APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1979, p. 67-72 Vol. 37, No. 1
0099-2240/79/01-0067/06$02.00/0

Influence of Substratum Characteristics on the Attachment of a Marine Pseudomonad to Solid Surfaces

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Received for publication 25 August 1978

The attachment of a marine *Pseudomonas* sp. to a variety of surfaces was investigated, and the number of bacteria which became attached was related to the surface charge and degree of hydrophobicity of the substratum. Large numbers of bacteria attached to hydrophobic plastics with little or no surface charge [Teflon, polyethylene, polystyrene, poly(ethylene terephthalate)]; moderate numbers attached to hydrophilic metals with a positive (platinum) or neutral (germanium) surface charge; and very few attached to hydrophilic, negatively charged substrata (glass, mica, oxidized plastics). The results suggest that both electrostatic and hydrophobic interactions are involved in bacterial attachment.

The attachment of bacteria to solid surfaces is an important phenomenon in many environments, including aquatic habitats (4), soils (24), the human mouth (14), and mammalian epithelial tissues (18). Some bacteria have special attachment structures, e.g., pili or holdfasts, but many have not and appear to attach by means of extracellular polymeric adhesives (11, 26). The attachment of a bacterium to a surface is dependent upon two events: (i) that the bacterium encounters a surface and comes close enough for attachment to occur and (ii) that the bacterial outer surface adheres to the substratum. The probability of a bacterium encountering a potential substratum is affected by a number of factors, e.g., culture concentration and bacterial motility (10), whereas attachment is dependent upon the chemical and physical interactions between the potential substratum, the bacterial surface, and the polymeric adhesive. Because these interactions are to some extent determined by the characteristics of the potential substratum, it was decided to investigate the attachment of bacteria to a number of different materials. The bacterium was a marine pseudomonad which appears to attach by means of an extracellular polymer (11). Some of its attachment properties have been described previously (9-13).

MATERIALS AND METHODS

Organism and growth. The marine *Pseudomonas* sp. (National Collection of Marine Bacteria [NCMB] 2021) was cultured as previously described (9) for 21 to 22 h, at which time the bacteria were in the late-log phase of growth. The organisms were then collected by centrifugation and resuspended in autoclaved, filtered (0.2-μm porosity) seawater to a concentration of 2.5×10^9 to 5×10^9 bacteria per ml. These suspensions remained stable during experiments and did not tend to form aggregates.

Substrata. Commercially available materials used as substrata included PTFE Teflon film (Dupont); polyethylene (PE) (Sterilin); polystyrene bacteriological (PS) and tissue culture (TC-PS) dishes (Falcon); poly(ethylene terephthalate) (PET) (Dupont Mylar film); freshly cleaved mica (Mica & Micanite Supplies); and glass microscope slides (Chance) and germanium (microcrystalline) plates polished to a mirror finish.

Specially prepared materials included the following: (i) Samples of PS and PET were treated for 10 min at maximum power at 250×10^{-3} torr of residual air pressure in a radiofrequency (RF) plasma cleaning device (Harrick, Ossining, N.Y.), resulting in an increase in water wettability; these are designated as RF-PS and RF-PET, respectively. Scanning electron microscopy showed that there was no change in surface roughness of treated PS and PET. (ii) Samples of PE and nylon 6.6 (Dupont) which were recrystallized against gold by the method of Schonhorn (30), so that the water wettability was increased without an increase in charge density (designated as Au-PE and Au-nylon, respectively). Nylon simultaneously compression molded against glass provided a smooth surface of lower wettability than Au-nylon and served as a control. After recrystallization, mercury was used to remove the gold, and only samples with no detectable residual gold or mercury were used. The method for metal detection was X-ray fluorescence analysis, kindly done by J. V. Gilfrich. (iii) A series of PS substrata of progressively higher water wettabilities, obtained by treatment in a RF plasma cleaning device for periods of ~0.25, ~0.5, ~1, 10, 60, and 600 s, was used. (iv) A high-purity epoxy resin, characterized previously (1), was prepared. (v) Platinum plates, me-

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tallographically polished to a mirror finish, were also used.

The substrata were cleaned as follows. (i) Plastics were washed in detergent and rinsed exhaustively with distilled water. (ii) Glass and mica were cleaned in hot nitric acid after detergent washing and rinsed with distilled water. (iii) Germanium was either heated to 600°C for several hours in a flowing hydrogen atmosphere (reducing conditions) and kept wrapped in aluminum foil, which had previously been cleaned in the RF plasma cleaner, or washed with detergent, rinsed with water and then acetone, oven dried after blotting with filter paper, and treated for several minutes in the RF plasma cleaner (oxidizing conditions). (iv) Platinum, after washing, was heated until red hot in a Bunsen flame and allowed to cool a few seconds before use.

Attachment assays. The substrata were exposed to the bacterial suspensions at ~18°C for 2 h without stirring to allow attachment of bacteria. The substrata were then gently rinsed three times with ~10 ml of sterile water to remove loosely attached and residual suspended organisms. In initial experiments distilled water was usually used for rinsing, although the number of bacteria which remained attached did not seem to depend on whether distilled or seawater was used. In later experiments seawater was used for rinsing. Attached bacteria were then fixed with Bouin fixative (71% [vol/vol] saturated aqueous picric acid–24% [vol/vol] Formalin–5% [vol/vol] acetic acid) and stained with either ammonium oxalate crystal violet or acridine orange (5-mg/liter solution in distilled water). Supplementary observations indicated that rinsing, fixing, and staining procedures did not influence results. Attached bacteria were counted by bright-field or fluorescence microscopy.

Attachment of bacteria to medium-coated substrata. A medium extract was prepared by filtering (0.2-μm porosity) the supernatant obtained by centrifuging the cultures (see above). The filtrate was allowed to adsorb onto PS and RF-PS (four each of 5-cm dishes) and platinum (four 1-cm^2 plates) by exposing the surfaces to the filtrate for 1 h, and they were then rinsed with sterile seawater. During this time, the bacteria were maintained in 100 ml of sterile seawater at 10°C. Portions (10 ml) of bacterial suspension (1.4×10^9 bacteria per ml) were immediately added to the treated dishes and to a dish containing the platinum plates, as well as to clean PS and RF-PS control dishes. Substrata were removed at intervals of 15, 60, 120, and 180 min and rinsed with sterile seawater, fixed, and stained as above. Control experiments with clean platinum were done separately because of lack of platinum substrata.

When possible, replicate test substrata were used, and all experiments were repeated at least once.

Contact angles. The contact angles of quartz-distilled water on the substrata were measured with a NRL-type contact angle goniometer (Ramé-Hart, New Jersey) to observe profiles of sessile drops at 20°C and 60% relative humidity. Droplets were transferred to the substrata with a freshly flamed platinum wire when observing advancing contact angles (θ_A). Receding contact angles (θ_R) were determined by removing water from the drops with clean filter paper.

RESULTS

Bacterial attachment to different substrata. The numbers of bacteria attached to different test substrata are given in Table 1, together with the measured water contact angles (θ) and critical surface tensions (γ_c) (another measure of wettability [35]), where known. The experiments were carried out over a period of months, introducing unavoidable and undefinable variations between successive batch cultures. To allow comparisons of a large number of experiments, PS substrata were included in each experiment, and the ratio of the mean number of bacteria attached to the test substratum to those attached to PS is given as the index of attachment (I_a). PS was chosen as a reference because it gives reliable and reproducible results in the short term.

The materials used as substrata can be grouped as follows according to their surface characteristics and suitability for attachment.

(i) Hydrophobic materials include nonpolar polymers with no ionogenic functional groups and whose surface charge, if any, arises from low levels of impurities or through adsorption (or desorption) of ions from a surrounding medium (17), i.e., PTFE (Teflon), PE, PS. These bore large numbers of attached bacteria; coverage of each surface with a monolayer of bacteria was usually obtained within ~2 h (I_a = 0.8 to 1.0) as was previously found (10).

(ii) Polymers of increased wettability may contain significant quantities of polar groups,

TABLE 1. *Number of bacteria attached to different surfaces after 2 h of exposure*

Surface	Advancing water contact angle (degrees)	Critical surface tension (10^{-4} Ncm^{-1})	$\bar{x}$ Bacteria per 100 μm^2 ± confidence limits (P = 0.05)	I_a[a]
PTFE	105	1.9	31.2 ± 0.3	0.8
PE	95	3.1	33.8 ± 0.4	0.8
PS	86	3.3	40.5 ± 0.4	1.0
PET	79	4.3	38.6 ± 0.4	0.95
Epoxy	57		15.5 ± 0.3	0.5
Nylon 6.6	66		24.0 ± 0.4	0.9
Au-nylon	56		14.5 ± 0.6	0.5
Au-PE	76		23.1 ± 0.2	0.7
TC-PS			4.7 ± 0.6	0.1
RF-PS	32		2.8 ± 0.5	0.1
RF-PET	40		2.4 ± 0.6	0.1
Mica	0	>7.4	2.6 ± 0.4	0.1
Glass	0	>7.4	0.8 ± 0.2	0.02
Ge (oxidized)	0	>7.4	1.8 ± 0.1	0.07
Pt	0	>7.4	13.4 ± 0.6	0.3
			15.5 ± 0.4	0.6
Ge (reduced)	0	>7.4	28.2 ± 0.7	0.7
			23.3 ± 0.6	0.6

[a] Ratio of the number of attached bacteria on the test substratum to those attached to a polystyrene control.

e.g., nylon 6.6, epoxy resin, and PET. Polar groups introduced by RF discharge onto PET and PS place them in this category also, and some of these polar groups may bear negative charge, e.g., carboxyl and phenolic groups (3, 29). PS and PET may be treated to the extent that their receding water contact angles approach zero by commercial processing, e.g., Falcon tissue culture ware, or by laboratory treatment with the RF discharge (RF-PS and RF-PET).

Recrystallization of polymers against gold can increase their surface density by transcrystallization, leading to a higher surface free energy and wettability without introducing ionogenic or additional polar groups (8, 30).

These polymers show a range of advancing water contact angles, with a strong correlation between increasing contact angle and bacterial attachment; I_a ranges from 0.1 to 10.95. Figure 1 illustrates the relation obtained between water contact angles on PS samples treated in the RF discharge for various times and bacterial attachment. The combined results of three separate experiments yield a strong positive correlation for both advancing (θ_A; $r = 0.91$) and receding (θ_R; $r = 0.82$) contact angles, with both significant at $P = 0.001$.

(iii) Inorganic, hydrophilic materials. The inorganic materials used are all hydrophilic with zero water contact angles.

The numbers of bacteria attached to glass, mica, and germanium discharge treated in air were very low ($I_a = 0.01$ to 0.02). Attachment to freshly flamed platinum was somewhat variable, with I_a from 0.3 to 0.6 in repeated experiments. Thus, bacteria consistently attached in smaller numbers to platinum than on the hydrophobic surfaces, but more attached than to the other hydrophilic surfaces glass, mica, or air-treated germanium. Germanium cleaned by heating in hydrogen, however, consistently had numbers of attached bacteria similar to, or slightly higher than, those obtained with platinum ($I_a = 0.6$ to 0.7).

Effect of adsorbed medium filtrate on attachment. Soluble components of the medium filtrate adsorbed onto platinum considerably reduced subsequent bacterial attachment, whereas contact with medium filtrate did not affect attachment to PS. Adsorption of medium components to PS was indicated by the PS becoming hydrophilic. However, rinsing with seawater can render such PS samples hydrophobic again, indicating easy desorption of hydrophilic medium components from the PS surface.

Attachment to these materials as a function of time is shown in Fig. 2.

DISCUSSION

There are several types of interactions which could affect attachment processes of cells in aqueous media. The balance of dispersion and electrostatic forces postulated in the theory of lyophobic colloid stability leads to a weak secondary minimum of repulsion forces between a cell and a potential substratum, allowing association at a slight distance from the surface (32). This was suggested as the basis for an initial reversible stage of bacterial attachment by Marshall et al. (26). These authors suggested that extracellular polymers then bridged the gap between bacterium and substratum, resulting in more permanent attachment. Studies of attachment of tissue cells of several types (2, 16, 22) have indicated that many show a preference for

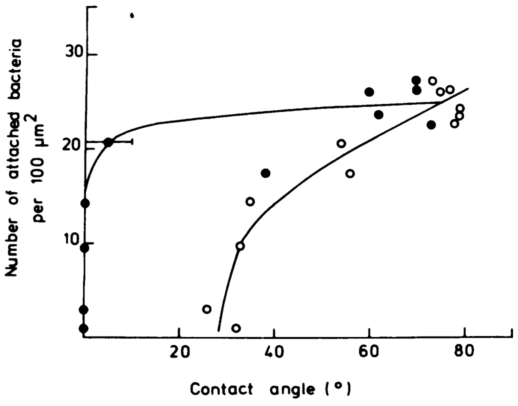

FIG. 1. *Relationship between numbers of attached bacteria and the advancing contact angles* (○) *and receding contact angles* (●) *of water on PS substrata. These different water wettabilities were obtained by treatment in the RF plasma cleaner.*

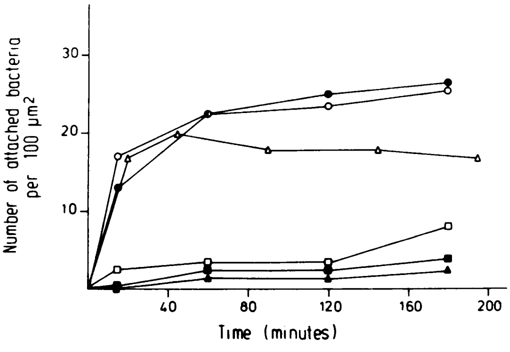

FIG. 2. *Influence of adsorbed medium filtrate on bacterial attachment to different substrata. Symbols:* ○, *PS;* ●, *filtrate-coated PS;* □, *RF-PS;* ■, *filtrate-coated RF-PS;* △, *platinum;* ▲, *filtrate-coated platinum.*

negatively charged, hydrophilic surfaces, in contrast to what was found here and contrary to what is expected if electrostatic and dispersion forces are the dominant factors in cell adhesion. Additional interactions possible in biological systems which might affect attachment include hydrophobic bonding (25), coordination with metals or other cations, polar group interactions, steric interferences (21, 23), and specific reactions between surface functional groups (5).

It is difficult to predict adherence of populations of bacteria in natural waters because simple generalizations based on first principles are prevented by (i) the presence of variable and undefined dissolved components which may alter substratum and bacterial attachment through adsorption; (ii) the presence of different bacteria which may possess different surface characteristics; and (iii) the different types of interactions possible. Thus, a number of studies have shown a greater tendency for marine bacteria (28, 31) and other aquatic organisms (7) to attach to hydrophobic surfaces, whereas one in situ investigation (6) has demonstrated higher numbers of organisms on hydrophilic substrata. The understanding of preferences of attaching organisms in particular circumstances, then, requires detailed information on the substrata, dissolved components and bacterial populations involved. Our laboratory studies describe the response of a particular bacterium, and further work will be required to establish the degree of generality of the pattern of attachment observed with this organism.

The relationship between bacterial attachment and water wettability in the series of polymeric materials used here (Fig. 3) gives a clear indication of the importance of this property. However, the variation in attachment to the different hydrophilic materials requires consideration. Glass and mica, which had low numbers of attached bacteria, have been observed by electrokinetic measurement to bear a negative surface charge in seawater. This charge is consistent with their chemical structure, involving oxyanions at the surfaces (27, 34). Platinum, which had higher numbers of attached bacteria, has been found to be electrokinetically positive in organic-depleted seawater (20). Germanium was cleaned in two ways (under oxidizing conditions by RF glow discharge in residual air and under reducing conditions by heating in hydrogen), resulting in surfaces which differed in their suitability for attachment. The reducing conditions can be expected to lead to less germanic acid (anionic) groups on the germanium surface (33) which is consistent with the essentially zero electrophoretic mobility of hydrogen-cleaned germanium (19). Because the glass, mica, and oxidized germanium had very low numbers of attached bacteria, whereas the positive platinum and neutral germanium had higher attached numbers, surface charge is suggested to be a significant factor.

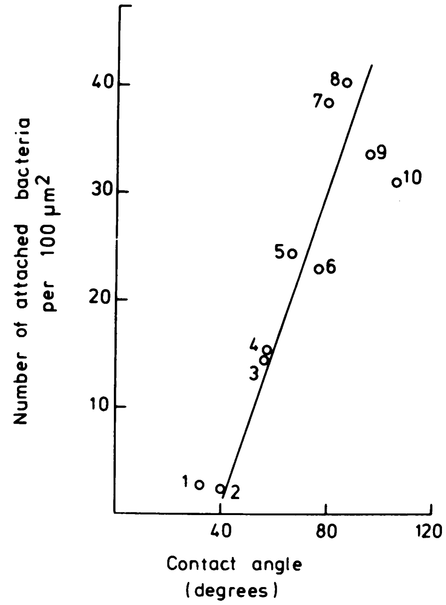

FIG. 3. *Relationship between numbers of attached bacteria and advancing water contact angles of water on different surfaces. The substrata are: 1 (RF-PS); 2 (RF-PET); 3 (Au-nylon); 4 (epoxy); 5 (nylon 6.6); 6 (Au-PE); 7 (PET); 8 (PS); 9 (PE); 10 (PTFE).*

Most bacteria appear to bear a negative surface charge (15), and the pseudomonad used in these studies has been shown to have an acidic hydrophilic surface polymer (which may be the attachment adhesive [11]). Thus, once a monolayer of attached bacteria has formed on a hydrophobic surface, this favorable attachment surface is converted to a hydrophilic, negatively charged (thus unfavorable) surface. This may account for the decrease in rate of bacterial attachment to a surface as it becomes covered with bacteria, as indicated by the leveling out of the curves in Fig. 2 (10).

The decreased attachment on platinum which had been exposed to cell-free medium is similar, because the peptone medium components which adsorb are expected to bear negative net charge at seawater pH. The lack of effect of medium on attachment to PS may be explained by its easy displacement from that surface.

Electrokinetic studies with hydrocarbons in aqueous media often indicate negative charges on the hydrocarbon kinetic units (32), and this is apparently due to a reduction in cation concentration in the liquid boundary region near the hydrocarbon, rather than from charges on the hydrocarbon (17). Thus, approach of charged bacteria to a nonionogenic hydrophobic surface may be expected to perturb easily the ionic distribution which leads to the apparent surface charge. This contrasts with the repulsion expected from the stable charges present at ionogenic surfaces.

Some of the polymer samples used in this study were oxidized by electrical glow discharge to increase their wettability. The hydrophilic properties of RF-PS and RF-PET may be partially associated with a surface charge component (in addition to the production of polar groups) because there is evidence of the formation of acid and phenolic groups on oxidized plastics (3, 29). However, the strong positive correlation between the number of attached bacteria and the hydrophobic nature of the polymer, as determined by water contact angle, is evident with both treated and untreated polymers. Thus, the present data illustrate the importance of charge on hydrophilic substrata and the importance of wettability on hydrophobic substrata.

The correlation of attachment with water contact angle strongly suggests the involvement of a hydrophobic interaction, but because the bacterial surface polymer is apparently an ionic hydrophilic material, the nature of the interaction is not obvious. Because, as noted previously, a number of other marine organisms attach preferentially to plastic substrata, the basic processes leading to this tendency may have wide applicability. This possibility and the nature of the interaction are being studied further.

ACKNOWLEDGMENTS

We thank J. V. Gilfrich and W. Bascom, Naval Research Laboratory, for the X-ray fluorescence analysis and for the epoxy resin sample, respectively. We are also grateful to N. G. Maroudas for the PTFE Teflon.

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