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Bacterial Adhesion under Static and Dynamic Conditions

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The deposition of various pseudomonads and coryneform bacteria with different hydrophobicities (water contact angles) and negative cell surface charges on negatively charged Teflon and glass surfaces was investigated. The levels of deposition varied between 5.0×10^4 and 1.6×10^7 cells cm^{-2} and between 5.0×10^4 and 3.6×10^7 cells cm^{-2} for dynamic column and static batch systems, respectively, indicating that there was a wide variation in physicochemical interactions. Batch and column results were compared in order to better distinguish between hydrodynamic and other system-dependent influences and method-independent physicochemical interactions. Despite the shorter suspension-solid contact time in columns (1 h) than in batch systems (4 h), the level of deposition (expressed as the number of cells that adhered) divided by the applied ambient cell concentration was 4.12 ± 1.63 times higher in columns than in batch sytems for 15 of 22 strain-surface combinations studied. This demonstrates that transport of microbial particles from bulk liquid to surfaces is more efficient in dynamic columns (transport dominated by convection and diffusion) than in static batch systems (transport by diffusion only). The relative constancy of this ratio for the 15 combinations shows that physicochemical interactions affect adhesion similarly in the two systems. The deviating deposition behavior of the other seven strain-surface combinations could be attributed to method-dependent effects resulting from specific cell characteristics (e.g., to the presence of capsular polymers, to an ability to aggregate, to large cell sizes, or to a tendency to desorb after passage through an air-liquid interface).

A better understanding and control of bacterial adhesion are needed for application of microorganisms in fixed-bed bioreactors (5) and in microbiological techniques used for biorestoring contaminated aquifers and soils (12), for improving the recovery of oil from the subsurface (16), for assessing the movement of pathogens or genetically engineered organisms in groundwater systems (38, 39), and for preventing bacterial colonization and biofilm formation on human teeth (27), prosthetic and other medical devices (28), and the inner walls of pipes in water supply and industrial systems (8).

The deposition of micron size particles, such as bacteria, on solid surfaces can be regarded as a two-step process: (i) the particles are transported close to the adhesive surface, and (ii) adhesion takes place under the control of physicochemical interactions and shear forces (41).

Transport of bacteria from bulk liquid to surfaces strongly depends on the hydrodynamics of the system studied. Some authors have investigated adhesion under defined particle flux and fluid shear conditions by using a rotating disk (19), a flat plate flow cell (35), or an impinging jet system (48). With the latter two methods, adhesion can be measured in situ, which avoids uncontrolled effects of transfer of substrata through the air-liquid interface (34).

In addition to these advanced techniques, there is a need for quick, inexpensive, and reliable procedures to measure bacterial adhesion. One problem often encountered when data from investigators who use such simple systems (1, 7, 22, 42, 43) are compared is that seemingly insignificant differences in methods lead to substantially different results. This is especially true when the hydrodynamics of deposition are not properly controlled or when the effects of transfer of substrata through the air-liquid interface are not taken into account. Also, with methods used in our laboratory (42, 43) erratic results have been obtained occasionally. To better distinguish between transport and other effects of methods on deposition and the actual physical chemistry of adhesion, we compared batch and column methods for a limited number of bacterium-substratum combinations. In this paper we focus on the transport effects and effects of methods; the physicochemical mechanisms involved in bacterial adhesion have been thoroughly analyzed in another study (29).

Greater hydrophobicity of cells and substrata results in greater attractive forces and higher levels of adhesion (29, 42), whereas smaller (more negative) electrokinetic potentials of cells and solids and lower levels of ionic strength (I) result in greater repulsive electrostatic interactions and lower levels of adhesion (29, 43, 44). We selected hydrophobic Teflon and hydrophilic glass, both of which are negatively charged, as the model surfaces. Various gram-negative pseudomonads and gram-positive coryneform bacteria with different cell surface hydrophobicities and negative electrophoretic mobilities (u) were used. Adhesion on submerged flat pieces of surfaces was studied in static batch systems. Dynamic column systems were used to study adhesion from suspensions percolated over water-saturated packed beds of substratum granules. Transport of cells from bulk liquid to surfaces is controlled by diffusion under static conditions (17, 18) and is governed by convection and diffusion in dynamic columns (10, 41). In this study, we also investigated adhesion and detachment during the transfer of substrata through the air-liquid interface or as a result of

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shear during washing procedures. Possible contamination of the solid surfaces by compounds excreted by the cells during the adhesion assays which may have affected attachment (23) was also studied.

MATERIALS AND METHODS

Aqueous media. The aqueous media used for all experiments were made with deionized (MilliQ-treated) water (Nanopure System D4700 apparatus; Barnstead/Thermolyne Co., Dubuque, Iowa). Phosphate-buffered saline (PBS) solutions having various I values were used (PBS having an I of 0.1 M contained 84.4 mmol of NaCl per liter, 2.1 mmol of KH_2PO_4 per liter, and 6.8 mmol of K_2HPO_4 per liter in deionized water and had a pH of 7.2). PBS degassed by decompression (−3.3 kPa for 10 min) was used to prevent air bubble formation on the Teflon supports.

Bacteria. The strains used are listed in Table 1. These strains were selected on the basis of their cell surface properties (2, 4), their environmental relevance (ability to degrade xenobiotic compounds) (9, 30, 46), and their potential applications in biotechnology (32, 47).

Bacterial cultivation and preparation. Rhodococcus strains C3 and C4 and all of the pseudomonad strains except P2 were cultivated in the mineral medium described by Schraa et al. (31), except that yeast extract was not added. Ethanol (50 mM) was used as the sole carbon and energy source for all strains except strain P3, which was grown with 5 mM 3-chlorobenzoate. The other strains were cultivated either in nutrient broth (Difco) (8 g/liter of deionized water) (strain P2) or in brain heart infusion broth (Merck) (40 g/liter of deionized water) (coryneform bacteria other than the rhodococci). The bacterial cells were harvested in the late exponential phase by centrifugation for 10 min at 20,000 × g at 4°C. Then the cells were washed three times by resuspension and centrifugation, using precooled PBS with the I required for each specific experiment. Finally, the cells were resuspended in an amount of PBS equal to 1% of the original culture volume and stored on ice until experiments were started (within 1 h).

Physicochemical and physical characterization of bacteria. The contact angles of drops of water (θ_w) placed on dried bacterial lawns were measured by using a microscope equipped with a goniometric eyepiece and the method of Van Loosdrecht et al. (42). The u values of bacterial cells in 0.010 M PBS were determined by using a laser-Doppler velocimetric device (Zetasizer 3; Malvern Instruments, Ltd., Worcestershire, Great Britain). The values given below for θ_w and u are the averages of the values from at least three independently grown cultures. The effective radii (R_e) (in meters) of the cells of the different bacterial strains were determined in the following two ways: (i) from the average geometric mean of the cell width (w) and length (l) determined for 50 cells by using a light microscope [$R_e = 0.5(wl)^{1/2}$], and (ii) with the Stokes-Einstein equation ($D_e = kT/6\pi\eta R_e$, where k is the Boltzmann constant [in joules per kelvin], T is the absolute temperature [in Kelvin], and η is the dynamic viscosity [in kilograms per meter per second]) by using effective diffusion coefficients (D_e) (in square meters per second) obtained from dynamic light-scattering (26) measurements and by using the Contin multiexponential fit of the autocorrelation function (24, 25).

Electron micrographs were obtained for negatively stained strain C2 and C3 cells. Prewashed cells from 1 drop of a suspension were allowed to attach to Formvar-coated grids, after which the specimens were washed in deionized water and air dried. The cellular proteins and lipids were stained by incubating the preparations in a 1% uranyl acetate solution (pH 4.7, adjusted with 0.1 M KOH) for 0.5 to 1 min. Finally, the specimens were washed with deionized water, air dried, and placed in an electron microscope for observation. Suspended strain C4 cells were examined with a light microscope (Diaplan 2000; Leitz, Wetzlar, Germany) and were tested for capsular material by negatively staining them with India ink.

Solid surfaces. Surfaces of PFA-Teflon (also registered as Teflon 350; a copolymer of perfluoroalkoxyheptafluoropropylene and polytetrafluoroethylene) were obtained from Fluorplast, Raamsdonksveer, The Netherlands. Transparent 0.1-mm-thick film and granules (type 9738) with diameters ranging from 250 to 500 μm (average, 375 μm) were used. Glass microscope coverslips (Rofa-Mavi, Beverwijk, The Netherlands) and Teflon film were cut to a size of 9 by 18 mm. Glass beads with a diameter of 450 ± 50 μm were used (Boom, Meppel, The Netherlands). The surfaces were cleaned by submerging them in concentrated chromosulfuric acid for 24 h at 60°C, after which they were well rinsed, first with a 0.5 M KCl solution and then with deionized water. Finally, they were air dried and stored in glass containers until they were used.

Physicochemical characterization of surfaces. Specific outer surface areas (A_{out}) of 49 and 73 $cm^2\ g^{-1}$ were calculated from the average radii of the glass and Teflon beads, respectively, assuming that the glass beads were nonporous. N_2 gas adsorption on the Teflon granules was determined and analyzed by using the Brunauer-Emmett-Teller method (13), which yielded a specific surface area of 1.2 $m^2\ g^{-1}$ (much larger than A_{out}) and an internal porosity value of 0.5% resulting from pores with diameters ranging from 20 to 200 nm. Electron micrographs of gold-coated beads also revealed pores with diameters of <300 nm. Bacterial cells cannot penetrate these pores.

Three measurements of θ_w for two samples of each solid were obtained by using a microscope equipped with a goniometric eyepiece (42). The electrokinetic potentials of the surfaces were determined by measuring streaming potentials in a parallel plate flow cell (21), using 0.010 M PBS as the electrolyte. A 17-g portion of Teflon beads submerged in 50 cm^3 of a degassed 0.01 M KNO_3 solution was titrated with acid (HNO_3) and base (KOH) as described by Fokkink et al. (11); this procedure revealed the presence of unidentified negatively charged groups with a pK_a of approximately 7 at an apparent surface charge density of 2.5 C/m^2 of surface available for N_2 adsorption as determined by the Brunauer-Emmett-Teller method (data not shown). Such high surface charges are never found (14, 37), and considering the hydrophobicity of the samples, we concluded that the majority of the charged groups are located inside the Teflon matrix and are accessible for protons but not for N_2 molecules.

The presence of surface-active compounds excreted by cells during adhesion was determined by measuring θ_w on the surfaces. To do this, we incubated suspensions of prewashed cells (cell concentration [c], 1 × 10^8 cells cm^{-3}) for 4 h at room temperature. After centrifugation two pieces of each type of substratum were incubated for 2 h in 9-cm^3 samples of the supernatants. The surfaces were air dried, and θ_w values were determined.

Batch adhesion experiments. Three batch adhesion methods were used for the batch adhesion experiments.

(i) **Method 1.** Glass vials (volume, 9 cm^3) with rubber stoppers were cleaned with a nonionic detergent solution, rinsed well with deionized water, boiled twice in large

TABLE 1. Physicochemical properties of the bacteria used in this study

Strain	Other designation	θ_w (°)[a]	u (10^{-8} m^2 V^{-1} s^{-1})[b]	Cell length (μm)[c]	Cell width (μm)[c]	R_e(GM) (μm)[c]	D_e(DLS) (10^{-13} m^2 s^{-1})[d]	R_e(DLS) (μm)[d]	Other properties[e]
Coryneform bacteria									
C1	*Arthrobacter* sp. strain DSM 6687	15 (1)[f]	−2.91	ND[g]	ND	ND	2.69 (0.10)	0.80 (0.03)	CNT, NA
C2	Coryneform strain DSM 6685	29 (1)	−2.18	1.0 (0.2)	0.7 (0.0)	0.41 (0.10)	5.90 (0.30)	0.36 (0.02)	Capsules, NA
C3	*Rhodococcus* sp. strain C125[h]	70 (5)	−3.34	2.9 (0.8)	1.9 (0.5)	1.17 (0.37)	1.74 (0.03)	1.24 (0.27)	No capsules, NA
C4	*Rhodococcus erythropolis* A177[h]	87 (5)	−3.15	3.2 (0.2)	1.2 (0.3)	0.97 (0.15)	1.95 (0.01)	1.10 (0.15)	No capsules, NA
C5	*Corynebacterium* sp. strain DSM 6688	89 (1)	−2.12	2.0 (0.8)	0.6 (0.2)	0.56 (0.21)	ND	ND	CNT, NA
C6	*Corynebacterium* sp. strain DSM 44016	103 (6)	−2.58	1.1 (0.5)	0.8 (0.3)	0.47 (0.19)	3.78 (0.13)	0.57 (0.02)	CNT, NA
C7	*Gordona* sp. strain 1775/15[i]	115 (5)	−2.47	ND	ND	ND	ND	ND	CNT, aggregates
C8	*Gordona* sp. strain DSM 44015	117 (4)	−2.89	2.0 (0.7)	0.9 (0.5)	0.66 (0.32)	2.61 (0.12)	0.82 (0.04)[j]	CNT, aggregates
Pseudomonads									
P1	*Pseudomonas oleovorans* ATCC 29347	17 (1)	−1.86	3.4 (2.6)	0.90 (0.05)	0.89 (0.37)	ND	ND	CNT, NA, long cells
P2	*Pseudomonas fluorescens* p62[k]	25 (1)	−1.03	1.3 (0.1)	1.1 (0.1)	0.59 (0.08)	3.75 (0.06)	0.57 (0.02)	CNT, NA
P3	*Pseudomonas* sp. strain B13[l]	32 (1)	−2.11	ND	ND	ND	4.33 (0.01)	0.49 (0.01)	CNT, NA
P4	*Pseudomonas putida* mt2[m]	40 (4)	−1.08	1.3 (0.1)	1.1 (0.1)	0.59 (0.07)	3.65 (0.05)	0.59 (0.01)	CNT, NA

[a] Results of at least three independent measurements.
[b] The standard deviation in all cases was less than 14%.
[c] Cell length and width were determined with a light microscope and were used to calculate R_e by using the geometric mean approach [R_e (GM)]. Cellular particles of strains C1, C3, and C4 were predominantly composed of two to four associated cells; only single cells were observed for all of the other nonaggregating strains.
[d] D_e and R_e values were obtained from dynamic light-scattering measurements [D_e (DLS) and R_e (DLS), respectively].
[e] The presence of capsules was determined either by electron microscopy (cells were negatively stained with uranyl acetate) (strains C2 and C3) or by light microscopy (cells were stained with India ink) (strain C4). CNT, presence of capsules not tested. Aggregation was tested at I values of 0.1 and 1 M. NA, aggregation not observed.
[f] The values in parentheses are standard deviations.
[g] ND, not determined.
[h] *Rhodococcus* sp. strain C125 and *R. erythropolis* A177 were reclassified; they were formerly named *Corynebacterium* sp. strain C125 and *Arthrobacter* sp. strain A177, respectively (30).
[i] See references 2 and 4.
[j] Dynamic light-scattering measurements were determined after cell aggregates had settled.
[k] See reference 44.
[l] See reference 9.
[m] See reference 46.

volumes of deionized water, rinsed again, and air dried. For each adhesion measurement sealed vials were prepared in triplicate as follows. A piece of either glass or Teflon film was placed into each vial, and the vials were then filled to the top with degassed PBS and sealed without a headspace by using the rubber stoppers. Aliquots (between 60 and 170 μl) of concentrated cell suspensions were gently injected into the vials in order to attain the appropriate initial suspended c. Values of c were determined by measuring optical density at either 280 or 660 nm (standard error, 2%), which was calibrated by determining direct counts with a light microscope and a counting chamber (standard error, 15%). The vials were immediately placed on a vertically positioned rotating wheel (8 rpm; amplitude, 10 cm) and incubated at room temperature (20 ± 3°C). The surfaces periodically moved slowly up and down at a maximum velocity of 6 mm s^{-1}. After incubation, 45 cm^3 of cell-free PBS was added to each vial, and the excess fluid was allowed to flow out freely. The flow inlet (internal diameter, 2 mm) was placed a few millimeters above the bottom of the flask, and the flow was not aimed directly at the solid support. The replacing fluid was added at a flow rate (Q) of either 15 or 100 cm^3 min^{-1}. The two Q values were used to test the effect of shear. Because the fluid in the batches was mixed completely by adding PBS to the vials, the c was reduced by a factor of $1/\exp(-45/9)$ (approximately 150) for a Q of 100 cm^3 min^{-1}. The dilution factor was much greater than 150 at a Q of 15 cm^3 min^{-1} because suspended cells were removed by plug flow wash-out. The glass and Teflon sheets were removed from the vials, placed on microscope slides (during which the glass remained wet but the Teflon dewetted at least partially), covered with coverslips, and examined with a microscope (magnification, ×250; Diaplan 2000; Leitz) that had a video camera (magnification, ×2; model LDK 12; Philips, Eindhoven, The Netherlands) mounted on top. The numbers of adhered cells were determined at six randomly chosen locations; these locations were not closer than 3 mm from the edges of the surfaces. This was because the diffusion layer was very narrow at the edges and did not provide local static conditions; hence, the levels of deposition at locations close to the edges were likely to be influenced by artifacts, whereas static conditions were maintained at the more central parts of the surfaces. The observed area was adjusted so that the number of cells counted per location ranged from 20 to 60. Levels of adhesion (Γ) (number of cells per square centimeter) were determined by averaging the values obtained for the three vials which, in turn, were determined from the mean for the six adhesion values obtained per vial.

The width (δ) of the diffusion boundary layer adjacent to the surfaces was defined as follows (17):

$$\delta = D_e^{1/3} (\eta/\rho)^{1/6} (x/v)^{1/2} \quad (1)$$

where ρ is the density of water (in kilograms per cubic meter), η is the viscosity of water (in kilograms per meter per second), x is the distance from the front or rear surface edge (in meters), and v is the velocity of the surface (in meters per second). The width of the diffusion boundary layer was calculated by using the D_e values obtained from dynamic light-scattering or geometric mean procedures (Table 1), the range of positions at which adhesion was determined (3 mm $\leq x \leq$ 9 mm), and a value of 6 mm s^{-1} for the velocity of the surface.

(ii) Method 2. Method 2 differed slightly from method 1; its objective was to detect the effects of transfer of substrata through the air-liquid interface in the presence of suspended cells. After incubation the surfaces were removed directly from the suspension. Nonattached cells were removed by transferring the substrata into 100 cm^3 of cell-free 0.1 M PBS, after which the substrate were moved gently forward and backward 10 times. The numbers of adhered cells were determined as described above for method 1.

(iii) Method 3. Sealed vials (see method 1) containing 5 g of degassed beads and 7 cm^3 of PBS were prepared in triplicate to assay adhesion to Teflon beads. The other procedures were similar to the method 1 procedures, except that levels of adhesion were determined from depletion data.

The methods and conditions used in the different adhesion experiments are summarized in Table 2. Adhesion to Teflon was studied as a function of time (t) at a c of 1×10^8 cells cm^{-3} and as a function of c after incubation for 2 h for strain P2 by using method 1 and for coryneform strains C3 and C4 by using methods 1 and 2 (Table 2, experiments 2 and 4). All of the other batch results were obtained after incubation for 4 h at a c of 5×10^8 cells cm^{-3}. Levels of adhesion were determined for all strain-surface combinations by using method 1 with a Q of 15 cm^3 min^{-1} (Table 2, experiment 1). The effect of fluid velocity during washing was tested by comparing levels of adhesion at Q values of 100 and 15 cm^3 min^{-1} for strains P1, P2, P4, and C1 on glass and for strains P1, P4, C5, and C8 on Teflon (Table 2, experiment 3). Levels of adhesion to Teflon beads (method 3) were determined for strains C3, C4, and P4 (Table 2, experiment 5). Desorption of strain C4 attached to Teflon film as determined by method 2 was studied in batch preparations in the presence and absence of 5 g of initially cell-free Teflon beads (Table 2, experiment 6).

Column experiments. Glass columns with an internal diameter of 1.0 cm and a length of 10 cm were used. Porous glass frits (thickness, 3 mm; pore size, 0.15 mm) separated the internal column space from the inlet and outlet parts. Any air between Teflon beads submerged in PBS was removed by decompression (−3.3 kPa for 10 min). Submerged glass or Teflon beads were transferred with a pipet to columns which were already filled with PBS, thus avoiding exposure of the beads to air. The columns were agitated during packing, which resulted in a reproducible length (9.0 ± 0.3 cm) and overall porosity (0.33 ± 0.03) of the granular bed. Total porosity values were estimated from breakthrough curves by using chloride as the conservative tracer. Chloride concentrations were measured with a microchlorocounter (Marius, Utrecht, The Netherlands). The influent was supplied to the vertical downflow columns by a peristaltic pump. The flow rate was kept constant (within 2%) for each column but varied between 16 and 21 cm^3 h^{-1} for different columns. Samples of the concentrated stock suspensions were diluted in 0.1 M PBS to an optical density at 280 nm of 0.60 ± 0.05 (10^7 cells $cm^{-3} < c < 10^8$ cells cm^{-3}). These suspensions were applied to the columns for 1 h, during which their optical densities at 280 nm remained constant. The influent was then changed to cell-free 0.1 M PBS, which was added to the columns for 45 min. The effluent of a single column was collected in one flask in which the c was determined at the end of the experiment. Adhesion was studied for all strain-surface combinations (except aggregating strain C7) (Table 2, experiment 7). Detachment from Teflon as a result of passing through the air-liquid interface was tested for the same strains by using the same set of columns containing Teflon as described above and a second set of similarly treated columns which were flushed with deionized water (I, <0.0001M) after flushing with

cell-free PBS (Table 2, experiment 8). The fluids from high-I and low-I columns were drained and collected in separate flasks. The level of adhesion (Γ) (in number of cells per square centimeter) and the fraction of cells desorbed as a result of passing through the air-liquid interface ($f_{a/l}$) (expressed as a percentage) were calculated as follows:

$$\Gamma = (V_i c_i - V_e c_e)/(m A_{out}) \quad (2)$$

$$f_{a/l} = (V_d c_d)/(V_i c_i - V_e c_e) \times 100\% \quad (3)$$

where the subscripts i, e, and d indicate influent, effluent, and drainage fluid, respectively, V is the suspension volume (in cubic centimeters), and m is the mass of the beads in the column (in grams). All results presented below were obtained from duplicate columns.

RESULTS

Characteristics of bacterial cells. The properties of the bacterial strains are shown in Table 1. The values for θ_w and u ranged from 15 to 117° and from -1.03×10^{-8} to -3.34×10^{-8} m^2 V^{-1} s^{-1}, respectively, indicating that there was wide variation in the levels of cell surface hydrophobicity and electrokinetic charges. The R_e values estimated by light microscopy did not differ much from those obtained from dynamic light scattering. Some microbial particles were found to be agglomerates (strains C1, C3, and C4) (Table 1). Capsular polymers were detected on the surface of strain C2 by electron microscopy. In an aqueous environment, these capsule polymers are likely to extend much farther than the approximately 2 μm observed when a dehydrated specimen was used. Electron micrographs of strain C3 and negative staining of C4 cells with India ink revealed no capsular material on either of the cell surfaces (Table 1). Strains C7 and C8 aggregated at I values of ≥0.1 M, which is consistent with their high levels of hydrophobicity.

Solid surface characteristics. The θ_w values are consistent with known properties; i.e., glass is hydrophilic (θ_w, 12 ± 2°), and Teflon is hydrophobic (θ_w, 105 ± 1°). The electrokinetic potentials were −44.8 ± 1.8 and −43.6 ± 1.7 mV for glass and Teflon, respectively. Although the fact that there is a negative charge on Teflon is well established, its origin is not known. Perhaps the negative charge is related to the presence of the ether oxygens in the alkoxy groups. The hydrophilicity of glass and the hydrophobicity of Teflon are not substantially altered by adsorption of compounds excreted by cells into a medium; θ_w increased by less than 6° on glass, and on Teflon θ_w was reduced by 7 and 10° for strains C3 and C8, respectively, and by less than 4° for all of the other organisms. Since dried adsorbed biopolymer films lead to surface-water contact angles between 20 and 40° (45), we concluded that the amounts of excreted products that may have adsorbed on the test substrata were very small and probably did not influenced adhesion.

Diffusion-controlled adhesion in batch experiments. Γ increased linearly with the square root of time ($t^{1/2}$) (Fig. 1A) and c (Fig. 1B) for strains C3 and P2 when batch method 1 was used (Table 2, experiment 2). This is consistent with the hypothesis that the rate of attachment is controlled by the diffusion of particles from bulk liquid toward the surface (18).

Effect of transfer through the air-liquid interface. The low level of adhesion of strain C4 compared with strain C3 as determined by method 1 (Table 2, experiment 2, and Fig. 1) was unexpected since these two strains had similar θ_w and u values (Table 1). The level of adhesion of strain C4 when

TABLE 2. Methods, conditions, and strain-surface combinations used for the adhesion experiments

Expt	Phenomenon investigated	Method	Conditions					Strain-substratum combination(s) tested
			Q (cm^3 min^{-1})	t_{batch} (h)	c (cells cm^{-3})	t_{column} (h)	I (M)	
1	Deposition under static conditions	Batch method 1	15	4	5×10^8	0.1	All	
2	Concn and time dependency of static deposition	Batch method 1	15	Various	Various	0.1	P2-Teflon, C3-Teflon, C4-Teflon	
3	Effect of shear during washing	Batch method 1	15 or 100	4	5×10^8	0.1	P1-glass, P2-glass, P4-glass, C1-glass, P1-Teflon, P4-Teflon, C5-Teflon, C8-Teflon	
4	Effect of passing through an air-liquid interface in the presence of suspended cells	Batch method 2	Various	Various	Various	0.1	C3-Teflon, C4-Teflon	
5	Deposition on colliding beads	Batch method 3		4	$10^7 < c < 10^8$	0.1	P4-Teflon, C3-Teflon, C4-Teflon	
6	Detachment induced by colliding beads	Batch method 2 with beads added		0.5	5×10^8	0.1	C4-Teflon	
7	Deposition under dynamic conditions	Column method			$10^7 < c < 10^8$	1	0.1	All except C7 combinations
8	Detachment upon passing through an air-liquid interface	Column method			approx 0	0.1 or <0.0001	All strains-Teflon except C7-Teflon and P3-Teflon at both I values and P4-Teflon at I of <0.0001 M	

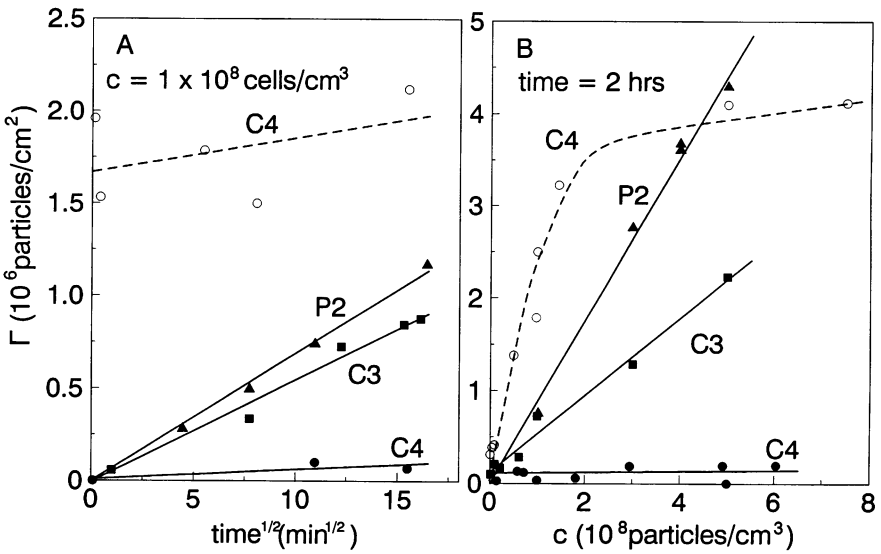

FIG. 1. Adhesion of strains C3 (■), C4 (●), and P2 (▲) to Teflon as determined by batch method 1 and adhesion of strain C4 as determined by method 2 (transfer of Teflon through the air-liquid interface in the presence of suspended cells) (○) as a function of time (A) and c (B).

batch method 2 was used (Table 2, experiment 4) was 20-fold higher than the level of adhesion when method 1 was used. The occurrence of a nondiffusive transport mechanism was demonstrated by the observation that adhesion instantaneously reached high levels and did not depend linearly on c. Similar results for strain C3 were observed when method 2 was used (data not shown). In additional tests in which a video camera mounted on a light microscope was used, it was demonstrated that strain C3 cells accumulated at the air-liquid interface (Fig. 2A) and preferentially attached to the solid surface at the three-phase boundary with contact times ranging from seconds (Fig. 2B) to minutes (Fig. 2A). Deposition without interference of the air-liquid interface was much slower (Fig. 2A) and resulted in a homogeneous distribution of adhered cells (Fig. 2C). Figure 3 shows detachment as a result of passing an air-liquid interface through columns (Table 2, experiment 8). At an I of 0.1 M the $f_{a/l}$ (equation 3) was less than 3% for 9 of 11 strains studied. Higher levels of desorption were observed for C1 (22.5%) and P2 (7.5%). $f_{a/l}$ values were higher for an I of <0.0001 M because of increased electrostatic repulsion, but most of the $f_{a/l}$ values remained below 15%; the only exception was the strain C1 $f_{a/l}$ (22.5%).

Effect of shear on adhesion in batch preparation. The effect of shear on adhesion in batch preparations was tested by adding 45 cm^3 of cell-free PBS to vials at different Q values (100 or 15 cm^3 min^{-1}) (method 1) (Table 2, experiment 3) and by studying adhesion in the presence of Teflon beads (Table 2, experiments 5 and 6). For hydrophilic strain P1 on both glass and Teflon and for the hydrophilic to intermediately hydrophobic organisms C1, P2, and P4 on glass, Γ was found to be reduced by the higher flow rate (Table 3). This indicates that weak adhesive bonds allowed shear forces to reduce adhesion. For Teflon and intermediately to highly hydrophobic bacteria (strains P4, C5, and C8) the opposite was found; Γ increased with Q. Apparently, the increased transport of particles from the bulk liquid to the surface was greater than the removal of adhered cells due to fluid shear. Shear forces induced by moving beads in mixed systems can also reduce adhesion. The experiments with the beads were performed to test to what extent results obtained with mixed batch systems containing granular substrata can be used to predict deposition under more quiescent conditions. The levels of adhesion (Γ/c) on Teflon beads (Table 2, experiment 5) (method 3, strains C3, C4, and P4) (data not shown) were found to be 48 to 65% of the levels of adhesion on Teflon film (Table 2, experiment 1) (method 1). Cells of strain C4 attached irreversibly to Teflon film as determined by method 2 but desorbed completely when Teflon beads were added (Table 2, experiment 5, and Fig. 4).

Adhesion in batch preparations and columns compared. Adhesion data for all strain-surface combinations were obtained from both batch and column experiments (Table 2, experiments 1 and 2) at an I of 0.1 M (Table 4). Direct comparisons of the two sets of data are not possible since adhesion in batch experiments was assayed at a c of 5×10^8 cells cm^{-3} and adhesion in column experiments was determined at 1×10^7 cells $cm^{-3} \leq c \leq 1 \times 10^8$ cells cm^{-3}. Therefore, Γ/c values were used for comparison (Fig. 5). The Γ/c values varied by more than 2 orders of magnitude for both systems. For 15 of the 22 strain-surface combinations studied, $\log(\Gamma/c)_{column}$ and $\log(\Gamma/c)_{batch}$ appeared to be related according to a linear regression line with a positive intercept and a slope that was not significantly different from unity ($P < 0.05$). Hence, $(\Gamma/c)_{column}/(\Gamma/c)_{batch}$ was nearly constant, and averaging the 15 ratios yielded:

$$(\Gamma/c)_{column} = (4.12 \pm 1.64)\ (\Gamma/c)_{batch} \quad (4)$$

Deviation of Γ/c values from the main trend was observed only when the bacterial cells aggregated (C8), were elongated (P1), produced capsules (C2), or displayed significant desorption from Teflon upon passing through an air-liquid interface (C1).

DISCUSSION

Adhesion in batch experiments under static conditions. Transport of bacteria from bulk liquid to surfaces was shown to be governed by diffusion for the batch systems studied by

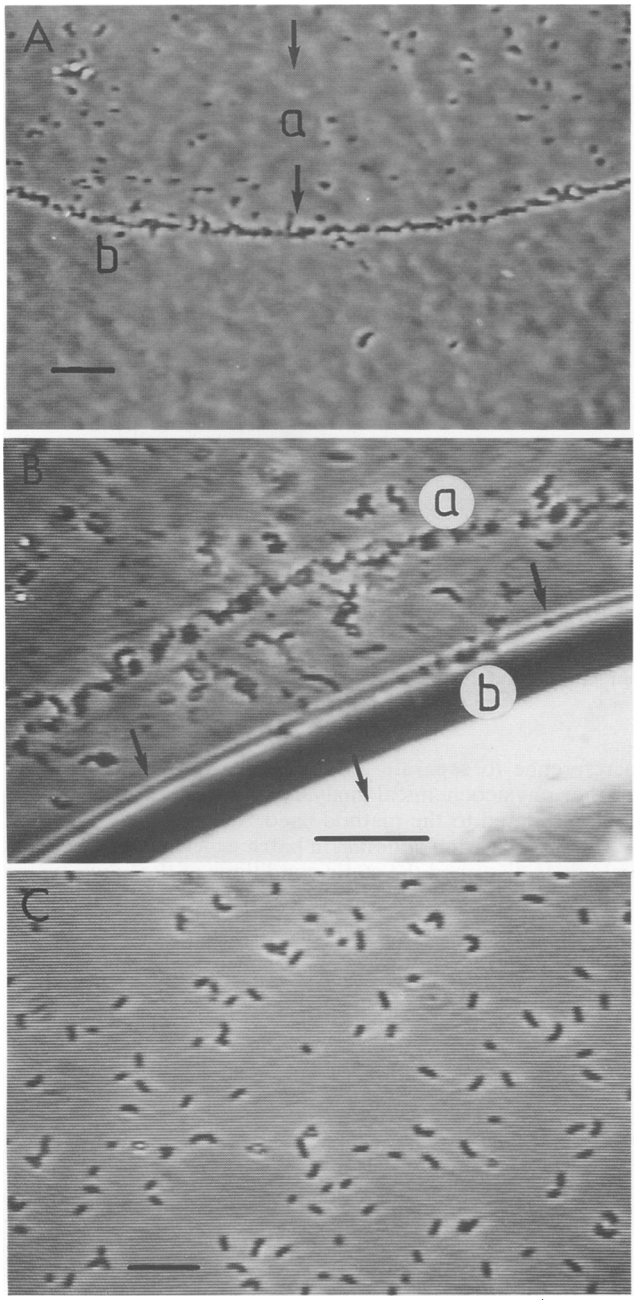

FIG. 2. Effects of the air-liquid interface on adhesion of strain C3 to Teflon. (A) On a surface placed in a suspension made with nondegassed PBS an air bubble developed, and the three-phase boundary moved through region a in the direction indicated by the arrows and stopped after 5 min at position b. Suspended cells were removed by washing (Q, 15 cm^3 min^{-1}), and the surface was placed under the microscope. Bar = 5 μm. (B) Air-suspension interface. A microscope equipped with a video camera was used for direct observations. The air-liquid interface was first situated at position b, moved to position a after the system had been agitated, and receded in the direction indicated by the arrows. Cells were deposited mainly at position a, and the cells at the air-liquid interface (position b) moved very fast, indicating high fluid dynamics. Bar = 5 μm. (C) Normal adhesion pattern of strain C3 observed with method 1 (Q, 15 cm^3 min^{-1}). Bar = 5 μm.

using method 1 (Q, 15 cm^3 min^{-1}) (Fig. 1). By using equation 1 the diffusion boundary layer thickness was calculated to range from 5 to 8 μm and from 7 to 11 μm for the largest cells used (C3) and the smallest cells used (C2), respectively. Apparently, fluid motion in the bulk liquid did not penetrate these thin diffusion layers; hence, static conditions prevailed. The number of bacteria transported by diffusion (N_T) (in number of cells per square meter) was calculated by using the D_e values given in Table 1 and the following equation (18):

$$N_T = 2c(D_e t/\pi)^{1/2} \quad (5)$$

N_T values of 3.3×10^6 particles cm^{-2} and 4.75×10^6 cells cm^{-2} were obtained for strains C3 and P2, respectively; these values are not significantly different from the Γ values (Table 4) obtained for C3 ($3.16 \times 10^6 \pm 0.25 \times 10^6$ particles cm^{-2}) and P2 ($5.65 \times 10^6 \pm 1.46 \times 10^6$ particles cm^{-2}). Γ was either not significantly different from or smaller than N_T for all other strain-surface combinations tested (data not shown) except strain C2-surface combinations. These findings indicate that (i) in general equation 5 correctly describes particle transport in these batch systems, (ii) the net physicochemical interaction is attractive and deposition is not retarded by a repulsive barrier when Γ is approximately the same as N_T, and (iii) repulsive interactions prevent 100% efficient adhesion when Γ is less than N_T. For strain C2, Γ/N_T values of 1.33 and 5.85 were obtained for glass and Teflon, respectively, which indicates that equation 5 is not applicable to this strain. The specific behavior of strain C2 may be a result of the capsular polymers that extend several micrometers (probably more than 10 μm) into the area surrounding the cells. The polymers may enhance deposition by penetrating the stagnant diffusion layer that separates the bulk liquid from the surface. The capsular polymers have a high affinity for both glass and Teflon; apparently, they do not impede adhesion by steric hindrance.

Comparison of adhesion in batch and column systems. Among the various combinations of strains and surfaces studied, the levels of adhesion (Γ/c) varied more than 2 orders of magnitude in both batch and column systems. The approximately similar ratios of level of adhesion in columns to level of adhesion in batch systems (equation 4) that were observed for 15 of 22 combinations of strains and surfaces indicate that the physicochemical origins of adhesion are the same for both systems. For a complete comparison of the batch and column results the solid-suspension contact times had to be taken into account; the solid-suspension contact time for columns (t_{column}) was 1 h, and the solid-suspension contact time for batch systems (t_{batch}) was 4 h. $(\Gamma/c)_{batch} \propto t^{1/2}$ (equation 5). For ideal deposition in columns, the effluent particle concentration and the rate of deposition are constant after the initial breakthrough (10); hence, $(\Gamma/c)_{column} \propto t$. Consequently, $[\Gamma/(c\ t)]_{column}$ and $[\Gamma/(c\ t^{1/2}]_{batch}$ are constants. From the slope of equation 4 and from the t_{batch} (4 h) and t_{column} (1 h), the following equation was derived:

$$(\Gamma/c)_{column} = (8.24 \pm 3.27)(t_{column}/t_{batch}^{1/2})(\Gamma/c)_{batch} \quad (6)$$

The factor (8.24 ± 3.27) $h^{-1/2}$ in equation 6 reveals that transport of microbial particles from bulk liquid to surfaces in columns (transport dominated by convection and diffusion) is more efficient than transport in batch systems (transport by diffusion only). This relationship may be extended to results obtained at various values of t_{batch} and t_{column} provided that additional tests confirm that ideal deposition occurs in columns.

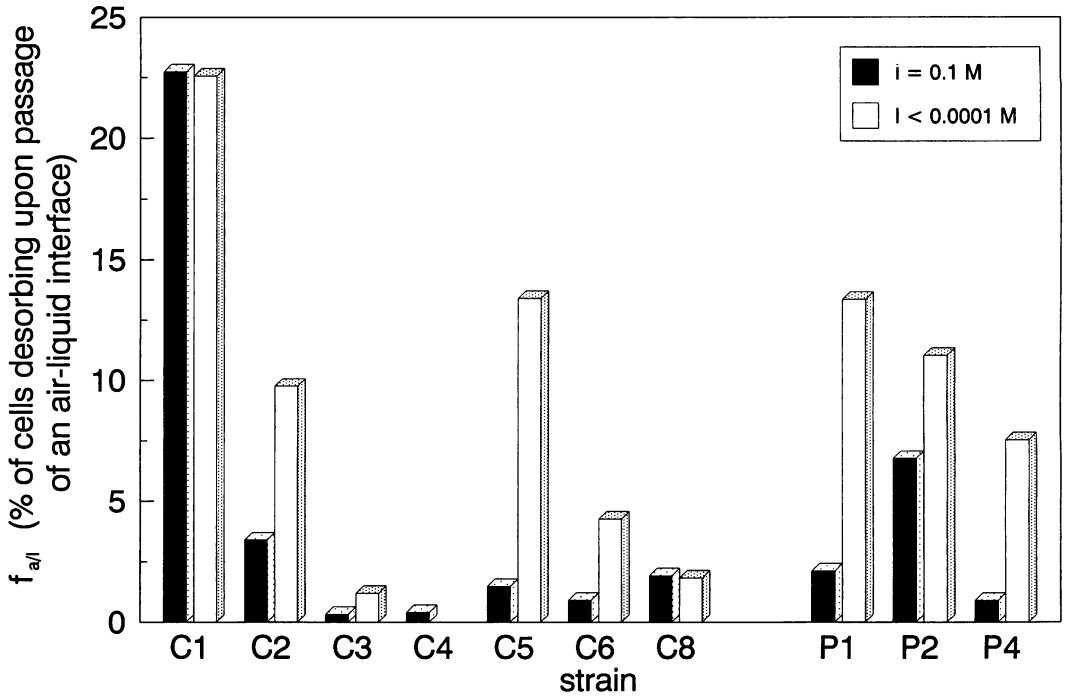

FIG. 3. Fraction of cells desorbing from Teflon beads in columns when an air-liquid interface was passed ($f_{a/l}$). The two aqueous media tested were 0.1 M PBS and deionized water (I, <0.0001 M).

Deviations from equation 4 (Fig. 5) can be explained in all cases except the combination of strain C1 and Teflon. Cell aggregates of strain C8 and the elongated cells of strain P1 (Table 1) are most likely physically retained between the beads, leading to a high level of uptake of cells by columns. In addition, aggregation such as that observed for strains C7 and C8 reduces adhesion in batch systems (equation 5). For strain C2 in column systems, Γ/c did not exceed the Γ/c values found for the other bacterial species, which indicates that the surface polymers of strain C2 can increase the level of adhesion only under static conditions. The deviating behavior of strain C1 on Teflon may have been partly an effect of desorption in batch experiments after Teflon surfaces were transported through the air-liquid interface, but this behavior may also have been influenced by other (unknown) factors since the measured desorption value of 22.5% (Fig. 3) was not sufficient to account for the observed deviation. The correlation between column and batch results shown in Fig. 5 and equation 4 may be used in the future as a reference to separate adhesion data directly suitable for further physicochemical analysis from results influenced by factors related to the method used.

TABLE 3. Γ in batch systems after washing with 45 cm^3 of PBS at a Q of 100 cm^3 min^{-1} compared with Γ after washing at a Q of 15 cm^3 min^{-1}, expressed as a ratio

Surface	Strain	Γ_{100}/Γ_{15}[a]
Glass	P4	0.05
	P2	0.20
	P1	0.60
	C1	0.67
Teflon	P1	0.68
	C8	1.18
	P4	1.24
	C5	1.86

[a] Γ_{100}, Γ after washing at a Q of 100 cm^3 min^{-1}; Γ_{15}, Γ after washing at a Q of 15 cm^3 min^{-1}.

Effect of shear on adhesion in batch experiments. High fluid velocities disturbed the static conditions of the batch system

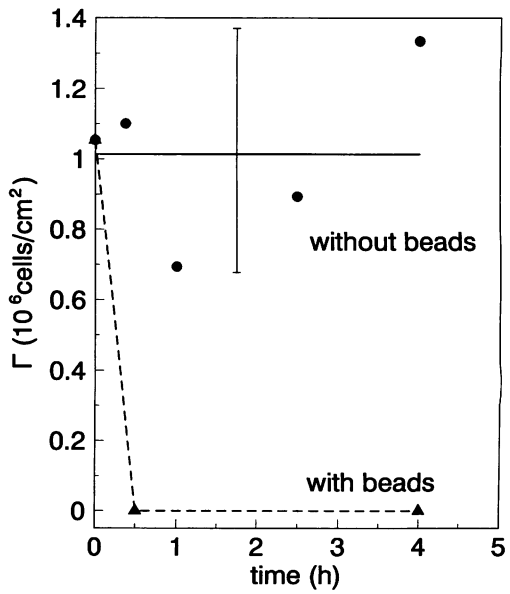

FIG. 4. Desorption of cells of strain C4 adhered to Teflon film as determined by batch method 2 in the presence (▲) and absence (●) of Teflon beads. The cells remained irreversibly attached in the absence of beads but desorbed completely after beads were added. The bar indicates the standard deviation for data obtained in the absence of beads.

TABLE 4. Γ in batch systems (method 1; Q, 15 cm^3 min^{-1}) and column systems

Strain	Γ (10^6 particles cm^{-2})			
	Batch system[a]		Column system[b]	
	Teflon	Glass	Teflon	Glass
C1	0.16 (0.02)	0.23 (0.03)	1.93	0.35
C2	35.49 (7.14)	8.05 (1.75)	15.50	5.94
C3	3.16 (0.25)	0.12 (0.04)	1.62	0.07
C4	0.34 (0.22)	0.06 (0.02)	0.20	0.05
C5	3.79 (0.09)	1.52 (0.18)	2.64	1.05
C6	4.44 (1.05)	2.02 (0.19)	3.25	2.32
C7	1.51 (0.60)	0.20 (0.08)	ND[c]	ND
C8	0.47 (0.07)	0.16 (0.03)	3.00	0.91
P1	0.22 (0.09)	0.050 (0.001)	8.03	1.59
P2	5.65 (1.46)	1.38 (0.31)	16.3	4.52
P3	0.17 (0.03)	0.113 (0.004)	0.50	0.50
P4	2.76 (0.52)	1.41 (0.16)	15.90	4.59

[a] The values in parentheses are standard deviations. The average standard deviation was 19% (excluding the results for strain C4 on teflon).
[b] The average standard deviation for duplicate column results was less than 2.5%.
[c] ND, not determined.

and led to either higher or lower levels of adhesion (Table 3). The lower levels of adhesion for hydrophilic strain-surface combinations at higher washing Q values indicate that there were weak adhesive bonds which were disrupted by shear forces (33, 48). The greater resistance to shear found for the more hydrophobic strains on Teflon indicates that there was strong attraction. High levels of adhesion and/or strong adhesion for more hydrophobic strain-surface combinations was also found in other studies (6, 22, 29, 42). The shear forces in batch systems with granular substrata were greater than the shear forces at high Q values since the levels of adhesion were also lower for combinations of hydrophobic surfaces and hydrophobic strains (method 3, strains C3, C4, and P4) (Fig. 4). One of the practical consequences of these findings is that the deposition of a bacterial species as measured in a mixed suspension of sediment grains cannot be used to predict its adhesion behavior in a natural sediment or in packed sediment columns, where shear forces are small or event absent. Not all investigators appear to be aware of these effects of ill-defined shear forces on adhesion on granular substrata in mixed batch systems (15, 36, 45).

Passing through the air-liquid interface. In general, the level of desorption when the air-liquid interface was passed (Fig. 3) was found to be lower than the standard deviations of 10 to 20% typical for most batch adhesion data (Table 4), which is at variance with the significant level of desorption predicted by Sjollema et al. (34). On the other hand, strong increases in the levels of adhesion of strains C3 and C4 to Teflon as a result of passing through the air-liquid interface were observed when method 2 was used (Fig. 1 and 2A and B). The low level of adhesion of strain C4 under submerged conditions in batch experiments (method 1) (Fig. 1A) and column experiments (Table 4 and Fig. 5) indicates that the high level of attachment of strain C4 determined by method 2 was a result of strong attraction triggered by the air-liquid interface. Neu and Poralla (20) identified a surface-active lipopolysaccharide on the cell surface of *Rhodococcus eryth-*

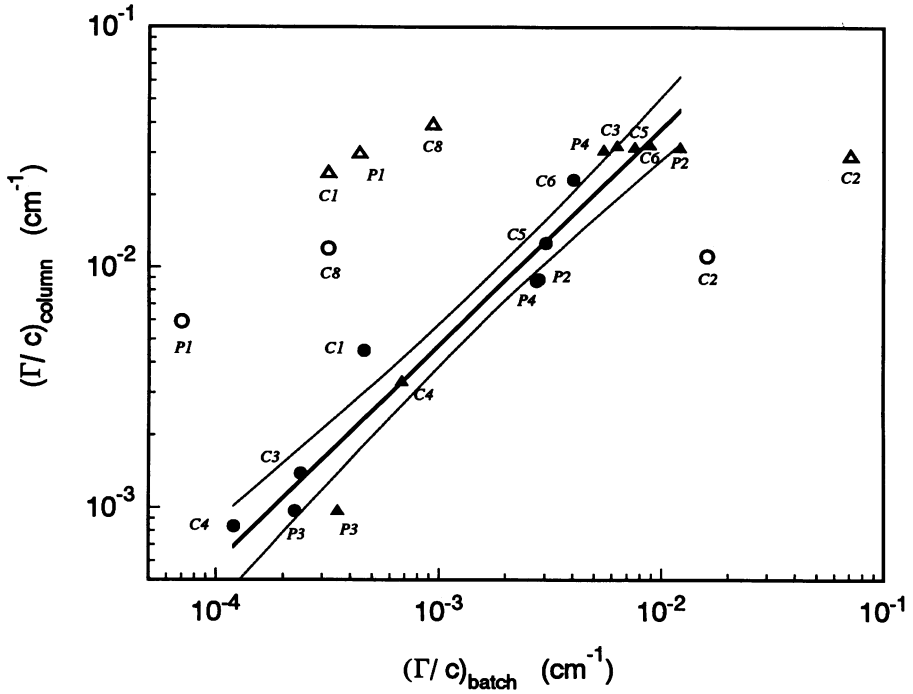

FIG. 5. Deposition in columns as a function of deposition in batch preparations for glass (circles) and Teflon (triangles). Deposition is expressed as Γ/c. The solid line corresponds to equation 4 and was obtained from linear regression analyses of the data (solid symbols) by excluding the results obtained for strains C1, C2, C8, and P1 on Teflon and for strains C2, C8, and P1 on glass (open symbols). These results were excluded for reasons explained in the text. The curves demarcate the confidence interval ($P < 0.05$). The slope close to unity indicates an approximately constant ratio of level of adhesion in columns to level of adhesion in batch preparations. The significant intercept shows that this ratio is greater than unity.

ropolis, and this compound may also be present on the exterior of *R. erythropolis* C4 cells. Such amphoteric polymers tend to orient their hydrophobic tails to the air side of the interface and their hydrophilic carbohydrate moieties into an aqueous environment. Steric hindrance between such hydrated polymers may prevent adhesion under submerged conditions. In addition, θ_w measured on dried bacterial lawns may not reflect the real hydrophobicity of the hydrated cell surface of strain C4, as has also been suggested for hydrophobic oral streptococci (40). The assumed hydrophilic outer polymer layer cannot be very thick since it could not be made visible by negative staining with India ink. Cells of C4 that adhered to the air-liquid interface may be able to contact the solid phase with the hydrophobic parts of their cell surface polymers, leading to the high level of adhesion observed when method 2 was used. Since the transfer of microorganisms with (partly) hydrophobic cell surface polymers from bulk liquid to the air-liquid interface is energetically favorable, these bacteria tend to accumulate at this interface (Fig. 2B). Moreover, the dynamics of the fluid near this interface facilitate the transport of cells to the substratum (Fig. 2A and B). Hence, a higher level of adhesion mediated by an air-liquid interface may result from (i) higher local cell concentration coupled with fast particle transport, and (ii) a change in the structure of the outer cell polymer layer. The increase in level of adhesion when the air-liquid interface is passed may be an important factor in the attachment and retention of bacteria in unsaturated soils (15, 38), aerated bioreactors, or waste gas biofilters (3).

Conclusions. The transport of microbial particles from bulk liquid to surfaces is a factor of 4.12 ± 1.63 more efficient in dynamic columns (transport dominated by convective diffusion) than in static batch systems (transport mediated by diffusion only) for the conditions used in this study. Our findings demonstrate that for any system used to study microbial deposition, the transport of cells from the bulk liquid to the substratum must be taken into account for a proper assessment of bacterial adhesion.

Comparing levels of bacterial deposition as measured by two independent hydrodynamically defined methods provides a way to distinguish between adhesion results that are suitable for further physicochemical analyses and adhesion results that are highly influenced by factors related to the method used. Selection of data is important since adhesion was influenced by system-dependent effects as a result of specific cell characteristics for 32% of the strain-surface combinations studied. Although these cases are regarded as less suitable for testing and developing general adhesion theories, the observed phenomena may have great importance for practical applications. For instance, aggregating and large cells are probably physically retained in porous media like soil, aquifers, and packed bed reactors and tend to clog the pores of such systems. Also, increased attachment as a result of passing through air-liquid interfaces may have great practical importance since this may be a dominant factor in the immobilization of hydrophobic microorganisms in unsaturated zones in soil and in aerated bioreactors.

The bacterium-substratum combinations that appeared to be not influenced by effects related to the method used (68% of all cases tested) were used for a thorough analysis of the physical chemistry and reversibility of bacterial adhesion (29).

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