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Adhesion and Motility of Gliding Bacteria on Substrata with Different Surface Free Energies

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The adhesion and motility of several aquatic and terrestrial gliding bacteria on slides differing in their critical surface energies have been examined. In general, adhesion was tenacious on low-critical surface energy (hydrophobic) surfaces and tenuous on hydrophilic surfaces. Gliding was inhibited on very hydrophobic substrata and skittish on very hydrophilic surfaces.

Motility of the gliding bacteria requires association with an interface, typically a solid or semisolid substratum. Neither the mechanism(s) of adhesion nor those of motility of this taxonomically heterogeneous assemblage of bacteria are understood (reviewed in references 9, 13, 25, 31, and 39). Several reports indicate that motility is affected by substratum characteristics. Reichenbach (37) reported that *Archangium violaceum* glides "shakily" in wet mounts and smoothly on agar. Certain motility mutants of *Cytophaga johnsonae* are unable to glide on glass but able to glide on agar (14). Additionally, some gliding bacteria require the presence of polyanions for motility on gel substrata (3).

Gliding bacteria in wet mounts prepared on standard microscope slides make contact with and adhere to both slide and cover slip surfaces; they begin to glide shortly thereafter. In some species, translocation is interrupted by flipping, spinning, or pendulumlike movements (10, 21, 28, 32, 38, 41). However, in our recent studies of *Flexibacter maritimus*, the motility of cells from comparable cultures was erratic when examined microscopically, leading to the hypothesis that differences in the surface characteristics of slides might influence the adhesion and motility of the bacteria. This report describes the adhesion and motility of *F. maritimus* and several other gliders on slides derivatized with a variety of silanes so as to modify their critical surface energies (CSEs) while maintaining identical substratum texture.

MATERIALS AND METHODS

Bacterial strains. The bacteria examined in this study and their origins and descriptions are presented in Table 1.

Culture conditions. *Myxococcus xanthus* was initially cultured without agitation in CT liquid medium (18) at 30°C, after which it was incubated overnight at 25°C on a rotary shaker (100 rpm). The other strains were incubated in shallow layers of culture media (Table 1) without agitation at 25°C.

Slide silanization. Borosilicate glass slides (Fisherfinest premium microscope slides, Fisher Inc.) were treated with silanizing reagents to covalently modify the surfaces. Before treatments were applied, all slides were baked (i.e., muffled) at 500°C for a minimum of 4 h. Muffled glass slides were used as one of the seven surface treatments. The other six surface treatments were aminopropyltrimethoxysilane (APS), 3-chloropropyltrimethoxysilane (ClPrS), diphenyldichlorosilane (DPS), *n*-octadecyldimethyl[3-(trimethoxysilyl)-propyl] ammonium chloride (ODAPQ), *N*-trimethoxysilylpropyl-*N*,*N*,*N*-trimethylammonium chloride (QAP), and (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (TDF). Silanization reagents were purchased from Petrarch Chemical Co.

Either 1 or 2% solutions of the silanization reagents were made in one of four solvents (Table 2). Solvent selection was dependent upon the specific reagent. All solvents were high-performance liquid chromatography grade. Once a silane reagent solution was made, it was used immediately. The slides were completely covered and incubated according to the procedure for the specific reagent. After the rinse step, the slides were dried at 60°C for 30 min. Before use, the slides were rinsed three times with high-performance liquid chromatography-grade methylene chloride, cured in a 105°C oven for 30 min, and stored overnight in a desiccator under vacuum. In some experiments, the muffled glass slides were not rinsed with methylene chloride.

APS-COOH was generated by treating APS-derivatized glass with excess succinic anhydride in acetone containing 0.25% triethylamine (46a).

Wettability measurements. The wettability coefficient (WC) of the slides was determined by measuring the spread of 25-μl drops of a series of solutions of water and methanol according to the method of D. Rittschof and J. D. Costlow (*in* J. Ros, ed., *Proceedings of the 22nd European Marine Biology Symposium*, in press). Solutions used were 100, 80, 60, 40, 30, 20, 10, and 0% distilled water in high-performance liquid chromatography-grade methanol. Drops were applied to the surface, and their longest horizontal dimensions were measured. When a drop spread ≥20 mm, all higher methanol concentrations were assigned a value of 20. Drop spread measurements were reduced to a single number and scaled from 0 to 100 according to the following equation:

$$WC = [(\frac{8}{1/W100 + 1/W80 + 1/W60 + 1/W40 + 1/W30 + 1/W20 + 1/W10 + 1/W0} - 4)/16] \times 100$$

where W100 is the millimeters over which a drop of 100% water spreads, W80 is the millimeters over which a drop of 80% water in methanol spreads, W60 is the millimeters over which a drop of 60% water in methanol spreads, etc., 8 is the number of solvent concentrations used, 4 is the minimum possible drop measurement in millimeters, and 16 is the measurable range in millimeters. The scaling results in values for surfaces with known wettabilities that are roughly

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TABLE 1. Bacteria examined in this study

Strain	Growth medium (reference)	Source (original description)
Cytophaga sp. strain U67	C62 (23)	J. Henrichsen (23)
Cytophaga sp. strain Adh3	C62	This laboratory (11)
Cytophaga sp.-like RB1058	CAS[a]	This laboratory
Flexibacter columnaris EK28	Cytophaga (33)	J. F. Bernardet (7)
Flexibacter maritimus ly1-1	CAS	D. Baxa (6)
Flexibacter sp. strain FS-1	YE/2 (16)	D. White (45)
Microscilla sp. strain RB-1	CAS	This laboratory
Myxococcus xanthus DK1622	CT (18)	D. Kaiser (26)

[a] Composed of 0.2% Casamino Acids (Difco Laboratories) in artificial seawater made up like formula 1 of Smibert and Krieg (46) with the exception that we employed 0.1 mg of $FeSO_4$ per liter. The final pH was 7.5.

comparable to the critical surface tensions obtained with a contact angle goniometer (4; Rittschof and Costlow, in press).

Observations. Droplets (10 μl) of actively motile cells in culture medium were applied to the derivatized slide surface and covered with a 12- by 12-mm cover slip (Assistent) that had been washed with 0.1 N HCl, rinsed with distilled water, and dried at 150°C. This provided a 70 μM column of cell suspension. Cells in the plane of focus of the slide were observed with a Wild phase-contrast microscope at a magnification of ×400. Microphotographs were taken with a Zeiss Universal phase-contrast microscope equipped with an Olympus OM-2 camera with Tmax 125 film.

Cells that no longer demonstrated Brownian motion were considered to be firmly adherent to the substratum. Weakly adherent cells demonstrated some Brownian motion but were not dislodged by streaming of the suspending medium. Gliding motility was assessed by observation of adherent bacteria moving in a direction parallel to the cell long axis. Flexing, flipping, and pivoting movements were also noted.

Adhesion quantitation. Slides were submerged for 1 min in a suspension of exponential-phase bacteria (A_{540} of 0.1) in growth medium. Loosely adherent bacteria were removed by two successive 5-s submersions in sterile culture medium. For those strains growing in marine salts-containing medium, the second rinse was in C62 medium (23). The slides were then carefully blotted dry with bibulous paper. The bacteria in five randomly selected 0.094-mm^2 fields were counted either on the dry slide or after rehydrating the fixed bacteria by preparing a wet mount with sterile culture medium.

TABLE 2. Procedures[a] for derivitization of microscope slides with silanes

Derivatized surface	% Silane	Coupling solvent[b]	Reaction interval (min)	Rinse step solvent(s)[b]
APS	2	95% ETOH	15–30	ETOH (twice), MEOH (once)
APS-COOH	—[a]	—[a]	15–30	Acetone (twice), H_2O (once)
ClPrS	1	95% ETOH + 0.2% HOAC	30	ETOH (twice), MEOH (once)
DPS	1	MECL	60	MECL (twice)
ODAPQ	2	H_2O	30	H_2O (twice)
QAP	2	95% ETOH	15–30	ETOH (twice)
TDF	1	MECL	60	MECL (twice)

[a] See Materials and Methods for procedural details.
[b] ETOH, Ethanol; HOAC, acetic acid; MEOH, methanol; MECL, methylene chloride.

TABLE 3. Adhesion and motility of bacteria on derivatized slides

Derivatized surface	WC[a]	Results with: *Cytophaga* sp. strain U67		*F. maritimus* ly1-1	
		Adh/Mot[b]	No. of adherent bacteria/field[c]	Adh	No. of adherent bacteria/field
Expt 1					
TDF	8	+/−	28 ± 3	+	107 ± 85
ODAPQ	14	+/−	68 ± 47	+	87 ± 54
APS	26	+/+	73 ± 38	+	66 ± 31
DPS	38	+/±	52 ± 18	+	31 ± 5
ClPrS	38	+/+	48 ± 18	+	42 ± 10
Muffled glass	92	±/±	24 ± 19	±	22 ± 5
Expt 2					
TDF	10	+/−	22 ± 5	+	220 ± 118
ODAPQ	15	+/+	85 ± 32	+	116 ± 49
APS	15	+/+	11 ± 4	+	154 ± 107
ClPrS	21	+/±	37 ± 5	+	77 ± 43
DPS	26	+/+	25 ± 14	+	54 ± 23
QAP	34	+/+	13 ± 6	+	20 ± 7
Muffled glass	94	+/±	28 ± 12	+	5 ± 3

[a] Scaled from 0 to 100.
[b] Adhesion/motility as determined by observations of viable bacteria. + and −, Positive and negative for adhesion or motility; ±, tenuous adhesion or limited motility.
[c] Mean ± standard deviation as described in Adhesion quantitation (see Materials and Methods).

RESULTS

Cytophaga sp. strain U67, a model bacterium in our studies, demonstrated distinct adhesion and motility behaviors across the range of derivatized slides of different CSEs tested (Tables 3 and 4). On TDF-derivatized glass, which had the lowest CSE of the series, cells initially made contact with the slide by one pole. This was immediately followed by a pivot, at the end of which the entire cell length was in contact with the slide. In the absence of detectable Brownian motion and gliding movement, we concluded that adhesion to the substratum was firm. On slides derivatized with ODAPQ and DPS, silanes conferring higher CSEs on the slides, initial contact and subsequent adhesion appeared similar to that on TDF. However, in some experiments,

TABLE 4. Adhesion and motility of bacteria on derivatized slides[a]

Derivatized surface	WC	Adhesion/motility of: *Cytophaga* sp. strain:		*Flexibacter* sp. strain:		*Cytophaga* sp.-like strain RB1058
		U67	Adh3[b]	EK28	FS-1	
TDF	8	+/−	±	+/−	+/+	+/−
ODAPQ	18	+/−	±	+/±	+/±	+/−
DPS	37	+/−	+	+/+	+/+	+/+
ClPrS	38	+/±	−	+/+	+/+	+/+
APS	44	+/+	−	+/±	+/±	+/+
APS-COOH	51	+/+	−	+/+	+/±	+/+
QAP	57	+/+	−	+/+	+/+	+/+
Muffled glass	64	±/++	−	+/++	+/++	±/+

[a] For scales, see footnotes *a* and *b* of Table 3. ++, Very strong motility.
[b] Results for strain Adh3 are for adhesion only.

limited gliding motility was observed. On slides of intermediate CSE (APS, ClPrS, QAP), motility was observed in almost all cases. It was difficult to assess the tenacity of adhesion since the bacteria, many of which had a fixed longitudinal curvature, rotated around their long axes during gliding (21). This gave an erratic or "bumpy" appearance to their translocation. On the very high CSE muffled glass, adhesion appeared to be tenuous; Brownian motion of the bacteria occurred in place. The bacteria did, however, resist being dislodged by streaming. For some muffled-glass wet mounts, it was difficult to assay motility because of weak adhesion and Brownian motion (Table 3). In others, gliding was very active; the bacteria appeared to skitter on the substratum (Table 4). Like the other bacteria in this study, *Cytophaga* sp. strain U67 adhered to and glided on the untreated cover slips (the WC was 18.7 in one set of measurements).

We attempted to quantitate adhesion by counting the *Cytophaga* sp. strain U67 cells remaining adherent to the silane-derivatized slides after submersion in bacterial suspension. The numbers of adherent bacteria had no correlation to the results from direct observations of motile cells (Table 3).

The behavior of *Cytophaga* sp. strain Adh3, an adhesion-defective mutant of *Cytophaga* sp. strain U67 (11), was distinct from that of its parent (Table 4). It adhered weakly or not at all to substrata of both low and high CSEs. Adhesion was observed consistently only on slides derivatized with DPS (WC range of 23 to 38). In one experiment, several observers detected gliding of Adh3 on DPS.

Adhesion of another freshwater member of the family *Cytophagaceae*, *Flexibacter columnaris* EK28, appeared to be comparable over the entire range of derivatized slides, distinguishing its behavior from that of *Cytophaga* sp. strain U67 (Table 4). However, the two strains were similar in that there was little or no motility on low-CSE substrata. *F. columnaris* cells were occasionally observed to pivot or spin continuously on one pole attached to the derivatized slides, including those on which they were unable to translocate.

A terrestrial isolate, *Flexibacter* sp. strain FS-1, demonstrated a pattern of adhesion similar to that of *Cytophaga* sp. strain U67. However, this strain was distinguished by its ability to glide and flex on all substrata examined (Table 4).

We have also observed the adhesion and motility behaviors of three marine strains of the family *Cytophagaceae*. *F. maritimus*, the cultures of which become detectably viscous because of an accumulation of extracellular polymers during static incubation (R. P. Burchard, unpublished results), demonstrated firm adhesion on all substrata examined except muffled glass (highest CSE), to which the cells appeared to attach tenuously. Gliding on any one derivatized surface was inconsistent. However, motility was observed on all of the substrata tested. Quantitative adhesion results for this strain are presented in Table 3. In contrast to the results from *Cytophaga* sp. strain U67, there was an ordered progression in the data; the density of adherent bacteria decreased with increasing CSE.

Adhesion and motility of *Cytophaga* sp.-like strain RB1058, another marine glider, were comparable to those of *Cytophaga* sp. strain U67 in that the bacteria adhered firmly to all substrata but muffled glass, to which adhesion was tenuous. Gliding did not occur on TDF (lowest CSE) and was sometimes inhibited on ODAPQ and DPS (Table 4). A third marine isolate, tentatively designated as *Microscilla* sp. strain RB1, demonstrated adhesion throughout the range of substrata tested. The *Microscilla* filaments appeared to adhere to low-CSE surfaces very tenaciously; initially straight, they were able to glide very slowly into slightly flexed postures. Gliding was more active and continuous on higher-CSE surfaces (Fig. 1B and C). On muffled glass, the filaments sometimes appeared to skitter quickly over the substratum.

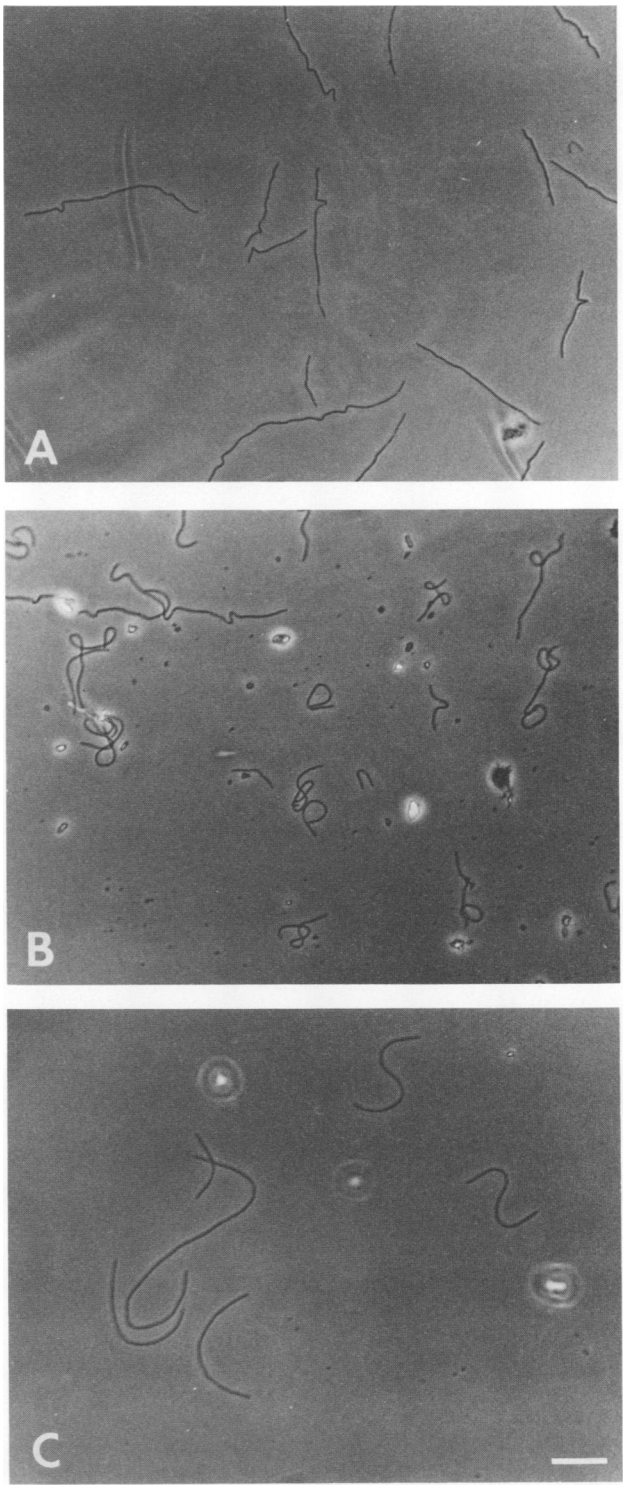

FIG. 1. *Microscilla* sp. strain RB1 on TDF (A), DPS (B), and muffled glass (C). Bar, 20 μm.

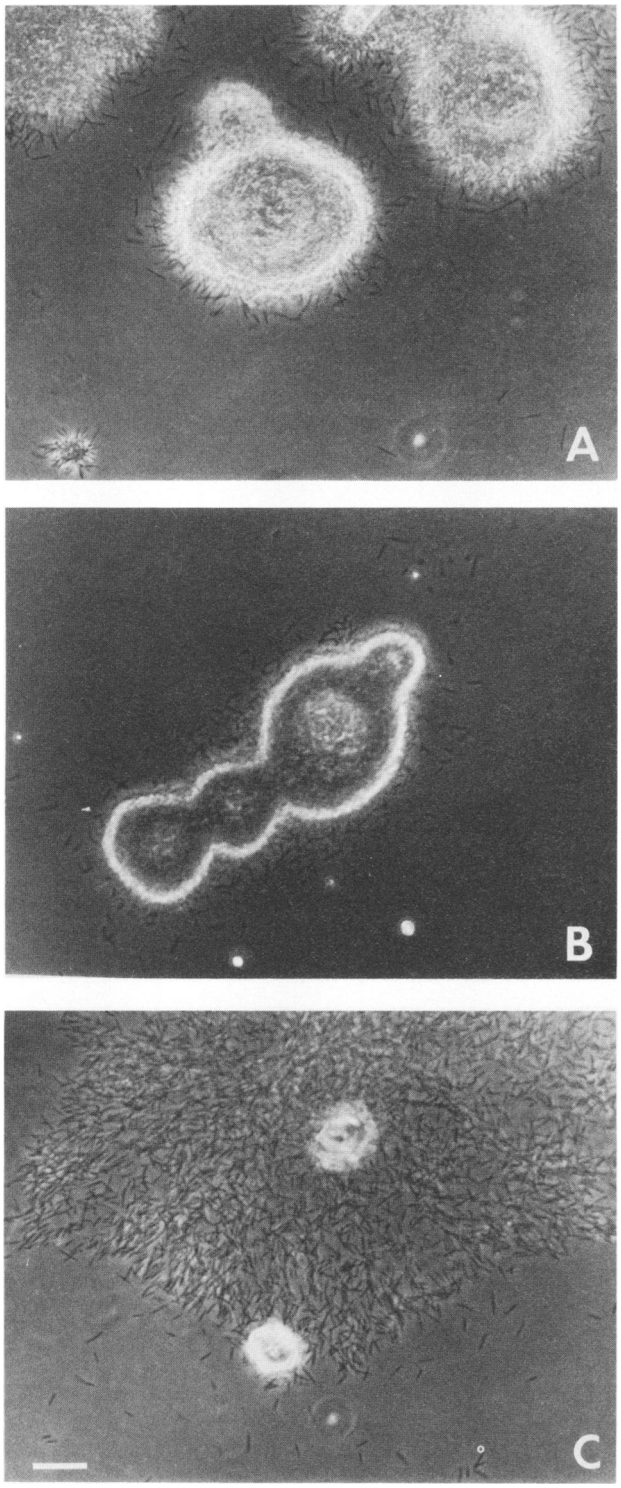

FIG. 2. *M. xanthus* DK1622 rosettes on muffled glass at $t = 0$ (A), muffled glass at $t = 50$ min (B), and ODAPQ at $t = 50$ min (C). Bar, 20 μm.

M. xanthus, a gliding bacterium that is unrelated to the family *Cytophagaceae* (39), demonstrated tenuous adhesion over the entire range of CSE substrata tested. These bacteria glided with velocities 1 to 2 orders of magnitude slower than those of members of the family *Cytophagaceae*, making it difficult to determine whether single cells were actively translocating on the derivatized slides. As an alternative assessment of motility, the displacement of bacteria from rosettes that had settled on the slides was determined (Fig. 2A). Rosettes are tight clusters of radially arrayed cells that form in rotary shake cultures of some *M. xanthus* strains (8, 40). After incubation periods of approximately 25 min, bacteria from the settled rosettes appeared to have moved out from the rosette on all substrata but muffled glass (Fig. 2B). The displacement of bacteria was likely to have been due to straight-line gliding, since most of them were still arrayed radially, as they had been in the intact rosettes.

DISCUSSION

Our observations demonstrate that the adhesion and motility of diverse gliding bacteria are affected by the surface energies of their substrata. These bacteria adhere more tenaciously to surfaces of low CSE than to hydrophilic ones, suggesting that one or more of the bacterial surface components that make contact with substrata are relatively hydrophobic. Some of these components appear to be cell envelope proteins (11; unpublished results). They may be localized in specific surface domains, since initial contact with hydrophobic substrata by *Cytophaga* sp. strain U67 and some of the other strains was typically made via the bacterial pole. This accords with an early observation of polar adhesion by a *Flexibacter* sp., suggesting that its poles are more hydrophobic than are the lateral surfaces (29).

Adhesion may be mediated, at least in part, by a water exclusion mechanism (49). On very hydrophobic substrata, water may be excluded so well that the bacteria stick and are prevented from gliding. In contrast, on very hydrophilic substrata (high CSE), water may be poorly excluded, preventing close bacterial contact and accounting for limited or skittering motility. As has been suggested for other bacteria (20, 34, 44), electrostatic interactions may account for the limited adhesion of gliding bacteria to hydrophilic substrata. The silanes that we employed to achieve intermediate-range CSEs were both charged and uncharged. We were unable to distinguish reproducible differences in adhesion and motility on slides derivatized with these silanes. Since adhesion was most tenacious on low-CSE surfaces, our results suggest a major role of van der Waals forces rather than charge-charge interactions. Once a gliding bacterium has attached to a substratum, whether tenaciously or weakly, it is not readily dislodged in a low-shear environment. It is apparent that the adhesion of gliding bacteria is biophysically complex, as is the case for other bacteria (e.g., references 1, 5, 27, and 43).

Cytophaga sp. strain U67, with its relatively hydrophobic cell surface (M. Sorongon and R. P. Burchard, unpublished data), typifies the behavior of other gliders in its progressively more tenacious adhesion to more-hydrophobic substrata. Its adhesion-defective mutant Adh3, selected by the inability to adhere to hexadecane in a bacterial adherence to hydrocarbon enrichment protocol (11, 42), had a relatively hydrophilic surface compared with that of its parent strain. Charge repulsion may account for the inability of the strain to adhere to more-hydrophilic (high-CSE) substrata. Poor exclusion of water may explain the loss of adhesion to low-CSE surfaces. Only on DPS-derivatized glass, intermediate in CSE, did *Cytophaga* sp. strain Adh3 adhere reproducibly. In one set of experiments, Adh3 was observed to glide on DPS, indicating that the motility apparatus is functional in this mutant. Since DPS-derivatized slides varied in CSE, the lack of reproducible motility suggests that

the mutant cells will glide only on substrata with very specific surface properties.

Other gliding bacteria examined in this study, including those that produce substantial amounts of extracellular polymers ("slime"), also have cell surface properties that permit them to adhere more firmly to substrata of low CSE than to those that are hydrophilic. The extracellular polymers do not appear to interfere with the cell envelope making direct contact with the substratum (11; unpublished data).

In a recent quantitative adhesion study of another *Flexibacter* sp., similar numbers of bacteria attached to both hydrophilic and hydrophobic polystyrene substrata (30). In contrast, the number of adherent *F. maritimus* cells increased with increasing hydrophobicity of the substratum. These data correlated with the adhesion and motility behavior of the bacteria in wet mounts. A variety of species of nongliding bacteria have also been reported to adhere in greater numbers to hydrophobic surfaces than to hydrophilic surfaces (e.g., references 2, 20, 44, and 47). However, this held true only when the surface tension of the test bacterium was less than that of the suspending medium (2) or only for relatively low concentrations of bacteria in the suspensions in which the substrata were submerged (47). Native populations of aquatic bacteria have been reported to demonstrate an adhesion minimum on substrata in the middle range of CSE (critical surface tension of $\sim 2.0 \times 10^{-4}$ to 2.5×10^{-4} N/cm). For marine bacteria, adhesion maxima were found for both higher and lower CSEs (17). Freshwater bacterial populations demonstrated an adhesion maximum and a second minimum on successively more hydrophilic substrata (36).

Quantitation of adhesion of *Cytophaga* sp. strain U67, a freshwater strain, demonstrated no correlation with observations of its apparent tenacity of adhesion and motility (Table 3). A similar lack of correlation between the numbers of adherent bacteria and hydrophobicity has been reported by others (27). These data exemplify the conclusion of Busscher et al. (12) and van Pelt et al. (47) that enumeration of bacteria adherent to a surface after a fixed period of submersion in cell suspension may not be an appropriate measure of binding strength. We are currently developing assays of the strength of adhesion of gliding bacteria.

The bacterial-adhesion literature illustrates that the presence of organic molecules, ions, and free radicals; ionic strength; and bacterial physiology all affect adhesion to surfaces (e.g., references 1, 19, 24, 30, 35, and 49). We minimized substratum effects due to the adsorption of organics and ions since most of our observations were made immediately after preparing the wet mount. However, preliminary observations suggest that physiology and culture medium affect the adhesion and motility of the gliding bacteria. For example, *Microscilla* sp. strain RB1 grown in HSM (0.2% tryptone, 0.05% yeast extract, 0.3% gelatin, 3% marine salts [Forty Fathoms Marine Enterprises, Towson, Md.]), a richer and much less defined medium prepared with an aquarium marine salts mixture, demonstrated more tenuous adhesion on both high- and low-CSE substrata than did comparable cells grown in CAS, a Casamino Acids (Difco Laboratories)-artificial seawater medium. Furthermore, *F. maritimus* grown in shake culture behaved differently on muffled glass than when grown without agitation. We are exploring some of these phenomena.

Tenacity of adhesion affects the expression of the machinery of gliding. The bacteria that we examined adhered firmly to but glided little if at all on low-CSE substrata. On highly hydrophilic (high-CSE) substrata, adhesion appeared to be tenuous and gliding, if it occurred, often appeared to be relatively fast; cells sometimes seemed to skitter across the muffled glass slides. The differences in cell contact and in motility were particularly striking with *Microscilla* sp. strain RB1 (Fig. 1). In contrast, the gliding diatom *Nitzschia linearis* adheres to glass (high CSE) more strongly than to low-CSE polystyrene (22). Another diatom demonstrated an adhesion minimum on glass derivatized with dichlorodimethylsilane (surface energy of 2.5×10^{-4} N/cm; 15). These studies suggest different mechanisms of adhesion and motility between the structurally distinct gliding bacteria and diatoms.

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