate flow chamber. J. Colloid Interface Sci. **164:**355–363.
27. **Morra, M., E. Occhiello, and F. Garbassi.** 1989. Contact angle hysteresis in oxygen plasma treated poly(tetrafluoroethylene). Langmuir **5:**872–876.
28. **Owens, N. F., D. Gingell, and P. R. Rutter.** 1987. Inhibition of cell adhesion by a synthetic polymer adsorbed to glass shown under defined hydrodynamic stress. J. Cell Sci. **87:**667–675.
29. **Owens, N. F., D. Gingell, and A. Trommler.** 1988. Cell adhesion to hydroxyl groups of a monolayer film. J. Cell Sci. **91:**269–279.
30. **Paul, J. H., and G. I. Loeb.** 1983. Improved microfouling assay employing a DNA-specific fluorochrome and polystyrene as substratum. Appl. Environ. Microbiol. **46:**338–343.
31. **Pitt, W. G., M. O. McBride, A. J. Barton, and R. D. Sagers.** 1993. Air-water interface displaces adsorbed bacteria. Biomaterials **14:**605–608.
32. **Porter, M. D., T. B. Bright, D. L. Allara, and C. E. D. Chidsey.** 1987. Spontaneously organized molecular assemblies. 4. Structural characterization of *n*-alkyl thiol monolayers on gold by optical ellipsometry, infrared spectroscopy, and electrochemistry. J. Am. Chem. Soc. **109:**3559–3568.
33. **Prime, K. L., and G. M. Whitesides.** 1993. Adsorption of proteins onto surfaces containing end-attached oligo(ethylene oxide): a model system using self-assembled monolayers. J. Am. Chem. Soc. **115:**10714–10721.
34. **Rijnaarts, H. H. M., W. Norde, E. J. Bouwer, J. Lyklema, and A. J. B. Zehnder.** 1993. Bacterial adhesion under static and dynamic conditions. Appl. Environ. Microbiol. **59:**3255–3265.
35. **Shreve, G. S., R. H. Olsen, and T. M. Vogel.** 1991. Development of pure culture biofilms of *P. putida* on solid supports. Biotechnol. Bioeng. **37:**512–518.
36. **Sjollema, J., H. J. Busscher, and A. H. Weerkamp.** 1988. Deposition of oral streptococci and polystyrene latices onto glass in a parallel plate flow cell. Biofouling **1:**101–112.
37. **Sjollema, J., H. J. Busscher, and A. H. Weerkamp.** 1989. Real-time enumeration of adhering microorganisms in a parallel plate flow cell using automated image analysis. J. Microbiol. Methods **9:**73–78.
38. **Sjollema, J., H. C. Van der Mei, H. M. W. Uyen, and H. J. Busscher.** 1990. The influence of collector and bacterial cell surface properties on the deposition of oral streptococci in a parallel plate flow cell. J. Adhesion Sci. Technol. **4:**765–777.
39. **Van Oss, C. J.** 1991. The forces involved in bioadhesion to flat surfaces and particles—their determination and relative roles. Biofouling **4:**25–35.
40. **Van Pelt, A. W. J., A. H. Weerkamp, M. H. W. J. C. Uyen, H. J. Busscher, H. P. de Jong, and J. Arends.** 1985. Adhesion of *Streptococcus sanguis* CH3 to polymers with different surface free energies. Appl. Environ. Microbiol. **49:**1270–1275.