APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1997, p. 1725–1731 Vol. 63, No. 5
0099-2240/97/$04.00+0

Cell Surface Characteristics of *Lactobacillus casei* subsp. *casei*, *Lactobacillus paracasei* subsp. *paracasei*, and *Lactobacillus rhamnosus* Strains

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Received 2 October 1996/Accepted 3 March 1997

Hydrophilic and electrostatic cell surface properties of eight *Lactobacillus* strains were characterized by using the microbial adhesion to solvents method and microelectrophoresis, respectively. All strains appeared relatively hydrophilic. The strong microbial adhesion to chloroform, an acidic solvent, in comparison with microbial adhesion to hexadecane, an apolar *n*-alkane, demonstrated the particularity of lactobacilli to have an important electron donor and basic character and consequently their potential ability to generate Lewis acid-base interactions with a support. Regardless of their electrophoretic mobility (EM), strains were in general slightly negatively charged at alkaline pH. A pH-dependent behavior concerning cell surface charges was observed. The EM decreased progressively with more acidic pHs for the *L. casei* subsp. *casei* and *L. paracasei* subsp. *paracasei* strains until the isoelectric point (IEP), i.e., the pH value for which the EM is zero. On the other hand, the EM for the *L. rhamnosus* strains was stable from pH 8 to pH 3 to 4, at which point there was a shift near the IEP. Both *L. casei* subsp. *casei* and *L. paracasei* subsp. *paracasei* strains were characterized by an IEP of around 4, whereas *L. rhamnosus* strains possessed a markedly lower IEP of 2. The present study showed that the cell surface physicochemical properties of lactobacilli seem to be, at least in part and under certain experimental conditions, particular to the bacterial species. Such differences detected between species are likely to be accompanied by some particular changes in cell wall chemical composition.

Lactobacilli are gram-positive, nonsporulated, and anaerobic bacteria (27). They are normal inhabitants of the oral cavity and the digestive tract in humans. Some *Lactobacillus* strains are used in food fermentations, and typical examples are found in the dairy industry for the production of cheese, yogurt, and other fermented milk products (12). In the latter case, these microorganisms may also have an adverse effect due to their adhesive properties resulting in a microbial biofilm formation, a well-known source of bacterial contamination and alteration of dairy products (7, 12, 14).

The bacterial adhesion depends partly on reversible and, subsequently, irreversible interactions (2, 6, 32, 35). The initial and reversible stage is mediated by a complex of physicochemical interactions, including hydrophobicity and charges (5, 13, 25, 32), which are ubiquitously thought to be nonspecific but important properties. A few studies have reported the involvement of physicochemical factors in the capacity of *Lactobacillus* strains to adhere to a cellular or inert support (18, 21, 22, 31, 38). Moreover, such factors seem sometimes to be involved in the pathogenesis of certain human infections, such as root surface caries (31) and endocarditis (22, 37). However, their implication in the mechanisms of attachment and colonization remains obscure. This is, in fact, complicated by an insufficient understanding of the physicochemical surface properties of lactobacilli.

The first aim of this study was therefore to determine the cell surface characteristics and the potential ability to generate physicochemical interactions with a support of eight *Lactobacillus* strains, including two *L. casei* subsp. *casei* strains, three *L. paracasei* subsp. *paracasei* strains, and three *L. rhamnosus* strains. Some of these strains were specifically isolated from various dairy products. The second aim was to eventually detect differences between the species used herein, allowing us to discriminate between them by establishing a set of physicochemical criteria with regards to three parameters, i.e., hydrophobic/hydrophilic character, Lewis acid-base interactions, and electrostatic properties. Investigations of these three aspects were performed by using the microbial adhesion to solvents (MATS) method, which has been recently developed (3), and microelectrophoresis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Eight *Lactobacillus* strains of three different species were studied. *L. paracasei* subsp. *paracasei* DN114.001 and DN114.003 and *L. rhamnosus* DN116.007 and DN116.030 were isolated from dairy products (Groupe Danone Collection) (CIRDC, Le Plessis-Robinson, France) (36, 41). The other four strains were American Type Culture Collection (Biovalley, Conches, France) or Institut Pasteur Collection (Institut Pasteur, Paris, France) reference strains (8, 22, 38). All strains were identified with biochemical analysis by using API 50CH identification systems (Biomérieux). They were stored at −70°C until use.

For each experiment, bacteria were thawed, subcultured overnight, and grown in de Man-Rogosa-Sharpe liquid reference medium (MRS broth) (Biokar Diagnostics) adjusted to pH 5.4 with HCl (11). Cultures were performed at 37°C for 24 h in static conditions. Growth curves showed that bacteria harvested beyond 20 h were in the stationary phase.

Chemicals. The salt KNO_3 (Sigma) and chemical products in the highest purity grade [chloroform (Sigma-Aldrich), *n*-hexadecane (Sigma), ethyl acetate (Aldrich), HNO_3 (Prolabo), and KOH (Prolabo)] were obtained commercially.

MATS method. MATS was measured according to the method originally proposed by Rosenberg et al. (40) and recently modified by Bellon-Fontaine et al. (3). Briefly, bacteria were harvested in the stationary phase by centrifugation at 5,000 × *g* for 10 min, washed twice, and resuspended to an optical density of

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TABLE 1. MATS

Species and strains	% of adhesion (± SD)[a] to:		
	Hexadecane	Chloroform	Ethyl acetate
L. casei subsp. *casei*			
ATCC 393	18.0 ± 2.1	76.8 ± 4.1	24.1 ± 1.2
ATCC 27139	2.7 ± 0.8	84.4 ± 1.8	13.9 ± 0.7
L. paracasei subsp. *paracasei*			
DN114.001	8.3 ± 1.5	82.3 ± 2.8	14.2 ± 0.7
DN114.003	9.6 ± 1.7	82.2 ± 1.0	13.2 ± 0.6
CIP 103138	8.2 ± 1.6	97.4 ± 0.8	22.3 ± 0.9
L. rhamnosus			
ATCC 7469	8.7 ± 0.5	79.9 ± 1.4	16.5 ± 1.1
DN116.007	5.8 ± 1.4	70.7 ± 1.5	11.3 ± 1.3
DN116.030	26.5 ± 2.2	85.2 ± 2.1	11.7 ± 1.8

[a] Means ± standard deviations of two measures of at least three separate experiments.

0.4 at 400 nm (A_0) (approximately 10^8 CFU ml^{-1} cell density) in 0.1 M KNO_3 (pH 6.2). This high concentration in electrolytes was used to avoid charge interferences, since it has been reported that some solvent droplets, especially hexadecane, are negatively charged in aqueous suspensions (17). To 1.2 ml of cell suspension was added 0.2 ml of solvent. After a 10-min preincubation at room temperature, the two-phase system was mixed on a vortex for 2 min. To allow complete phase separation of the mixture, the aqueous phase was removed after 15 min and its optical density at 400 nm (A_1) was measured. The percentage of microbial adhesion to solvent was calculated as $(1 - A_1/A_0) \times 100$.

Three different solvents were tested in this study: hexadecane, which is an apolar solvent; chloroform, a monopolar and acidic solvent; and ethyl acetate, a monopolar and basic solvent (3). Only microbial adhesion to hexadecane reflects cell surface hydrophobicity or hydrophilicity because electrostatic interactions are absent, as noted above. The values of MATS obtained with the two other solvents, chloroform and ethyl acetate, were regarded as a measure of electron donor/basic and electron acceptor/acidic characteristics of bacteria, respectively (3, 43, 44). Furthermore, it should be noted that all these three solvents have similar van der Waals properties. As a consequence of their surface tension characteristics, differences in the results between hexadecane-chloroform and hexadecane-ethyl acetate enabled us to indicate the existence of Lewis acid-base interactions at the bacterial cell surface (3, 43, 44).

The analysis of an eventual bacterial lysis under the action of the three solvents was previously monitored with lactic acid bacteria by phase-contrast microscopy. No deleterious effects were observed (3).

Microelectrophoresis. Electrophoretic mobility (EM) was measured to determine the cell surface net charge of the bacteria (4, 9). Immediately prior to the measurements, cells in the stationary phase were harvested by centrifugation at $5{,}000 \times g$ for 10 min, washed twice, and resuspended in KNO_3.

The EM as a function of pH was first determined in 1 mM KNO_3 (ionic strength of 1 mM) at a concentration of around 10^7 cells/ml. KNO_3 solution is the reference medium commonly used to avoid nonspecific absorption of ions on cell surfaces. The pH of the resuspending fluid was adjusted to 2, 3, 4, 5, 6, 7, and 8 by addition of KOH or HNO_3. Electrophoretic mobilities of the bacteria with the appropriate pH values were measured at room temperature on a ZM 77 Zetameter model (Zetameter Inc., New York, N.Y.). The EMs, expressed in 10^{-8} $m^2 \cdot V^{-1} \cdot s^{-1}$, were derived from the velocities of the bacteria in suspension under an applied electric field of 100 V.

The influence of the ionic strength of the resuspending fluid on the bacterial surface charge was also investigated. The effects of three ionic concentrations (1, 100, and 160 mM KNO_3) were studied. In order to ascertain the highest ionic concentrations that did not affect (i) bacterial viability and (ii) results about EM, cell counts were performed. No modifications were observed.

Statistical analysis. A Student's *t* test for paired or unpaired samples was used to compare the results (30).

RESULTS

MATS method. Three different solvents were employed by using the MATS method to evaluate the hydrophobic/hydrophilic cell surface properties of lactobacilli and their Lewis acid-base characteristics. The results are reported in Table 1.

First, direct measurements of the cell surface hydrophobicity and hydrophilicity were carried out by the partitioning of cells between aqueous and hexadecane at a high ionic strength of 0.1 M (pH 6.2). The very low percentages of bacteria which adhered to this apolar solvent, ranging from 2.7 to 26.5%, demonstrated a hydrophilic surface, regardless of the *Lactobacillus* strains tested.

Microbial adhesion to chloroform was then examined (Table 1). The results showed an overall strong affinity of lactobacilli to this acidic solvent and electron acceptor. These higher values of adhesion were compared with those obtained for hexadecane because both solvents possess the same van der Waals properties. The important difference observed was due to the implication of Lewis acid-base interactions resulting from the electron donor and basic character of bacterial strains.

The data obtained for ethyl acetate, which is a strongly basic solvent and electron donor, produced results contrary to those encountered with chloroform: the bacterial adhesion to this third solvent was low, ranging from 11.3 to 24.1%. It confirmed the nonacidic character of the bacterial strains studied.

Moreover, whatever the solvent, there were no significant differences between the three bacterial species studied.

Microelectrophoresis. The cell surface net charge of the bacteria was examined by microelectrophoresis, which measures the EM of microorganisms in the stationary phase.

EM was first determined in 1 mM KNO_3 at various pHs, ranging from 2 to 8. Figures 1 to 3 represent the pH-dependent EM measurements of *L. casei* subsp. *casei*, *L. paracasei* subsp. *paracasei*, and *L. rhamnosus* strains, respectively. The results obtained with EM as a function of pH revealed four different features. (i) All bacterial strains were negatively charged at high pH values (alkaline pH) and progressively became positively charged with decreasing pH values (acidic pH). (ii) The electronegativity was variable between strains at a given pH. (iii) The profiles of the EM in relation to the pH were nearly identical for the different strains of the same species. Indeed, the EM was progressively modified with lower pH for both *L. casei* subsp. *casei* and *L. paracasei* subsp. *paracasei* with a variation of the charge sign at the isoelectric points (IEPs), i.e., the pH values for which the EM is zero (Fig. 1 and 2). For the *L. rhamnosus* strains, the EM was almost constant from pH 8 to pH 3 until an important and quick shift of the surface charge at the IEP (Fig. 3). (iv) The IEP was also relatively similar for the different strains of the same species (Table 2). The highest IEPs were measured for *L. casei* subsp. *casei* and *L. paracasei* subsp. *paracasei* strains (IEP of 4), whereas *L. rhamnosus* strains acquired a lower IEP at around pH 2.

Second, the effects of the ionic strength of the resuspending fluid were evaluated (Table 3). The EM, for a single strain, was influenced by the ionic concentration in KNO_3. As expected, the EM was drastically reduced with increasing ionic strength. As a consequence of their low electronegativity, neither *L. casei* subsp. *casei* nor *L. paracasei* subsp. *paracasei* exhibited any EM because their charges may have been masked by an ionic strength of ≥100 mM. The high charge of *L. rhamnosus* strains decreased progressively as a function of the ionic strength and was evident even at a very high concentration in electrolytes, especially for the ATCC 7469 and DN116.007 *L. rhamnosus* strains. On the other hand, differences were observed in various bacterial species at 1 mM KNO_3 (Table 3). *L. rhamnosus* strains were significantly the most electronegative bacteria tested in this study, compared to *L. paracasei* subsp. *paracasei* strains ($P < 0.05$) and *L. casei* subsp. *casei* strains ($P < 0.001$). Furthermore, *L. paracasei* subsp. *paracasei* strains were significantly more electronegative than *L. casei* subsp. *casei* strains ($P < 0.001$).

Relationships between MATS method and microelectrophoresis. Investigations were performed to establish correlations between the electrophoretic mobility of bacteria and the

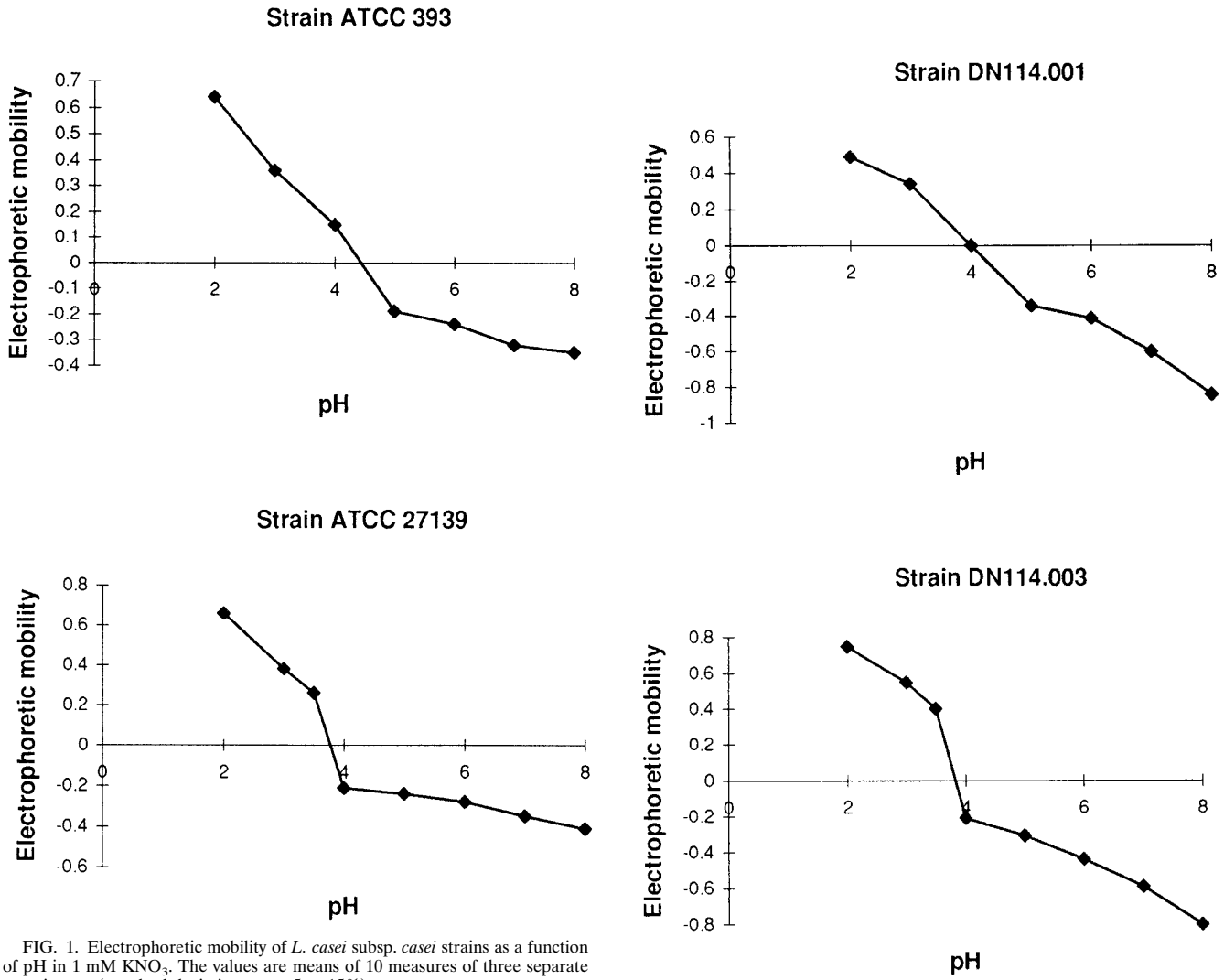

FIG. 1. Electrophoretic mobility of *L. casei* subsp. *casei* strains as a function of pH in 1 mM KNO_3. The values are means of 10 measures of three separate experiments (standard deviation was ≤5 to 15%).

hydrophilic character or Lewis acid-base properties. Few correlations were found between cell surface charges and the hydrophilicity of lactobacilli, as determined by using a correlation coefficient of 0.318 (Fig. 4). Furthermore, no relationships seemed to exist between the electrostatic properties and the electron donor profile of the microorganisms ($r = 0.103$).

DISCUSSION

In spite of several studies on the cell surface hydrophobicity and charges of lactobacilli, these physicochemical aspects remain poorly understood. Several techniques are usually employed to assess cell surface properties, such as microelectrophoresis (4, 9, 10, 16), contact angle measurements (9, 38, 39) or microbial adhesion to hexadecane in a two-phase system (10, 22, 38), Fourier transform infrared spectroscopy (9), and X-ray photoelectron spectroscopy (9, 10, 29, 33).

In the present study, we used two physicochemical techniques to determine the cell surface characteristics and the potential ability to adhere to a support of eight *Lactobacillus* strains, of which four were isolated from various dairy products and the other four were reference strains. These strains belonged to three different species: *L. casei* subsp. *casei*, *L. pa-*

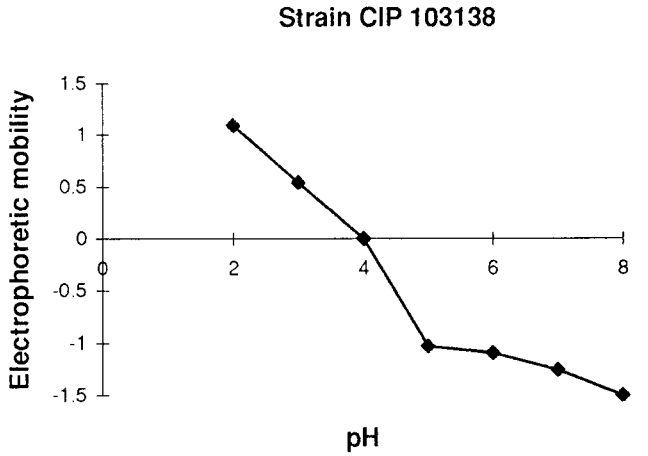

FIG. 2. Electrophoretic mobility of *L. paracasei* subsp. *paracasei* strains as a function of pH in 1 mM KNO_3. The values are means of 10 measures of three separate experiments (standard deviation was ≤5 to 15%).

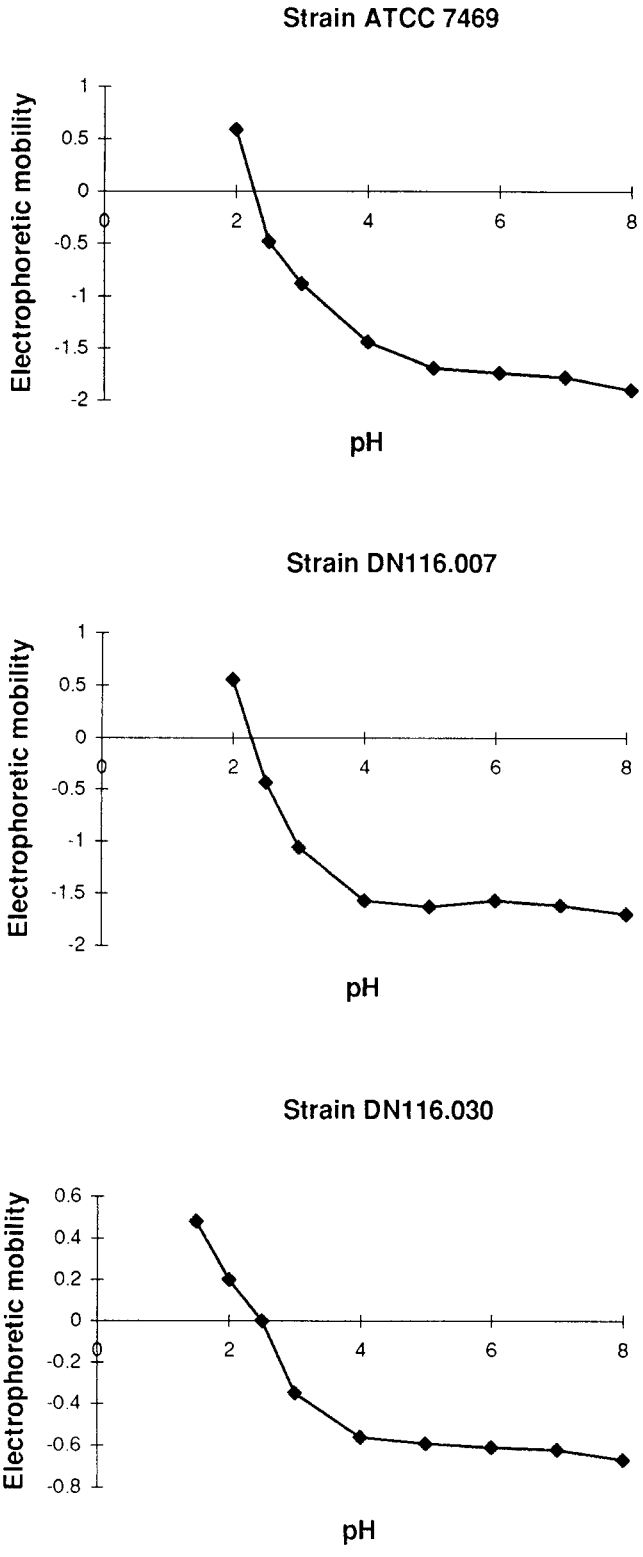

FIG. 3. Electrophoretic mobility of *L. rhamnosus* strains as a function of pH in 1 mM KNO_3. The values are means of 10 measures of three separate experiments (standard deviation was ≤5 to 15%).

TABLE 2. IEP of *Lactobacillus* strains in 1 mM KNO_3

Species and strains	IEP[a]
L. casei subsp. *casei*	
ATCC 393	4.4
ATCC 27139	3.7
L. paracasei subsp. *paracasei*	
DN114.001	4.0
DN114.003	3.8
CIP 103138	4.0
L. rhamnosus	
ATCC 7469	2.3
DN116.007	2.3
DN116.030	2.4

[a] The IEP was evaluated with an error of ±0.1 pH unit.

racasei subsp. *paracasei*, and *L. rhamnosus*. These species are among the most commonly encountered species in the dairy industry. The aim of our work was to expand knowledge about the cell surface properties of lactobacilli and, potentially, detect differences between the species used that would allow the establishment of discriminating criteria.

First, cell surface hydrophobicity was examined by using a classical microbial adhesion to hexadecane test. The results indicated that the microorganisms studied were relatively hydrophilic (Table 1). Similar results concerning the two strains *L. casei* subsp. *casei* ATCC 393 and *L. rhamnosus* ATCC 7469 were previously obtained by Harty et al.; they had hydrophilicity values of 24.6 ± 7.7 and 9.0 ± 8.2, respectively (22). *L. casei* subsp. *casei* ATCC 27139 and *L. rhamnosus* DN116.007 were fully hydrophilic. This hydrophilic nature of lactobacilli, regardless of the species, has often been encountered in previous studies (1, 9, 22, 38).

Microbial adhesion to two other solvents was also investigated (Table 1). This simple MATS method was recently developed to assess the Lewis electron donor/electron acceptor properties of bacterial surfaces (3). All lactobacilli tested here displayed maximal affinity for an acidic solvent such as chloroform and low affinity for a basic solvent such as ethyl acetate. These results demonstrated that lactobacilli are strong electron donors and weak electron acceptors, as confirmed by their hydrophilic cell surface properties. In other words, lactobacilli have a strong basic and a weak acidic character. The quantitatively important existence of chemical groups such as $-COO^-$ and $-HSO_3^-$ at the surface of microorganisms could explain their strong electron donor character.

We subsequently studied electrostatic cell surface properties of lactobacilli by measuring the EM in microelectrophoresis, which is a common method to determine cell surface charges (24, 32). The bacterial charge is attributed to cell wall constituents, e.g., phosphate and carboxylate groups, proteins, etc (19). First, microelectrophoresis was performed as a function of pH. Cells were suspended in 1 mM KNO_3 (low ionic strength) at different pH values, resulting in various degrees of protonation of surface chemical groups, therefore affecting the cell surface charges and, thereby, the EM. The EM was variable between strains and species and depended on the pH value. Lactobacilli in this study must be regarded as having surfaces with a slightly negative charge at alkaline pH. This is in accordance with the results previously obtained by Harty et al. (22) and Cuperus et al. (10). Furthermore, the IEPs seemed to be relatively specific for the *Lactobacillus* species. Therefore, the IEP might be a characteristic of a certain bacterial

TABLE 3. Bacterial EM (10^{-8} $m^2 \cdot V^{-1} \cdot s^{-1}$) in various ionic concentrations at pH 7.4 for *Lactobacillus* strains

Species and strain	EM (mean ± SD) at indicated ionic concn of KNO_3		
	1 mM	100 mM	160 mM
L. casei subsp. *casei*			
ATCC 393	−0.34 ± 0.02	0	0
ATCC 27139	−0.36 ± 0.03	0	0
L. paracasei subsp. *paracasei*			
DN114.001	−0.72 ± 0.11	0	0
DN114.003	−0.70 ± 0.09	0	0
CIP 103138	−1.34 ± 0.20	0	0
L. rhamnosus			
ATCC 7469	−1.84 ± 0.06	−0.61 ± 0.05	−0.32 ± 0.05
DN116.007	−1.66 ± 0.07	−0.61 ± 0.08	−0.38 ± 0.04
DN116.030	−0.65 ± 0.02	0	0

species (Table 2). The IEPs for the *L. casei* subsp. *casei* and *L. paracasei* subsp. *paracasei* strains were rather high, ranging from 3.7 to 4.4. On the other hand, *L. rhamnosus* strains possessed a low IEP of 2.3 to 2.4, which was much more in agreement with hydrophilic cell surface properties previously identified (Table 1). Similar results for the *L. rhamnosus* strains with respect to the IEP were obtained by other authors (9, 10). For example, Cuperus et al. found an IEP of 2.0 for *L. rhamnosus* ATCC 7469 (9).

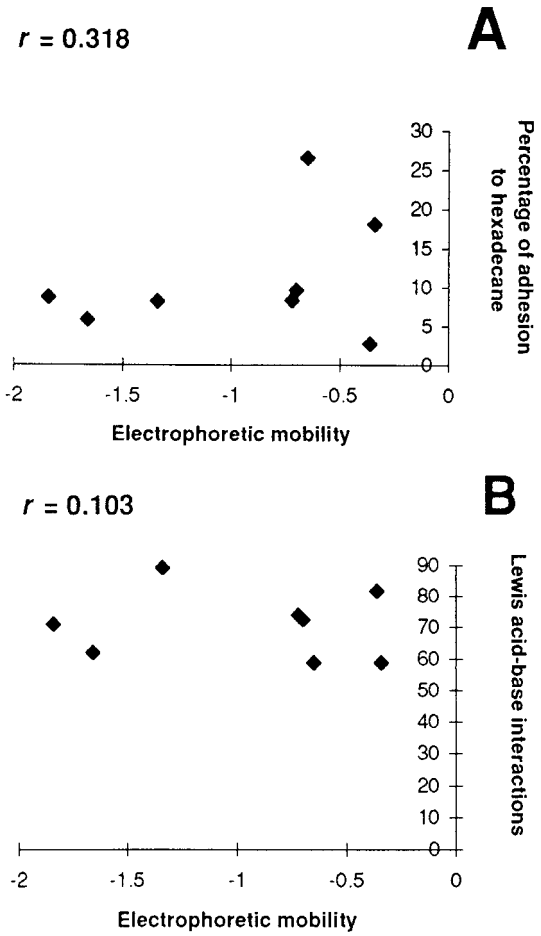

FIG. 4. Relationships between electrostatic properties and hydrophilicity (percentage of adhesion to hexadecane) (A) or Lewis acid-base interactions (difference between microbial adhesion to chloroform and microbial adhesion to hexadecane) (B). *r*, coefficient of linear correlation.

The profile of the EM in relation to the pH appeared also to be relatively characteristic of the *Lactobacillus* species. Both *L. casei* subsp. *casei* and *L. paracasei* subsp. *paracasei* had an electrophoretic profile progressively modified with lower pH with a variation of the charge sign at the IEP (Fig. 1 and 2). We speculate that the progressive reduction in cell surface electronegativity with decreasing pH might be due to the gradual degrees of protonation of several and various chemical groups. In this case, each level would indicate the respective pK from the different groups. The shift observed at the IEP could signify the last pK displayed. For the *L. rhamnosus* strains, the EM was relatively constant between pH 8 and pH 3 until an important and quick shift of the surface charge at the IEP at a more acidic pH (Fig. 3). This could mean that a predominant chemical group was present and was protonated at its pK. So, the change of EM as a function of pH is essentially reminiscent of a titration of the charged surface groups. These observations are in accordance with the 1979 report by James of typical pH mobility curves for model surfaces, especially with carboxyl groups (23).

The cell surface charge in relation to the ionic strength of the resuspending fluid was also investigated (Table 3). Cell surfaces became less electronegative with increasing ionic strengths. This reduction of the charge was attributed to the important adsorption of cations, which led to the neutralization of components mediating the surface charges. Significant differences were observed between bacterial species with regard to their electronegativity at given pH and ionic concentration.

Another aim of this work was to compare the possible relationships between electrostatic properties and hydrophilic or Lewis acid-base properties. A lack of marked correlation was evident. A similar observation has also been put forward by other authors concerning a correlation between cell surface hydrophobicity and charge (15, 26). On the other hand, numerous previous studies on the physicochemistry of microbial cell surfaces have shown relationships between surface charges, hydrophobicity, and elemental surface compositions of the cells (9, 20, 42). They indicate that the presence of (glyco-)proteinaceous material at the cell surface results in a higher hydrophobicity (9), whereas a hydrophilic surface was associated with the presence of polysaccharides. Lipoteichoic acids and other outer cell wall substances might have an effect on hydrophobicity as well, but it is unclear.

The notable difference of bacterial affinity for hexadecane and chloroform, solvents having identical van der Waals forces (3), showed the importance of the Lewis acid-base interactions at the cell surface of *Lactobacillus* strains. These data therefore demonstrate the capacity of lactobacilli to establish some interactions with a support other than those of van der Waals, for example. Hence, this physicochemical parameter must in fact occupy an important place among the physiological properties of lactobacilli and should be considered with much more attention in further studies of industrial processes. Indeed, microbial biofilm formation responsible for contamination and alteration of food products is a good example thereof, since physicochemical factors are recognized to play an important role with respect to bacterial adhesion. If we consider the potential ability of the lactobacilli used in this study to adhere by a nonspecific mechanism, the almost identical hydrophilic character of the strains leads us to suppose similar attachment

in conditions with a high ionic strength. On the contrary, lactobacilli will probably have different adhesion behaviors in the function of their various electrostatic properties in a low ionic strength medium.

The carbohydrate metabolism of lactobacilli usually allows a taxonomic differentiation between species, as seen in API systems (12, 27). Indeed, this differential fermentation has been exploited by microbiologists for a long time. Alternative technologies are also being developed, such as soluble protein patterns (28), DNA-DNA hybridizations (8), and rRNA probes (34). To our knowledge, no information exists about a possible classification of microorganisms with regard to their physicochemical cell surface properties. Therefore, our findings showing similar physicochemical properties among strains of the same species, regardless of the electrophoretic profile and the isoelectric point, might eventually be used in a physicochemical-taxonomic perspective as additional information for microbial classification. However, further investigations are necessary to validate this concept.

In conclusion, the results presented here demonstrated the tendency of lactobacilli to have an important electron donor and basic character and their capacity to generate Lewis acid-base interactions with a support. Similarities between strains of the same bacterial species with regard to their physicochemical properties have never been encountered in previous studies. Systematic changes were observed for all isolates representing a certain species, especially cell surface charges. They therefore allow us to conclude that these strains probably have similar surface architectures.

ACKNOWLEDGMENTS

We are greatly indebted to Tuomo Karjalainen for comments and critical reading of the manuscript, Vanessa Tagher for her English corrections, and Marie-Christine Degivry for providing us with bacterial strains.

This work was supported by Groupe Danone.

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