

Cell Surface Analysis Techniques: What Do Cell Preparation Protocols Do to Cell Surface Properties?

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Cell surface analysis often requires manipulation of cells prior to examination. The most commonly employed procedures are centrifugation at different speeds, changes of media during washing or final resuspension, desiccation (either air drying for contact angle measurements or freeze-drying for sensitive spectroscopic analysis, such as X-ray photoelectron spectroscopy), and contact with hydrocarbon (hydrophobicity assays). The effects of these procedures on electrophoretic mobility, adhesion to solid substrata, affinity to a number of Sepharose columns, structural integrity, and cell viability were systematically investigated for a range of model organisms, including carbon- and nitrogen-limited *Psychrobacter* sp. strain SW8 (glycocalyx-bearing cells), *Escherichia coli* (gram-negative cells without a glycocalyx), and *Staphylococcus epidermidis* (gram-positive cells without a glycocalyx). All of the cell manipulation procedures severely modified the physicochemical properties of cells, but with each procedure some organisms were more susceptible than others. Considerable disruption of cell surfaces occurred when organisms were placed in contact with a hydrocarbon (hexadecane). The majority of cells became nonculturable after air drying and freeze-drying. Centrifugation at a high speed ($15,000 \times g$) modified many cell surface parameters significantly, although cell viability was considerably affected only in *E. coli*. The type of washing or resuspension medium had a strong influence on the values of cell surface parameters, particularly when high-salt solutions were compared with low-salt buffers. The values for parameters obtained with different methods that allegedly measure similar cell surface properties did not correlate for most cells. These results demonstrate that the methods used to prepare cells for cell surface analysis need to be critically investigated for each microorganism so that the final results obtained reflect the nature of the in situ microbial cell surface as closely as possible. There is an urgent need for new, reliable, nondestructive, minimally manipulative cell surface analysis techniques that can be used in situ.

The surfaces of microbial cells are vital to the organisms' survival, since it is via them that the bacteria interact with the environment. Characterization of these surfaces is a rapidly expanding field of microbiology and encompasses both the macromolecular constitution and, on a more generic level, physicochemical properties, such as hydrophobicity and surface charge. Quantitative and qualitative measurement of these properties under laboratory conditions requires methods which provide data that is representative of the microorganism's natural environment. The techniques routinely employed for characterizing the physicochemical nature of microbial cell surfaces include techniques involving sessile liquid drop contact angle measurement, microbial adhesion to hydrocarbon (MATH), infrared spectroscopy, X-ray photoelectron spectroscopy (XPS), electrophoretic mobility, electron microscopy, retention on chromatographic resins, and adhesion to inanimate materials. Most of these methods require cell preparation prior to analysis.

For contact angle measurement, a layer of bacterial cells is deposited on a membrane filter, and the contact angle of a drop of a diagnostic liquid on the bacterial filter cake is measured with a goniometer (5, 50, 56). In this system, the values of water contact angles depend on the degree of dehydration of the cells in the filter cake (1). Contact angles change continuously as the filter cake dries until the level of dehydration allows the angle at the liquid-surface interface to remain stable

for 3 to 12 s. Excessive drying should be avoided as this may lead to collapse of hydrophobic structures on the cell surface and hence erratic contact angle values (52).

Cell surface hydrophobicity is often assessed by using the MATH assay (42). A cell suspension is mixed with hydrocarbon for a predetermined period to allow optimal interaction of the bacteria with the hydrocarbon phase (52). As a result, cells may remain in the liquid phase or partition either into the liquid-hydrocarbon interface or into the hydrocarbon phase, depending on their hydrophobicity. Many investigators have modified the original MATH test and have found that seemingly small variations in experimental conditions, such as the diameter of the test tubes, the pH of the suspension medium, and the volume of hydrocarbon used, can significantly alter the results (4, 17, 30). Aliphatic hydrocarbons are used in most cases, since aromatic hydrocarbons cause lysis of some bacterial species (55).

High-vacuum chemical analytical techniques, such as XPS, are increasingly used for chemical analysis of bacterial cells (43). The XPS technique requires that microorganisms be washed in desalted water and freeze-dried under a vacuum. Correlations between the data obtained with this cell surface technique and the data obtained with some of the other physicochemical methods have been reported for various yeasts and bacteria (8, 9, 24, 34), and many workers believe that these results accurately represent the properties of the hydrated cell envelope. However, other authors have suggested that the high vacuum necessary for the XPS technique and the resulting dehydration disrupt the cell surface to the extent that they seriously compromise the validity of the cell surface analysis in relation to hydrated samples (32).

Marshall et al. (32) pointed out the inherent dangers in

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extrapolating data from laboratory studies to microbial ecology. Any variation in or loss of surface polymers *in vitro* may completely alter the attachment mechanisms of an organism compared to its behavior in the natural environment. Considering the degree of variability introduced into any analytical protocol through operator error, as well as the delicate nature of microbial outer surface structures, it is questionable whether the parameters measured by the techniques used represent true microbial cell surface properties and whether the protocols used for cell preparation introduce artifacts and, therefore, additional sources of error. To our knowledge, there has not been a study which has comprehensively addressed how standard cell preparation procedures, which are integral to many cell surface analysis protocols, affect cell surface characteristics, structural integrity, and the survival of prokaryotic organisms.

In this study, we examined changes in the physicochemical surface properties of organisms subjected to high-speed centrifugation, air drying, and freeze-drying, which are techniques commonly used in many cell surface analysis protocols. These tests were performed with the following three microorganisms, each of which is representative of a different type of cell surface structure: *Escherichia coli* (a gram-negative rod), *Staphylococcus epidermidis* (a gram-positive coccus), and *Psychrobacter* sp. strain SW8 (a gram-negative coccobacillus with an extensive constitutive glycocalyx). The phenotypic variability of the results was assessed with carbon- and nitrogen-limited *Psychrobacter* sp. strain SW8. The structural integrity and viability of manipulated cell samples were scrutinized by using electron microscopy and viable cell counting. Changes in cell surface characteristics after treatment were assessed by assays for MATH, electrophoretic mobility, adhesion to solid substrata, and affinity to Sepharose gel columns. The latter two assays were employed because the interactions of cells with substrata depend essentially on the constitution of the outermost molecular layer of the cell surface. These methods are, therefore, sensitive indicators of the integrity of this part of the microbial interface.

MATERIALS AND METHODS

Reagents. All of the chemicals used were analytical grade reagents obtained from BDH, Sigma, or Merck unless otherwise stated. A sodium chloride standard solution containing 24 g of NaCl per liter and buffered with 1 mM Na_2HPO_4 (pH 7.5) was prepared with water obtained from a Milli-Q ZFMQ 23004 reverse-osmosis unit (Millipore, Bedford, Mass.). The water used for resuspending cell preparations was also buffered with 1 mM Na_2HPO_4 to pH 7.5.

Microorganisms and cultivation. The gram-negative marine organism *Psychrobacter* sp. strain SW8 was grown in continuous culture with an artificial seawater (ASW) minimal medium, as described by Schneider and Marshall (46). The chemostat temperature was 20°C, and feedback-controlled addition of 1 M HCl maintained the pH at 7.5. Sterile air was supplied to fermentors (culture volume, approximately 1 liter in a 1.5-liter vessel) at a rate of 3 liters of air per min, and the impeller speed was 500 rpm. A continuous supply of ASW medium was provided at a dilution rate of 0.08 h^{-1} by using peristaltic pumps (Watson-Marlow, Falmouth, Cornwall, England). The growth-limiting substrates were acetate for carbon-limited organisms and ammonia for nitrogen-limited cells. *E. coli* ATCC 8739 and *S. epidermidis* NCTC 11047 were grown in batch cultures with nutrient broth (Oxoid catalog no. CM3). Single loopfuls of either *E. coli* or *S. epidermidis* grown on nutrient agar slants were used to inoculate 250-ml Erlenmeyer flasks containing 20 ml of nutrient broth, which were then incubated for 16 h at 30°C on an orbital shaker. A 10% (vol/vol) inoculum from each culture was transferred into a flask containing fresh nutrient broth and grown to the mid-logarithmic phase (6 h for *E. coli* and 8 h for *S. epidermidis*). The optical densities at 546 nm (OD_{546}) of the cultures were determined with a spectrophotometer (Ultraspec II; LKB, Bromma, Sweden).

Preparation of bacterial suspensions for analyses. A flow diagram illustrating the procedure used for cell sample preparation is shown in Fig. 1. Unless otherwise stated, all bacterial cultures were harvested by centrifugation at $5,000 \times g$ for 10 min. Each cell pellet was resuspended in either the NaCl solution or water and then centrifuged and washed twice before final resuspension either in fresh NaCl solution or water. Abbreviations are used below to indicate the washing and resuspension media used for preparation of cell sam-

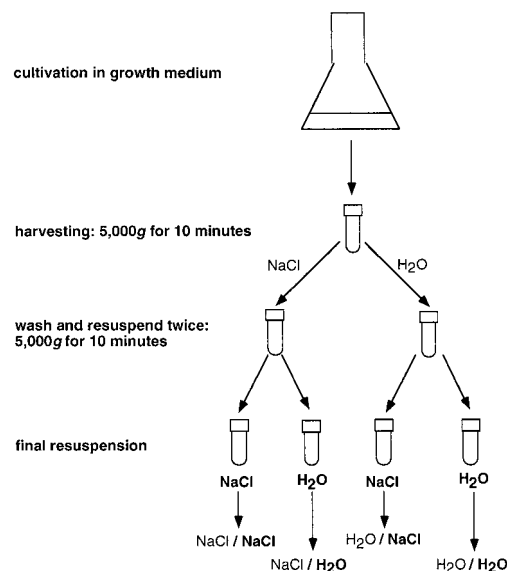


FIG. 1. Schematic representation of the harvesting protocol and washing sequences used to prepare microorganisms for cell surface analysis. In each case the washing medium is listed before the resuspension medium (i.e., wash medium/resuspension medium).

ples; for example, NaCl/H₂O indicates that the cell sample was washed twice in the NaCl solution before final resuspension in water. The abbreviations used are shown in Fig. 1. The buffered NaCl solution contained the same concentration of NaCl as ASW medium. The OD_{546} of the cell suspensions were adjusted to 1.0 for bacterial filter cake preparation (5) and to 0.3 for MATH assays (34), and a slightly turbid cell suspension (OD_{546} , 0.1) was used for electrophoretic mobility measurements.

Air-dried bacterial samples were prepared by the method described by van Oss et al. (56). Briefly, negative-pressure filtration was used to deposit cells onto cellulose acetate filters (pore size, 0.2 μm ; diameter, 45 mm; Sartorius, Göttingen, Germany), and the filter cakes were subsequently dried on agar plates to avoid excessive desiccation. Sessile liquid droplets placed on bacterial cell lawns were used to measure contact angles with a manually adjusted goniometer. The optimum time to obtain measurements was determined by monitoring the stability of contact angles as a function of drying time (5). Filter cakes of each of the bacterial species tested provided stable contact angle values after approximately 90 min of air drying.

The protocol described by Amory et al. (2) was used as a model of the procedure used to prepare cells for analysis by high-vacuum techniques, such as XPS. Harvested bacterial suspensions were washed and concentrated (100:1) in water, and 0.05-ml aliquots were subsequently transferred to acid-washed glass vials (diameter, 8 mm; length, 100 mm). The cells were rapidly frozen by immersing the vials in liquid nitrogen for 15 min prior to vacuum desiccation (268 K) with a Speedvac centrifugal freeze-drier (model 5PS; Edward's High Vacuum Ltd., Crawley, England). Freeze-dried samples were stored under a vacuum before they were resuspended in either the NaCl solution or water.

MATH test. Cell surface hydrophobicity was measured by the MATH test, as modified by Neu and Marshall (36). Briefly, 4-ml samples of a cell suspension were transferred to individual test tubes (diameter, 1.7 cm; length, 15 cm), each of which contained 1 ml of dodecane. The test tubes were vortexed at full speed for 2 min (Vortex Genie; Scientific Industries Inc., Springfield, Mass.) and then left to stand for 15 min to allow phase separation. The OD_{546} of the aqueous phase was determined, and partitioning of the bacterial suspension was expressed as the percentage of cells adsorbed by the hydrocarbon phase: percent partitioning = $[(0.3 - \text{OD}_{546} \text{ of aqueous phase})/0.3] \times 100$. The mean percentage of partitioning of an organism into the dodecane phase was calculated by using triplicate samples.

Electrophoretic mobility. Cell surface charge was measured by using a Delsa Zetameter and Delsa 440 software (Coulter Instruments, Hialeah, Fla.). The electrophoretic mobilities of triplicate samples of cell preparations resulting from each cell surface analysis technique were determined by the four-angle Doppler shift method, which is based on application of the Smoluchowski equation (23). The results were expressed in micrometer-centimeters per volt-second.

Bacterial cell counting. Bacterial suspensions resulting from each cell preparation technique were serially diluted in order to determine the total number of cells per milliliter with a hemocytometer (Improved Neubauer; Gordon-Keeble GP, Barton Mills, England). To determine viable cell counts, six 0.05-ml drops

from 10^{-5} , 10^{-6} , and 10^{-7} dilutions of each sample were placed on nutrient agar plates and incubated at 30°C for 60 h. For each dilution series, the numbers of CFU per milliliter were counted, and the average based on triplicate plate counts was determined. The percentage of surviving organisms was determined by dividing CFU by the total count.

Bacterial adhesion to inanimate substrata. Commercially available aluminum, stainless steel, perspex, and polypropylene were cut into coupons having uniform dimensions (38 by 19 by 3 mm) and were polished to a substratum surface roughness of less than 0.1 μm , as described by Schneider and Marshall (46). Each coupon was immersed in 95% ethanol for 1 h (or 10 s for the perspex coupons), thoroughly rinsed with water, and then transferred to a heated 10% (wt/vol) sodium dodecyl sulfate solution. After 1 h, the substrata were washed twice in boiling water and air dried under a protective hood. The residual organic contaminants remaining on the aluminum and stainless steel coupons after this procedure were removed by glow discharge for 90 s under a vacuum at 8 kV and 100 Torr with a Speedvac coating unit (Edward's High Vacuum Ltd.).

Clean coupons were mounted in perspex chambers that had been cleaned with 95% ethanol and rinsed with boiling water; these chambers were constructed to have laminar fluid flow characteristics (channel depth, 1 mm) (46). The chambers were mounted vertically to eliminate the influence of gravity on cell attachment. A multichannel peristaltic pump (Ismatec, Glattpbrugg, Switzerland) equipped with a pulsation dampening device was used to pump solutions to three parallel flow cells at a constant flow rate of 0.36 cm/s in each chamber. The chambers were equilibrated by flushing them with the appropriate solution (either the NaCl solution or water; both solutions were buffered with 1 mM phosphate buffer at pH 7.4) for at least 5 min before the substrata were exposed to bacterial suspensions for 10 min. Unattached cells were removed by washing the chambers with fresh suspension medium for an additional 15 min. The chambers were drained by reverse flushing at the same flow rate, and the substrata were allowed to dry.

Attached organisms were stained with a fluorochrome solution containing 1.0 μg of 4',6'-diamidino-2-phenylindole (DAPI) per ml. Fluorescing organisms were examined at a magnification of $\times 500$ under oil immersion (area of field of view, $2.01 \times 10^{-2} \text{ mm}^2$) by using an epifluorescence microscope (model BH2; Olympus, Tokyo, Japan) equipped with a type UG-1 exciter filter and a type L-420 barrier filter. Ten randomly selected fields were counted for each coupon. Statistically invalid field counts were identified and eliminated by using the method described by Schneider and Marshall (46). These counts occurred primarily when a randomly selected microscopic field coincided with a spot containing a large number of cells deposited from a liquid droplet that remained on the substratum after washing. First, the mean number of bacterial cells found attached to the 10 randomly selected fields counted for each test substratum was determined. Counts for individual fields that differed significantly from the mean were eliminated by using Grubb's outlier test at $P = 90\%$ (15), and a new average for cells attached to the substratum was calculated; the procedure was repeated until no further outliers were found. In these experiments, 6.3% of individual field counts were eliminated. Grubb's outlier test uses the following equation: $Y = (|X^* - \bar{X}|)/\sigma$, where \bar{X} is the mean of a series with n components, X^* is the value most different from the mean value for the series, and σ is the standard deviation. rN is the highest value for Y at which there is 90% probability that X^* is a valid value in a series with n components. If $Y < rN$, X^* is within the main series; if $Y > rN$, X^* is an outlier and is removed from the main series. In these cases, calculations were repeated for a new series with $(n - 1)$ components. The cell counts obtained for each of the three substratum samples used in each experiment were compared by using Student's t test at a P of < 0.01 to identify possible inconsistencies in the results obtained with the different replicates of the same material, but no significant differences in attachment of cells were found. The mean and standard deviation for the number of microbes retained on a substratum in a particular experiment were then calculated by combining the field counts obtained from the three replicate coupons. Results obtained in adhesion assays were statistically reproducible, as evidenced by replicate experiments conducted throughout the study period with cells of the three organisms that had been treated at $5,000 \times g$.

Sepharose gel interaction chromatography. Chromatography with Sepharose gels reveals interactions of microbial cells in columns packed with agarose beads. The surface chemistry of the beads can be modified in a controlled manner by covalent binding of aliphatic or charged residues. A range of commercially available beads with different well-characterized surface properties allows probing of a variety of cell surface parameters, such as hydrophobicity (33) and surface charge (24). Since bacteria may become bound to or entrapped by the Sepharose gel networks themselves (1), unsubstituted gels are used as controls. Sepharose gel interaction chromatography experiments were performed by using a modified method based on the technique developed by Smyth et al. (49). Pasteur pipettes plugged with glass wool were filled with 1.0 ml (bed volume) of either Sepharose CL-4B, phenyl-Sepharose CL-4B, octyl-Sepharose CL-4B, DEAE-Sepharose CL-6B, or carboxymethyl-Sepharose CL-6B gel (Pharmacia, Uppsala, Sweden) and equilibrated with either the NaCl solution or water. The void volume for all gels was determined to be 0.5 ml by using dextran blue. A 1-ml aliquot of a bacterial suspension (approximately 8×10^8 cells/ml) was applied to each column and was subsequently eluted with 1 ml of the appropriate resuspension medium. The OD_{546} of the eluates collected from each column were determined. Each test was performed in triplicate, and the means were

calculated. The amounts of bacterial cells not retained in the gel were determined from the OD_{546} of the eluates. The affinity of each bacterial species for each gel was expressed as the percentage of cells initially retained based on the total number of cells applied to the column: % retention = $[(\text{OD}_{546} \text{ of original SW8 suspension} - \text{OD}_{546} \text{ of initial eluate})/(\text{OD}_{546} \text{ of original SW8 suspension})] \times 100$. The data resulting from different cell preparation techniques were compared by using Student's t test ($P < 0.01$).

Electron microscopy. Following each treatment cell morphology was examined by transmission electron microscopy of negatively stained specimens. Carbon-coated copper grids (400 mesh) were immersed for 20 s in bacterial suspensions (OD_{546} , 0.3) resulting from each cell preparation technique and then stained with a 2% aqueous sodium phosphotungstate solution for 10 s. The excess liquid was removed by carefully touching the edges of the grids to filter paper and air drying the grids for 5 min prior to visualization with a model H-7000 transmission electron microscope (Hitachi, Tokyo, Japan).

Statistical comparison of data from different protocols. A comparative analysis of data was performed by using CricketGraph, version 1.3.2. (Computer Associates, Islandia, N.Y.) for Macintosh. Graphs were constructed by using data obtained from the various cell surface analysis techniques, and correlation coefficients were calculated by the linear regression method.

RESULTS

Reference states. Comparative analysis of the effects of different cell preparation protocols on cell surface properties requires the arbitrary choice of a reference state. Cells harvested by centrifugation at $5,000 \times g$ were used as reference cells (Table 1) because they most closely resembled unmanipulated organisms in the parameters that could be measured without extensive cell manipulation (e.g., viability and electrophoretic mobility). Organisms harvested by centrifugation at $5,000 \times g$ were viable and negatively charged (Table 1). Carbon- and nitrogen-limited reference *Psychrobacter* sp. strain SW8 and *S. epidermidis* were very hydrophobic, as determined by the MATH assay, but *E. coli* was only moderately hydrophobic (Table 1). The retention of the two *Psychrobacter* sp. strain SW8 phenotypes on hydrophilic surfaces (stainless steel and aluminum) was between two and three times greater than the retention of this organism on hydrophobic surfaces (perspex and polypropylene) (Table 1). Similar numbers of *E. coli* cells attached to perspex, stainless steel, and aluminum, but significantly fewer *E. coli* cells attached to polypropylene. *S. epidermidis* was retained poorly on polypropylene, but the numbers of *S. epidermidis* cells that attached to aluminum were between two and three times greater than the numbers of *S. epidermidis* cells that attached to stainless steel and perspex. The affinity for DEAE-Sepharose of all $5,000 \times g$ -treated organisms was high. The affinities of the two phenotypes of *Psychrobacter* sp. strain SW8 were high for octyl-Sepharose, moderate for phenyl-Sepharose, low for carboxymethyl-Sepharose, and either moderate (carbon-limited cells) or low (nitrogen-limited cells) for Sepharose (Table 1). The affinities of *E. coli* for the four non-DEAE-Sepharose gels were generally moderate, but the affinities of *S. epidermidis* were low.

The protocols used for manipulation of cells significantly modified cell surface properties in a manner which depended on the type of treatment, the nature of the microorganisms, and the type of washing or resuspension medium employed (Tables 2 through 7 and Fig. 2 through 4).

High-speed centrifugation. Centrifugation at $15,000 \times g$ in many instances did not alter the viability of *Psychrobacter* sp. strain SW8 or *S. epidermidis*, but the culturability of *E. coli* was significantly diminished (Fig. 2 and Table 2). High-speed centrifugation affected hydrophobicity almost exclusively when water was used as the resuspension medium (e.g., in the combinations NaCl/H₂O and H₂O/H₂O) (Table 2). In general, this cell manipulation procedure did not influence the electrophoretic mobility of *Psychrobacter* sp. strain SW8, but it reduced the surface charges of *E. coli* and *S. epidermidis* (Fig. 2

TABLE 1. Reference values for parameters used for comparative analysis of the effects of cell preparation protocols on cell surface properties^a

Organism	Electrophoretic mobility ($\mu\text{m-cm/Vs}$)	Viability ^b (%)	Hydrophobicity ^c (%)	Attachment (10^3 cells/cm ²) to:					% Retention on Sepharose columns			
				Stainless steel	Aluminum	Perspex	Polypropylene	Sepharose	Phenyl-Sepharose	Octyl-Sepharose	DEAE-Sepharose	Carboxymethyl-Sepharose
Carbon-limited strain SW8	-0.38 ± 0.01	85.4 ± 3.8	92.8 ± 1.9	5.2 ± 1.2	6.9 ± 2.4	2.0 ± 0.7	3.1 ± 0.9	57.4 ± 5.8	60.7 ± 7.7	80.6 ± 1.9	100	19.1 ± 3.3
Nitrogen-limited strain SW8	-0.51 ± 0.01	94.4 ± 3.8	92.2 ± 4.6	3.1 ± 0.9	3.7 ± 1.0	1.7 ± 0.6	1.2 ± 0.5	24.7 ± 1.6	41.9 ± 0.7	94.7 ± 0.6	100	22.6 ± 3.1
<i>E. coli</i>	-3.33 ± 0.01	89.1 ± 3.7	77.6 ± 7.5	5.9 ± 2.2	7.5 ± 2.0	7.9 ± 1.6	0.9 ± 0.6	42.8 ± 2.3	45.3 ± 3.7	39.4 ± 1.0	91.3 ± 1.8	53.7 ± 5.1
<i>S. epidermidis</i>	-1.04 ± 0.01	92.4 ± 3.8	91.3 ± 1.6	4.2 ± 1.2	8.2 ± 1.3	3.3 ± 1.1	0.4 ± 0.2	20.0 ± 2.7	26.3 ± 1.7	36.3 ± 2.1	97.7 ± 0.2	14.8 ± 2.3

^a Cells were centrifuged at $5,000 \times g$ and then washed and resuspended in buffered solutions containing NaCl.

^b Percent viability [(number of CFU on nutrient agar)/(total number of cells)] $\times 100$.

^c Percent hydrophobicity (MATH assay) = [(final optical density of the aqueous phase)/(optical density of the initial suspension prior to mixing with dodecane)] $\times 100$.

and Table 2). This technique had little effect on the adhesion of *Psychrobacter* sp. strain SW8 or *S. epidermidis* to solid surfaces, but for *E. coli* about two-thirds of the attachment results were different after high-speed centrifugation, with increased attachment and decreased attachment occurring at similar frequencies (Table 3 and Fig. 2). High-speed centrifugation modified about 30% of the Sepharose results obtained for *Psychrobacter* sp. strain SW8 and *E. coli* and approximately 70% of the Sepharose results obtained for *S. epidermidis* (Fig. 2). The majority of the effects occurred with the combinations NaCl/NaCl (*E. coli* and *S. epidermidis*), NaCl/H₂O (*Psychrobacter* sp. strain SW8), and H₂O/NaCl, as well as H₂O/H₂O (*S. epidermidis*) (Table 4).

Freeze-drying. Freeze-drying reduced the viabilities of the gram-negative organisms *Psychrobacter* sp. strain SW8 and *E. coli* by between 83 and 97% and the viability of the more resistant gram-positive organism *S. epidermidis* by approximately 60% (Table 2 and Fig. 3). No additional measurements could be obtained with freeze-dried *S. epidermidis* as attempts to resuspend desiccated samples in either the NaCl solution or water caused the majority of cells to aggregate and sediment. Freeze-drying significantly reduced the electrophoretic mobility of cells resuspended in water; the surface charge of nitrogen-limited *Psychrobacter* sp. strain SW8 was even reversed to slightly positive values (Table 2). When cells were resuspended in the NaCl solution, however, the cell surface charge of *E. coli* remained the same, while the charge of nitrogen- and carbon-limited *Psychrobacter* sp. strain SW8 was either strongly increased or reduced. Resuspension of freeze-dried cells in water reduced the hydrophobicity of *Psychrobacter* sp. strain SW8 but increased the hydrophobicity of *E. coli* (Table 2). Resuspension of the same cells in the NaCl solution had no effect on the hydrophobicity of *E. coli*, reduced the hydrophobicity of carbon-limited *Psychrobacter* sp. strain SW8, and increased the hydrophobicity of the nitrogen-limited phenotype. Significantly lower numbers of freeze-dried cells of *Psychrobacter* sp. strain SW8 attached to substrata in most experiments; in contrast, the *E. coli* attachment behavior was affected by freeze-drying in only a few experiments (Fig. 3). Freeze-drying also affected the interaction of microorganisms with Sepharose resins and generally increased their retention in the columns (Fig. 3). For the most part these effects did not occur in clear patterns; the exception was the consistently complete retention of all cells applied to DEAE-Sepharose columns (Table 4).

Air drying. Air drying affected microbes in a manner similar to freeze-drying, but the effects were less intense (Fig. 4 and Tables 2 through 4). The viabilities of all of the microbes were reduced, but the effects were less dramatic than the effects of freeze-drying. The gram-positive organism *S. epidermidis* exhibited the best resistance to the process; the reductions in viability for *S. epidermidis* were relatively small (12 to 25%) compared to the reductions in viability observed for *Psychrobacter* sp. strain SW8 (35 to 54%) and *E. coli* (80%). Air drying modified virtually every electrophoretic mobility value measured and generally reduced the value (Fig. 4 and Table 2). The effects of air drying on hydrophobicity varied depending on the microbe and on the resuspension medium (Fig. 4 and Table 2). Using water as the resuspension medium for air-dried *Psychrobacter* sp. strain SW8 resulted in reduced hydrophobicity, but no such effect occurred with the NaCl solution. Air drying reduced the hydrophobicity of *E. coli* in all assays but had no effect on the hydrophobicity of *S. epidermidis*. Air drying altered the attachment of *E. coli* and *S. epidermidis* to surfaces in the majority of assays, but no clear relationship between increases or decreases in retention and the nature of the surface or of the suspension medium was identified (Fig.

TABLE 2. Impact of cell preparation protocols on viability, electrophoretic mobility, and hydrophobicity of organisms when different combinations of washing and resuspension media were used

Organism	Treatment	% of reference value ^a											
		Viability				Electrophoretic mobility				Hydrophobicity			
		NaCl/NaCl ^b	NaCl/H ₂ O	H ₂ O/NaCl	H ₂ O/H ₂ O	NaCl/NaCl	NaCl/H ₂ O	H ₂ O/NaCl	H ₂ O/H ₂ O	NaCl/NaCl	NaCl/H ₂ O	H ₂ O/NaCl	H ₂ O/H ₂ O
Carbon-limited strain SW8	15,000 × g ^c	100	100	100	100	100	100	119	100	100	27	100	100
	Freeze-drying	— ^d	— ^d	3	3	— ^d	— ^d	83	0	— ^d	— ^d	47	11
	Air drying	65	60	56	46	82	85	71	80	100	100	40	45
	MATH	33	37	23	17	29	5	19	3				
Nitrogen-limited strain SW8	15,000 × g ^c	100	100	100	100	100	100	100	100	100	20	100	72
	Freeze-drying	— ^d	— ^d	5	5	— ^d	— ^d	220	−104 ^e	— ^d	— ^d	100	17
	Air drying	64	60	55	60	124	70	135	65	100	100	53	52
	MATH	36	42	28	31	69	1	55	4				
<i>E. coli</i>	15,000 × g ^c	79	77	66	66	15	72	112	74	100	125	100	55
	Freeze-drying	— ^d	— ^d	2	17	— ^d	— ^d	100	80	— ^d	— ^d	100	170
	Air drying	25	32	24	23	29	82	91	84	70	23	50	0
	MATH	25	15	21	15	23	82	294	79				
<i>S. epidermidis</i>	15,000 × g ^c	100	100	100	86	20	63	286	59	100	100	118	116
	Freeze-drying	— ^d	— ^d	47	38	— ^d	— ^d	— ^f	— ^f	— ^d	— ^d	— ^f	— ^f
	Air drying	76	75	88	75	79	1	133	36	100	100	100	100
	MATH	100	78	100	72	100	2	88	47				

^a The values are percentages of reference values determined after centrifugation at 5,000 × g.

^b Washing medium/resuspension medium.

^c Centrifugation at 15,000 × g.

^d The bacterial cells used for XPS analysis were not washed in the NaCl solution.

^e The sign of the cell surface charge was reversed.

^f Freeze-dried samples of *S. epidermidis* could not be resuspended.

4). For the most part the effects of air drying on the interactions of the two phenotypes of *Psychrobacter* sp. strain SW8 with solid substrata were similar. The exceptions were the interactions with stainless steel and aluminum; in these cases attachment of the two phenotypes subjected to NaCl/NaCl and H₂O/NaCl treatments differed significantly (Table 3). Air drying also modified the interactions of cells with most of the Sepharose columns; the exceptions were the DEAE-Sepharose column experiments, in which complete retention of cells was always observed (Fig. 4 and Table 4). With most other column materials, air drying increased the retention of nitrogen-limited *Psychrobacter* sp. strain SW8 but reduced the retention of the carbon-limited phenotype (Table 4). The interactions of air-dried *E. coli* with Sepharose gels were generally not affected; the exceptions were cells washed in water, which exhibited increased affinity for all of the Sepharose gels other than the DEAE-Sepharose gels. Air drying consistently reduced the affinity of NaCl/H₂O-treated *S. epidermidis* for Sepharose columns, but with the other medium combinations tested significant effects occurred only occasionally.

MATH. Contact between cells and hydrocarbon reduced the viability of *Psychrobacter* sp. strain SW8 and *E. coli* by between 63 and 85% (Table 2) but either had no effect or caused only a small reduction of the viability of *S. epidermidis*, depending on the resuspension medium. The electrophoretic mobilities of both phenotypes of *Psychrobacter* sp. strain SW8 were reduced by approximately 95% when the cells were resuspended in water, a reduction similar to the reduction observed for NaCl/H₂O-treated *S. epidermidis* (Table 2). Most of the reductions in electrophoretic mobility were smaller when the cells were resuspended in NaCl; the only exception was the H₂O/NaCl-

treated *E. coli* experiment, in which a large increase in electrophoretic mobility was observed after the cells were exposed to dodecane.

Change of resuspension medium. The cell surface properties of organisms resuspended in the NaCl solution were used as references for comparisons with similarly prepared cells resuspended in water (Fig. 5 and Tables 5 and 6). Resuspension of microorganisms in water did not change the viability of the microbes significantly (Fig. 5). Final resuspension of NaCl solution- or H₂O-washed organisms in water increased their electrophoretic mobility considerably (by up to 700%) compared to cells resuspended in the NaCl solution (Table 5). Reductions in electrophoretic mobility were observed with freeze-dried and hydrocarbon-treated *Psychrobacter* sp. strain SW8, as well as with air-dried and hydrocarbon-treated *S. epidermidis* (Table 5). In most cases the hydrophobicity of cells was considerably reduced by resuspension in water; the exceptions were most *S. epidermidis* preparations, whose hydrophobicities were similar in the two resuspension media (Table 5). Switching the suspension medium from the NaCl solution to H₂O altered the attachment of organisms to substrata in most instances and generally reduced the retention of organisms on surfaces (Fig. 6). Increased retention also occurred, but it occurred less frequently and primarily to aluminum (Fig. 6). With *Psychrobacter* sp. strain SW8 (but not *E. coli* or *S. epidermidis*), the washing medium appeared to determine whether attachment increased or decreased. With the NaCl solution as the washing medium, reductions in attachment predominated (83% of the results), but with H₂O as the washing medium, reduced attachment (33%) was observed as frequently as increased attachment (29%). Retention of cells on Sepharose

TABLE 3. Impact of cell preparation protocols on the attachment to different substrata of test organisms exposed to different combinations of washing and resuspension media

Organism	Treatment	% of reference value ^a															
		NaCl/NaCl ^b				NaCl/H ₂ O				H ₂ O/NaCl				H ₂ O/H ₂ O			
		Stainless steel	Aluminum	Perspex	Polypropylene	Stainless steel	Aluminum	Perspex	Polypropylene	Stainless steel	Aluminum	Perspex	Polypropylene	Stainless steel	Aluminum	Perspex	Polypropylene
Carbon-limited strain SW8	15,000 × g ^c	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	40	13	33	17	5	4	25	4
	Air drying	54	17	35	13	100	100	100	100	28	22	100	100	20	9	25	0
Nitrogen-limited strain SW8	15,000 × g ^c	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	0
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	48	100	18	100	97	15	9	40
	Air drying	100	100	29	0	100	100	100	100	352	656	100	100	26	10	0	0
<i>E. coli</i>	15,000 × g ^c	29	100	100	522	13	5	100	100	1,300	2,028	35	100	3,827	196	0	0
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	100	100	13	100	18	ag ^e	100	100
	Air drying	100	100	20	256	42	12	36	100	1,466	557	60	100	291	9	0	100
<i>S. epidermidis</i>	15,000 × g ^c	100	100	100	100	100	100	100	100	100	100	413	100	100	209	100	100
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^f	— ^f	— ^f	— ^f	— ^f	— ^f	— ^f	— ^f
	Air drying	390	100	21	400	100	190	19	— ^g	196	143	488	100	51	39	0	— ^g

^a The values are percentages of reference values determined after centrifugation at 5,000 × g.

^b Washing medium/resuspension medium.

^c Centrifugation at 15,000 × g.

^d The bacterial cells used for XPS analysis were not washed in the NaCl solution.

^e ag, cells aggregated on the surface.

^f Freeze-dried *S. epidermidis* cells could not be resuspended.

^g No cells adhered to the reference substratum.

columns was affected by changes of the resuspension medium more than attachment to solid surfaces (Fig. 7). The change from the NaCl solution to H₂O reduced the affinity of cells particularly to the hydrophobic columns (phenyl-Sepharose

and octyl-Sepharose), but lower affinities were also observed for Sepharose and carboxymethyl-Sepharose. No effect or increased affinity occurred with the DEAE-Sepharose column, a column that often retained all of the organisms applied to it.

TABLE 4. Impact of cell preparation protocols on the retention on Sepharose columns of test organisms exposed to different combinations of washing and resuspension media

Organism	Treatment	% of reference value ^a																			
		NaCl/NaCl ^b					NaCl/H ₂ O					H ₂ O/NaCl					H ₂ O/H ₂ O				
		Sepharose	Phenyl-Sepharose	Octyl-Sepharose	DEAE-Sepharose	Carboxymethyl-Sepharose	Sepharose	Phenyl-Sepharose	Octyl-Sepharose	DEAE-Sepharose	Carboxymethyl-Sepharose	Sepharose	Phenyl-Sepharose	Octyl-Sepharose	DEAE-Sepharose	Carboxymethyl-Sepharose	Sepharose	Phenyl-Sepharose	Octyl-Sepharose	DEAE-Sepharose	Carboxymethyl-Sepharose
Carbon-limited strain SW8	15,000 × g ^c	100	100	100	100	100	51	60	48	90	215	100	100	88	100	100	100	100	74	100	100
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	136	146	100	100	100	100	100	67	100	100
	Air drying	55	35	65	100	100	25	26	50	100	178	52	63	90	100	100	38	21	17	100	73
Nitrogen-limited strain SW8	15,000 × g ^c	100	1,714	100	100	100	100	151	275	100	100	100	138	100	100	100	55	100	52	100	100
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	243	161	100	100	178	304	408	352	100	234
	Air drying	167	213	100	100	299	210	215	379	100	165	100	142	100	100	148	203	227	224	100	171
<i>E. coli</i>	15,000 × g ^c	169	159	208	76	143	100	192	100	100	100	100	100	100	100	211	100	100	100	100	100
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	66	100	100	100	143	364	166	178	100	333
	Air drying	57	100	100	100	100	100	100	20	100	100	100	100	100	100	178	276	134	182	100	189
<i>S. epidermidis</i>	15,000 × g ^c	209	360	273	100	262	133	100	87	100	100	40	92	83	44	55	401	169	156	100	38
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^e	— ^e	— ^e	— ^e	— ^e	— ^e	— ^e	— ^e	— ^e	— ^e
	Air drying	100	147	100	100	100	21	18	12	100	44	58	100	100	72	47	224	100	100	100	49

^a The values are percentages of reference values determined after centrifugation at 5,000 × g.

^b Washing medium/resuspension medium.

^c Centrifugation at 15,000 × g.

^d The bacterial cells used for XPS analysis were not washed in the NaCl solution.

^e Freeze-dried *S. epidermidis* cells could not be resuspended.

TABLE 5. Impact of modification of the resuspension medium on viability, electrophoretic mobility, and hydrophobicity of test organisms subjected to different cell preparation protocols

Organism	Treatment	% of reference value					
		Viability		Electrophoretic mobility		Hydrophobicity	
		NaCl/H ₂ O (NaCl/NaCl) ^a	H ₂ O/H ₂ O (H ₂ O/NaCl)	NaCl/H ₂ O (NaCl/NaCl)	H ₂ O/H ₂ O (H ₂ O/NaCl)	NaCl/H ₂ O (NaCl/NaCl)	H ₂ O/H ₂ O (H ₂ O/NaCl)
Carbon-limited strain SW8	5,000 × g ^b	100	100	271	240	86	74
	15,000 × g ^c	100	100	246	210	24	69
	Freeze-drying	— ^d	100	— ^d	0	— ^d	17
	Air drying	100	100	281	270	82	100
	MATH	100	100	45	38		
Nitrogen-limited strain SW8	5,000 × g ^b	100	100	490	490	73	88
	15,000 × g ^c	100	100	460	458	14	75
	Freeze-drying	— ^d	100	— ^d	−108 ^e	— ^d	17
	Air drying	100	100	276	236	88	100
	MATH	100	100	9	32		
<i>E. coli</i>	5,000 × g ^b	100	87	145	564	50	66
	15,000 × g ^c	100	100	708	375	58	38
	Freeze-drying	— ^d	100	— ^d	478	— ^d	100
	Air drying	100	100	418	521	16	0
	MATH	55	100	505	151		
<i>S. epidermidis</i>	5,000 × g ^b	100	100	435	328	93	100
	15,000 × g ^c	100	100	1,362	672	100	100
	Freeze-drying	— ^d	100	— ^d	— ^d	— ^d	— ^d
	Air drying	100	100	5	88	91	100
	MATH	83	100	9	177		

^a Washing medium/resuspension medium (washing medium/resuspension medium with which the reference value was obtained).

^b Centrifugation at 5,000 × g.

^c Centrifugation at 15,000 × g.

^d The bacterial cells used for XPS analysis were not washed in the NaCl solution.

^e Reversal of the electrophoretic mobility sign resulted in a reduction in the value.

Change of washing medium. In an analysis of the effects of washing media on microbial cell surfaces, the properties of organisms initially washed in the NaCl solution were compared to the properties of similarly treated cells initially washed in water. Using different washing media generally did not alter cell viability (Tables 7 and 8 and Fig. 8). The electrophoretic mobilities determined for *Psychrobacter* sp. strain SW8 were similar for the two types of washing media (Fig. 8). The cell surface charge of *E. coli* was not modified when the washing medium was changed from the NaCl solution to water and the cells were resuspended in water but was significantly altered when the cells were resuspended in the NaCl solution (Table 7). A change of the washing medium from the NaCl solution to water always increased the electrophoretic mobility of *S. epidermidis*, irrespective of the final resuspension medium (Table 7). The same change of washing medium generally reduced the hydrophobicity of *Psychrobacter* sp. strain SW8, did not affect the hydrophobicity of *S. epidermidis*, and either increased or reduced the hydrophobicity of *E. coli* (Table 5 and Fig. 6). Replacing the NaCl solution with H₂O as the washing medium generally reduced the attachment of cells to the hydrophobic substrata polypropylene and perspex and increased the attachment of cells to the hydrophilic substrata stainless steel and aluminum (Table 8). Changing the washing medium had less impact on the affinity of cells for Sepharose than changing the resuspension medium (Fig. 7). The interactions of *E. coli* and *S. epidermidis* with Sepharose resins were affected more by

changing the washing medium than the interactions of *Psychrobacter* sp. strain SW8 (Fig. 8). Changing the washing medium from the NaCl solution to water when the NaCl resuspension medium was used generally increased the affinities of cells for the Sepharose columns, while the opposite occurred when H₂O was used as the resuspension medium (Table 8).

Electron microscopy. The gram-negative coccobacillus *Psychrobacter* sp. strain SW8, which occurs mostly as a doublet and has no flagella or fimbriae, is approximately 2 to 3 μm long (Fig. 9). Both carbon- and nitrogen-limited cells possess a thick constitutive glycocalyx which appears as a distinct structure external to the cytoplasm and cell wall in negatively stained preparations (Fig. 9A). The outermost region of the glycocalyx appears to be electron dense. No difference in the thickness of the glycocalyx was observed when the two SW8 phenotypes were compared. Centrifugation at 15,000 × g resulted in loss of the electron-dense outer layer of the glycocalyx of carbon-limited cells but not of the electron-dense outer layer of the glycocalyx of nitrogen-limited cells (Fig. 9B). Furthermore, blebbing of the outer cell membrane was observed in carbon-limited cell preparations resuspended in water (Fig. 9B). This effect was not observed in nitrogen-limited cells. Air-dried preparations of the organism appeared to have lost most of their glycocalyx structure, which left the outer cell membrane exposed (Fig. 9C). This was most apparent when we examined the surface morphology of carbon-limited cells, which was significantly modified following air drying. Similarly, cells of *Psy-*

TABLE 6. Impact of modification of the resuspension medium on interaction with Sepharose columns and attachment to solid substrata of test organisms subjected to different cell preparation protocols

Organism	Treatment	% of reference value																	
		NaCl/H ₂ O (NaCl/NaCl) ^a						H ₂ O/H ₂ O (H ₂ O/NaCl)				NaCl/H ₂ O (NaCl/NaCl)			H ₂ O/H ₂ O (H ₂ O/NaCl)				
		Sepharose	Phenyl-Sepharose	Octyl-Sepharose	DEAE-Sepharose	Carboxymethyl-Sepharose	Sepharose	Phenyl-Sepharose	Octyl-Sepharose	DEAE-Sepharose	Carboxymethyl-Sepharose	Stainless steel	Aluminum	Perspex	Polypropylene	Stainless Steel	Aluminum	Perspex	Polypropylene
Carbon-limited <i>Psychrobacter</i> sp. strain SW8	5,000 × g ^b	100	100	109	100	100	100	100	100	100	100	13	13	10	13	100	175	400	383
	15,000 × g ^c	57	62	57	90	218	100	100	90	100	27	13	8	0	42	100	500	200	
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	75	62	67	100	195	— ^d	— ^d	— ^d	— ^d	100	100	100	100
	Air drying	53	100	100	100	211	100	37	20	100	100	29	100	14	100	100	100	100	0
Nitrogen-limited <i>Psychrobacter</i> sp. strain SW8	5,000 × g ^b	72	63	26	100	137	57	33	24	100	65	32	46	24	17	100	300	100	100
	15,000 × g ^c	100	56	69	100	184	35	20	13	100	52	40	38	12	0	52	225	100	0
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	71	84	85	100	86	— ^d	— ^d	— ^d	— ^d	30	100	100	22
	Air drying	100	64	95	100	75	100	53	55	100	75	34	56	100	100	11	5	0	0
<i>E. coli</i>	5,000 × g ^b	56	49	178	110	50	11	26	22	104	31	266	532	35	0	100	2,642	10	100
	15,000 × g ^c	35	59	73	144	45	15	19	23	109	15	100	33	42	0	540	256	0	0
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	58	36	34	100	73	— ^d	— ^d	— ^d	— ^d	25	— ^e	40	100
	Air drying	63	68	32	100	100	32	34	38	118	33	100	46	100	43	36	44	0	25
<i>S. epidermidis</i>	5,000 × g ^b	403	317	257	100	666	14	15	18	108	56	100	63	100	0	136	100	100	0
	15,000 × g ^c	237	82	82	103	256	143	27	34	246	38	212	66	100	0	100	100	39	0
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^f	— ^f	— ^f	— ^f	— ^f	— ^f	— ^d	— ^d	— ^d	— ^f	— ^f	— ^f	— ^f
	Air drying	100	39	25	100	269	55	13	12	150	58	43	100	100	100	35	22	0	100

^a Washing medium/resuspension medium (washing medium/resuspension medium with which the reference value was obtained).

^b Centrifugation at 5,000 × g.

^c Centrifugation at 15,000 × g.

^d The bacterial cells used for XPS analysis were not washed in the NaCl solution.

^e No cells adhered to the reference substratum.

^f Freeze-dried samples of *S. epidermidis* could not be resuspended.

Psychrobacter sp. strain SW8 which remained intact following the freeze-drying procedure were for the most part devoid of a glycocalyx. However, the intact cells represented only a very small proportion of the sample viewed, as the morphology of most organisms was either grossly modified or totally disrupted by this protocol, particularly in the case of nitrogen-limited *Psychrobacter* sp. strain SW8 (Fig. 9E and F). Exposure of *Psychrobacter* sp. strain SW8 to dodecane in MATH assays resulted in complete removal of the glycocalyx, but the cells remained intact (Fig. 9D).

E. coli ATCC 8739 is a gram-negative rod which does not possess a glycocalyx or any distinguishable extracellular appendages. This organism is approximately 5 μm long and has an irregular outer cell surface (Fig. 10A). The surface of *E. coli* was compromised by high-speed centrifugation at 15,000 × g as distortion was clearly evident, particularly in preparations resuspended in water; however, the cells appeared not to disintegrate during the procedure. The significantly less dense intracellular material evident in electron micrographs of freeze-dried *E. coli* cells suggests that the cells lysed during the freeze-drying protocol and lost most of their plasma (Fig. 10B). The cells also had a tendency to aggregate when they were resuspended in liquid media. Air-dried *E. coli* cells had a strong tendency to aggregate, but otherwise these cells appeared to be similar to the controls. Micrographs of cells exposed to dodecane clearly showed damage to the *E. coli* cell surface; it appeared that the outermost surface of the organism was pulled away from the cell membrane during this procedure (Fig. 10C and D).

S. epidermidis NCTC 11047 is a gram-positive coccus with a diameter of 1 to 2 μm. This organism has a smooth surface and a solid spherical appearance (Fig. 11A). Cell morphology was unchanged after centrifugation at 15,000 × g. In contrast, the freeze-drying process caused significant structural damage to many *S. epidermidis* cells (Fig. 11B). Intact freeze-dried cells comprised less than 20% of the fields of view examined; the bulk of the sample consisted of completely disrupted cells and cell debris. While desiccation of *S. epidermidis* air drying resulted in severe modification of the cell surface, it appeared to be less harsh than freeze-drying and resulted in cell aggregation rather than lysis. Exposure to dodecane in MATH assays clearly affected the cell surface of this gram-positive bacterium, particularly organisms resuspended in water (Fig. 11C). Electron micrographs suggested that material was removed from the surface of the organism during this protocol.

Correlation between different methods of cell surface analysis. Since the results of both MATH assays and assays to measure retention on hydrophobic Sepharose columns (octyl-Sepharose and phenyl-Sepharose) depend on microbial cell surface hydrophobicity, we expected these parameters to be correlated with each other. However, good linear correlations ($R \geq 0.9$) were found only occasionally (2 of 32 correlations) and occurred as often as good correlations with data which we did not expect to correlate, such as data for retention on plain Sepharose (39). Similarly, the surface charges of microbes should determine the interactions of the microbes with charged resins. Again, the number of linear correlations between affinity for DEAE-Sepharose or carboxymethyl-Sepha-

TABLE 7. Impact of modification of the washing medium on viability, electrophoretic mobility, and hydrophobicity of test organisms subjected to different cell preparation protocols

Organism	Treatment	% of reference value					
		Viability		Electrophoretic mobility		Hydrophobicity	
		H ₂ O/NaCl (NaCl/NaCl) ^a	H ₂ O/H ₂ O (NaCl/H ₂ O)	H ₂ O/NaCl (NaCl/NaCl)	H ₂ O/H ₂ O (NaCl/H ₂ O)	H ₂ O/NaCl (NaCl/NaCl)	H ₂ O/H ₂ O (NaCl/H ₂ O)
Carbon-limited strain SW8	5,000 × g ^b	100	100	100	100	81	71
	15,000 × g ^c	100	100	122	100	75	219
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d
	Air drying	100	100	100	100	30	31
	MATH	100	49	100	100		
Nitrogen-limited strain SW8	5,000 × g ^b	86	100	100	100	72	86
	15,000 × g ^c	100	100	100	100	59	304
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d
	Air drying	100	100	100	100	39	39
	MATH	100	100	100	100		
<i>E. coli</i>	5,000 × g ^b	100	100	26	100	115	152
	15,000 × g ^c	100	100	194	100	100	67
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d
	Air drying	100	100	81	100	82	0
	MATH	100	100	321	100		
<i>S. epidermidis</i>	5,000 × g ^b	100	100	163	123	86	100
	15,000 × g ^c	100	100	2,305	114	100	100
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d
	Air drying	100	100	273	4,925	100	100
	MATH	100	100	142	2,911		

^a Washing medium/resuspension medium (washing medium/resuspension medium with which the reference value was obtained).

^b Centrifugation at 5,000 × g.

^c Centrifugation at 15,000 × g.

^d The bacterial cells used for XPS analysis were not washed in the NaCl solution.

rose and electrophoretic mobility was lower (6 of 32) than the number of correlations between data which we did not expect to be determined by surface charge (13 of 44 [39]). Affinity for Sepharose columns generally did not correlate with attachment to substrata having similar properties (e.g., hydrophobic columns and hydrophobic substrata or vice versa [39]).

DISCUSSION

The results of this systematic study clearly illustrate the shortcomings of techniques which may form part of a protocol for the preparation of microbial cells used for surface analysis. Both physicochemical and morphological properties of cells were altered during cell preparation. The MATH test, which is commonly used to assess cell hydrophobicity, was shown to physically change the constitution of microbial surfaces. Desiccation of microorganisms caused cellular disruption and reduced the numbers of viable organisms significantly. Harvesting by high-speed centrifugation generally reduced the net surface charge of *E. coli* or *S. epidermidis* but not the net surface charge of an organism with a dense glycocalyx, *Psychrobacter* sp. strain SW8. Washing and resuspending microorganisms in clean water also altered the physicochemical constitution of microbial cell surfaces. The considerable influence that the cell preparation protocol had on the outcome of cell surface analysis was most evident in the results obtained in adhesion assays and in Sepharose column experiments. These two techniques by themselves do not require additional cell manipulation and are sensitive primarily to the properties of

the cell-medium interface, the primary objective of cell surface analysis. In summary, the results obtained with a set of microorganisms that represent the different types of cell surfaces in the microbial world suggest that techniques that involve the cell manipulations which we examined are not suitable for obtaining information about physicochemical properties of unmanipulated cell surfaces.

The physicochemical parameters of the organisms used in these experiments have been measured previously by other workers. The hydrophobicity, as determined by the MATH test, and electrophoretic mobility of *Psychrobacter* sp. strain SW8 controls were consistent with previous values for these parameters obtained for this organism (25, 46). The electrophoretic mobilities determined for *E. coli* and *S. epidermidis* at the time of harvesting were comparable to the electrophoretic mobilities previously reported for the same strains by Gilbert et al. (18). The results of the MATH assays performed with *E. coli* and *S. epidermidis* fell within hydrophobicity ranges for different strains of these organisms determined by Harkes et al. (22) and Cuperus et al. (9). The attachment characteristics of *Psychrobacter* sp. strain SW8 to stainless steel, aluminum, perspex, and polypropylene PP were consistent with the results of previous assays conducted with this organism (46). The strains of *E. coli* and *S. epidermidis* used in this study were also used in gel interaction chromatography experiments by Gilbert et al. (18), who reported retention levels of 60 and 92%, respectively, for these organisms on octyl-Sepharose. Our results fell within the ranges of hydrophobicity values previously reported

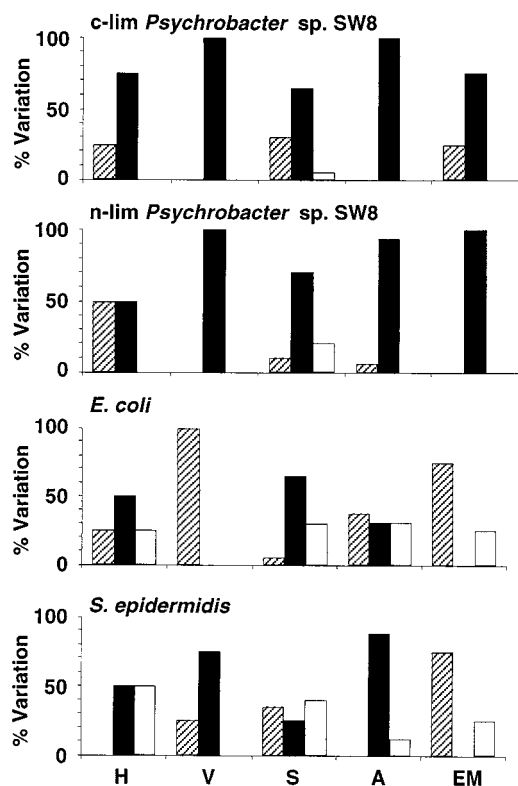


FIG. 2. Effects of centrifugation at $15,000 \times g$ on microbial physicochemical cell surface properties. H, hydrophobicity; V, viability; S, Sepharose column assays; A, attachment to solid substrata; EM, electrophoretic mobility. The percentages of experiments where the values of the parameters after treatment were reduced (cross-hatched bars), unchanged (solid bars), and increased (open bars) are shown. For information on reference data and modifications of the parameters see Tables 1 through 8. c-lim, carbon limited; n-lim, nitrogen limited.

for these organisms; discrepancies in the raw data were primarily due to the significantly higher concentration of NaCl in the elution buffers used by Gilbert et al. (18).

MATH. The significantly reduced survival rates of organisms exposed to dodecane in MATH tests, as well as electron micrographs of morphologically modified cell surfaces, clearly illustrate the destructive effect of mixing bacteria with this hydrocarbon. Vanhaecke and Pijck (55) also obtained evidence that cell lysis occurs in a study in which 10 different strains of gram-negative bacteria were exposed to hexadecane, octane, and xylene. These authors measured the level of ATP in the medium as an indirect lysis indicator and found that hexadecane was the hydrocarbon which caused the least lysis of the microorganisms tested. Some cells were disrupted by simple vortexing. Proteins, polysaccharides, fatty acids, and other cell components released by lysis or removed from the cell surface during vortexing are likely to adsorb to the surfaces of both cells and hydrocarbons, thus modifying their physicochemical properties in a manner analogous to the physicochemical changes observed after adsorption of organic films on solid substrata (45). The great reductions in the electrophoretic mobilities of all of the organisms after mixing with dodecane indicate the importance of this process, which in this case led to a significant loss of charged groups at the cell surface and/or to the masking of these charged components by lysed cell debris. In addition, hydrocarbon droplets are likely to remain negatively charged after conditioning with cell material. Geertsema-Doornbusch et al. (17) reported that hexadecane drop-

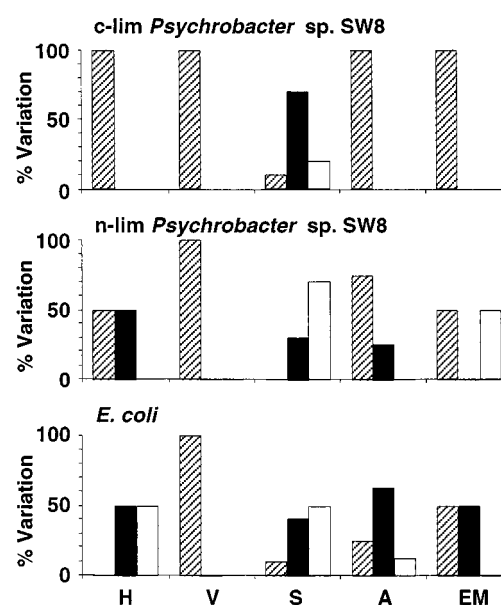


FIG. 3. Effects of freeze-drying on microbial physicochemical cell surface properties. H, hydrophobicity; V, viability; S, Sepharose column assays; A, attachment to solid substrata; EM, electrophoretic mobility. The percentages of experiments where the values of the parameters after treatment were reduced (cross-hatched bars), unchanged (solid bars), and increased (open bars) are shown. For information on reference data and modifications of the parameters see Tables 1 through 8. c-lim, carbon limited; n-lim, nitrogen limited.

lets are negatively charged at pH 7, while van der Mei et al. (53) demonstrated that the electrophoretic mobility of hexadecane droplets is reduced after adsorption of proteins. The MATH assay is, therefore, unsuitable for measuring cell surface hydrophobicity in view of both the destructive nature of the protocol and the fundamental physicochemical inconsistencies of the test (use of negatively charged droplets to measure hydrophobicity).

Centrifugation at $15,000 \times g$. The relatively small effects of centrifugation at $15,000 \times g$ on the physicochemical parameters of *Psychrobacter* sp. strain SW8 are surprising in view of the considerable alterations to the cell surface structure. These effects contrasted with the loss of viability of *E. coli* and the significant alterations of many cell surface parameters of *S. epidermidis*. The disappearance of the electron-dense outer layer of the glycocalyx of carbon-limited *Psychrobacter* sp. strain SW8 observed by electron microscopy had practically no effect on the cell surface parameters of this organism. We have to consider, however, that the technique employed for morphological analysis of cells, electron microscopy, is itself prone to introduction of artifacts since cells have to be desiccated prior to analysis (32). The artifacts introduced by this analysis, however, are consistent for each type of cell. Differences in morphology between cells of the same organism subjected to different treatments are, therefore, indicative of real effects of the treatment rather than artifacts introduced by electron microscopy. The disappearance of the electron-dense layer may represent both a loss of material and a conformational alteration introduced during centrifugation. Hydrostatic pressures inside a 15-ml laboratory centrifuge tube centrifuged at $10,000 \times g$ may be as high as 10 atm (20). The ensuing high shear rates could strip material from the cell surface and thus generate a new and very different microbe-environment interface. The physicochemical properties of this new interface would be similar to those of the original cell surface only if the

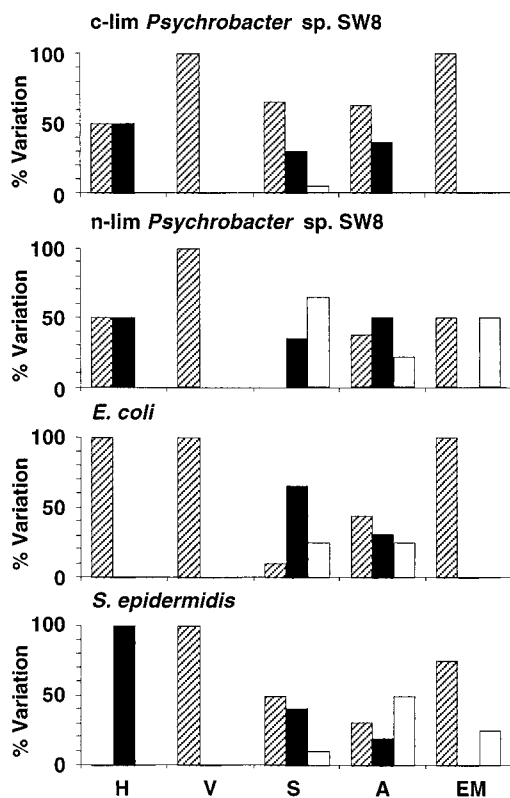


FIG. 4. Effects of air drying on microbial physicochemical cell surface properties. H, hydrophobicity; V, viability; S, Sephacrose column assays; A, attachment to solid substrata; EM, electrophoretic mobility. The percentages of experiments where the values of the parameters after treatment were reduced (cross-hatched bars), unchanged (solid bars), and increased (open bars) are shown. For information on reference data and modifications of the parameters see Tables 1 through 8. c-lim, carbon limited; n-lim, nitrogen limited.

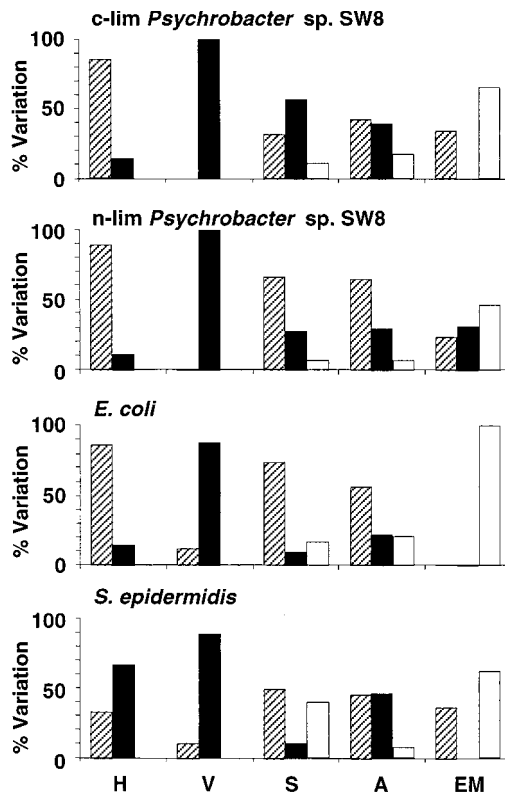


FIG. 5. Effects of changes in resuspension medium on microbial physicochemical cell surface properties. H, hydrophobicity; V, viability; S, Sephacrose column assays; A, attachment to solid substrata; EM, electrophoretic mobility. The percentages of experiments where the values of the parameters after treatment were reduced (cross-hatched bars), unchanged (solid bars), and increased (open bars) are shown. For information on reference data and modifications of the parameters see Tables 1 through 8. c-lim, carbon limited; n-lim, nitrogen limited.

stripped material was replaced by material with similar physicochemical properties from inside the glycocalyx. The blebbing observed in some cells indicated that weakening of the cell wall occurred during centrifugation, which eventually caused the cell wall to rupture and release the cell contents during desiccation for electron microscopy. The considerable decrease in viability and the widespread modifications of physicochemical cell surface properties suggest that *E. coli* cell surfaces did not resist centrifugation at $15,000 \times g$. Gilbert et al. (20) reported decreases of 25 and 40% in the viability of exponential-phase *Pseudomonas aeruginosa* following centrifugation at $5,000 \times g$ and $10,000 \times g$ respectively, and that centrifugation of *E. coli* at more than $10,000 \times g$ increased the susceptibility of this organism to biocides (19). Most damage to cells occurred in the first few minutes of centrifugation (20). Adsorption of material released from dead cells may have contributed to modification of the cell surfaces since the alterations were more pronounced when a considerable proportion of the population had been inactivated by high-speed centrifugation.

Effects of desiccation. The major cell disruptions caused by freeze-drying or air-drying procedures evident in electron micrographs and the significant decreases in viability should be interpreted with caution. Analyses of viability, of physicochemical parameters, and of electron microscopy results all require rehydration of dehydrated cells. This process may cause considerable damage to microbes, although it is well-known from electron microscopy and from recent investigations performed with environmental scanning microscopes that severe dehydra-

tion causes the collapse of biological macromolecular structures (3). If the conformation of cell structures were preserved in the dry state, then rehydration would not affect cell integrity. Damage to cell structures during the rehydration process, therefore, indicates that the constitution of these structures is altered when they are dry. A greater proportion of *S. epider-*

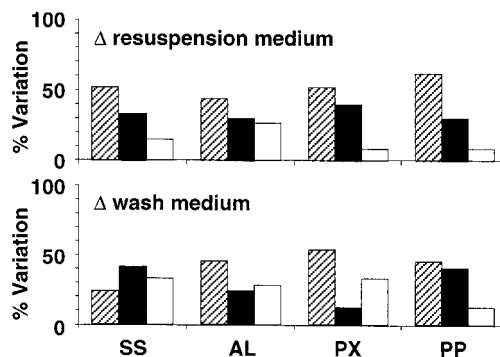


FIG. 6. Effects of changes of the resuspension and washing media on attachment of cells to substrata. SS, stainless steel; AL, aluminum; PX, perspex, PP, polypropylene. The percentages of experiments where the values of the parameters after treatment were reduced (cross-hatched bars), unchanged (solid bars), and increased (open bars) are shown. For information on reference data and modifications of the parameters see Tables 1 through 8.

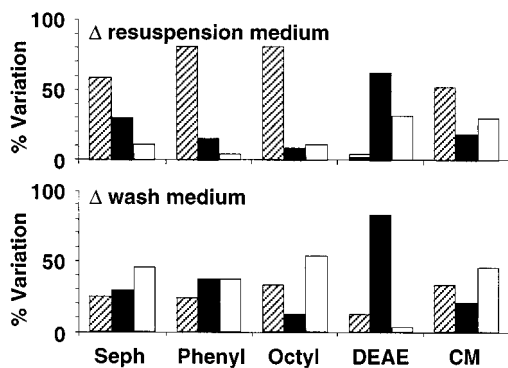


FIG. 7. Effects of changes of the resuspension and washing media on the retention of cells in Sepharose columns. Seph, unsubstituted Sepharose; Phenyl, phenyl-Sepharose; Octyl, octyl-Sepharose; DEAE, DEAE-Sepharose; CM, carboxymethyl-Sepharose. The percentages of experiments where the values of the parameters after treatment were reduced (cross-hatched bars), unchanged (solid bars), and increased (open bars) are shown. For information on reference data and modifications of the parameters see Tables 1 through 8.

midis than of the gram-negative organisms survived the desiccation processes primarily because highly cross-linked peptidoglycan constitutes approximately 50% of the gram-positive cell wall and provides these organisms with greater structural rigidity (58). The cell envelope of gram-negative organisms contains significantly less peptidoglycan, and consequently *Psychrobacter* sp. strain SW8 and *E. coli* had a much greater tendency to rupture during either desiccation process or when

dried samples were resuspended in liquid media (51, 57). Preparation of lyophilized microbial stock cultures usually includes the use of a cryoprotectant, such as sucrose; however, this procedure is not suitable for preparing samples for cell surface analysis by high-vacuum techniques as the cryoprotectant coats the cells, thus masking surface components (2). In the case of freeze-dried *S. epidermidis*, which could not be resuspended, exposure of macromolecules in the peptidoglycan layer of this organism due to dehydration resulted in cross-linking of wall components between adjacent cells. Leaked intracellular material was also evident in electron micrographs of *S. epidermidis*, and the molecules may have contributed to the aggregation of resuspended organisms observed. Severe alterations of the physiological characteristics and viability of microorganisms subjected to freeze-drying have been demonstrated previously (10, 28, 51). The cytoplasmic membrane is considered the main site of dehydration damage due to changes in the physical state of membrane lipids and/or modification of the structure of key proteins (29, 31). Rehydration of freeze-dried cultures in liquid media is usually completed within a few seconds, which subjects the cells to very fast changes in the hydration state (11). Following rehydration, cells of *Lactobacillus bulgaricus* did not control the entry of sodium chloride or prevent the loss of internal constituents, such as β -galactosidase (6). Investigating the effects of freeze-drying on yeast cells, Dengis and Rouxhet (10) observed a variety of surface morphologies within the same species. These authors observed that some cell surface modification, such as removal of polymers or of hairlike structures, occurred during the first centrifugation of the cells. Cooling of cell samples had to be strictly monitored during the

TABLE 8. Impact of modification of the washing medium on interaction with Sepharose columns and attachment to solid substrata of test organisms subjected to different cell preparation protocols

Organism	Treatment	% of reference value																								
		H ₂ O/NaCl (NaCl/NaCl) ^a						H ₂ O/H ₂ O (NaCl/H ₂ O)						H ₂ O/NaCl (NaCl/NaCl)				H ₂ O/H ₂ O (NaCl/H ₂ O)								
		Sepharose	Phenyl-Sepharose	Octyl-Sepharose	DEAE-Sepharose	Carboxymethyl-Sepharose	Sepharose	Phenyl-Sepharose	Octyl-Sepharose	DEAE-Sepharose	Carboxymethyl-Sepharose	Sepharose	Phenyl-Sepharose	Octyl-Sepharose	DEAE-Sepharose	Carboxymethyl-Sepharose	Stainless steel	Aluminum	Perspex	Polypropylene	Stainless steel	Aluminum	Perspex	Polypropylene		
Carbon-limited <i>Psychrobacter</i> sp. strain SW8	5,000 × g ^b	100	100	115	100	241	100	100	112	100	203	100	27	15	19	629	778	600	575							
	15,000 × g ^c	100	100	110	100	197	167	140	174	100	100	100	100	100	8	21	100	600	500	100						
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d		
	Air drying	100	203	260	100	155	146	100	37	100	83	39	100	43	100	100	100	100	0							
Nitrogen-limited <i>Psychrobacter</i> sp. strain SW8	5,000 × g ^b	136	146	106	100	188	100	100	100	100	100	100	37	100	33	310	282	275	250							
	15,000 × g ^c	154	134	101	100	240	100	47	19	100	68	165	43	59	100	213	257	550	100							
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d			
	Air drying	79	100	100	100	100	100	80	58	100	100	255	256	400	— ^e	100	22	0	0							
<i>E. coli</i>	5,000 × g ^b	219	188	221	100	100	42	100	27	100	53	10	9	51	33	7	46	14	100							
	15,000 × g ^c	130	126	110	132	125	58	40	34	100	43	459	233	26	13	2,005	1,911	0	100							
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d			
	Air drying	362	213	207	100	194	182	100	245	100	78	167	37	150	17	48	35	0	10							
<i>S. epidermidis</i>	5,000 × g ^b	404	326	272	94	588	14	17	9	100	49	100	65	24	100	100	100	46	100							
	15,000 × g ^c	78	94	83	42	123	47	31	34	100	19	100	70	220	100	100	100	100	100							
	Freeze-drying	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d			
	Air drying	263	249	208	67	256	151	100	100	100	55	66	100	557	19	54	17	0	37							

^a Washing medium/resuspension medium (washing medium/resuspension medium with which the reference value was obtained).

^b Centrifugation at 5,000 × g.

^c Centrifugation at 15,000 × g.

^d The bacterial cells used for XPS analysis were not washed in the NaCl solution.

^e No cells adhered to the reference substratum.

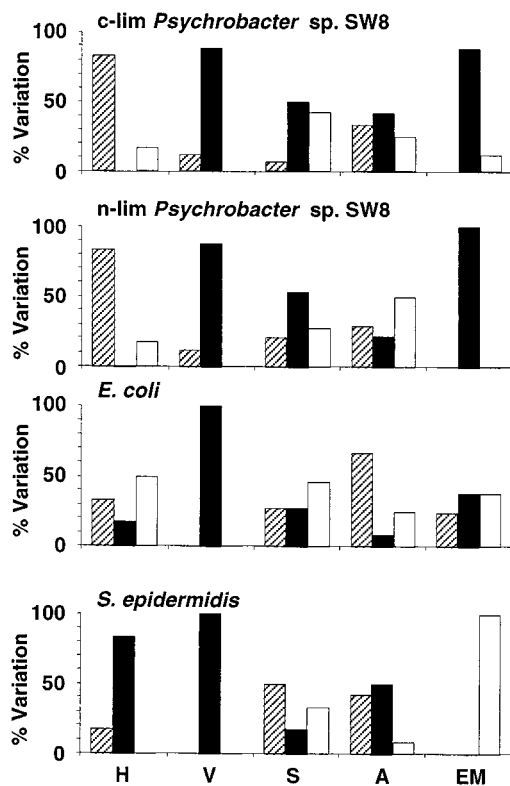


FIG. 8. Effects of changes of washing medium on microbial physicochemical cell surface properties. H, hydrophobicity; V, viability; S, Sepharose column assays; A, attachment to solid substrata; EM, electrophoretic mobility. The percentages of experiments where the values of the parameters after treatment were reduced (cross-hatched bars), unchanged (solid bars), and increased (open bars) are shown. For information on reference data and modifications of the parameters see Tables 1 through 8. c-lim, carbon limited; n-lim, nitrogen limited.

freeze-drying process to improve the reproducibility of analyses. Scanning electron microscope analysis of cells that were either melted or mechanically disrupted prior to freeze-drying indicated that variation in the results occurred primarily because of the release of intracellular material from lysed cells.

Organisms survived air drying considerably better than they survived freeze-drying, probably because of the lower level of dehydration. The better survival of *Psychrobacter* sp. strain SW8 than of *E. coli* following air drying was probably due to the protection against desiccation afforded to the former organism by its glycocalyx. Similarly, Ophir and Gutnick (38) found that mucoid strains of *E. coli*, *Acinetobacter calcoaceticus*, and *Erwinia stewartii* were significantly more resistant to desiccation than corresponding isogenic nonmucoid mutants of these organisms. When results obtained with air-dried cells are analyzed, it is important to consider that these organisms were deposited as a thick layer, the filter cake, on a membrane. Desiccation would have affected primarily the cells located in the top layers of the filter cake. The cells at the center and at the bottom of the filter cake remained hydrated to some extent, and these cells were probably the cells that survived the procedure. Therefore, the cell populations used to analyze cell surface parameters of air-dried organisms were heterogeneous and included cells exposed to different degrees of dehydration. The majority of the cells in a filter cake are embedded in the structure and are not exposed at the surface. These cells therefore determine the physiological properties of the population. With the exception of hydrophobicity of *S. epidermidis*, all of

the parameters measured were significantly modified by air drying. Clearly, even cells embedded in filter cakes were affected considerably by the procedure.

Influence of resuspension medium. Organisms resuspended in the NaCl solution were compared to organisms resuspended in water to determine the effects of modification of the resuspension medium on cell surface properties. The organisms used in this study had a net negative surface charge at pH 7.4. The negative charge of bacterial cells is derived from ionogenic groups which include amino and carboxylic acids and phosphate groups (22). Not all of these groups need to be exposed at the cell surface, however. In fact, ionogenic groups embedded in the cell wall may contribute significantly to the surface charge (54). The negatively charged cell surface attracts positively charged ions from the medium and repels the negatively charged counterparts. This process generates the Stern layer at the medium-cell interface that contains sorbed counterions (48). The thickness of this layer corresponds to the average diameter of the hydrated sorbed counterions. Further into the medium, the Stern layer is replaced by the diffuse double layer, a region of charge imbalance, where cations are accumulated and anions are depleted. The thickness of this region increases when the ion concentration in the medium decreases, since ions required for compensation of the charge on the cell surface have to be recruited from a larger volume of solution around the cell.

Most physicochemical parameters measured in this work required contact between the analyte (hydrocarbon droplet, solid substratum, Sepharose gel) and the cell surface. All analyte surfaces except the positively charged carboxymethyl-Sepharose surface were negatively charged in the buffers used. Attachment of cells to these surfaces, therefore, involved the contact of two negatively charged interfaces. This physicochemical phenomenon is best described by the DLVO theory (48), which is based on the energy changes that occur when two charged particles attach to each other in an electrolyte. Attachment requires that the forces of attraction (van der Waals forces, hydrogen bonds, ionic bonds) overcome the forces of repulsion (primarily electrostatic forces) in the immediate vicinity of the cell surface. The repulsive forces decrease exponentially with distance from the cell surface, while the attractive forces are maximal upon contact. Replacement of a phosphate-buffered solution containing NaCl at the concentration found in seawater by a medium containing the same amount of phosphate buffer but no salt should modify the electrostatic component of the interfaces involved in the process significantly. This obviously should influence the value of contact-dependent parameters of cells, such as adhesion to solid substrata, retention in Sepharose columns, and contact with hydrocarbon.

The DLVO theory and the Helmholtz-Smoluchowski equation predict that the change from a high-salt medium to a low-salt medium, such as phosphate-buffered H₂O, should increase the electrophoretic mobility of the organisms in absolute terms (e.g., change the values to more negative numbers) and reduce hydrophobicity, affinity to Sepharose, and adhesion to substrata. These effects were indeed observed in the majority of our assays. However, situations in which the values of the parameters remained unchanged or even increased suggest that the interactions of cells with analytes in the assays were not solely determined by electrostatic forces. Reductions in electrophoretic mobility were observed with most cells exposed to hydrocarbons in the MATH assay and with some freeze-dried and air-dried cells (Table 5). Preferential stripping of charged cell surface components attached to the hydrocarbon droplets may have been responsible for the reduction in cell

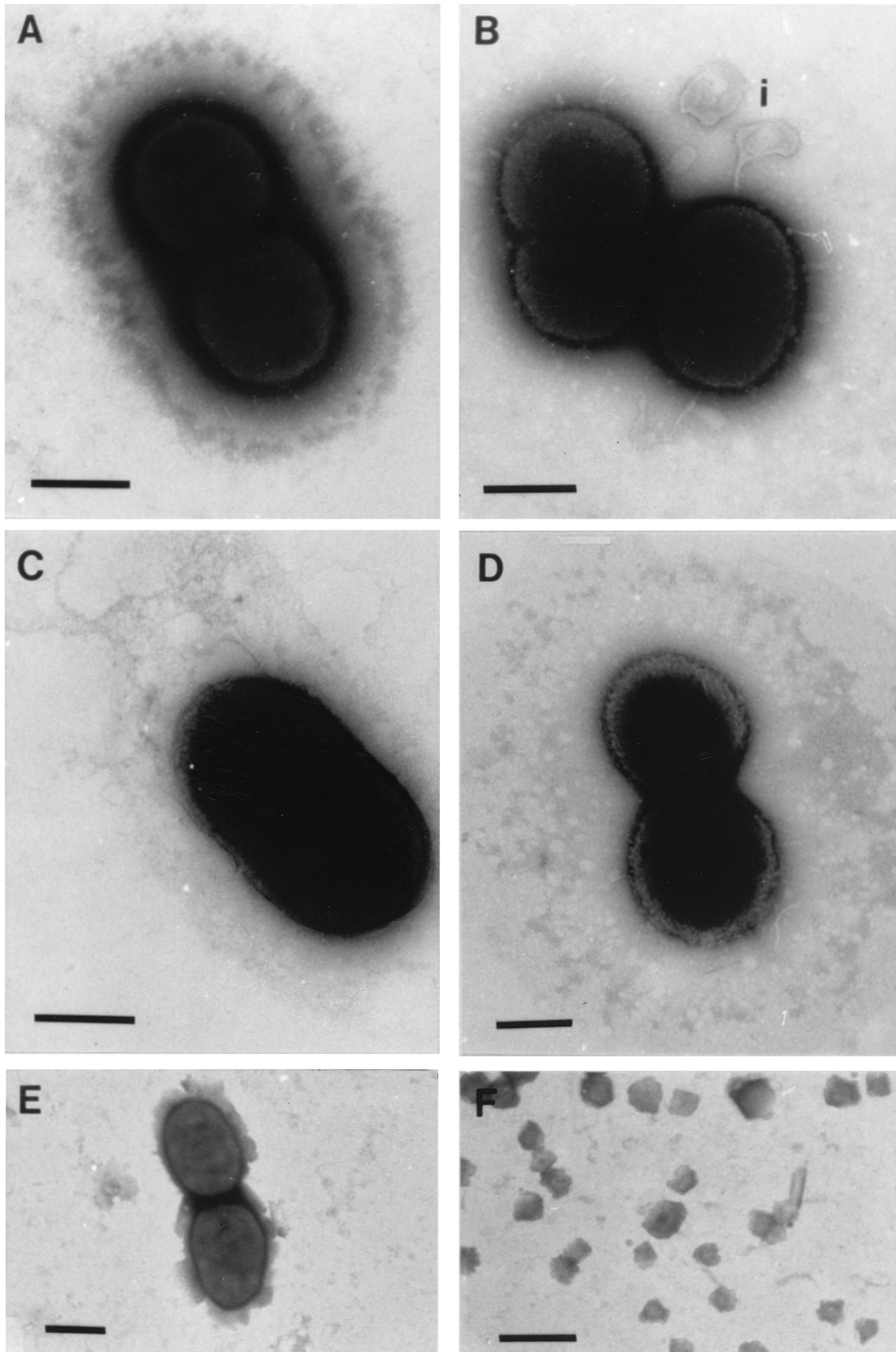


FIG. 9. Negatively stained electron micrographs of *Psychrobacter* sp. strain SW8. (A) Cells harvested by centrifugation at $5,000 \times g$. Bar = $1 \mu\text{m}$. (B) Carbon-limited phenotype harvested by centrifugation at $15,000 \times g$. Bar = $1 \mu\text{m}$. i, blebbing of cell envelope. (C) Cells air dried on cellulose acetate filters. Bar = $2 \mu\text{m}$. (D) Cells after mixing with dodecane in the MATH assay. Bar = $1 \mu\text{m}$. (E and F) Freeze-dried cells. Bars = 2 and $5 \mu\text{m}$, respectively.

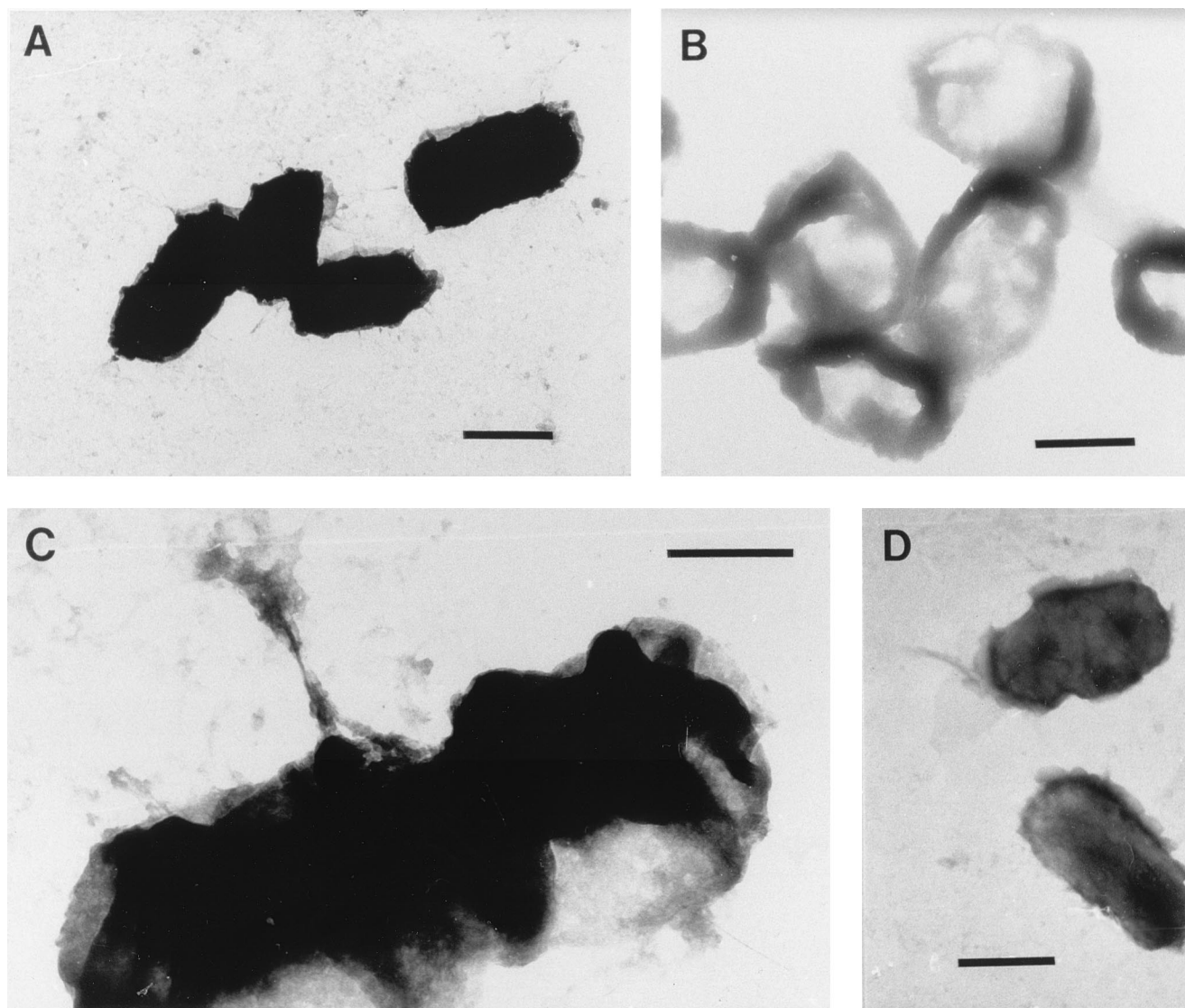


FIG. 10. Negatively stained electron micrographs of *E. coli* ATCC 8739. (A) Cells harvested by centrifugation at $5,000 \times g$. Bar = $2 \mu\text{m}$. (B) Freeze-dried cells. Bar = $2 \mu\text{m}$. (C and D) Cells after mixing with dodecane in the MATH assay. Bars = 1 and $2 \mu\text{m}$, respectively.

surface charge of the MATH-treated organisms. Reduced cell surface charges of air-dried or freeze-dried cells were most probably caused by a different process. The polymers of microbial cell surfaces are sufficiently flexible to allow their reorientation at the cell surface, depending on environmental conditions. Air and vacuum are hydrophobic phases (7). In such environments, a dynamic polymer surface such as the cell surface would reorientate to expose hydrophobic groups at the interface (7) in a manner analogous to the modification of the surface chemical composition of hydrogels, which depends on the hydration state of the polymers (40). When the surfaces of some freeze-dried cells were rehydrated, the macromolecules at the interface, which were in equilibrium with a hydrophobic medium, may not have been capable of reverting back rapidly to the hydrophilic interface characteristic of hydrated media. These cells would thus remain less charged and more hydrophobic than the original cells. DEAE-Sepharose is a positively charged surface. The fact that the affinity for this type of column was scarcely affected or even improved in some instances when the resuspension medium was changed from the

NaCl solution to water reflects the strong attractive force between the surface of the DEAE-Sepharose column and the negatively charged cells. An additional factor that needs to be considered when the results of adhesion assays or Sepharose column experiments are analyzed is the fact that cell manipulation protocols, such as freeze-drying, centrifugation, and hydrocarbon contact, may disrupt the cell surface structure so that cell surface roughness is increased and macromolecular appendages (polysaccharides or proteins) protrude into the medium. Under certain conditions and on certain substrata, some of these appendages may bridge the repulsive electric double layer and establish sufficiently strong links to initiate adhesive contacts.

Influence of washing buffer. Organisms resuspended in the NaCl solution were compared to organisms resuspended in water to determine the effects of modification of the washing medium on cell surface properties. The washing medium can have an effect on the cell surface properties of a microorganism only via modification of the cell surface itself, since it is not the final interaction environment. Osmotic effects are one pos-

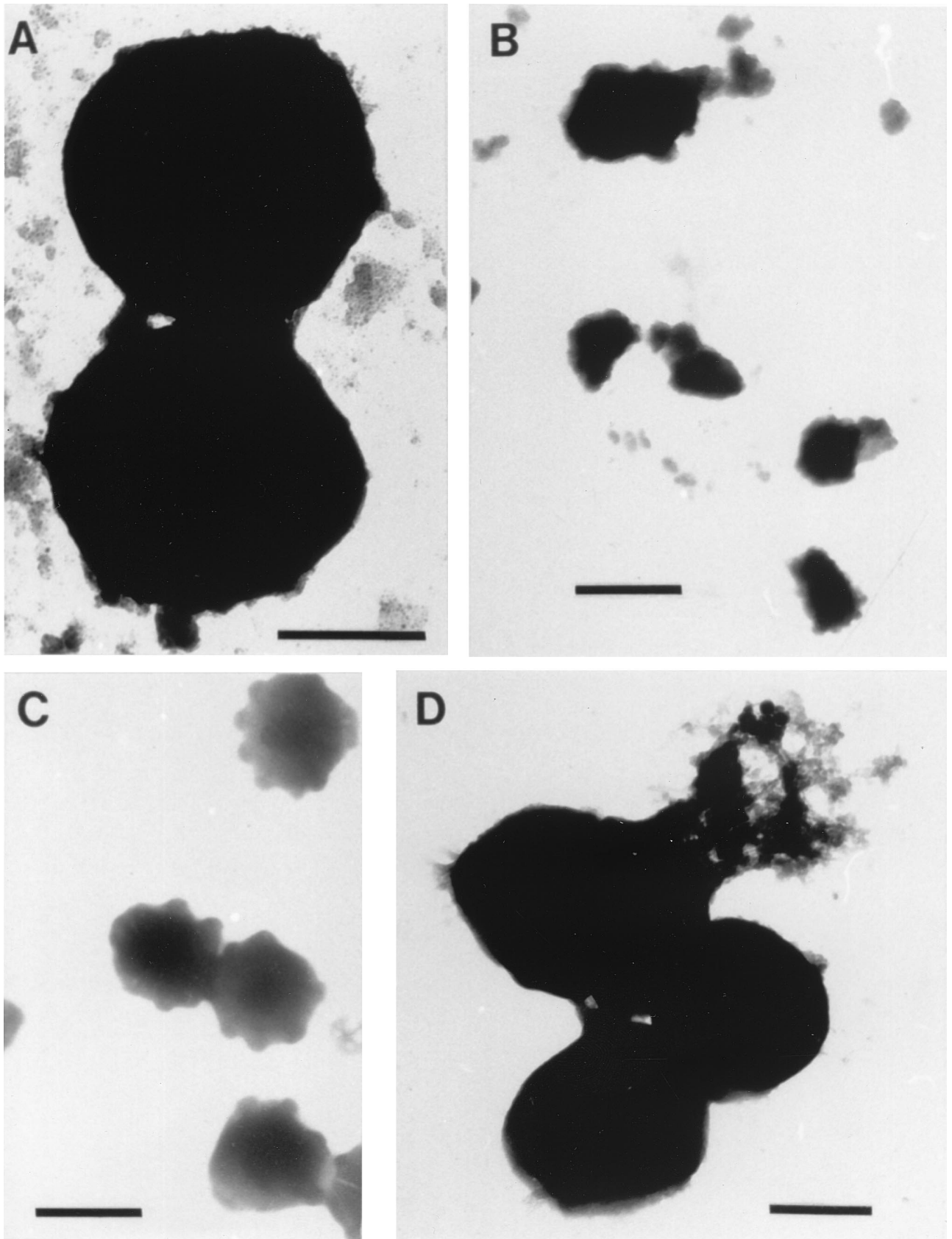


FIG. 11. Negatively stained electron micrographs of *S. epidermidis* NCTC 11047. (A) Cells harvested by centrifugation at $5,000 \times g$. Bar = $1 \mu\text{m}$. (B) Freeze-dried cells. Bar = $2 \mu\text{m}$. (C) Cells air dried on cellulose acetate filters. Bar = $2 \mu\text{m}$. (D) Cells after mixing with dodecane in the MATH assay. Bar = $1 \mu\text{m}$.

sible mechanism by which a change from a high-salt washing medium to a low-salt medium may change the cell surface of a microbe. Microorganisms in aquatic environments have high intracellular concentrations of potassium ions and metabolites which produce cytoplasmic turgor pressure (27). This internal pressure is borne by the peptidoglycan layer and is maintained by the passive and active influx and efflux of ions from the surrounding liquid medium (57). Consequently, sudden exposure of microorganisms to lower-ionic-strength conditions disturbs the balance of ion transfer and results in conformational changes in the cell envelope. If these changes include modifications to the cell surface and if the modifications are stable in the resuspension buffer, then physicochemical cell surface properties are altered. The modifications can also result in the leakage of internal cell components, such as periplasmic and cytoplasmic enzymes and ions, as has been extensively reported previously for *E. coli* (33, 47, 59). These effects were not sufficiently strong to significantly affect the viability of the cells in this study. Coating of cell surfaces with leaked components, however, could have significantly modified their properties. Adsorption of ions from high-salt solutions or the lack of adsorption of ions is a third mechanism by which the washing medium may affect the cell surface properties of microbes. Adsorption of counterions may destabilize cell surfaces exposed to high shear stress during centrifugation, to dehydration during freeze-drying or air drying, and to vortexing and hydrocarbon contact during MATH. Carryover of adsorbed cations from a high-salt washing buffer into a low-salt resuspension medium should affect the cell surface properties measured in this medium compared with the properties of cells washed in low-salt medium. The type and density of adsorbed ions can vary greatly depending on the chemical composition of the cell wall, the presence of extracellular surface structures, the growth conditions, and the cell preparation protocols (13, 44). Cell surfaces damaged during cell preparation may therefore adsorb different ions, a factor which may exacerbate the effects of the cell manipulation procedure on cell surface properties.

Correlations between cell surface analysis techniques. Poor correlation between different parameters which should measure similar properties of cell surfaces is a common problem (12, 14, 35, 41). It may appear surprising that tests in which cells are supposedly exposed to the same chemical groups, such as straight-chain hydrocarbons in MATH and octyl-Sepharose tests, produce results which do not correlate with each other, as has often been observed by other workers (12, 16, 26, 35, 50). This lack of correlation is in part a consequence of often missing or inaccurate information about the mechanism underlying the method. For example, the negative charge of hexadecane droplets at pH 7 implies that electrostatic interactions play a major role in the MATH assay. A cell surface charge measured by electrophoretic mobility includes charges generated by materials buried in the cell membrane or cell wall and may not accurately reflect the charge at the microbe-medium interface. The mechanisms of a cell surface analysis method need to be carefully considered to avoid overinterpretation of the data obtained with it. For example, cells used in adhesion assays may be centrifuged at $5,000 \times g$, but the same cells are subjected to vortexing in the presence of hydrocarbons before MATH tests. As our results clearly show, these two types of cells may have very little in common on their surfaces, and the MATH results may, therefore, not reflect the properties of the cell surface of the organisms used in the adhesion assay (or Sepharose column chromatography). Microbial cell surface analysis requires new methods that do not depend on extensive cell manipulation prior to measurement and that allow deter-

minations of cell surface parameters of organisms obtained directly from the environment. Flow cytometry (37) and atomic force microscopy in the tapping mode in situ (21) in combination with appropriate cell surface markers or appropriately modified cantilever tips may permit nondestructive and non-invasive analysis of cell surface parameters.

Conclusion. All of the microbes investigated in this work were affected to some degree by cell preparation techniques, but in each technique some organisms or different phenotypes of the same organism were more susceptible than others. Cells may even lose their viability after manipulation by some protocols. Therefore, it is important that the effects of cell surface analysis protocols on microbes be ascertained in every investigation pertaining to bacterial cell surface characteristics to ensure that cell samples are subjected to the least disruptive preparation methods and that the final results reflect the nature of the true microbial cell surface as realistically as possible. A second, very important issue that needs to be considered when cell surface analysis data are interpreted is the actual mechanism by which cell surface parameter data are obtained. The standard protocol used for harvesting bacteria, centrifugation at $5,000 \times g$, was found to be the protocol that was least disruptive to the structural integrity of cells. The composition of the buffer used in the cell surface analysis test should resemble the composition of the environment of the microbe as closely as possible. Nondestructive Sepharose column assays are preferable for hydrophobicity determinations. Last but not least, reliable, nondestructive cell surface analysis techniques need to be developed, perhaps by using some of the powerful new techniques, such as flow cytometry and atomic force microscopy.

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