Cells and Materials

Volume 2 | Number 4 Article 1

1992

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CELLS AND MATERIALS, Vol. 2, No. 4, 1992 (Pages 271-280) 1051-6794/92$3.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

THE EFFECT OF SERIAL PASSAGING OF LACTOBACILLI IN LIQUID MEDIUM ON THEIR PHYSICO-CHEMICAL AND STRUCTURAL SURFACE CHARACTERISTICS

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(Received for publication June 24, 1992, and in revised form November 22, 1992)

Abstract

The aim of this study was to examine the effect of serial passaging in liquid medium on physico-chemical and structural surface characteristics of two lactobacillus strains. To this end, starting cultures (p = 1) of urogenital isolate *L. acidophilus* RC14 and poultry isolate *L. fermentum* B54 were serially passaged in liquid medium up to 20 times (p = 20). In both strains, similar physico-chemical and structural changes were observed following serial passaging. According to water contact angle measurements, cells in p = 20 cultures became less hydrophobic. Microelectrophoresis showed that isoelectric points shifted towards the acidic region upon serial passaging. In addition, the strains had become heterogeneous with respect to the pH dependence of their zeta potentials. According to FTIR and XPS, the surfaces of p = 20 cultures of both strains showed decreasing amounts of proteinaceous material and increasing amounts of polysaccharide-like substances as compared to p = 1 cultures. Heterogeneity of p = 20 strains was also reflected in structural features of the cell surface at the electron microscopic level. In p = 1 cultures of both strains, all cells had a stained layer: in contrast, in p = 20 cultures about half of the cells became devoid of this layer. The study clearly showed that surface characteristics of lactobacilli may change following serial passaging in liquid medium. Knowledge of these changes is of importance because these microorganisms are considered for use in biomedical applications like the restoration of a healthy vaginal microflora by cell seeding.

Key words: Lactobacilli, serial passaging, zeta potential, contact angle, hydrophobicity, XPS, FTIR, surface composition, RR-staining layer.

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Introduction

Lactobacilli play a dominant role in the vaginal flora of healthy women [6]. The acidic environment of the vagina, resulting in part from the production of lactic acid, is advantageous for these strains. They can therefore successfully compete with other microorganisms including, for example, *Escherichia coli*. This natural barrier against infection is believed to be enhanced by the production of hydrogen peroxide by some lactobacillus strains [7]. The healthy urogenital ecosystem is vulnerable and may be disturbed easily by, for examples, changes in the hormonal status or by catheterization [1, 5]. Pathogens, which normally would have no access to the system in which lactobacilli act as a protective coat, may then become dominant.

Surface characteristics of both urogenital epithelia and lactobacilli as well as interactions between these different kinds of surfaces play an important role in their mutual adhesion. These characteristics comprise net surface charge, hydrophobicity and the presence of surface appendages and specific receptor sites. Eisen and Reid [3] emphasized that knowledge of surface characteristics of these microorganisms is of importance to eventually design a cocktail of strains that could be useful to restore a healthy vaginal flora. Surface characteristics of bacteria may, however, change with culture conditions [3], and the outcome of clinical trials may then be different than expected.

Recently, 23 lactobacillus strains of different origin have been investigated with respect to the effect of serial passaging on hydrophobicity [10]. It was found that the hydrophobicities differed considerably between strains. Furthermore, some strains lost their hydrophobicity after serial passaging in liquid medium. Although only some strains showed a clear loss of hydrophobicity, it was hypothesized that in cultures of other strains only a proportion of the cells had lost their hydrophobicity and that these cultures had become heterogeneous. This hypothesis developed from the observations that a relation was lacking between the outcomes of two different hydrophobicity tests. Physico-chemical surface properties of seven strains were further characterized by microelectrophoresis, Fourier transform infrared

spectroscopy (FTIR), and X-ray photoelectron spectroscopy (XPS) [2] in order to obtain information with respect to net surface charge, molecular composition, and elemental surface composition, respectively. Differences in hydrophobicity and zeta potentials of the strains could be explained in terms of the chemical composition of the cell surfaces, while neglecting, at this stage, possible differences in structural features of the cell surface and their influence on the physico-chemical characteristics of the cell surfaces [2].

If lactobacillus strains are to be employed in an attempt to force restoration of a healthy vaginal flora [9], it is of importance to know the effects of serial passaging on those surface characteristics of strains of interest that determine their adhesion.

The aim of this study is, therefore, to further examine the physico-chemical and structural changes brought about at the surfaces of two strains that showed, in prior studies, the largest decrease in hydrophobicity upon serial passaging in liquid medium [10]. To this end, microelectrophoresis, water contact angle measurements, FTIR, XPS and electron microscopy were done on starting cultures (first isolates) and on strains after 20 serial passages in liquid medium. At this point, it should be noted that only microelectrophoresis and electron microscopy are techniques that allow one to distinguish individual cells in a culture which has become heterogeneous with respect to surface properties.

Materials and Methods

Bacteria and Culture Conditions

Lactobacilli investigated in this study were *L. acidophilus* RC14 (a human urogenital isolate) and *L. fermentum* B54 (a poultry isolate). In this text, these isolates will be further referred to by their strain number only.

Strains were thawed, streaked on Lactobacillus agar according to De Man, Rogosa and Sharp (MRS-agar, Merck, Germany) and incubated for 24 hours at 37°C in an atmosphere containing 5% CO_2. Subsequently, an inoculation was made in 10 ml MRS-broth and cultured under the same conditions (p = 1). Passaging of strains comprised of a daily repetitive inoculation of 150 μl of the culture into 10 ml fresh broth over a total period of three weeks (p = 20 culture). For experiments with p = 1 and p = 20 cultures, about 3 ml of these were inoculated into 50 ml fresh broth and grown overnight for 18 hours. Cells were harvested and washed twice in Millipore Q water by centrifugation at 10,000 *g* for 10 minutes.

Microelectrophoresis

Washed bacterial cells were resuspended in 10 mM potassium phosphate buffer at a concentration of 10^7-10^8 cells.ml^{-1}. The pH of the suspending fluid was adjusted to 2, 3, 4, 5, 7 and 9 by the addition of HCl or KOH, respectively. Zeta potentials were measured of four independently grown cultures of every strain at each pH with a Lazer Zee meter 501 (PenKem, Bedford Hills, NY, USA). The calculation of apparent zeta potentials from the measured electrophoretic mobilities, as done here, is based on the assumed validity of the Helmholtz-Smoluchowski equation [4] and isoelectric points (IEP) estimated by linear extra- or interpolation of their pH dependence.

The principle of manual operation of the apparatus is that a camera transfers a microscopic image from the bacteria in suspension, obtained by scattering of laser light, to a TV monitor via a rotating prism. Depending on the velocity of the bacteria in suspension under an applied electric field of 150 V, adjustment of the rotational speed of the prism enables the operator to 'freeze' movement of bacteria on the monitor. The rotational speed of the prism can then be used to calculate the apparent zeta potential. If the bacterial culture is heterogeneous, different bacterial speeds may be present, and the operator will have to record two or more zeta potentials for the different types of bacteria in suspension. This procedure was, in a few cases, also performed by a recently developed, operator independent automated image analysis system connected with the Lazer Zee meter [8].

Contact Angle Measurements

Water contact angles (WCA) were measured essentially as described by Van der Mei *et al.* [11] and originally proposed by Van Oss and Gillman [14]. Briefly, bacterial lawns with a thickness of about 50 layers on cellulose acetate membrane filters (Millipore; pore diameter 0.45 μm) were prepared by negative pressure filtration of a suspension of washed cells. Filters with bacterial lawns were quickly glued to a thin layer of dental wax just above its melting point on an aluminium disc and then immediately fixed by placing the aluminium disc in contact with ice. This procedure prevented wrinkling and crack formation of the lawns. Discs with mounted filters were dried at 37°C for 2 to 3 hours in order to obtain so-called plateau contact angles [13]. Of every bacterial strain, two independent cultures were grown. Of each culture, two bacterial lawns were prepared on which water contact angles were recorded. On each lawn, 3-4 water contact angles were measured whereafter the values were averaged. The average for the two lawns was taken as the final value. Using this technique, only the hydrophobicity of a culture as a whole can be assessed.

Fourier Transform Infrared Spectroscopy (FTIR)

Pellets of washed cells were frozen in liquid nitrogen and freeze-dried in a Leybold Hereaus Combitron CM30. Bacterial samples were hereafter combined with KBr (1:50 by weight; total weight of mixture approximately 100 mg), grounded and pressed for 30 seconds to obtain translucent pellets. Infrared absorption spectra were recorded on a MX-S spectrometer from Nicolet Instruments (spectral resolution and wavenumber accuracy 4 cm^{-1} and 0.01 cm^{-1}, respectively). A KBr pellet was used as background reference and all measurements were composed of 500 scans. Quantitation of

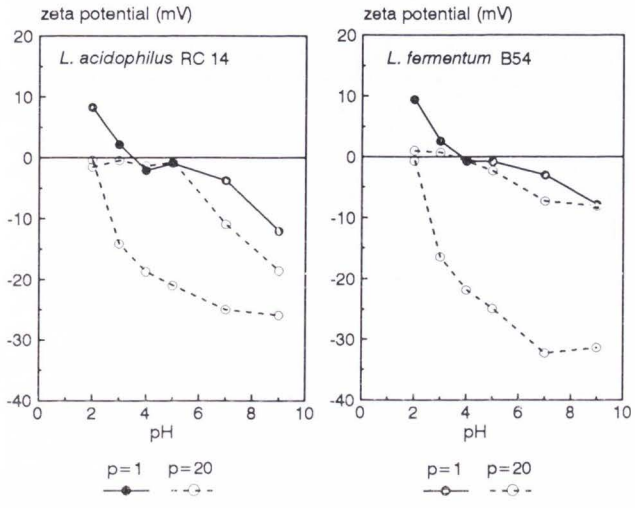

Figure 1. Zeta potentials of *Lactobacillis acidophilus* RC14 and of *Lactobacillis fermentum* B54 as a function of pH in 10 mM potassium phosphate buffer measured manually are shown. For the p = 20 cultures, two differently charged populations were present within one culture, indicated by the dotted lines. Vertical bars denote for each pH the mean standard deviations over observations on four independently grown p = 1 and p = 20 cultures at that pH.

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a spectral region of interest was obtained by integration of the region and normalization with respect to the CH absorption band region around 2930 cm^{-1}. Using this technique, only the average molecular composition of the freeze-dried material can be determined.

X-ray Photoelectron Spectroscopy (XPS)

Freeze-dried bacterial samples were obtained as described above. Bacterial samples were then pressed in a stainless steel trough and left overnight in a Vacuum Generators ESCA 3 Mk II instrument to reach a vacuum better than 10^{-8} Torr. The instrument was equipped with a Tracor Northern TN 1710 signal averager to enhance the signal to noise ratio. X-ray emission was generated by a magnesium anode at 14 kV, 20 mA. After a scan of the overall spectrum, peaks were recorded in the following order: C_{1s}, O_{1s}, N_{1s}, P_{2p} and again C_{1s} to check for the absence of time dependent variation due to sample deterioration and/or contamination. The area under each peak after linear background subtraction was used to calculate peak intensities using the Wagner sensitivity factors [15] and yield elemental surface concentration ratios N/C, O/C and P/C. The carbon peak was decomposed by a least square fitting program into three Gaussian components set at 285.0 eV, 286.6 eV, and 288.4 eV, representing carbon in C-C and C-H bonds (C1 fraction), C-O and C-N bonds (C2 fraction), and -(C=O)-NH bonds (C3 fraction), respectively. The oxygen peak was decomposed into two components: one set at 533.2 eV, thought to be representative for oxygen in C-O bonds (O2 fraction) as in polysaccharides, and the other component set at 531.3 eV, thought to be representative for other functional groups (O1 fraction) as C=O. This technique allows only for the elemental surface composition of entire samples under investigation and not for the ones of possible sub-populations.

Electron Microscopy

Washed cells were fixed in a 1:1:1 mixture of Ruthenium red in double distilled water (1.5 mg/ml), 3.5% glutaraldehyde in double distilled water and 0.2 M sodium cacodylate, pH 6.5 for 1 hour at 0°C. Cells were then washed three times in 0.07 M cacodylate buffer. Postfixation was carried out in a mixture as described above, the aldehyde being replaced by 4% osmium tetroxide and for 3 hours at 27°C. Following one washing step in 0.07 M cacodylate buffer, cells were pelleted in 2% agarose in 0.07 M cacodylate buffer to facilitate further manipulation. Small blocks of agar containing the bacteria were then dehydrated over a graded series of ethanol (50%, 70%, 96% and 100% twice). Propylene oxide-Epon 812 mixtures of increasing polymer content preceded embedding in pure Epon 812 (Serva, Heidelberg, New York). Thin sections were cut with a diamond knife on a LKB 2128 Ultrotome. They were poststained on copper grid with uranyl acetate and lead citrate. Samples were observed in an Akashi 002A transmission electron microscope operated at 80 kV.

Results and Discussion

Microelectrophoresis

Fig. 1 shows the pH dependence of the zeta potentials of p = 1 and p = 20 cultures of both lactobacillus strains. Note that standard deviations increase with increasing pH values. The pH dependence of the zeta potentials is roughly similar for the p = 1 cultures of both strains. For p = 20 cultures, however, a clear dichotomy in the pH dependence of the zeta potentials occurs within each strain, i.e., the cultures have become heterogeneous. In p = 20 cultures of both strains, the IEP shifted towards the acidic region (see also Fig. 3). Results in Fig. 1 were obtained manually and contain no information on the fraction of cells with a given zeta potential. Such information can be obtained from the automated, image analysis based measurements, as shown in Fig. 2, for *L. fermentum* B54 at pH 4 only. This confirms the dichotomy observed manually.

Water Contact Angles

In both strains, water contact angles on bacterial lawns of p = 1 cultures are high (102 and 105 degrees for RC14 and B54, respectively), which indicates a hydrophobic surface. In the p = 20 cultures, a large decrease in water contact angles on lawns of the heterogeneous populations occurs with respect to the homogeneous p = 1 cultures (Fig. 3).

Fourier Transform Infrared Spectroscopy (FTIR)

The IR spectra are basically the same for the two strains and their p = 1 and p = 20 cultures. The most

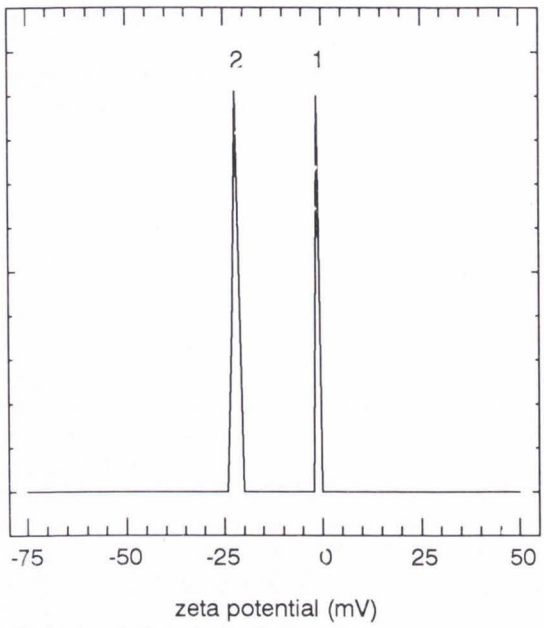

Figure 2. Zeta potential distribution, measured by means of automated image analysis of *Lactobacillus fermentum* B54 in 10 mM potassium phosphate buffer at pH 4, confirmed the observed dichotomy in zeta potentials of p = 20 cultures by manual measurements. Analysis involved 30-40 individual bacteria; peak 1 represents 40% of the cells, whereas peak 2 represents 60%.

Table 1. Chemical composition data for *Lactobacillis acidophilus* RC14 and *Lactobacillis fermentum* B54 and their changes upon serial passaging, i.e., p = 1 and p = 20 cultures, including infrared absorption band ratios[1], and elemental surface concentration ratios[2] and chemical functionalities in which carbon and oxygen containing groups are involved.

	RC14 p = 1	RC14 p = 20	B54 p = 1	B54 p = 20
AmI/CH	9.5	8.8	9.6	8.3
AmII/CH	3.1	2.7	3.2	2.5
PI/CH	1.3	1.4	1.5	1.3
PII/CH	4.8	7.2	5.6	6.4
N/C	0.076	0.055	0.082	0.065
O/C	0.449	0.615	0.494	0.590
P/C	0.007	0.004	0.007	0.004
C1	0.38	0.26	0.36	0.28
C2	0.46	0.59	0.49	0.57
C3	0.17	0.15	0.15	0.15
O1	0.13	0.27	0.14	0.23
O2	0.87	0.73	0.86	0.77

[1]duplicate cultures coincided within 5%.
[2]duplicate cultures coincided within 10%.

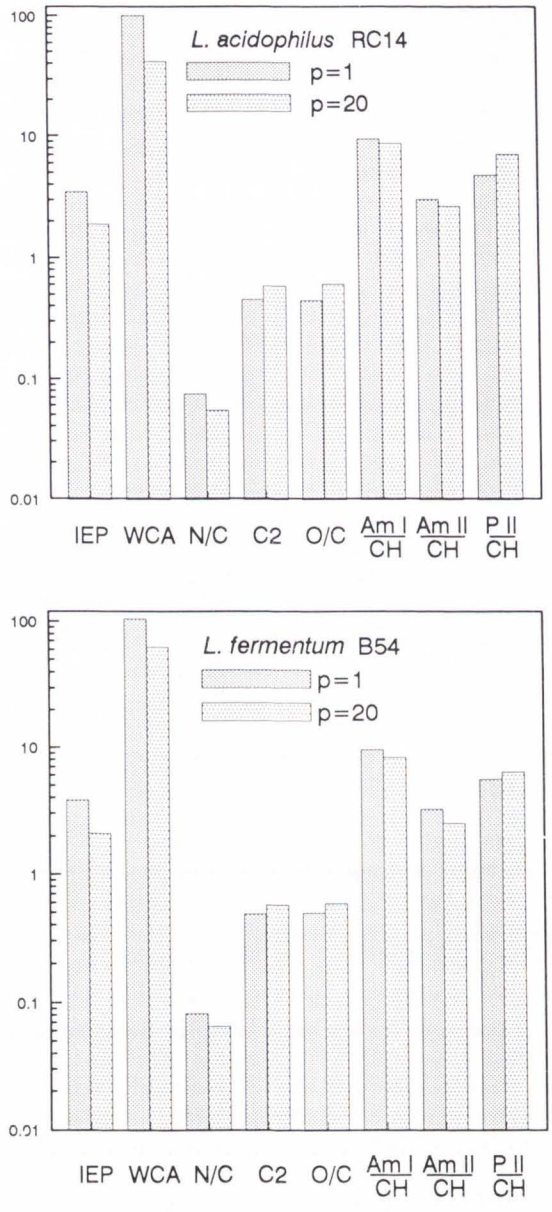

Figure 3. Physico-chemical surface characteristics of *Lactobacillus acidophilus* RC14 and *Lactobacillus fermentum* B54 showing the largest changes upon serial passaging. Units: WCA (degrees), all other parameters are dimensionless.

important bands are located at 2932 cm^{-1} (CH band: CH_2-CH_3 stretching), 1656 cm^{-1} (AmI band: C=O stretching in proteins), 1541 cm^{-1} (AmII band: N-H bending in proteins), 1239 cm^{-1} (PI: phosphates) and at 1072 cm^{-1} (PII: polysaccharides). The relative areas under the various absorption bands, however, differ between strains and cultures after serial passaging. The absorption band ratios of AmI, AmII, PI and PII with respect to the CH-band around 2932 cm^{-1} are summarized in Table 1. Quantitative differences in molecular composition of the two strains and cultures are clearly

present. Following serial passaging, the AmI/CH and AmII/CH absorption band ratios, indicative for proteins, decrease in both strains, whereas the PII/CH absorption band ratio, indicative for polysaccharides, increases. Trends for the PI/CH absorption band ratio (phosphates) are not obvious. Major changes in IR absorption band ratios upon serial passaging are included in Fig. 3.

X-ray Photoelectron Spectroscopy (XPS)

Table I also summarizes the elemental surface concentration ratios determined by XPS, as well as the results of the decomposition of the C_{1s} and O_{1s} peaks.

Only minor differences in elemental surface composition of strains B54 and RC14 become obvious from the XPS analysis, but for both strains the p = 1 culture shows major differences with the p = 20 culture. Elemental surface concentration ratios N/C decrease in both strains upon serial passaging, whereas the reverse holds true for O/C. In both strains, C2 and O1 fractions increase upon serial passaging. Values for N/C, O/C and C2, both prior to and after serial passaging, are plotted in Fig. 3.

Electron Microscopy

Fig. 4 shows differences between the p = 1 and p = 20 culture of RC14 at the transmission electron microscopic level. In the p = 1 culture, all cells are covered with a relative thick stained layer (SL, see Figs. 4a and 4b). In the p = 20 culture, however, two cell types are present. One cell type has a SL, similar to the p = 1 culture, whereas the other cells are devoid to a smaller or greater extent of such a thick SL (Figs. 4c, 4d and 4e). Exactly the similar structural difference between p = 1 and p = 20 cultures at the transmission electron microscopic level can be seen for B54 in Fig. 5.

As mentioned before, only microelectrophoresis and electron microscopy distinguish between cell types within one culture. Yet, also the other techniques, probing only average characteristics per culture, indicate major differences for the p = 1 and p = 20 cultures, despite the fact that the heterogeneity in a culture comprised approximately 50% of the cells. Therefore, it may well be that the differences in chemical composition, isoelectric point and hydrophobicity between the two cell types in the p = 20 cultures are about twice as large as indicated in Fig. 3.

Although it is known that cells can change their surface characteristics upon serial passaging, this has not been studied hitherto by a combination of physico-chemical and electron microscopic methods for lactobacilli. Since the loss of a SL of these cells upon serial passaging is concurrent with a decrease in IEP and in water contact angle, we suggest, also on the basis of previous work with oral streptococci [12], that the cells lose glyco-proteinaceous material from their surfaces upon serial passaging. This suggestion is confirmed by the decrease in N/C as by XPS and in AmI/CH and AmII/CH as by FTIR. Most likely this loss of glyco-proteinaceous material is accompanied by an increased exposure of surface polysaccharides, indicated by increased values for both O/C and C2 as by XPS and PII/CH as by FTIR.

The development of heterogeneous populations upon serial passaging in liquid medium, confirms our previous suggestion made in this direction [10]. The argument for that suggestion was indirect, though: The absence of a correlation between water contact angles and adhesion to hexadecane of these lactobacilli. Continued serial passaging might eventually result in the loss of the SL by all cells in a culture and therewith in a population which is homogeneous again with respect to its surface characteristics.

The observations presented here are of importance for those working with lactobacilli in order to develop techniques involving seeding of the urogenital tract with these cells, since only 20 passages of a culture in liquid medium may result in significantly altered surface properties. Furthermore, the observations attest to the usefulness of microelectrophoresis, when desired in combination with electron microscopy, for the study of heterogeneous bacterial populations. Additionally, in the present case, those techniques only probing average culture characteristics were able to pick up the changes in surface characteristics induced by serial passaging of the isolates.

Acknowledgements

This work was supported in part by grant MA 9994 from the Medical Research Council of Canada. Furthermore, the support of "Department of Education and Scientific Research - Belgium" ("Concerted Action Physical Chemistry of Interfaces and Biotechnology") is gratefully acknowledged. The authors are greatly indebted to Mrs. Marjon Schakenraad-Dolfing and Mrs. Ellen van Drooge for manuscript preparation.

References

1. Catheter-acquired urinary tract infection (1991). The Lancet, **338**, 857-858, (editorial).

2. Cuperus PL, Van der Mei HC, Reid G, Bruce AW, Khoury AH, Rouxhet PG, Busscher HJ (1993). Physico-chemical surface characteristics of urogenital and poultry lactobacilli. J. Coll. Interface Sci., in press.

3. Eisen A, Reid G (1989). Effect of culture media on *Lactobacillus* hydrophobicity and electrophoretic mobility. Microb. Ecol. **17**, 17-25.

4. Hiemenz PC (1977). Electrophoresis and other electrokinetic phenomena. In: Principles of Colloid and Surface Chemistry. Lagowski JJ (ed.), Marcel Dekker, New York, p. 452-487.

5. Kunin CM, McCormick RC (1966). Prevention of catheter-induced urinary tract infections by sterile closed drainage. N. Engl. J. Med. **274**, 1155-1161.

6. Mårdh P-A (1991). The vaginal ecosystem. Am. J. Obstet. Gynecol. **165**, 1163-1168.

7. Nagy E, Petterson M, Mårdh P-A (1991). Antibiosis between bacteria isolated from the vagina of women with and without signs of bacterial vaginosis. APMIS **99**, 739-744.

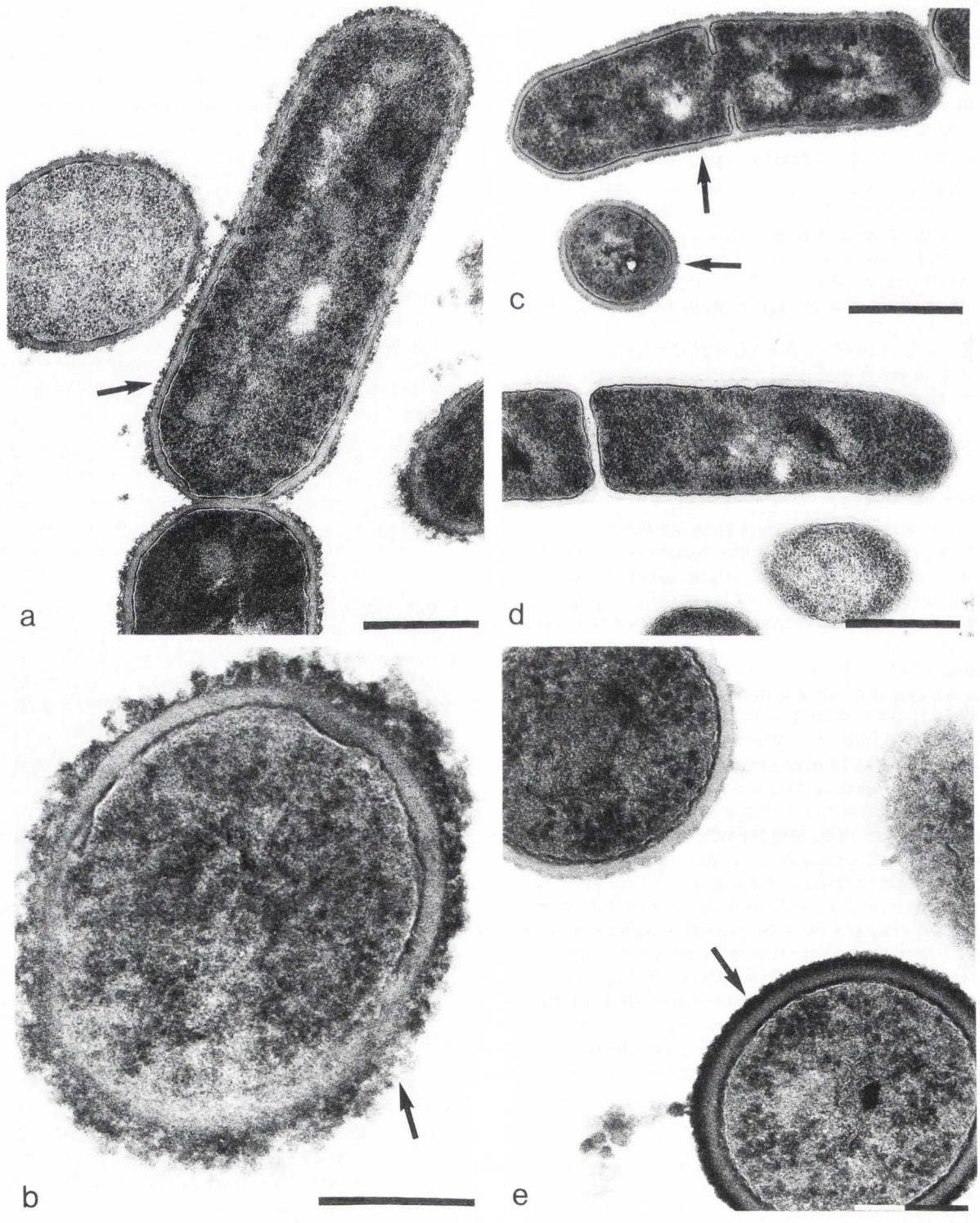

Figure 4. Transmission electron micrographs of sectioned, ruthenium red/uranyl acetate stained *Lactobacillus acidophilus* RC14, showing the differences between p = 1 (**a**, **b**) and p = 20 (**c**, **d**, **e**) cultures. In p = 1 cultures, all cells are covered with a stained layer (a, b; arrows). In the heterogeneous p = 20 cultures, approximately 50% of the cells may be covered with this layer (c, e; arrows), whereas others have become devoid of it (d, e). Bars in Figs. **a**, c, d = 0.5 μm; and in Figs. b, e = 0.2 μm.

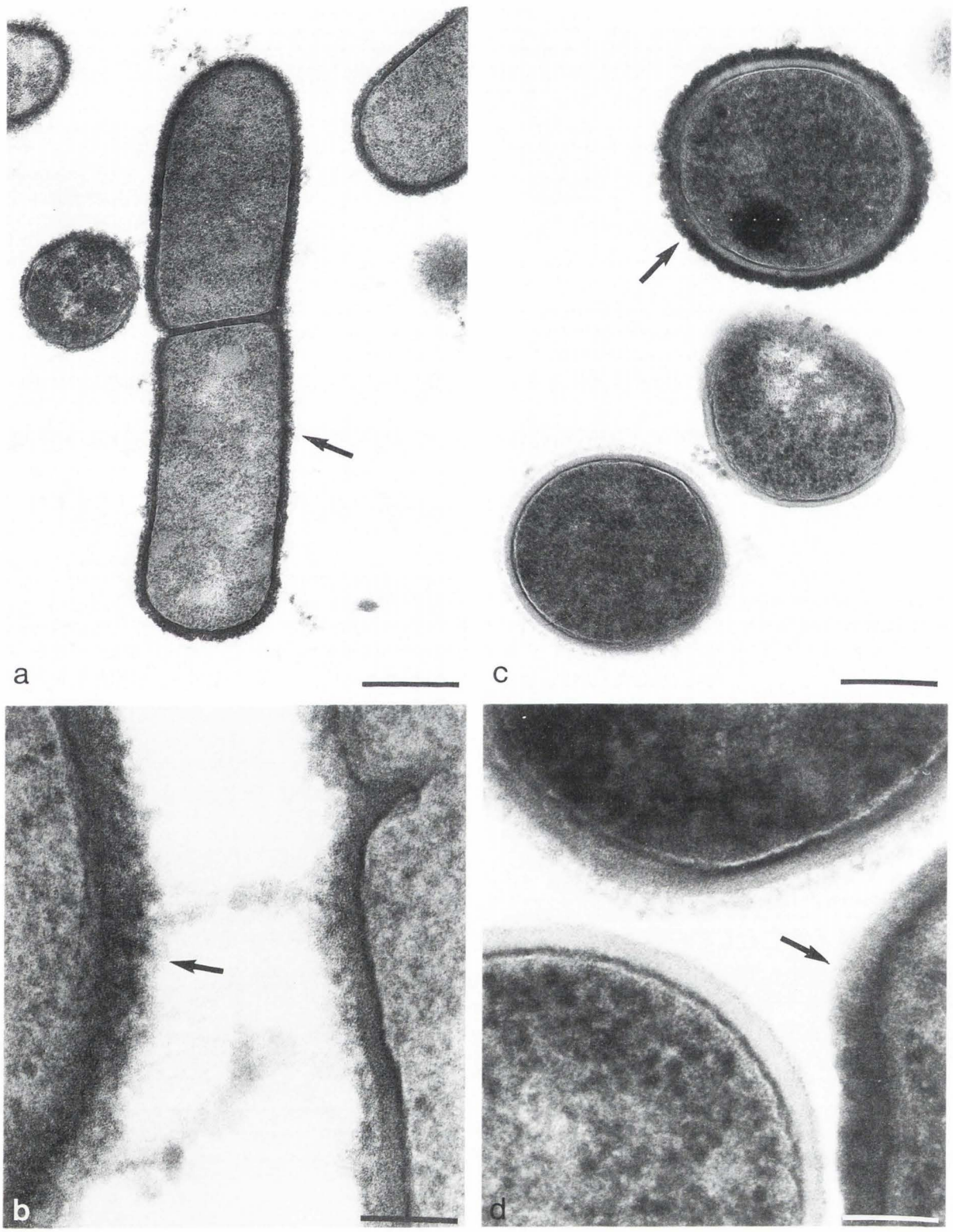

Figure 5. Transmission electron micrographs of sectioned, ruthenium red/uranyl acetate stained *Lactobacillus fermentum* B54, showing the differences between p = 1 (**a**, **b**) and p = 20 (**c**, **d**) cultures. In p = 1 cultures, all cells are covered with a stained layer (a, b; arrows). In p = 20 cultures, about half of the cells are covered with this layer (c, d; arrows), whereas the others have become devoid of it. Bars in Figs. a, c = 0.5 μm; in Figs. b, d = 0.1 μm.

8. Noordmans J, Kempen J, Busscher HJ (1993). Automated image analysis to determine zeta potential distributions in particulate microelectrophoresis. J. Coll. Interf. Sci., in press.

9. Reid G, Bruce AW, McGroarty JA, Cheng K-J, Costerton JW (1990). Is there a role for lactobacilli in prevention of urogenital and intestinal infections? Clin. Microbiol. Rev. **3**, 335-344.

10. Reid G, Cuperus PL, Bruce AW, Van der Mei HC, Tomeczek L, Khoury AH, Busscher HJ (1992). Comparison of contact angles and adhesion to hexadecane of urogenital, dairy and poultry lactobacilli: Effect of serial culture passages. Appl. Environ. Microbiol. **58**, 1549-1553.

11. Van der Mei HC, Weerkamp AH, Busscher HJ (1987). A comparison of methods to determine hydrophobic properties of streptococcal cell surfaces. J. Microbiol. Meth. **6**, 277-287.

12. Van der Mei HC, Leonard AJ, Weerkamp AH, Rouxhet PG, Busscher HJ (1988). Properties of oral streptococci relevant for adherence: zeta potential, surface free energy and elemental composition. Coll. Surf. **32**, 297-305.

13. Van der Mei HC, Rosenberg M, Busscher HJ (1991). Assessment of microbial cell surface hydrophobicity. In: Microbial Cell Surface Analysis - Structural and Physicochemical Methods. Mozes N, Handley PS, Busscher HJ, Rouxhet PG (eds.), VCH Publishers, New York, p. 262-287.

14. Van Oss CJ, Gillman CF (1972). Phagocytosis as a surface phenomenon. I. Contact angles and phagocytosis of nonopsonized bacteria. J. Reticuloendothel. Soc. **12**, 283-292.

15. Wagner CD, Davis LE, Zeller MV, Taylor JA, Raymond RH, Gale LH (1981). Empirical atomic sensitivity factors for quantitative analysis by electron spectroscopy for chemical analysis. Surf. Interf. Anal. **3**, 211-225.

Discussion with Reviewers

T.A. Fassal: Does the MRS broth and culture conditions simulate the environment that the cells will encounter on seeding sufficiently? How can these observations from serial passage in these culture conditions be related to a potentially different set of environmental growth conditions?
Authors: Without going into details, MRS broth of Merck consists mainly of universal peptone, meat extract, yeast extract, glucose dipotassium hydrogen phosphate, diammonium hydrogen phosphate and sodium acetate. According to P.-A. Mårdh [6], vaginal fluid "may contain various portions of cervical secretion, uterine, follicular, and peritoneal fluid, as well as exfoliated epithelial cells, bacteria and bacterial products. Occasionally semen, contraceptive and hygiene products are added to the vaginal contents". Thus, it is clear that MRS-broth does not simulate the vaginal environment. However, we did not aim for that at this stage, but merely wanted to investigate the effect of serial passaging on surface characteristics of lactobacilli and to which end we needed an appropriate culture medium of constant composition.

T.A. Fassal: How may the surface characteristics be selected by different growth conditions?
Authors: We do not know up to what extent surface characteristics may be selected by different growth conditions. We showed that surface characteristics do change upon serial passaging and we anticipate, without proof, that similar changes would have been found in a different medium.

T.A. Fassal: Were the strains selected because they are likely candidates for restoration purposes?
Authors: *L. fermentum* B54 has indeed been used in clinical studies (Reid *et al.* text ref. 9; and McGroarty and Reid, Microbial Ecology in Health and Disease **1**: 215-219, 1988). Since *L. acidophilus* RC14 also showed a clear loss of hydrophobicity, similar to *L. fermentum* B54 upon serial passaging [10], we investigated that strain in addition for comparative reasons.

T.A. Fassal: You found heterogeneity with respect to zeta potentials in p = 20 cultures of both strains, but quantified this only once using an automated image analysis system. Why did you not apply the system on all observations?
Authors: The system was still in development for routine operation, and was only occasionally available.

T.A. Fassal: To show the difference between cells with and without a SL layer, we used ruthenium red-lysine (Fassal *et al.*, Cells and Materials **2**: 37-48; 1992). This staining procedure may show the difference even more dramatic than does ruthenium red alone.
Authors: This may well be also the case for the strains we investigated. The ruthenium red staining, however, proved to be sufficient to demonstrate the difference between cells in p = 20 cultures in our case.

W.H. Wilborn: Vaginal epithelium of the human female in her reproductive years is moist (non-keratinized) stratified squamous. It produces glycogen in response to estrogen stimulation, sheds its superficial cells as squames, and is sometimes coated with mucus produced by cells of the endocervix. What characteristics, other than lactic acid production, would be desirable for lactobacilli to have, in order for them to survive in the vagina and to serve the most beneficial role in respect to protecting and maintaining the integrity of the vaginal epithelium?
Authors: Based primarily on our published *in vitro* data and clinical understanding, we believe that for lactobacilli to survive in the vagina and aid in the protection against infection, they must have: **a**) an adequate nutritional supply; **b**) be able to colonize the mucus and epithelium; **c**) produce substances which interfere, to some

extent, with the growth and colonization of other organisms, for example, bacteriocins, acids, possibly hydrogen peroxide, or via competitive exclusion; and **d**) be able to coaggregate with other bacteria to form a balanced flora. The ability to resist the action of antibiotics and nonoxynol-9 in spermicides could also be important, as use of these agents disrupts the flora. It is unclear if lactobacilli alter the actual cell integrity, although from studies over 75 women, implantation with these bacteria does not appear to harm the normal vaginal epithelium.

W.H. Wilborn: You described lactobacilli with and without ruthenium red-positive cell coats. Did you observe other ultrastructural differences between lactobacilli with and without the cell coats?
Authors: No, we did not. It should not be excluded, however, that upon very extensive investigation intracellular differences are present as well, but the aim of this study was not pointed at this.

W.H. Wilborn: Are you aware of strains of lactobacilli that can withstand topical medications known to be effective for treating vaginal candidiasis?
Authors: A US patent has described lactobacilli which inhibit the growth of yeast. However, we are not aware of an actual commercial lactobacillus product or strain that withstands topical medications for yeast or other vaginal infections. It is feasible that strains do exist which resist agents such as nystatin. We have strains in our collection which resist the action of antibacterials and spermicide, the latter of which is a topical agent which invariably destroys the indigenous lactobacillus flora.

M. Fletcher: Do you think the loss of protein material was a rapid (i.e., within a few generations) or gradual process? Was there evidence of cells in a transition phase?
Authors: We think it is a gradual process. We did, however, not investigate p = 5 or 15 cultures, for example. In p = 20 cultured we indeed found cells in a transition phase according to their cell surface morphology. The occurrence of these cells was not frequent, though.

M. Fletcher: Is it possible that some strains lost already some of their "*in vivo*" properties in the laboratory process of isolation?
Authors: This should not be excluded completely. The difference between p = 1 and p = 20 cultures is evident, nevertheless, and we think that our p = 1 culture is very close to the "*in vivo*" strain as it was obtained after minimal culturing.

M. Fletcher: You suggest that the protein and carbohydrate are in one molecule and that you are dealing with a glycoprotein. Bacterial glycoproteins are very rare, however.
Authors: We did not want to suggest that we are dealing with glycoproteins and, therefore, used the term "glyco-proteinaceous material" which includes the option that the material is comprised of two different molecules.

M. Fletcher: Why should all cells eventually become deficient in the SL as stated in the Discussion? Many bacteria are known to undergo phase variation and produce progeny of two different phenotypes. This can, in some cases, increase the possibility of success of the organism by increasing the possibility of producing a phenotype that can take advantage of uncertain or fluctuating environmental conditions.
Authors: This is an interesting comment. In fact, we cannot tell whether all cells would become devoid of the SL layer in the end. Indeed, it may well be that the heterogeneity observed will remain unchanged following further serial passaging and we agree that bacteria may well adapt such heterogeneity in order to have better means available to adhere to different substrata.

S. Denyer: Differences in growth rate between p = 1 and p = 20 cultures and harvesting of cells in different growth phases may have a profound effect on surface characteristics and may explain for the differences you found in the strains you investigated.
Authors: Harvesting of cells was always done in the stationary phase, whereas further handling was carried out in a similar way in all experiments. Therefore, we attribute the changes observed to the effects of serial passaging only.

S. Denyer: Do you have any information on the surface character of intermediates between p = 1 and p = 20 cultures, and are any particular surface features more sensitive to passage number than others?
Authors: We have no information about what happens between p = 1 and p = 20 cultures. Most likely, electron microscopy will be the most sensitive technique to pick up any heterogeneity, followed by microelectrophoresis.

S. Denyer: Did you explore colony morphology and appearance of p = 1 and p = 20 cultures. If colonial variants were observed in the p = 20 culture, then subpopulations could be separated and surface characteristics determined. Furthermore, potential for spontaneous variation could be explored.
Authors: We never saw any difference in colony morphology.

S. Denyer: For p = 1 cultures, surface protein is relatively exposed through the diffuse exopolymeric (probably exopolysaccharide) "slime" layer. With repeated passage the exopolymeric layer is consolidated into a more identifiable "capsule-type" layer more effectively masking the underlying protein. This would then imply no loss of synthetic capability, but only a more effective cross linking process.

Authors: Looking at the electron microscopic images, we conclude that a clearly visible surface layer is being lost in part of the cells following serial passaging. If a diffuse exopolymeric layer would have been consolidated and become more concrete, we think that this layer should have been visible in the electron micrographs. The reverse happened, however: visible information disappeared in part of the cells.