APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1987, p. 1893–1897
0099-2240/87/081893-05$02.00/0

Vol. 53, No. 8

The Role of Bacterial Cell Wall Hydrophobicity in Adhesion

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Received 17 February 1987/Accepted 27 May 1987

In this study, the adhesion of bacteria differing in surface hydrophobicity was investigated. Cell wall hydrophobicity was measured as the contact angle of water on a bacterial layer collected on a microfilter. The contact angles ranged from 15 to 70°. This method was compared with procedures based upon adhesion to hexadecane and with the partition of cells in a polyethylene glycol-dextran two-phase system. The results obtained with these three methods agreed reasonably well. The adhesion of 16 bacterial strains was measured on sulfated polystyrene as the solid phase. These experiments showed that hydrophobic cells adhered to a greater extent than hydrophilic cells. The extent of adhesion correlated well with the measured contact angles (linear regression coefficient, 0.8).

Since the beginning of this century, different reports have been published which suggested that solid-liquid interfaces can have a considerable effect on bacterial physiology. As early as 1913, Söhngen (17) showed that inorganic colloids may influence a variety of microbial processes in soil (e.g., nitrogen fixation, denitrification, etc.). In the 1940s, Zobell (25) inferred that solid surfaces are beneficial to bacteria in dilute nutrient solutions. This view was supported by Stotzky and Rem (19), who found a stimulating effect of montmorillonite clay on the activity of a number of bacteria. In recent years, these and other observations have led to more detailed investigations concerning the influence of solid surfaces on microbial activity (4). Despite the recognition that solid surfaces may influence microbial activities, a good explanation for the observed phenomena is still lacking. Even the adhesion behavior of bacteria is not yet fully understood.

A few authors have described bacterial adhesion in terms of surface free energy (1, 3, 5, 6). Surface free energy was calculated from the contact angle of a drop of water or another liquid on a given surface or on a closed layer of bacteria. The contact angle (θ) of a drop of liquid (L) on a solid surface (S) is a function of the three different surface free energies involved and may be quantified in terms of the three surface tensions (γ, expressed in $N \cdot m^{-1}$) through Young's equation (Fig. 1) $\gamma_{LV} \cos\theta = \gamma_{SV} - \gamma_{SL}$.

Experimentally, it is not possible to determine the surface tensions of the solid-liquid (γ_{SL}) and solid-vapor (γ_{SV}) interfaces independently. Therefore, a second relationship in addition to Young's equation 1 is needed. Fowkes (7) proposed a (nonthermodynamic) relationship in which the interfacial tension is the geometric mean of the surface tension of the two interacting phases. Two approaches based on this assumption are usually used to estimate solid surface tension, namely the geometric mean (3) and the equation of state (1). In the former approach, it is assumed that the total surface tension is the sum of a dispersive part (because of London-van der Waals interactions) and a term comprising all other interactions (e.g., dipole-dipole, hydrogen bonding, etc.). For the theoretical backgrounds of both approaches, see Fowkes (7), Good (9), and Neumann et al. (12). Since there is some controversy regarding the relative merits of both approaches (2, 18), we will compare them to show that the practical results are comparable.

From the above it becomes evident that solid surface free energy as a thermodynamic quantity cannot be calculated from the contact angle but can only be estimated by making some nonthermodynamic assumptions. The contact angle, however, is a relative measurement of the hydrophobicity of the surface which in most cases shows a correlation with the surface free energy (the surface free energy decreases with increasing hydrophobicity). Nevertheless, the data in this paper are solely interpreted in terms of hydrophobicity (because this is what is measured by contact angles), and the terms surface free energy or surface tension will be used only when referring to the work of other authors who consistently use this term in their publications.

In addition to the contact angle method, the hydrophobicity of bacteria can also be determined by partitioning bacteria between two aqueous phases (8) or by quantifying the number of bacteria adhering to droplets of organic solvents (13). The former method is based on the partitioning of bacterial cells between a polyethylene glycol (PEG) and a dextran (DEX) phase. A simple calculation shows that, theoretically, most of the cells will move to one phase, depending on their surface free energy (other interactions, e.g., steric or electrical, are neglected). Partitioning of particles over two phases is defined by the equation $\ln K = \Delta_{part}F/RT$, where K is the partition coefficient, $\Delta_{part}F$ is the difference in surface free energy of the particle surfaces between the two phases (expressed in $J \cdot mol^{-1}$), and R and T have their usual meaning. The quantity $\Delta_{part}F$ can be computed by multiplying the total surface area of 1 mol of bacteria (A) with the difference between the molar surface free energies of the bacteria in the two different phases. Since the surface area of 1 mol of cells is about $2 \cdot 10^{12}$ m^2, the partition coefficient will reach extreme values, even for very small differences in surface free energy. As a result, all of the cells will move to either one of the phases rather than distribute themselves more or less evenly over the two phases. Only in one special case will all cells move to the interface. This occurs if the product of the contact area of the bacterium located at the interface with PEG (A_{PEG}) and

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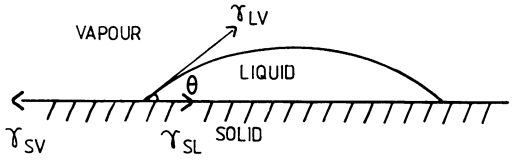

FIG. 1. Relationship between the contact angle and the different interfacial tensions.

the difference between the surface free energies of cell-PEG (F_{BPEG}) and cell-DEX (F_{BDEX}) is smaller than the product of the area occupied by the bacterium in the PEG-DEX interface (A_i) and the surface free energy of PEG-DEX (F_{PEGDEX}): $A_{PEG}(F_{BPEG} - F_{BDEX}) < A_i F_{PEGDEX}$. Using this equation and an $F_{PEG/DEX}$ of 0.06 mJ · m^{-2} (16), it can be calculated that bacteria move to the interface if the difference between the surface free energies of the bacteria in the two phases is smaller than 0.036 mJ · m^{-2}. This condition is satisfied when the bacterial surface free energy is about 58 to 62 mJ · m^{-2} (F_{DEX} = 60 mJ · m^{-2} and F_{PEG} = 59 mJ · m^{-2}), which would correspond to a contact angle of 34 to 41° (11). The finding that a specific bacterial population concentrates at the interface can be used to check the quantitative validity of contact angle measurements.

In this paper, data on the hydrophobicity of 23 different bacterial strains are presented, and this hydrophobicity is related to the adhesion of the cells to negatively charged polystyrene. In addition, the mentioned methods of measuring hydrophobicity are compared, and their applicability is critically evaluated.

MATERIALS AND METHODS

Preparation of bacterial suspensions. All strains investigated in this study were obtained from the culture collection of the Department of Microbiology, Agricultural University, Wageningen, The Netherlands. The following strains were used: *Acinetobacter* sp. 210A, *Agrobacterium radiobacter*, *Alcaligenes* sp. A157, *Arthrobacter globiformis* Ac8, *Arthrobacter simplex* A20, *Arthrobacter* sp. A177, *Arthrobacter* sp. A127, *Azotobacter vinelandii* A59, *Corynebacterium* sp. C125, *Escherichia coli* (NCTC 9002 and K-12), *Micrococcus luteus* M59, *Mycobacterium phlei* M9, *Pseudomonas fluorescens* P9, *Pseudomonas aeruginosa* P8, *Pseudomonas putida* P11, *Pseudomonas* sp. 26-3, *Pseudomonas* sp. (P52), *Pseudomonas* sp. P80, *Rhizobium leguminosarum* (R6), *Rhodopseudomonas palustris*, and *Thiobacillus versutus* (ATCC 25364).

Bacteria were grown in mineral salts medium containing the following (per liter of distilled water): 1.93 g of KH_2PO_4, 7.93 g of K_2HPO_4, 0.75 g of NH_4Cl, 0.05 g of $MgSO_4$, and 1 ml of trace element solution (24). Ethanol (4 ml/liter) was used as the sole carbon and energy source because it has minimal interactions with surfaces (it is uncharged and has a low octanol-water coefficient). Strains showing no growth on ethanol (*A. vinelandii*, *E. coli*, and *M. luteus*) were grown on nutrient broth. The incubation temperature was 30°C.

After 40 h of incubation, bacteria were harvested by centrifugation and washed twice in 0.1 M phosphate-buffered saline containing 0.29 g of KH_2PO_4 per liter, 1.19 g of K_2HPO_4 per liter, and 4.93 g of NaCl per liter. For adhesion experiments, cells were suspended in phosphate-buffered saline to a final concentration of 1×10^9 to 3×10^9 cells per ml. Before the cell suspensions were used, they were filtered through an 8-μm (pore size) micropore filter (Sartorius, Göttingen, Federal Republic of Germany) to remove large cell agglomerates.

Measurement of bacterial hydrophobicity. (i) Contact angle measurement. Bacterial surfaces for measuring contact angles were prepared by collecting bacterial cells on 0.45-μm (pore size) micropore filters (Sartorius). Filters with a continuous bacterial layer were mounted on glass slides and dried in a desiccator for 0.5 to 3 h. Then the contact angle of a 0.1 M NaCl solution with the bacterial surface was measured. No change in contact angle occurred between 0.5 and 3 h. This is in accordance with findings of Absolom et al. (1) and Busscher et al. (3). Incidentally, a method developed by Absolom et al. (1) was used in which a bacterial film was prepared on agar instead of on a micropore filter. Contact angles were measured directly with the aid of a microscope with a goniometric eyepiece (Krüss GmbH, Hamburg, Federal Republic of Germany). Each reported contact angle is the mean of at least six independent measurements.

(ii) Partitioning of cells in two-phase systems. Relative bacterial hydrophobicity measurements developed by Rosenberg (13) and Gerson (8) were compared with contact angle measurements. The first method is based on adhesion of cells to hexadecane droplets. The second method is based on the partitioning of cells in a two-phase system of an 8% DEX (Pharmacia T500)–6% PEG (Merck 6000) solution in water. The surface tensions of the PEG-DEX solutions were measured with a Wilhelmy plate tensiometer.

Preparation of polystyrene disks. Negatively charged polystyrene latex (containing OSO_3^- groups) was prepared as described by Goodwin et al. (10), and the initiator 3 mM $K_2S_2O_8$ was used. The latex obtained was dialyzed, freeze-dried, and subsequently dissolved in toluene (7%, wt/wt). A 30-ml portion of this solution was poured into a glass petri dish (diameter, 12 cm) with a flat bottom which was mounted horizontally. The toluene was allowed to evaporate slowly for 3 days. The polystyrene film obtained was cut into disks (diameter, 1 cm) which were stored dust free. For adhesion experiments, the air-dried side of the disks was used (20). This side has a contact angle for water of 70°. The number of charged groups per unit of surface area could not be established. From the electrophoretic mobility of the original latex particles (-7.8×10^{-8} m/Vs in 0.01 M phosphate-buffered saline), it could be inferred that the polystyrene disks had a considerable negative surface potential.

Adhesion experiments. Freshly prepared bacterial cell suspensions were incubated together with polystyrene disks on a rotary shaker at 25°C. After incubation for 0.5 h, the disks were taken from the suspension and rinsed gently for 30 s in 0.1 M phosphate-buffered saline to remove nonattached cells. The rinsing was performed by moving the disks slowly through the water to prevent detachment of cells due to shear forces. Possible transfer of the cells from the polystyrene surface to the air-water interface during the washing procedure could not occur because a drop of liquid always remained on the disk during the washing procedure. Rinsed disks were dried and colored with Erythrocyne red. The number of cells adhering to the surface were counted under a light microscope with a calibrated eyepiece. Surface coverage was calculated by multiplying the number of cells per square meter by the cross-sectional area of the cell.

RESULTS

In a first attempt, we tried to measure contact angles of bacterial deposits by the method described by Absolom et al. (1). Although the procedure was followed closely, we were

TABLE 1. Contact angles of water for different bacteria

Strain no. and name	Contact angle (°)
1 *Pseudomonas fluorescens*	21.2 ± 1.5
2 *Pseudomonas aeruginosa*	25.7 ± 0.9
3 *Pseudomonas putida*	38.5 ± 1.0
4 *Pseudomonas* sp. strain 26-3	20.1 ± 0.8
5 *Pseudomonas* sp. strain 52	19.0 ± 1.0
6 *Pseudomonas* sp. strain 80	29.5 + 0.5
7 *Escherichia coli* NCTC 9002	15.7 ± 1.2
8 *Escherichia coli* K-12	24.7 ± 0.4
9 *Arthrobacter globiformis*	23.1 ± 0.7
10 *Arthrobacter simplex*	37.0 ± 0.9
11 *Arthrobacter* sp. strain 177	60.0 ± 1.5
12 *Arthrobacter* sp. strain 127	38.0 ± 1.3
13 *Micrococcus luteus*	44.7 ± 0.9
14 *Acinetobacter* sp. strain 210A	32.6 ± 0.5
15 *Thiobacillus versutus*	26.8 ± 0.8
16 *Alcaligenes* sp. strain 175	24.4 ± 0.5
17 *Rhodopseudomonas palustris*	34.3 ± 0.5
18 *Agrobacterium radiobacter*	44.1 ± 0.5
19 *Bacillus licheniformis*	32.6 ± 0.5
20 *Corynebacter* sp. strain 125	70.0 ± 3.0
21 *Azotobacter vinelandii*	43.8 ± 0.5
22 *Rhizobium leguminosarum*	31.0 ± 1.0
23 *Mycobacter phlei*	70.0 + 5.0

not able to obtain reasonable contact angles. The bacteria were washed away from the agar by the drop of water placed on them. The measured contact angles (about 17°) did not differ very much for the bacteria tested and closely resembled the contact angle of clean agar. Other investigators (H. J. Busscher, personal communication) have had the same experience. Measurement of contact angles on bacteria collected on micropore filters gave more meaningful results.

The results of these contact angle measurements with 0.1 M NaCl solution and a range of different strains are summarized in Table 1. The variation in contact angle was relatively small (±1°), indicating that the bacterial film surface was rather homogeneous. The contact angles for different strains can deviate strongly from one to another, even within the same genus. No direct correlation between contact angles for gram-positive and gram-negative cell walls was observed. To test the agreement between the geometric mean (3) and equation of state (1) approaches for estimation of surface tension (γ_{SV}) or its dispersive part (γ^d), the contact angles of α-bromonaphthalene (a completely apolar liquid) and 0.1 M NaCl solution on a bacterial layer were measured. Both approaches gave almost identical results (Table 2). This is not surprising since the geometric mean and equation of state approaches have essentially the same theoretical basis (model proposed by Fowkes [7]).

To examine to what extent the preparation procedure of a bacterial layer for contact angle measurements influences cell surface hydrophobicity, a comparison was made between the contact angle measurement and the behavior of bacteria in two different two-phase systems. The experimental setup of both measurements is shown in Fig. 2. From the relationship between the contact angle measurements and the adhesion to hexadecane droplets (Fig. 3), we concluded that bacteria with a contact angle below 30° do not adhere to the hydrocarbon phase. Above this critical contact angle, adhesion increased concomitantly with the contact angle. Although important deviations occur, the general trend in the partition of bacteria in the PEG-DEX system approximately follows the contact angle measurements (Fig. 4). Three of four bacterial strains expected to concentrate at the interface actually did so. The contact angle measurements also have a predictive value for the adherence of bacteria to negatively charged polystyrene (Fig. 5). Correlation between coverage of a surface and contact angle measurements on these surfaces has also been reported elsewhere (1, 3, 5, 14, 23). A good correlation between bacterial adhesion and the hexadecane test has already been reported earlier (13).

DISCUSSION

Measurement of bacterial hydrophobicity can be of importance in many research areas, e.g., biofouling, oral microbiology (3), phagocytosis (22), soil microbiology, etc. Therefore, a good measure for bacterial hydrophobicity is needed. The use of a broad range of various tests (14) makes it difficult to compare the outcomes of the different studies. It may be worthwhile to initiate some test series in different laboratories with a few reference strains. A thorough evaluation of the results may lead to a generally accepted standard hydrophobicity test. In the following part we will evaluate the three methods used to measure surface hydrophobicity and discuss possible practical problems and shortcomings.

The measurement of contact angles of an aqueous 0.1 M NaCl solution with a layer of bacteria gave reproducible results, although the bacterial layer had to be dried slightly before measurements could be performed. Contact angles correlated relatively well (r^2, 0.8) with the adhesion of bacteria to negatively charged polystyrene (Fig. 5). From these findings and the data reported in the literature (1, 3), it can be concluded that contact angles are very useful for estimating the hydrophobicity of the cell surface of a given organism and consequently provide an important factor for predicting its adhesion to various surfaces. Analyses of such data in terms of individual surface free energies or surface tensions, as done in the equation of state and geometric mean approaches, involves a nonthermodynamic assumption and should therefore be avoided; the more so as the use of surface free energies to calculate adhesion energy (1, 3) is restricted to cases in which bacteria and a solid make direct contact whereby the original phase boundaries are replaced by new ones. In the experiments reported here, cells may adhere at a certain distance from the solid surface, at the so-called secondary minimum of the DLVO theory (15, 21).

TABLE 2. Comparison of calculated surface free energies by the equation of state the geometric mean approaches[a]

Organism	Contact angle (°)		Equation of state approach (mJ · m^{-2})		Geometric mean approach (mJ · m^{-2})	
	α-Bromo-naphthalene	Water	γ^d	Water γ_{SV}	γ^d	Water γ_{SV}
Pseudomonas sp. strain 26-3	25	20	41	68	40	70
Arthrobacter globiformis	20	23	42	67	42	72
Arthrobacter sp. strain 177	37	60	36	47	36	48
Micrococcus luteus	31	44	38	56	39	60
Veillonella alcalescens	57	20	28	68	27	68
Streptococcus sanguis	41	42	34	57	34	59
Streptococcus salivarius	44	26	33	65	33	67
Streptococcus mitior	31	55	38	49	38	53

[a] The data for *V. alcalescens*, *S. sanguis*, *S. salivarius*, and *S. mitior* were taken from Busscher et al. (3) and are used here as additional data to show the agreement between the equation of state and geometric mean approaches.

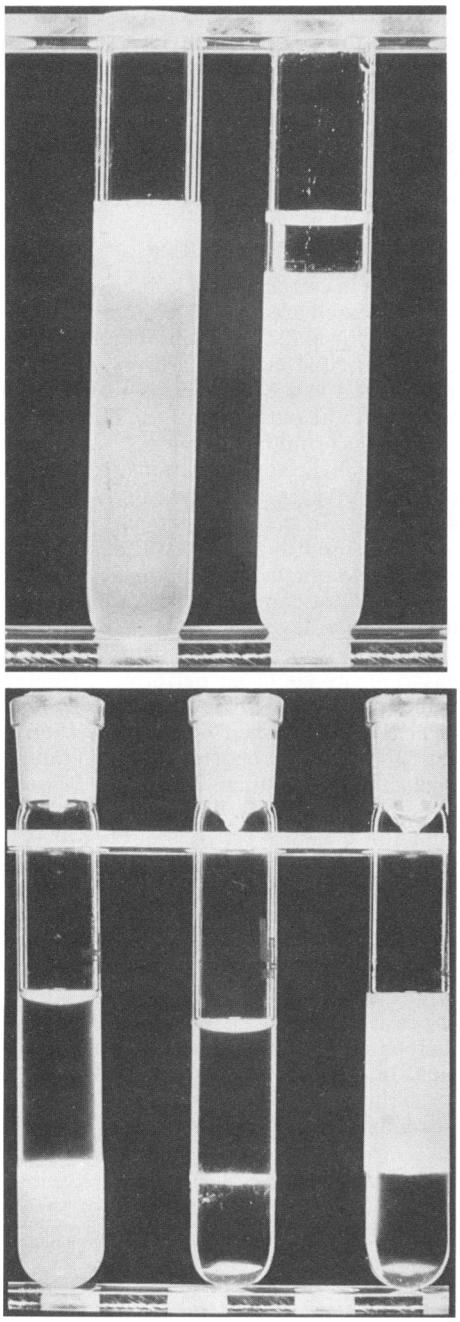

FIG. 2. Experimental setup of two hydrophobicity tests. (A) Adhesion of *Arthrobacter* sp. strain 177 (left) and *Pseudomonas* sp. strain 26-3 (right) to hexadecane. (B) Partitioning of *Arthrobacter* sp. strain 177 (left), *Arthrobacter* sp. strain 127 (center), and *Pseudomonas* sp. strain 26-3 (right) in a PEG-DEX two-phase system.

In that case, no new boundaries are formed and a balance of surface free energies will overestimate the adhesion free energy.

In the hexadecane test, removal of cells from the aqueous suspension depends on their adhesion to the hydrocarbon phase. Thus, this method is very sensitive to the amount of surface area created during mixing of the two liquid phases. This surface area in turn is dependent on the size and

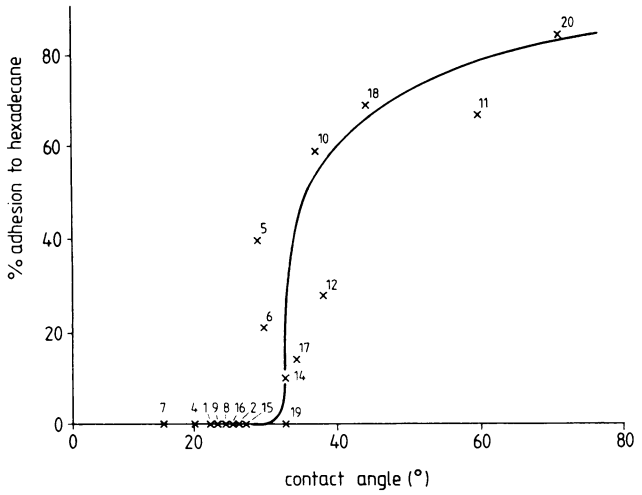

FIG. 3. Relationship between contact angle and adhesion of bacteria to hexadecane. Numbers refer to the numbering of the different bacteria in Table 1.

number of hexadecane droplets obtained in the aqueous phase. Droplet formation is influenced by mixing conditions like temperature, type of mixing vessel, etc. Since this method is not standardized, data obtained in different laboratories might show some deviations. A second problem is the formation of small hexadecane droplets stabilized by bacteria (Pickering stabilization) which do not leave the water phase. This emulsion may affect the measurement because adhesion is measured as a decrease in extinction. However, this can be circumvented by microscopically counting the bacteria in the water phase. Several bacterial strains showed a tendency to form stable emulsions, especially *Micrococcus luteus*. Besides these technical problems, quantification of hydrophobicity may be affected by the extraction of cell surface components by hexadecane. A further disadvantage of the hexadecane method is its insensitivity toward differences in hydrophobicity in rather hydrophilic bacteria (Fig. 3).

Partitioning of cells in the two-phase PEG-DEX system is very sensitive to details in surface structures because $\Delta_{part}F$ is determined by a delicate balance of surface free energies and steric, electrical, and various other interactions which are not all determined in the contact angle measurement. On the basis of the contact angle measurements, three of four

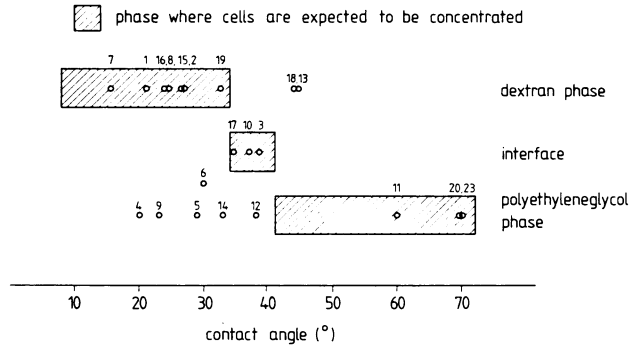

FIG. 4. Relationship between contact angle and partitioning of cells in a PEG-DEX two-phase system. Numbers refer to the numbering of the different bacteria in Table 1.

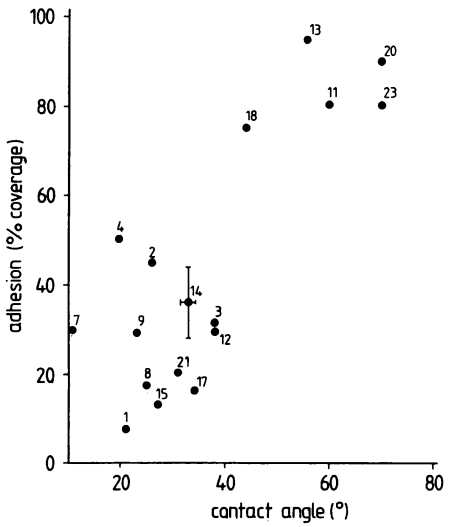

FIG. 5. Relationship between bacterial hydrophobicity as determined by contact angle measurements and adhesion to negatively charged polystyrene. Numbers refer to the numbering of the different bacteria in Table 1. Bars indicate the average standard deviation in the measurements.

bacteria expected to concentrate at the interface were actually found there. Not all bacteria behaved as expected from the contact angle measurement, which indicates that interactions other than hydrophobicity may also play a role in the partitioning of bacteria. A practical problem is that both phases are relatively viscous, which means that the mixture needs to be shaken very intensively and the time to allow phase separation must be long (24 h). If the two conditions are not entirely fulfilled, an incorrect partition equilibrium will be obtained. Also in this case, microscopy can help to determine quickly to which phase the bacteria have been transferred without having to wait for full separation.

In conclusion, we can say that contact angles are a good measurement of bacterial hydrophobicity and have a predictive value for adhesion. Because of the shortcomings of the existing models in generating absolute values for the hydrophobicity of bacterial cells, interpretation of such data in terms of bacterial surface free energy is suspicious. Because of the importance of bacterial adhesion in a great variety of technologies and natural processes, there is an urgent need to come to one generally accepted method for the measurement of cell hydrophobicity. Based on the data reported in the literature and our own findings, we propose use of the water contact angle measurement to quantify cell hydrophobicity.

ACKNOWLEDGMENT

This investigation was supported by a special grant from the Agricultural University, Wageningen, The Netherlands.

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