APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1992, p. 1549–1553
0099-2240/92/051549-05$02.00/0

Vol. 58, No. 5

Comparison of Contact Angles and Adhesion to Hexadecane of Urogenital, Dairy, and Poultry Lactobacilli: Effect of Serial Culture Passages

GREGOR REID,[1,2]* PIETER L. CUPERUS,[3] ANDREW W. BRUCE,[2] HENNY C. VAN DER MEI,[3] LISA TOMECZEK,[2,4] ANTOINE H. KHOURY,[5] AND HENK J. BUSSCHER[1,3]

Department of Microbiology and Immunology, University of Western Ontario, London, Ontario N6A 5B8,[1] and Urology Research, Toronto General Hospital,[2] Department of Microbiology, University of Toronto,[4] and Division of Urology, Hospital for Sick Children,[5] Toronto, Ontario, Canada, and Laboratory for Materia Technica, University of Groningen, 9713 AV Groningen, The Netherlands[3]

Received 9 December 1991/Accepted 27 February 1992

The aim of this study was to examine the hydrophobicities of 23 urogenital, dairy, poultry, and American Type Culture Collection isolates of lactobacilli and to determine the effect on hydrophobicity of serially passaging the strains in liquid medium. To this end, strains were grown after isolation and identification and then serially passaged up to 20 times. Hydrophobicity was assessed through contact angle measurements on lawns of cells by using water, formamide, methylene iodide, 1-bromonaphthalene, and hexadecane as wetting agents and through measurement of their partitioning in a hexadecane-water system. The hydrophobicities of these strains varied widely, with *Lactobacillus casei* strains being predominantly hydrophilic and *L. acidophilus* strains being mostly hydrophobic. For some isolates, serial passaging was accompanied by a clear loss of hydrophobic surface properties, whereas for other strains, cultures became heterogeneous in that some cells had already lost their hydrophobic surface properties while others were still hydrophobic. Adhesion of this collection of lactobacilli to hexadecane droplets in microbial adhesion to hexadecane (MATH) tests was driven by their aversion to water rather than by their affinity for hexadecane, as concluded from the fact that hexadecane contact angles were zero for all strains. Furthermore, adhesion of the lactobacilli to hexadecane in MATH tests occurred only when the water contact angle on the cells was above 60°.

Lactobacilli are the dominant organisms in the healthy adult intestine, vagina, and perineum, where their presence has been associated with some degree of resistance against infection (8, 9). For the urogenital tract, although many factors have been demonstrated and proposed for this protective function, it can be generally stated that adhesion to surfaces is the most important factor identified to date (7). This adhesion not only provides resistance against urinary washout from the urethra but also provides a means to colonize the urogenital mucosa and to compete with other organisms attempting to colonize.

Adhesion of microorganisms is generally thought to be mediated by a complicated interplay of hydrophobic and charge properties of the interacting surfaces (5), possibly complemented by a distinct influence of the absence or presence of specific receptor sites and appendages on the microbial cell surface (1). The hydrophobicity of the microbial cell surface is ubiquitously accepted as a major factor in adhesion (12, 13). Over the years, several methods to measure the hydrophobicity of microbial cells, including contact angle methods, microbial adhesion to hydrocarbons (MATH), hydrophobic interaction chromatography, and salting out, have been proposed (15). Despite the fact that all methods claim to measure the hydrophobicity of the cell surface, there is (in general) no relation between the outcomes of various tests (3, 6, 12, 16). Van der Mei et al. (16) have argued that a relation between the outcomes of various tests exists only when the tests are conducted with closely related strains, whereas other authors found relations only when considering extremely hydrophobic and hydrophilic strains (3, 6). Van Loosdrecht et al. (19) calculated the surface Gibbs energy of partitioning of colloidal particles and hydrocarbons in water and concluded that only cells with water contact angles of greater than 30 to 40° could adhere to hydrocarbon droplets in MATH. This conclusion was experimentally verified for their collection of microbial strains, and a clear sigmoidal relation between the percent adhesion to hexadecane in MATH and water contact angles was found. Later, Vanhaecke et al. (18) found a similar relation for 15 isolates of *Pseudomonas aeruginosa*, although in their study a significant adhesion to hexadecane was observed only when water contact angles were above 60° rather than 30 to 40°.

Previous studies with lactobacilli have shown variability of surface hydrophobicity (4) and a linear correlation between hydrophobicity and *Lactobacillus acidophilus* T13 binding to hydrophobic polymers (10). In order to continue the study of these organisms, we determined the following: (i) the hydrophobicities of 23 isolates of lactobacilli by contact angle measurements and MATH, (ii) the effect of serial passages of the strains in liquid medium on their hydrophobicity, and (iii) whether there is a relation between water contact angles and the percent adhesion of these strains to hexadecane in MATH tests.

MATERIALS AND METHODS

Bacteria. Twenty-three isolates of lactobacilli were selected on the basis of various characteristics (9, 10). It was our intent to test strains isolated up to 9 years before our study as well as fresh isolates that had been stored for less

* Corresponding author.

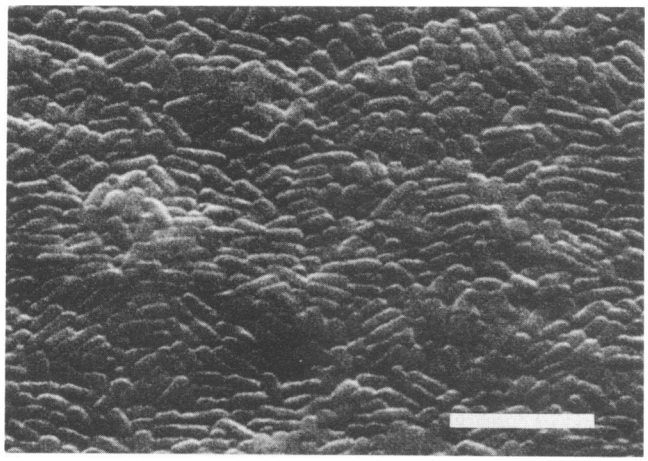

FIG. 1. Scanning electron micrograph of a lawn of *L. casei* ATCC 7469 deposited on a cellulose acetate filter. Bar, 5 μm.

TABLE 1. Hydrophobicities of isolates of lactobacilli passaged once, as assessed by contact angles and MATH

Species or subspecies	Strain	Contact angle (°)[a]				% Adhesion in MATH[b]
		θ_W	θ_F	θ_M	$\theta_{1\text{-}B}$	
L. acidophilus	68	74	39	52	35	32
	75	66	56	50	39	13
	RC14	102	47	55	38	55
	T13	80	39	46	27	29
L. casei	55	36	27	44	33	0
	8	30	33	48	31	0
	43	46	29	55	38	0
	36	19	29	44	33	0
	62	19	30	50	32	0
	65	58	32	46	33	0
	70	43	26	44	23	0
L. casei subsp. *rhamnosus*	ATCC 7469	34	28	51	31	0
	RC15	52	29	48	27	0
	RC17	54	39	47	30	0
	GR1	33	38	44	26	0
	81	86	37	52	37	39
L. fermentum	A60	29	27	54	33	0
	B54	105	46	55	38	50
L. gasseri	56	90	46	47	29	70
	60	67	43	47	24	35
L. jensenii	RC28	87	40	47	30	36
L. plantarum	RC6	25	31	49	24	0
	RC20	79	43	45	30	31

[a] Data are averages for three separate bacterial cultures. Standard deviations were 14, 7, 5, and 5° for contact angles (θ) with water (W), formamide (F), methylene iodide (M), and 1-bromonaphthalene (1-B), respectively. Hexadecane contact angles were 0° for all strains.

[b] Average standard deviation was 15%.

than a month. The strains were identified to the species and strain levels under microaerophilic conditions by using API CH50 strips and fermentation tests according to specifications of the Virginia Polytechnic Institute manual (4a) and subsequently stored at −70°C in skim milk. They included the following strains isolated between 1982 and 1986 from the healthy urogenital tract of adults and stored with minimal numbers of passages: *L. casei* subsp. *rhamnosus* GR1, RC15, and RC17; *L. acidophilus* RC14; *L. plantarum* RC6 and RC20; and *L. jensenii* RC28. The other previously isolated strains were the dairy strain *L. casei* subsp. *rhamnosus* 81, poultry isolates *L. fermentum* B54 and A60 and *L. acidophilus* T13, and American Type Culture Collection strain *L. casei* subsp. *rhamnosus* ATCC 7469. In addition, 11 fresh isolates were tested, including 6 from healthy women (*L. casei* 55, 8, and 70; *L. gasseri* 60; and *L. acidophilus* 68 and 75) and 5 from women with histories of urogenital infections (*L. casei* 43, 36, 62, and 65 and *L. gasseri* 56).

Culture conditions. Stored strains were thawed, streaked on MRS agar (Merck, Darmstadt, Germany) and incubated overnight at 37°C in a 5% CO_2 incubator. Subsequently, an inoculum in 10 ml of MRS broth was prepared and cultured under the same conditions. After 24 h, 150 μl of the culture was inoculated into 10 ml of fresh broth. Bacteria harvested from this culture were designated cells passaged once. Inoculation into fresh broth was repeated up to 20 times and cells were harvested for measurements after 10 and 20 serial passages. All experiments were done in triplicate with three separate bacterial cultures.

Contact angle measurements. Contact angles were measured essentially as described by van der Mei et al. (14) and originally proposed by Van Oss and Gillman (21). Briefly, cells were harvested and washed twice in Millipore Q water by centrifugation at 10,000 × *g* for 10 min. Bacterial lawns were then prepared on cellulose acetate membrane filters (Millipore; pore diameter, 0.45 μm) by negative pressure filtration of a bacterial suspension. Filters with bacterial lawns were quickly glued to a thin layer of dental wax just above its melting point on an aluminum disc and then immediately fixed by placing the aluminum disc in contact with ice. This procedure prevented wrinkling and crack formation on the lawns. Next, discs with mounted filters were dried at 37°C for 2 to 3 h in order to obtain so-called plateau contact angles (14).

Occasionally, scanning electron microscopy was done in order to check the homogeneity of the bacterial lawns on the filters. To this end, bacterial preparations were exposed to osmium tetroxide vapor overnight. Subsequently, they were air dried, sputtered with gold, and examined. Figure 1 shows a representative example of a lawn of lactobacilli used for contact angle measurements.

Contact angles were measured with water, formamide, methylene iodide, 1-bromonaphthalene, and hexadecane (two droplets of each liquid on one filter).

MATH. The MATH test was originally proposed by Rosenberg (11), and the method used in our study was that of van der Mei et al. (14). Briefly, cells were harvested and washed twice in 10 mM phosphate buffer (pH 7.0) by centrifugation and resuspended in the same buffer to an optical density, A_0 (at 600 nm), of between 0.4 and 0.6. Next, 150 μl of hexadecane was added to 3 ml of bacterial suspension, and the two-phase system was vortexed for two periods of 30 s with an interval of 5 s between periods. Subsequently, 10 min was allowed for phase separation, and the optical density (A) was measured again. The percentage of cells in the hexadecane fraction was calculated by the formula percent adhesion = $[1 - (A/A_0)] \times 100$ and used as a measure of hydrophobicity.

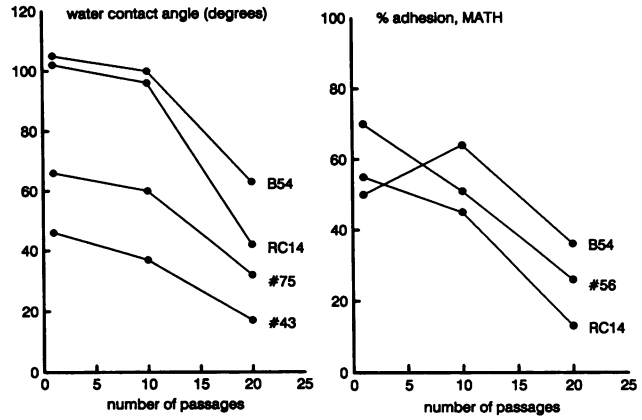

FIG. 2. Hydrophobicities expressed by water contact angles and percent adhesion of lactobacilli to hexadecane in MATH as a function of the number of serial passages of the strains in liquid medium. Data are given only for those strains showing a significant ($P < 0.05$ for paired analysis by the Student t test between passages 1 and 20) decrease in hydrophobicity. The average standard deviation for the separate bacterial cultures was 14° for water contact angles and 15% for the percent adhesion.

RESULTS

Table 1 summarizes the contact angles of the five liquids used and the percent adhesion of the lactobacilli to hexadecane. As can be seen, the hydrophobicities of the strains vary within wide ranges, with the *L. acidophilus* strains being relatively hydrophobic and the *L. casei* strains being relatively hydrophilic. For the other species, different hydrophobicities for the strains tested were observed. Note that hexadecane contact angles were zero for all strains (Table 1, footnote *a*).

Figure 2 shows that only some of the isolates lost their hydrophobic properties after serial passaging in liquid medium. Thus, most isolates can be considered stable with respect to hydrophobicity.

In Fig. 3, the relationships between contact angles with polar (water versus formamide) and nonpolar (methylene iodide versus 1-bromonaphthalene) liquids are given. Interestingly, a relationship is less evident for strains after serial passaging than for the strains subcultured only once.

Figure 4 presents the hydrophobicity of the strains as measured by MATH as a function of the water contact angle for lactobacilli after 1 and 20 serial passages. Prior to serial passages, the percent adhesion to hexadecane was zero for strains having water contact angles below 60°. When water contact angles on the strains exceeded 60°, the percent adhesion to hexadecane of those strains increased proportionally with the water contact angles. Although a similar pattern existed for cells after serial passages, the percent adhesion to hexadecane began to increase for water contact angles above 30°, and the data showed more scatter than they showed for the cultures passaged only once.

DISCUSSION

A number of properties of some of the lactobacillus strains studied have been measured previously with a view toward understanding more about their role in the healthy flora of the intestine and urogenital tract. Their adhesiveness to epithelia is important and appears to be mediated by lipoteichoic acids (2) and extracellular proteinaceous adhesins. The

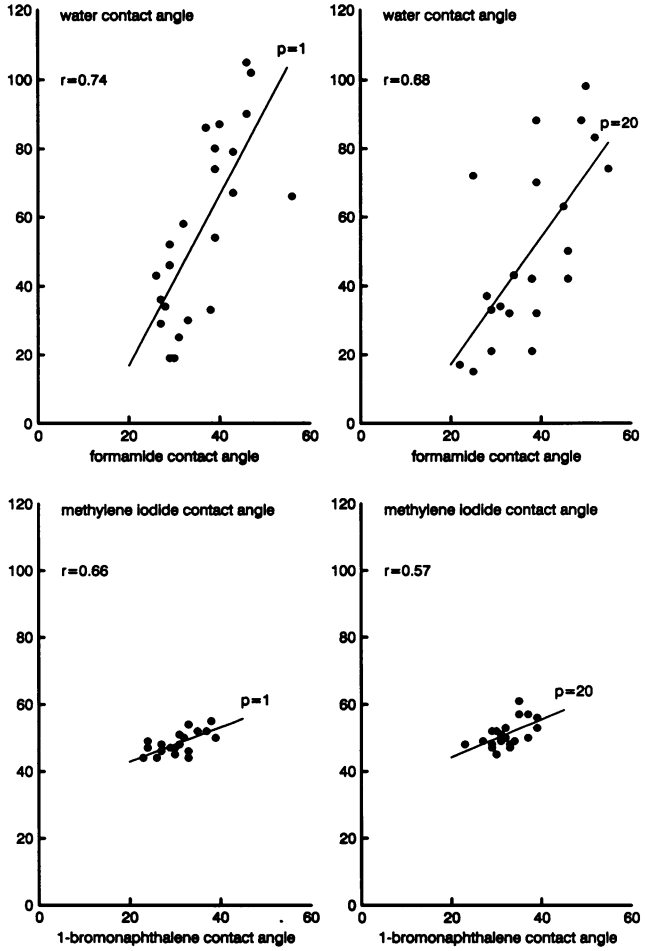

FIG. 3. Water contact angles as a function of formamide contact angles and methylene iodide contact angles as a function of 1-bromonaphthalene contact angles for isolates of lactobacilli passaged once (p=1) and after 20 serial passages (p=20). The lines represent the results of a linear least-square fit of the data (r denotes the coefficient of linear correlation). All contact angles are in degrees. For standard deviations of the data, see Table 1.

effect that these components and other outer cell wall substances have on hydrophobicity is not known but is probably of importance in the adhesion process. In the present study, serial passaging in liquid medium clearly induced a loss of hydrophobic properties for *Lactobacillus* strains 43, 56, 75, RC14, and B54 (Fig. 2). A secondary observation was that there existed a reduced correlation, for strains cultured 20 times compared with those cultured only once, between the contact angles for different liquids (Fig. 3) as well as between water contact angles and their hydrophobicities as derived from the MATH test (Fig. 4). In addition, cultures passaged once which had water contact angles of less than 60° did not adhere to hexadecane droplets used for the MATH test. After 20 passages, however, some of the lactobacilli whose lawns had water contact angles between 30 and 40° degrees did show adhesion to hexadecane. As a speculative explanation for the above result, we propose that serial passaging of lactobacilli may cause a clear loss of hydrophobic properties for some strains but that for the majority of strains, heterogeneous cultures, in each of which a fraction of cells have already lost their hydrophobicity, will

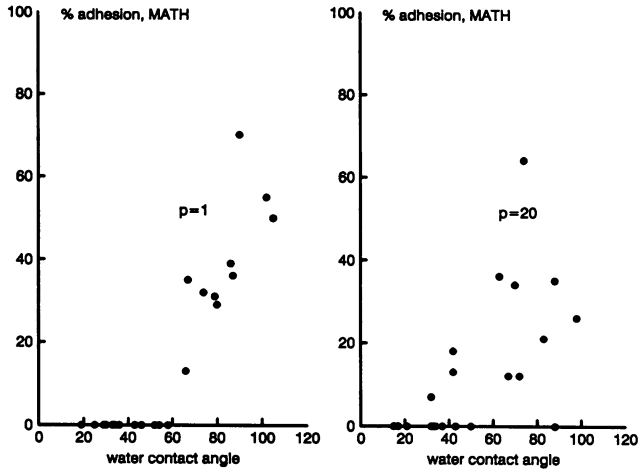

FIG. 4. Percent adhesion to hexadecane in MATH as a function of water contact angles for isolates of lactobacilli passaged once (p=1) and after 20 serial passages (p=20). The average standard deviation for three separate bacterial cultures was 14° for water contact angles and 15% for the percent adhesion.

develop. This culture heterogeneity causes a range of responses for the individual cells, while the contact angle measures an average property for the culture, thereby yielding scattered relations between the outcomes of the hydrophobicity tests. It is possible that for some strains, the loss of hydrophobic properties in MRS broth reflects the lack of a need to adhere to a surface in order to survive.

Several authors have speculated on the actual mechanism of MATH and its relation with contact angles. Van Loosdrecht et al. (19) used strictly physicochemical arguments based on the surface Gibbs energy of partitioning and indeed found, using 20 widely different strains, adhesion to hexadecane droplets in MATH to occur for cells with water contact angles above 30 to 40°. However, the arguments of van Loosdrecht et al. (19) ignore typical features of microbial cell surfaces, such as surface heterogeneity and the possible presence of surface appendages. This most likely explains why van der Mei et al. (16) could not find a relation between the MATH hydrophobicity and water contact angles for a collection of oral streptococcal strains unless they confined the analysis to a series of closely related mutants of *Streptococcus salivarius* HB or, as in the study of Vanhaecke et al. (18), to isolates of one species. In this respect, it is interesting to note that van Loosdrecht et al. (19) found their relation between water contact angles and MATH for a collection of seemingly unrelated, widely different strains but that in their study an unintentional selection was made for nonaggregating strains showing no wall growth in culture (23).

The positive correlation between MATH hydrophobicity and water contact angles found by Vanhaecke et al. (18) for 15 *P. aeruginosa* strains and in this study for 23 lactobacillus strains is broadly similar to that predicted and found by van Loosdrecht et al. (19), although in their study adhesion to hexadecane started to occur only for water contact angles of about 60° (19). However, for nine *Streptococcus mitis* strains, no relation between the outcomes of the two hydrophobicity tests was evident (17). Therefore, any conclusion concerning MATH and contact angles based on a given collection of strains should not be generalized for other strains.

It is interesting to note that hexadecane contact angles were zero on all strains, despite differential outcomes of MATH. This supports a recent conclusion of van der Mei et al. (14) based on studies with *Acinetobacter calcoaceticus* and *Serratia marcescens* strains which showed that MATH does not probe the affinity of cells for hexadecane but rather probes their aversion to water.

Both water and formamide are polar liquids with hydrogen-donating and hydrogen-accepting properties (20, 22), which is reflected by the relation between the contact angles of the two liquids on our bacterial lawns (Fig. 3). In contrast, methylene iodide, 1-bromonaphthalene, and hexadecane are completely apolar, but because of their relatively low surface tensions, the relation between their contact angles (Fig. 3) is slightly weaker than for the high-surface-tension liquids. The surface tension of hexadecane is much lower than those of the other two apolar liquids, explaining the complete spreading of hexadecane on all strains.

Although the main aim of this study did not involve a clinical outcome, the data indicate that for some lactobacilli targeted for human suppositories or dairy or up-scale industrial fermentation, care should be taken to monitor surface changes during serial passaging. In addition, these properties should be monitored over the time span during which experiments are carried out with these organisms.

In summary, three key points have been demonstrated. (i) The hydrophobicity of lactobacillus strains varies within wide ranges on the basis of both MATH and water contact angles. (ii) Serial passaging of clinical isolates in liquid medium may cause a loss of surface hydrophobicity, but most of the strains examined were relatively stable. (iii) The MATH test is based on the aversion of cells to water rather than on an affinity for hexadecane. Partitioning of cells in MATH does not occur when water contact angles are below 60° for the present collection of lactobacillus strains when the cells are passaged only once.

ACKNOWLEDGMENT

This work was supported by grant MA9994 from the Medical Research Council of Canada.

REFERENCES

1. **Busscher, H. J., and A. H. Weerkamp.** 1987. Specific and nonspecific interactions in bacterial adhesion to solid substrata. FEMS Microbiol. Rev. **46**:165–173.
2. **Chan, R. C. Y., G. Reid, R. T. Irvin, A. W. Bruce, and J. W. Costerton.** 1985. Competitive exclusion of uropathogens from human uroepithelial cells by *Lactobacillus* whole cells and cell wall fragments. Infect. Immun. **47**:84–89.
3. **Dillon, J. K., J. A. Fuerst, A. C. Hayward, and G. H. G. Davis.** 1986. A comparison of five methods for assaying bacterial hydrophobicity. J. Microbiol. Methods **6**:13–19.
4. **Eisen, A., and G. Reid.** 1989. Effect of culture media on *Lactobacillus* hydrophobicity and electrophoretic mobility. Microb. Ecol. **17**:17–25.

4a. **Holdeman, L. V., E. P. Cato, and W. G. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed., p. 63–71. Virginia Polytechnic Institute and State University, Blacksburg.

5. **Mozes, N., F. Marchal, M. P. Hermesse, J. L. van Haecht, L. Reuliaux, A. J. Léonard, and P. G. Rouxhet.** 1987. Immobilization of microorganisms by adhesion; interplay of electrostatic and non-electrostatic interactions. Biotechnol. Bioeng. **30**:439–450.
6. **Mozes, N., and P. G. Rouxhet.** 1987. Methods for measuring hydrophobicity of microorganisms. J. Microbiol. Methods **6**:99–112.
7. **Reid, G., and A. W. Bruce.** 1991. Development of lactobacilli therapy to prevent recurrent urinary tract infections in females.

Int. Urogynecol. J. **2**:40–43.

8. **Reid, G., A. W. Bruce, J. A. McGroarty, K.-J. Cheng, and J. W. Costerton.** 1990. Is there a role for lactobacilli in prevention of urogenital and intestinal infections? Clin. Microbiol. Rev. **3**:335–344.
9. **Reid, G., R. L. Cook, and A. W. Bruce.** 1987. Examination of strains of lactobacilli for properties that may influence bacterial interference in the urinary tract. J. Urol. **138**:330–335.
10. **Reid, G., L. A. Hawthorn, R. Mandatori, R. L. Cook, and H. S. Beg.** 1988. Adhesion of lactobacilli to polymer surfaces *in vivo* and *in vitro*. Microb. Ecol. **16**:241–251.
11. **Rosenberg, M.** 1984. Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. FEMS Microbiol. Lett. **22**:289–295.
12. **Rosenberg, M., and R. J. Doyle.** 1990. Microbial cell surface hydrophobicity: history, measurement, and significance, p. 1–37. *In* R. J. Doyle and M. Rosenberg (ed.), Microbial cell surface hydrophobicity. American Society for Microbiology, Washington, D.C.
13. **Rosenberg, M., and S. Kjelleberg.** 1986. Hydrophobic interactions: role in bacterial adhesion. Microb. Ecol. **9**:353–393.
14. **Van der Mei, H. C., M. M. Cowan, and H. J. Busscher.** 1991. Physico-chemical and structural studies on Acinetobacter calcoaceticus RAG-1 and MR-481. Two standard strains in hydrophobicity tests. Curr. Microbiol. **23**:337–341.
15. **Van der Mei, H. C., M. Rosenberg, and H. J. Busscher.** 1991. Assessment of microbial cell surface hydrophobicity, p. 263–290. *In* N. Mozes, P. S. Handley, H. J. Busscher and P. G. Rouxhet (ed.), Microbial cell surface analysis—Structural and physico-chemical methods. VCH Publishers, Inc., New York.
16. **Van der Mei, H. C., A. H. Weerkamp, and H. J. Busscher.** 1987. A comparison of various methods to determine the hydrophobicities of streptococcal cell surfaces. J. Microbiol. Methods **6**:277–287.
17. **Van der Vegt, W., H. C. Van der Mei, J. Noordmans, and H. J. Busscher.** 1991. Assessment of bacterial biosurfactant production through axisymmetric drop shape analysis by profile. Appl. Microbiol. Biotechnol. **35**:766–770.
18. **Vanhaecke, E., J.-P. Remon, M. Moors, F. Raes, D. De Rudder, and A. Van Peteghem.** 1990. Kinetics of *Pseudomonas aeruginosa* adhesion to 304 and 316-L stainless steel: role of cell surface hydrophobicity. Appl. Environ. Microbiol. **56**:788–795.
19. **van Loosdrecht, M. C. M., J. Lyklema, W. Norde, G. Schraa, and A. J. B. Zehnder.** 1987. The role of bacterial cell wall hydrophobicity in adhesion. Appl. Environ. Microbiol. **53**:1893–1897.
20. **Van Oss, C. J., M. K. Chaudhury, and R. J. Good.** 1988. Interfacial Lifshitz-Van der Waals and polar interactions in macroscopic systems. Chem. Rev. **88**:927–941.
21. **Van Oss, C. J., and C. F. Gillman.** 1972. Phagocytosis as a surface phenomenon. I. Contact angles and phagocytosis of nonopsonized bacteria. J. Reticuloendothel. Soc. **12**:283–292.
22. **Van Oss, C. J., R. J. Good, and M. K. Chaudhury.** 1986. The role of Van der Waals forces and hydrogen bonds in "hydrophobic interactions" between biopolymers and low energy surfaces. J. Colloid Interface Sci. **111**:378–390.
23. **Zehnder, A. J. B.** Personal communication.