APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1990, p. 788–795
0099-2240/90/030788-08$02.00/0

Vol. 56, No. 3

Kinetics of *Pseudomonas aeruginosa* Adhesion to 304 and 316-L Stainless Steel: Role of Cell Surface Hydrophobicity

ERWIN VANHAECKE,[1]* JEAN-PAUL REMON,[2] MICHEL MOORS,[3] FILIEP RAES,[4] DIRK DE RUDDER,[2] AND ARSENE VAN PETEGHEM[3]

Laboratory for Pharmaceutical Microbiology and Hygiene,[1] Laboratory for Pharmaceutical Technology,[2] and Laboratory for Non-ferrous Metallurgy and Electrometallurgy,[3] State University, and Department of Fixed Prosthodontics and Periodontology, University Hospital,[4] Ghent, Belgium

Received 18 August 1989/Accepted 12 December 1989

Fifteen different isolates of *Pseudomonas aeruginosa* were used to study the kinetics of adhesion to 304 and 316-L stainless steel. Stainless steel plates were incubated with approximately 1.5×10^7 CFU/ml in 0.01 M phosphate-buffered saline (pH 7.4). After the plates were rinsed with the buffer, the number of adhering bacteria was determined by a bioluminescence assay. Measurable adhesion, even to the electropolished surfaces, occurred within 30 s. Bacterial cell surface hydrophobicity, as determined by the bacterial adherence to hydrocarbons test and the contact angle measurement test, was the major parameter influencing the adhesion rate constant for the first 30 min of adhesion. A parabolic relationship between the CAM values and the logarithm of the adhesion rate constants (ln *k*) was established. No correlation between either the salt aggregation or the improved salt aggregation values and the bacterial adhesion rate constants could be found. Since there was no significant correlation between the bacterial electrophoretic mobilities and the ln *k* values, the bacterial cell surface charge seemed of minor importance in the process of adhesion of *P. aeruginosa* to 304 and 316-L stainless steel.

Bacterial colonization of solid surfaces has been demonstrated in a wide variety of environments. The importance of avoiding bacterial adhesion to surgical implants (4, 11, 14, 15, 21), tap water distribution (13) and cooling (2, 4) systems, milk transfer pipelines (22), power plant condenser tubes (12), or water for injection distribution and storage systems (4, 8, 13, 36) is generally recognized. Although the use of stainless steel is widely applied in the pharmaceutical industry, bacterial adhesion to stainless steel is poorly documented (10, 37, 43). *Pseudomonas aeruginosa* was chosen as the test microorganism in this study because it is both widely found in aquatic environments and generally recognized as a frequent contaminant of metal implants (1, 4, 5, 8, 21, 26). Stanley has examined the process of adhesion of one *P. aeruginosa* strain to one batch of 304 stainless steel (37). It should be clear, however, that results obtained with a single strain on a single stainless steel surface cannot be a solid basis for the understanding of the process of adhesion of *P. aeruginosa*. It is generally accepted that electropolished surfaces are more resistant to bacterial adhesion than are those with a higher surface roughness (6, 46). Because data supporting this view are lacking, we studied the characteristics of the adhesion of endotoxin-producing, gram-negative bacteria to stainless steel. The impact of the cell hydrophobicity, as measured by four different methods, and the cell surface charge on the kinetics of adhesion of *P. aeruginosa* to 304 and 316-L stainless steel was evaluated by using 15 different isolates.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* PA53, 220, 220-R2, and PAO-1 were kindly provided by S. Cryz, Swiss Serum and Vaccine Institute, Berne, Switzerland. *P. aeruginosa* 9027 and 27853 were obtained from the American Type Culture Collection, Rockville, Md. *P. aeruginosa* B13 was a waterborne microorganism. The other strains were isolates from patients with urinary tract infections or otitis media.

To avoid phenotypic alterations of the strains, a dense stock suspension in saline, stored at −196°C, was used to inoculate new stock cultures on tryptic soy agar (TSA) (Oxoid Ltd, Basingstoke, England) every 3 months. Daily subcultures were made from those stock cultures on TSA stored at 4°C for 14 days. After 7 h of growth on TSA at 37°C, an entire nutrient agar plate (Oxoid) was inoculated. After growth for 17.5 h, the bacteria were scraped off the agar plates, collected by centrifugation, washed twice with 0.01 M phosphate-buffered saline (PBS) (8.5000 g of NaCl, 1.4196 g of Na_2HPO_4, 1.3609 g of KH_2PO_4, double-deionized water to 1.0 liter, pH 7.4) and adjusted to a density of 1.5×10^7 CFU/ml by optical density measurement.

Measurement of bacterial cell hydrophobicity. (i) BATH test. The bacterial adherence to hydrocarbons (BATH) test was performed as described by Rosenberg et al. (30–32) and Weiss et al. (44), using hexadecane and octane as organic test fluids. Xylene was excluded from the test because it was found to produce unreliable hydrophobicity results (38).

(ii) SAT. Bacteria were grown on TSA for 7 h and on nutrient agar for 17.5 h (37°C), scraped off the agar plates, collected by centrifugation, washed twice with 0.002 M sodium phosphate buffer (pH 6.8), and diluted to 5×10^9 bacteria per ml. Serial dilutions of 4 M $(NH_4)_2SO_4$ in buffer, differing by 0.2 M increments (from 0.2 to 3.0 M), were made. Bacterial suspensions (20 μl) were mixed with equal volumes of ammonium sulfate solute. The lowest concentration of ammonium sulfate giving visible aggregation was scored as the salt aggregation hydrophobicity test (SAT) value (17, 23, 24, 29, 33).

* Corresponding author.

(iii) Improved SAT (ISAT). Bacterial cells were prepared as described above for the SAT. Cell aggregation was examined in the presence of methylene blue and increasing concentrations of ammonium sulfate (34). The interpretation criteria of the SAT were followed.

(iv) Contact angle measurement (CAM) method. Bacterial cell layers were prepared as described by Weerkamp et al. (43) by collecting the cells on a cellulose triacetate filter (pore diameter, 0.20 μm) (Sartorius, Göttingen, Federal Republic of Germany). The prepared membrane filters were stored overnight on an agar surface containing 10% (vol/vol) glycerol, air dried, and mounted on stainless steel plates. The contact angles of drops of PBS on the dried bacterial cell layers were measured and calculated by using a self-made computer program based on the Tchebycheff equation (18).

Measurement of electrophoretic mobility. Bacteria were suspended in 0.01 M PBS (pH 7.4). Electrophoretic mobility was determined by laser Doppler velocimetry with a Zeta Sizer (Malvern Instruments, Malvern, England). The mobility was used as a measurement of the electrostatic state of the bacteria, without converting it into the theoretical Zeta potential (7, 16, 40).

Stainless steel plates. Stainless steel plates (45 by 45 mm, 1.5 mm), one 304 batch and two 316-L batches, were kindly provided by Ateliers A. Deprest (Rupelmonde, Belgium). Before use, the plates were boiled in 0.1% sodium dodecyl sulfate (technical grade), extensively washed with deionized water and ethanol, rinsed with chloroform and ethanol, stored overnight in 30% HNO_3 (passivation), washed again with deionized water and ethanol, and finally heated for 1 h at 170°C. To evaluate the influence of the surface roughness of the stainless steel materials on the adhesion behavior of *P. aeruginosa* cells, materials with different surface roughnesses were included in the adhesion experiments. The electropolished surfaces were characterized by the lowest surface roughness as indicated by the R_a values (μm), which are the arithmetical means of the absolute distances calculated from the middle line of all measured surface heights and depths. The lowest R_a value corresponds to the lowest surface roughness, characterizing the 120-grit materials as the roughest material.

Adhesion experiments. The metal plates were statically incubated at room temperature with a *P. aeruginosa* cell suspension in 0.01 M PBS (pH 7.4) adjusted to an optical density of 1.0 at 400 nm (Pye-Unicam SP1800 spectrophotometer) corresponding to approximately 1.5×10^7 viable cells (CFU) per ml. At fixed time intervals, from 30 s to 2 h, they were rinsed with 50.0 ml of 0.01 M PBS (pH 7.4) and 10.0 ml of deionized water. To remove the excess fluid, the plates were immediately dried under nitrogen and stored for 15 min in a desiccator. The edges of the stainless steel plates were subsequently taped off, leaving in the center an untaped space of about 2 to 3 cm^2, which was measured exactly after the quantification of the number of adhering cells. The number of bacteria adhering to the uncovered surface was determined by a bioluminescence assay.

Bioluminescence assay. (i) Instrumentation and reagents. Light production was measured by using Lumit-PM (luciferin-luciferase reagent) in a Lumac Biocounter M2010 (Lumac bv, Landgraaf, The Netherlands) (20, 27, 38). Nucleotide-releasing reagent for microbial cells (NRB) (Lumac) was used as the bacterial ATP-releasing agent.

(ii) Measurement of the number of adhering bacteria. A 100-μl portion of NRB was added to the uncovered stainless steel surface and immediately recovered. A 50-μl portion of Tris buffer (pH 7.75) was added twice to the surface to effect a quantitative transfer of the extracted bacterial ATP. The total amount of ATP present in the NRB-Tris mixture was measured and referred to a standard curve of known numbers (CFU) of viable bacteria plotted against the number of relative light units (RLE), which are an instrument-specific digital expression of the amount of ATP. Therefore, although RLE were measured in the biocounter, the adhesion results were expressed as CFU per square centimeter.

To evaluate the effectiveness of the NRB extraction procedure, 20 μl of a suspension of *P. aeruginosa* 848/25 and 803/4 in PBS was dropped on an electropolished (lowest surface roughness) and a 120-grit-treated 316-L stainless steel surface (highest surface roughness) and dried in a desiccator after evaporation under nitrogen. The extraction procedure was applied, and the extracted amounts of ATP were compared with those found for equal amounts of bacterial cells in suspension.

The stability of the bacterial ATP during the adhesion experiments was measured for each of the bacterial strains. The change in bioluminescence values during storage was taken into account for the conversion of the RLE produced by the adhering bacteria to the number of viable adhering cells (CFU per square centimeter): $A=[(P \cdot f_t \cdot C)/B]$, where A is the number of viable adhering bacteria per square centimeter, P is the number of RLE produced by the adhering bacteria per square centimeter, f_t is the conversion factor introduced as compensation for the change in bioluminescence of the bacterial ATP during storage, C is the concentration of the bacterial cells in suspension, and B is the number of RLE produced by 1.0 ml of the bacterial suspension.

Stainless steel roughness measurement. The surface roughness measurement was performed with a perthometer (Perthen, Göttingen, Federal Republic of Germany). This apparatus consisted of a diamond needle (diameter, 2 to 3 μm) screening the metal surface and so revealing surface irregularities.

Chronopotentiometric measurements. A metal plate submerged in an electrolyte immediately takes an electrical potential, which may vary or be constant in time (42, 45). This potential was measured as a function of time versus a reference electrode (e.g., a saturated calomel electrode) also submerged in the electrolyte.

Stereomicroscopic analysis. To confirm the results obtained with the luminometric assay, stereomicroscopic counts of the bacteria adhering to the stainless steel surfaces were made with a Wild Heerbrug microscope (Van Hopplynus, Brussels, Belgium) at a magnification of ×500.

Calculation of the adhesion rate constants. The kinetic model of others was used to determine the adhesion rate constants over the first 30 min of adhesion (3, 35, 41). By plotting $\ln[\Phi_t/(\Phi_t-\Phi_f)]$ against time t, a linear correlation was obtained and the adhesion rate constant (k) could be calculated as

$$\ln \frac{\Phi_t}{\Phi_t - \Phi_f} = \ln \frac{\Phi_o - \Phi_f}{\Phi_o} + k \cdot t \frac{\Phi_f}{\Phi_o - \Phi_f}$$

where Φ_t is the fraction of nonadhering cells at time t, Φ_0 is the initial fraction of nonadhering cells (which equals one), and Φ_f is the fraction of nonadhering cells at equilibrium at the given cell concentration. The Φ_i value could be calculated by $\Phi_i=[(n_{max}-N_i)/n_{max}]$, where i denotes the contact time between the stainless steel plates and the bacterial suspension. The maximum number of adhering cells per square centimeter (n_{max}) was determined by a Scatchard

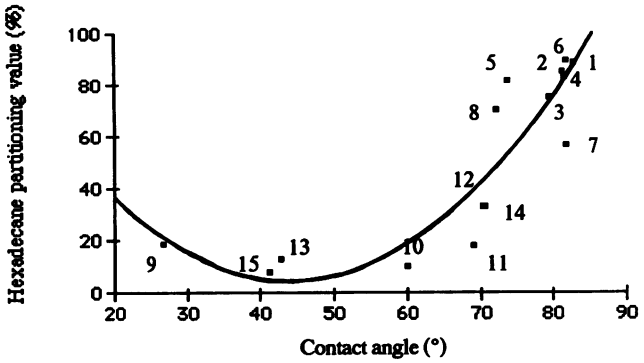

FIG. 1. Curvilinear relationship between the contact angle values and the hexadecane-partitioning values of all 15 *P. aeruginosa* isolates (second-degree polynomial; $r = 0.8990$; $P < 0.01$). The numbers correspond to those shown in the stub of Table 2.

analysis as described by Klotz (19). One-point adhesion experiments ($t = 30$ min) were performed for each bacterial strain at bacterial cell densities ranging from 5×10^4 to 5×10^9 CFU/ml. By plotting the logarithm of the nonadhering cells versus the number of adhering cells (CFU per square centimeter), an accurate determination of the n_{max} value was made.

Chemicals. Hexadecane was obtained from UCB, Leuven, Belgium. All other chemicals were purchased from Janssen Chimica (Beerse, Belgium) unless otherwise indicated.

Statistical analysis. The goodness of fit of the polynomial curve was computed by using a program (Statworks; Cricket Software Inc., Philadelphia) written for the Apple Macintosh computer.

RESULTS

Determination of the number of adhering cells. A linear correlation between the total number of CFU per milliliter in suspension and the number of RLE per milliliter ($r = 0.9997$; $P < 0.01$) was obtained. For the conversion of the amount of RLE produced by the adhering bacteria per square centimeter to the number of CFU adhering per square centimeter, the decrease in bioluminescence production of the bacterial ATP during the adhesion experiments was taken into account (Fig. 1). From the experiments involving the extraction of 20 μl of a bacterial suspension added to the stainless steel surfaces, the efficiency of the NRB-Tris-EDTA extraction procedure for the determination of the ATP of the adhering bacteria was equal to 89.8 ± 2.2% [SD]; $n = 4$), independently of the surface roughness of the stainless steel material. Other extraction procedures, such as trichloric acid or microwave extraction, were less reliable and efficient than the NRB-Tris-EDTA extraction procedure.

Cell surface hydrophobicity values. Table 1 shows the hydrophobicity values of all bacterial strains tested. No correlation between the SAT or ISAT results and the CAM or hexadecane-partitioning values existed. (i) *P. aeruginosa* 9027, U856/3, and 8111/87 were highly hydrophobic according to the ISAT but appeared hydrophilic with the BATH test. (ii) *P. aeruginosa* PAO-1, 220, and 220-R2 were highly hydrophobic in the partitioning test but behaved like hydrophilic strains in the SAT and ISAT. On the other hand, a curvilinear relationship (second-degree polynomial, $r = 0.8980$; $P < 0.01$) between the CAM and the hexadecane-partitioning values (Fig. 1) was observed.

Relationship between cell surface hydrophobicity and cell surface charge. Bacterial cells with a high hexadecane-partitioning value, except for *P. aeruginosa* 220-R2, tended to have a high negative surface charge (Table 1). The electrophoretic mobilities of *P. aeruginosa* 27853 and 9027, both characterized by a low hexadecane-partitioning and CAM value, were about twice as high as those calculated on the basis of the curvilinear correlation existing between the surface charge and the BATH values for the other 11 *P. aeruginosa* strains. No correlation between the SAT or ISAT values and the electrophoretic mobilities for the 15 strains was observed.

TABLE 1. Cell surface hydrophobicity and charge values of *P. aeruginosa* isolates[a]

Strain no.	Strain designation	Values obtained with: BATH		SAT	ISAT	CAM	Electrophoretic mobility[b]
		Hexadecane	Octane				
1	B13	88.61	84.21	1.6	2.0	82.80	−1.74
2	803/4	85.20	88.89	1.6	1.4	81.13	−1.63
3	220-R2	75.00	64.55	3.0	3.0	79.45	−0.70
4	220	82.80	67.73	1.8	4.0	81.60	−1.24
5	PA-53	81.72	86.86	1.6	0.0	73.76	−1.34
6	PAO-1	89.64	71.16	4.0	4.0	81.63	−1.58
7	107	56.48	30.30	2.0	3.0	81.61	−0.48
8	821/21	70.05	74.10	1.6	4.0	81.61	−1.51
9	9027	18.40	19.60	2.0	0.0	26.04	−1.13
10	ER3	10.19	20.88	4.0	4.0	60.02	−0.43
11	U856/3	17.55	35.71	0.0	0.0	69.07	−0.58
12	805	33.00	17.80	2.0	2.0	70.41	−0.82
13	27853	12.80	12.19	3.0	2.0	42.61	−1.03
14	8111/87	33.20	14.50	0.0	0.0	70.57	−0.39
15	848/25	7.69	26.82	4.0	4.0	41.27	−0.32

[a] Each experiment was performed three times. A hydrophobic strain should be characterized by a high BATH value (maximum value, 100%; maximum SD, 11.4%), a low SAT or ISAT value (minimum value, 0 M; maximum SD, 0.2 M), or by a high CAM value (maximum value, 180°; maximum SD, 2.1°).

[b] Expressed in 10^{-8} m^2/V per s and are indicative for the bacterial cell surface charge (maximum SD, 0.15×10^{-8} m^2/V per s). A highly charged cell surface is characterized by a high negative electrophoretic mobility.

Chronopotentiometric behavior of the stainless steel. The chronopotentiometric behavior of each of the stainless steel batches was quite different. In contrast to the highly stable chronopotentiometric properties of one batch of 316-L stainless steel material, an unstable potentiometric behavior was observed for the other 316-L batch and especially for the 304 batch. With reference to the saturated calomel electrode, both a negative and a positive potential had been measured for the 304 batch. One batch of 316-L stainless steel was characterized by spontaneous changes of the potential during measurement. Such equilibrium disturbances were not observed for the other batches of 316-L stainless steel.

Influence of surface roughness on the kinetics of adhesion. Electropolished (lowest surface roughness) and 400-, 320-, and 120-grit (highest surface roughness) materials (Table 2) of one batch of 304 and one batch of 316-L stainless steel were submitted to the adhesion experiments. Six strains were used: three with high BATH and CAM values (*P. aeruginosa* 803/4, PA53, and PAO-1), two with low BATH and CAM values (*P. aeruginosa* 27853 and 848/25), and one strain characterized by intermediate hexadecane-partitioning and high CAM values (*P. aeruginosa* 821/21) (Table 1). Measurable adhesion, even to the electropolished surfaces, occurred within 30 s (Fig. 2). The kinetics of adhesion of strains with low hexadecane-partitioning and CAM values (*P. aeruginosa* 27853 and 848/25) were drasti-

TABLE 2. Surface roughness values of the 304 and one batch of 316-L stainless steel[a]

Type of steel	Roughness value (μm) of stainless steel	
	304	316-L
Electropolished[b]	0.115	0.100
400-grit treated	0.330	0.160
320-grit treated	0.360	0.225
120-grit treated[c]	0.600	0.900

[a] Mean R_a values (n = 6) of the 304 and one batch of 316-L stainless steel treated with different grits. The R_a value is defined as the mean of the absolute distances calculated from the middle line of all measured surface heights and depths. The higher the surface roughness, the higher the corresponding R_a value.
[b] Lowest surface roughness.
[c] Highest surface roughness.

cally influenced by the surface roughness of the substrata. The adhesion rate constants to the electropolished 316-L plates were about 100 times lower than those to the 120-grit surfaces (Fig. 3). When the test microorganism was characterized by a high hexadecane-partitioning or CAM value, the difference in the rate of adhesion to the electropolished and the 120-grit plates was minimal (Fig. 3). This was reflected by the lack of a significant difference between the number of cells adhering to different surfaces after 30 min of incubation with the stainless steel plates (Fig. 2). Stereomicroscopic counts of the bacteria adhering to the stainless steel plates confirmed these observations.

Influence of NaCl concentration on the number of adhering cells. One strain (803/4) characterized by high and one strain (848/25) characterized by low CAM and hexadecane-partitioning values were suspended in 0.01 M PBS with varying NaCl concentrations (0 to 0.85%) and incubated with a 304 and 316-L electropolished plate for 60 min. Within the NaCl concentration range tested (0 to 0.85% in 0.01 M PBS), no major influence of the NaCl concentration on the number of adhering bacteria was observed (Fig. 4). For *P. aeruginosa* 803/4, a pronounced reduction in the number of adhering bacteria occurred when the bacteria were suspended in sterile deionized water.

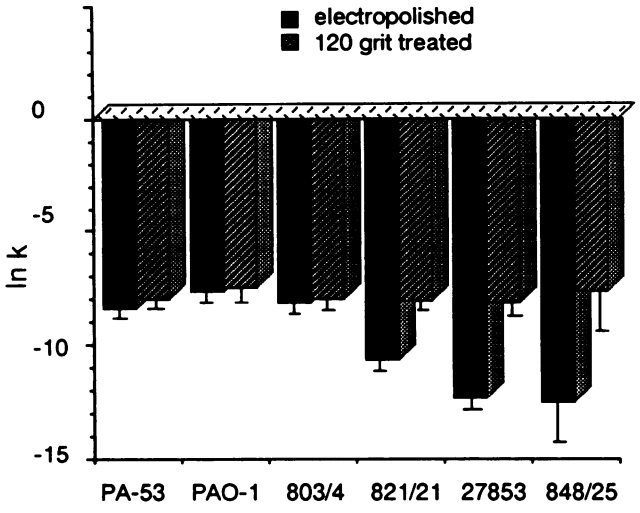

FIG. 3. Logarithm of the adsorption rate constants (k, min^{-1}) of six *P. aeruginosa* strains (high hexadecane-partitioning and CAM values, *P. aeruginosa* PAO-1, PA-53, and 803/4; moderate partitioning and high CAM values, strain 821/21; and low values, isolates 27853 and 848/25; 1.5×10^7 CFU/ml) to an electropolished (lowest surface roughness) and a 120-grit-treated (highest surface roughness) batch of 316-L stainless steel. A small ln k value corresponds to low numbers of adhering bacterial cells within a 30-min contact period (n = 3 ± SD).

Influence of the pH on the number of adhering cells. *P. aeruginosa* 803/4 and 848/25 were suspended in 0.01 M PBS (pH 5.0, 6.0, 7.4, and 8.0) and incubated for 60 min with a

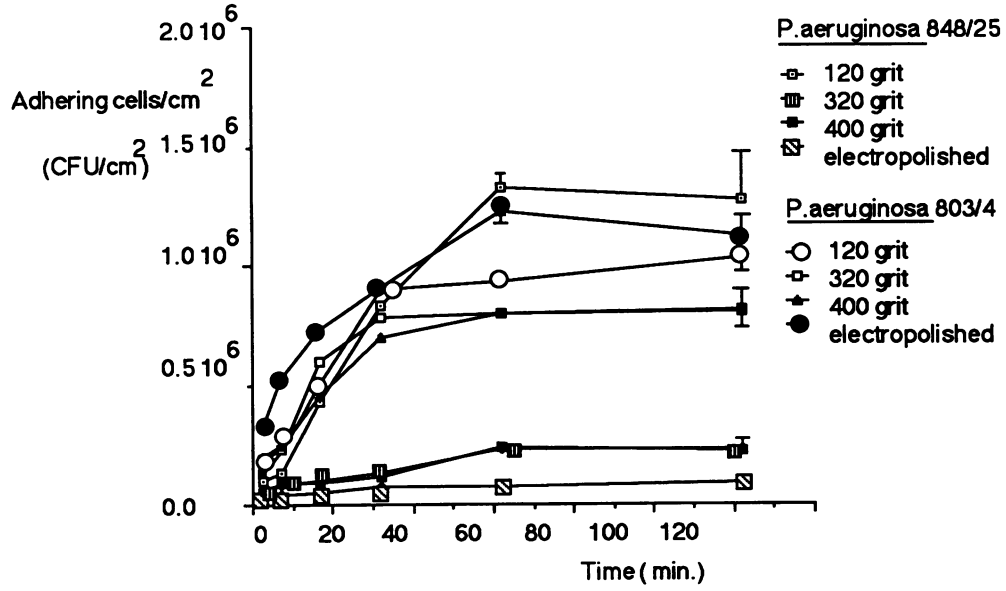

FIG. 2. Kinetics of adhesion of a *P. aeruginosa* strain characterized by low (strain 848/25) or high (strain 803/4) hexadecane-partitioning and CAM values to 316-L stainless steel materials with different surface roughness (n = 4 ± SD). The electropolished stainless steel plates have the lowest surface roughness. Of the nonelectropolished materials, the 120-grit-treated plates have the highest and the 400-grit-treated plates have the lowest surface roughness.

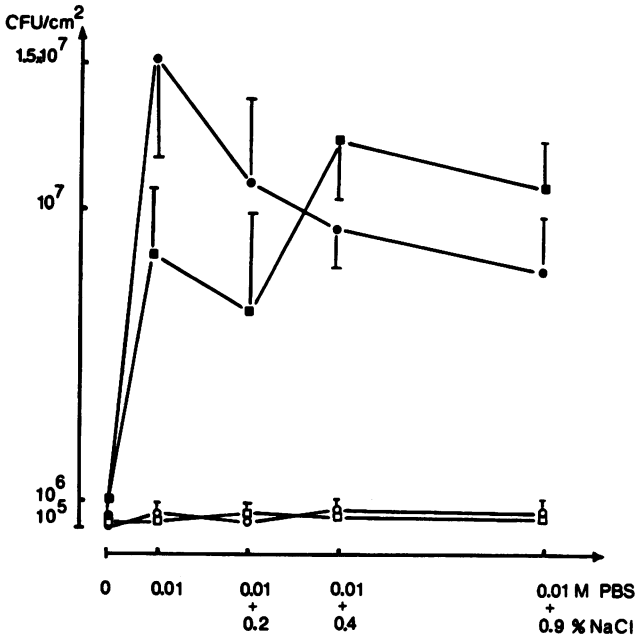

FIG. 4. Effect of increasing concentrations of NaCl in 0.01 M PBS (pH 7.4) on the adhesion of *P. aeruginosa* cells (strain 848/25, open symbols; strain 803/4, closed symbols) to electropolished 316-L (□) and 304 (○) stainless steel (contact time, 60 min.; $n = 3 \pm$ SD). Point zero on the x axis represents the adhesion experiment in deionized water.

304 and 316-L stainless steel electropolished plate. For strain 803/4, a maximum adhesion occurred between pHs 6.0 and 7.4 (Fig. 5). The influence of the pH on the adhesion of *P. aeruginosa* 848/25 to 304 and 316-L stainless steel was less pronounced.

Kinetics of adhesion to electropolished 304 and 316-L stainless steel. A kinetic analysis of the adhesion of each of the 15 bacterial strains to one 304 and two 316-L batches was performed. The maximum number (CFU) of bacteria adhering per square centimeter at saturating cell densities (5×10^9 CFU/ml) of the bacterial suspensions was, for all strains, very similar and independent of the stainless steel type or of the surface roughness of the material (Table 3). No significant correlation between the cell surface charge values and the adhesion rate constants could be found. A curvilinear correlation (without *P. aeruginosa* 107 and 821/21: $r = 0.9540$; $P < 0.01$; third-degree polynomial) between the hexadecane-partitioning values and the ln k to one batch of electropolished 316-L stainless steel (Fig. 6) was observed. By plotting the CAM against the ln k values obtained for the *P. aeruginosa* strains adhering to that batch of 316-L elec-

TABLE 3. n_{max} values of *P. aeruginosa* strains adhering to stainless steel[a]

Strain no.	Strain designation	Values (± SD) obtained with:		
		Electropolished 304 steel	Electropolished 316-L steel	120-grit 316-L steel
1	B13	1.1 ± 1.7	2.0 ± 0.6	2.6 ± 0.9
2	803/4	2.7 ± 1.9	3.1 ± 0.8	2.1 ± 0.7
3	220-R2	1.4 ± 0.7	2.3 ± 1.3	1.9 ± 1.0
4	220	3.1 ± 1.3	3.0 ± 1.8	2.1 ± 1.1
5	PA-53	2.0 ± 1.0	1.9 ± 0.9	2.8 ± 0.5
6	PAO-1	2.8 ± 0.9	3.1 ± 1.3	1.7 ± 1.2
7	107	2.4 ± 0.6	1.2 ± 1.1	2.9 ± 1.4
8	821/21	3.0 ± 0.6	3.1 ± 1.1	2.1 ± 1.5
9	9027	1.1 ± 1.0	3.0 ± 0.9	2.5 ± 2.1
10	ER3	2.5 ± 1.3	2.2 ± 0.4	2.9 ± 0.3
11	U856/3	2.1 ± 0.5	1.7 ± 1.3	1.6 ± 0.9
12	805	1.1 ± 0.9	1.5 ± 1.4	2.9 ± 0.5
13	27853	3.4 ± 1.1	3.8 ± 1.0	3.1 ± 0.5
14	8111/87	2.7 ± 0.7	3.6 ± 0.9	2.0 ± 1.7
15	848/25	3.7 ± 1.4	2.5 ± 0.5	2.4 ± 0.7

[a] Maximum number per square centimeter (n_{max}, 10^7 CFU/cm^2) of the different *P. aeruginosa* strains adhering to electropolished (lowest surface roughness) 304 and 316-L stainless steel and 120-grit-treated (highest surface roughness) 316-L stainless steel. The values were determined by a Scatchard analysis as described by Klotz and are larger than the saturation values obtained after the stainless steel plates were incubated with approximately 1.5×10^7 CFU/ml in suspension for 120 min.

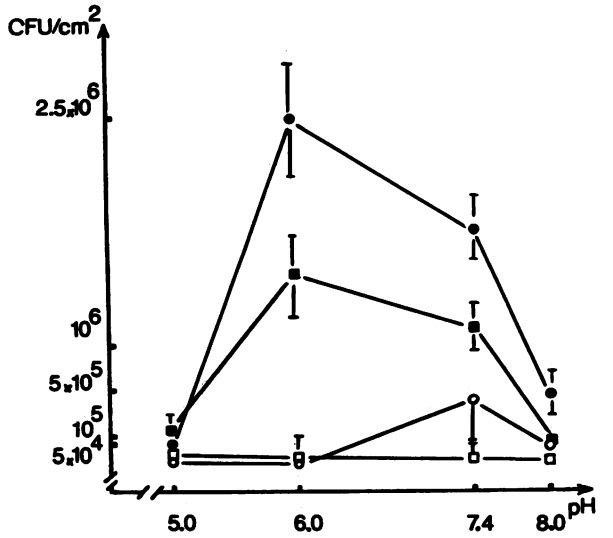

FIG. 5. Effect of pH on the adhesion of *P. aeruginosa* cells (strain 848/25, open symbols; strain 803/4, closed symbols) to electropolished 316-L (□) and 304 (○) stainless steel (contact time, 60 min.; $n = 3 \pm$ SD).

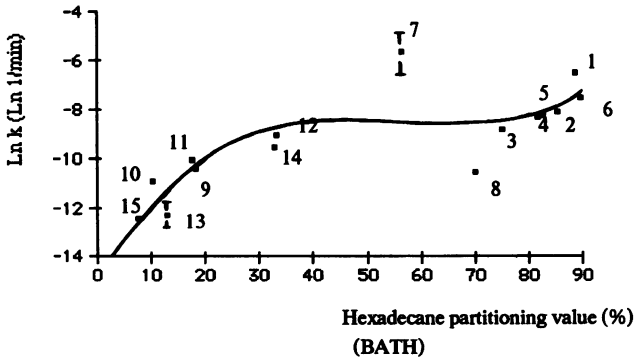

FIG. 6. Relationship between the bacterial cell surface hydrophobicity values, as measured by the hexadecane-partitioning test, and the logarithm of the constants (ln k) of the adsorption rate to one batch of 316-L electropolished stainless steel. Each value represents the mean of three observations. The numbers correspond to those shown in the stub of Table 2. A high (less negative) ln k value corresponds to a high rate constant of bacterial adhesion to the stainless steel surface, which is reflected by a higher number of adhering bacteria per square centimeter within the 30-min contact time.

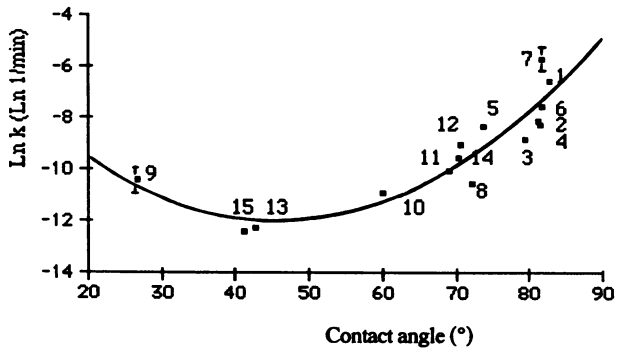

FIG. 7. Relationship between the bacterial CAM values and the logarithm of the constants (ln k) of the adsorption rate to one batch of 316-L electropolished stainless steel. The numbers correspond to those shown in the stub of Table 2. A high (less negative) ln k value corresponds to a high rate of bacterial adhesion to the stainless steel surface, which is reflected by a higher number of adhering bacteria per square centimeter within the 30-min contact time.

tropolished stainless steel, a second-degree polynomial relationship could be established ($r = 0.9290$; $P = 0.05$) (Fig. 7). No such relationship existed with the SAT (or ISAT) values. A significant curvilinear relationship between the CAM values and the ln k to the 304 stainless steel (without *P. aeruginosa* 9027 and 848/25: second degree; $r = 0.9200$; $P = 0.05$) and to the other 316-L stainless steel (without *P. aeruginosa* ER3 and 848/25: second degree; $r = 0.8920$; $P = 0.10$) was observed.

DISCUSSION

Contradicting results concerning the role of cell hydrophobicity in the process of bacterial adhesion to solid surfaces have been reported (9, 14, 15, 28, 30, 39). The controversy is mostly due to the use of different cell surface hydrophobicity measurement methods. Therefore, the cell surface hydrophobicity of each of the bacterial strains was determined by four methods. According to which method was chosen, major differences in the measured cell surface hydrophobicities of the bacterial strains were found. Except for the high correlation obtained between the CAM and the BATH (hexadecane-partitioning) results, no correlation existed between the cell surface hydrophobicity results of the *P. aeruginosa* strains. Therefore, evaluating the role of cell hydrophobicity on the process of bacterial adhesion to solid surfaces necessitates the use of several cell surface hydrophobicity measurement methods (25, 29, 30, 32).

After an extensive standardization, the bioluminescence technique proved to be highly useful in determining the number of bacteria adhering to solid surfaces. By using 15 different *P. aeruginosa* isolates, it has become possible to evaluate the rate of adhesion of *P. aeruginosa* strains to electropolished stainless steel plates, in comparison with those with a higher surface roughness.

A significant curvilinear correlation between the bacterial cell surface hydrophobicity values, as determined by the hexadecane-partitioning test, and the adhesion rate constants to one 316-L batch was observed (Fig. 6). Two strains characterized by a moderate hexadecane-partitioning value, *P. aeruginosa* 821/21 and 107 (hexadecane-partitioning values of 70.05 and 56.48%, respectively), did not follow the general relationship found for the other isolates. For the other 316-L and 304 electropolished stainless steel materials, which were characterized by a higher chronopotentiometric instability, such a correlation between the hexadecane-partioning and the ln k values was not observed. This was mainly due to the scattering of the adhesion rate constants to the 316-L batch and the behavior of *P. aeruginosa* 107 to the 304 material. Factors other than cell surface hydrophobicity, as measured by the BATH test, may therefore be of importance in the process of adhesion of *P. aeruginosa* to electropolished stainless steel. A parabolic relationship between the bacterial contact angle values and the logarithm of the adhesion rate constants was found for all strains adhering to one batch of 316-L stainless steel and for 13 of the 15 strains adhering to the other batches of 316-L and the 304 electropolished stainless steel. Whether the potentiokinetic state of the stainless steel plates influenced the adhesion behavior of the bacterial cells remains to be investigated.

A curvilinear or linear correlation between the cell surface charge and the ln k values was not observed. The bacterial cell surface charge seemed of minor importance in the process of bacterial adhesion to stainless steel. This was confirmed by performing a multiple linear regression analysis with the different cell surface hydrophobicity data and the electrophoretic mobilities as independent variables and the ln k as dependent variables. In none of the combinations could a positive contribution be attributed to the cell surface charge values in order to improve the significance of the correlation between the cell surface hydrophobicities and the ln k values.

The influence of the NaCl concentration on the extent of bacterial adhesion appeared to be minimal. This was in contrast to the results obtained by Stanley (37). Using one *P. aeruginosa* strain adhering to a batch of 304 stainless steel, Stanley observed an increase in the adhering fraction up to 10 mM NaCl or $CaCl_2$. The influence of the ionic strength of the suspending medium on the adhering fraction seems strain dependent. In agreement with Stanley, however, a major reduction in the number of adhering bacteria was observed with *P. aeruginosa* 803/4 when the cells were suspended in deionized water. This could be expected because electrostatic repulsions, which are pronounced in solutions with low ionic strength, reduce the adhesion of bacteria on the metal surface (9, 37). It remains unclear why this phenomenon was seen only with *P. aeruginosa* 803/4. In accordance with the results obtained by Stanley (37), maximal adhesion occurred around neutral pH. Additional experiments are certainly required to understand the NaCl and pH effect. As no change in cell surface hydrophobicity was measured upon changing the NaCl concentration or pH of the medium, factors other than cell surface hydrophobicity may influence the adhesion of *P. aeruginosa* cells to stainless steel.

From these adhesion experiments one may conclude the following. (i) Adhesion of *P. aeruginosa* cells to stainless steel, even to electropolished surfaces, occurs within 30 s. (ii) Although the SAT and the ISAT may measure the cell hydrophobicity accurately, they are not useful in predicting the adhesion of *P. aeruginosa* cells to electropolished 304 and 316-L stainless steel. (iii) Although the possibility cannot be excluded that bacterial cell surface properties other than the cell hydrophobicity may be measured by the CAM test and the BATH test, both methods are useful for predicting the adhesion of *P. aeruginosa* cells to electropolished stainless steel. (iv) If the *P. aeruginosa* strain is hydrophilic according to the BATH and CAM tests, the adhesion to electropolished surfaces appears to be minimal. (v) The adhesive properties of nitric acid-treated (passivated) electropolished 316-L stainless steel surfaces are batch dependent.

ACKNOWLEDGMENTS

We thank Ateliers A. Deprest for providing the metal plates, M. Vermeulen for the surface roughness measurements, K. Van den Haesevelde for the valuable discussions, and H. Stevens for some mathematical interpretations.

LITERATURE CITED

1. **Baier, R. E.** 1982. Comments on cell adhesion to biomaterial surfaces: conflicts and concerns. J. Biomed. Mater. Res. **16:** 173–175.
2. **Bott, T. R., and P. C. Miller.** 1983. Mechanisms of biofilm formation on aluminum tubes. J. Chem. Technol. Biotechnol. **33B:**177–184.
3. **Characklis, W. G.** 1984. Biofilm development: a process analysis, p. 137–157. *In* K. C. Marshall (ed.), Microbial adhesion and aggregation. Springer-Verlag KG, Berlin.
4. **Costerton, J. W., K.-J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. **41:**435–464.
5. **De Vicente, A., J. J. Borrego, F. Arrabal, and P. Romero.** 1986. Comparative study of selective media for enumeration of *Pseudomonas aeruginosa* from water by membrane filtration. Appl. Environ. Microbiol. **51:**832–840.
6. **Dunsmore, D. G., A. Twomey, W. G. Whittlestone, and H. W. Morgan.** 1981. Design and performance of systems for cleaning product-contact surfaces of food equipment: a review. J. Food Prot. **44:**220–240.
7. **Einolf, C. W., and E. L. Carstensen.** 1967. Bacterial conductivity in the determination of surface charge by microelectrophoresis. Biochim. Biophys. Acta **148:**506–516.
8. **Exner, M., G.-J. Tuschewitzki, and J. Scharnagel.** 1987. Influence of biofilms by chemical disinfectants and mechanical cleaning. Zentralbl. Bakteriol. Hyg. B **183:**549–563.
9. **Fattom, A., and M. Shilo.** 1984. Hydrophobicity as an adhesion mechanism of benthic cyanobacteria. Appl. Environ. Microbiol. **47:**135–143.
10. **Gordon, A. S.** 1987. Influence of alginate on attachment of *Vibrio* spp. to stainless steel surfaces in seawater. Appl. Environ. Microbiol. **53:**1175–1177.
11. **Gristina, A. G., and J. W. Costerton.** 1985. Bacterial adherence to biomaterials and tissue. J. Bone J. Surg. Am. Vol. **67:** 264–273.
12. **Hendrickson, D. A., and T. S. McComish.** 1980. Analysis of selected variables for predicting bacterial adhesion to power plant condenser tubes, p. 517–521. *In* R. C. W. Berkely, J. M. Lynck, J. M. Melling, P. R. Rutter, and B. Vincent (ed.), Microbial adhesion to surfaces. Ellis Horwood Ltd., Chichester, Great Britain.
13. **Herson, D. S., B. McGonigle, M. A. Pouger, and K. H. Baker.** 1987. Attachment as a factor in the protection of *Enterobacter cloacae* from chlorination. Appl. Environ. Microbiol. **53:**1178–1180.
14. **Hogt, A. H., J. Dankert, and J. Feijen.** 1985. Adhesion of *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* to hydrophobic biomaterial. J. Gen. Microbiol. **131:**2485–2491.
15. **Hogt, A. H., J. Dankert, and J. Feijen.** 1986. Adhesion of coagulase-negative staphylococci on methacrylate polymers and copolymers. J. Biomed. Mater. Res. **20:**533–545.
16. **James, A. M.** 1982. The electrical properties and topochemistry of bacterial cells. Adv. Coll. Interfac. Sci. **15:**171–221.
17. **Jonsson, P., and T. Wadström.** 1984. Cell surface hydrophobicity of *Staphylococcus aureus* measured by the salt aggregation test (SAT). Curr. Microbiol. **10:**203–210.
18. **Keeping, E. S.** 1962. The binomial, Poisson and normal distributions, 52–77. *In* E. S. Keeping (ed.), Introduction to statistical inferences. D. Van Nostrand Co., Princeton, N.J.
19. **Klotz, I. M.** 1982. Numbers of receptor sites from Scatchard graphs: facts and fantasies. Science **217:**1247–1249.
20. **Lai-King, N. G., D. E. Taylor, and M. E. Stiles.** 1985. Estimation of *Campylobacter* spp. in broth culture by bioluminescence assay of ATP. Appl. Environ. Microbiol. **49:**730–731.
21. **Leake, E. S., A. G. Gristina, and M. J. Wright.** 1982. Use of chemotaxis chambers for studying in vitro bacterial colonization of biomaterials. J. Clin. Microbiol. **15:**320–323.
22. **Lewis, S. J., and A. Gilman.** 1987. Microflora associated with the internal surfaces of rubber and stainless steel milk transfer pipeline. J. Appl. Bacteriol. **62:**327–333.
23. **Lindahl, M., A. Faris, T. Wadström, and S. Hjerten.** 1981. A new test based on salting out to measure relative surface hydrophobicity of bacterial cells. Biochim. Biophys. Acta **677:** 471–476.
24. **Mamo, W., F. Rozgonyi, A. Brown, S. Hjerten, and T. Wadström.** 1987. Cell surface hydrophobicity and charge of *Staphylococcus aureus* and coagulase-negative staphylococci from bovine mastitis. J. Appl. Bacteriol. **62:**241–249.
25. **Martin, A., J. Swarbrick, and A. Cammarata (ed.).** 1983. Physical pharmacy, p. 463–468. Lea & Febiger, Philadelphia.
26. **Mayer, K. H., and S. H. Zinner.** 1985. Bacterial pathogens of increasing significance in hospital-acquired infections. Rev. Infect. Dis. **7:**S371–S379.
27. **McWalter, P. W.** 1984. Determination of susceptibility of *Staphylococcus aureus* to methicillin by luciferin-luciferase assay of bacterial adenosine triphosphate. J. Appl. Bacteriol. **56:**145–150.
28. **Minagi, S., Y. Miyake, Y. Fujioka, H. Tsuru, and H. Suginaka.** 1986. Cell-surface hydrophobicity of *Candida* species as determined by the contact-angle and hydrocarbon-adherence methods. J. Gen. Microbiol. **132:**1111–1115.
29. **Mozes, N., and P. G. Rouxhet.** 1987. Methods for measuring hydrophobicity of microorganisms. J. Microbiol. Methods **6:** 99–112.
30. **Rosenberg, M.** 1984. Ammonium sulphate enhances adherence of *Escherichia coli* J-5 to hydrocarbon and polystyrene. FEMS Microbiol. Lett. **25:**41–45.
31. **Rosenberg, M., D. Gutnick, and E. Rosenberg.** 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. **9:**29–33.
32. **Rosenberg, M., and S. Kjelleberg.** 1986. Hydrophobic interactions: role in bacterial adhesion, p. 353–392. *In* K. C. Marshall (ed.), Advances in microbial ecology, vol. 9. Plenum Publishing Corp., New York.
33. **Rozgonyi, F., K. R. Szitha, S. Hjerten, and T. Wadström.** 1985. Standardization of salt aggregation test for reproducible determination of cell surface hydrophobicity with special reference to *Staphylococcus* species. J. Appl. Bacteriol. **59:**451–457.
34. **Rozgonyi, F., K. R. Szitha, A. Ljungh, S. B. Baloda, S. Hjerten, and T. Wadström.** 1985. Improvement of the salt-aggregation test to study bacterial cell-surface hydrophobicity. FEMS Microbiol. Lett. **30:**131–139.
35. **Rutter, P. R., and B. Vincent.** 1984. Physicochemical interactions of the substratum, microorganisms, and the fluid phase, p. 21–38. *In* K. C. Marshall (ed.), Microbial adhesion and aggregation. Springer-Verlag KG, Berlin.
36. **Shaw, J. C., B. Bramhill, N. C. Wardlaw, and J. W. Costerton.** 1985. Bacterial fouling in a model core system. Appl. Environ. Microbiol. **49:**693–701.
37. **Stanley, P. M.** 1983. Factors affecting the irreversible attachment of *Pseudomonas aeruginosa* to stainless steel. Can. J. Microbiol. **29:**1493–1499.
38. **Vanhaecke, E., and J. Pijck.** 1988. Bioluminescence assay for measuring the number of bacteria adhering to the hydrocarbon phase in the BATH test. Appl. Environ. Microbiol. **54:**1436–1439.
39. **Van Loosdrecht, M. C. M., J. Lyklema, W. Norde, G. Schraa, and A. J. Zehnder.** 1987. The role of bacterial cell wall hydrophobicity in adhesion. Appl. Environ. Microbiol. **53:**1893–1897.
40. **Van Loosdrecht, M. C. M., J. Lyklema, W. Norde, G. Schraa, and A. J. Zehnder.** 1987. Electrophoretic mobility and hydrophobicity as a measure to predict the initial steps of bacterial adhesion. Appl. Environ. Microbiol. **53:**1898–1901.
41. **Vincent, B., M. Jafellier, P. F. Luckham, and T. F. T. Tadros.** 1980. Adsorption of small positive particles onto large negative particles in the presence of polymer, part II. J. Chem. Soc. Faraday Trans. I **76:**674–682.
42. **Von Fraunhofer, J. A., and C. H. Banks.** 1972. Potentiostat and

its applications. Butterworths, London.
43. **Weerkamp, A. H., H. C. Van der Mei, and H. J. Busscher.** 1985. The surface free energy of oral streptococci after being coated with saliva and its relation to adhesion in the mouth. J. Dent. Res. **64**:1204–1210.
44. **Weiss, E., R. Rosenberg, H. Judes, and E. Rosenberg.** 1982. Cell-surface hydrophobicity of adherent oral bacteria. Curr. Microbiol. **7**:125–128.
45. **West, J. M.** 1971. Metals in equilibrium, p. 1–26. *In* J. M. West (ed.), Electrodeposition and corrosion processes. Van Nostrand Reinhold Company Ltd., London.
46. **Zoltai, P. T., E. A. Zottola, and L. L. McKay.** 1981. Scanning electron microscopy of microbial attachment to milk contact surfaces. J. Food Prot. **44**:204–208.