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A Novel Image-analysis Technique for Measurement of Bacterial Cell Surface Tension

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Abstract

Cell-surface hydrophobicity is different for *Staphylococcus epidermidis* cells grown under different environmental conditions; this might influence attachment and colonization of surfaces. Although a wide variety of techniques has been employed to measure bacterial surface hydrophobicity, including contact angle determinations, adherence to hydrocarbons, hydrophobic-interaction chromatography and salt aggregation, many of these either require large numbers of cells or do not yield comparable quantitative data. This study describes a novel, quantitative method for the determination of bacterial surface tension on the basis of image analysis of cell–cell interactions.

S. epidermidis (strains 900 and 901) were suspended in different concentrations of propanol of known surface tension and examined by bright-field microscopy linked via a charge-couple device (CCD) camera to an image analyser. Frames were chosen randomly and the data recorded as a ratio of count/percentage coverage for each frame. The results showed that for strains 900 and 901 this ratio was maximum at surface tensions of 67 and 61 $mN\,m^{-1}$ respectively. At these values of minimal interaction the surface tension of the liquid was equal to the bacterial cell surface tension. The results were in close agreement with those obtained from contact angles. The advantage of surface tension measurements is that, irrespective of the method used, the results generated are quantitative values and are therefore directly comparable.

The method reported is reliable, reproducible and is of particular value because the number of cells required is, typically, at least two orders of magnitude lower than is required for commonly used alternative methods.

Bacterial attachment and colonization of solid surfaces is substantially influenced by the nature of the substrate surface, the suspending medium, and the surface properties of the bacterium. Each of these can vary with changes in environmental conditions. In particular, structures on the bacterial surface involved in the initial adherence and colonization of indwelling host devices and host tissue can change. The intrinsic plasticity of the bacterial cell envelope (Ellwood & Tempest 1972; Brown & Williams 1985; Costerton 1988) has enabled the pathogenic organism to invade and adapt to changing in-vivo environmental conditions. *Staphylococcus epidermidis* has the ability to survive in body fluids under CO_2-replete conditions (Denyer et al 1990a). Carbon dioxide tension can alter the balanced functioning of metabolic pathways and induce or repress enzyme systems. This might in turn alter the biosynthesis and phenotypic expression of envelope components which might be involved in adherence. Denyer et al (1990b) observed significant differences between the surface chemistries of *S. epidermidis* grown in air and in air supplemented with 5% CO_2. By use of X-ray photoelectron spectroscopy and SDS-PAGE (sodium dodecylsulphate-polyacrylamide-gel electrophoresis) they discovered a significant increase in exposed carbohydrates and proteins after growth in CO_2 and in some strains this was linked to a change in adherence behaviour (Wilcox et al 1991).

Studies have shown that the cell envelope is also dependent on the composition and form (solid or liquid) of the culture medium (Al-Hiti & Gilbert 1983). Expression of high molecular-weight

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proteins by *Staphylococcus aureus* has been shown to be enhanced when the bacterium is grown on a solid medium compared with growth on the same medium in the liquid form (Cheung & Fischetti 1988); the bacterium was able to alter these surface proteins when passaged alternately on liquid and solid media. The availability of various nutrients can also influence the bacterial surface. Iron-repressible proteins are associated with the wall and membrane of *S. epidermidis* and these are expressed when the bacterium is grown in iron-deplete nutrient broth (Williams et al 1988; Modun et al 1992).

It is apparent from the literature that hydrophobic interactions between microorganisms and polymeric biomaterials are particularly important in the early stages of adhesion (Rozgonyi et al 1990; Skvarla 1993). Hydrophobic interactions arise because the molecules of a polar liquid arranged around a non-polar solute are more highly ordered than within the bulk of the liquid. The establishment of a hydrophobic interaction with a surface displaces the liquid molecules from around the solute, increasing the disorder of the system—in other words there is an increase in entropy. The strength of the interactions are determined by the hydrophobicity of the surfaces involved. Many hydrophobic components, primarily proteins and lipids (including lipoteichoic acids), are located on the surface of bacterial cells. Coagulase-negative staphylococci are known to have only a small amount of exposed surface protein (Hogt et al 1985; Reifsteck et al 1987) and this is, therefore, likely to play only a small part in contributing to the hydrophobicity of the surface.

Hydrophobicity, being a surface characteristic, is influenced by growth environment, and hydrophobicity varies for *S. epidermidis* cells grown on, or in, different culture media (Rozgonyi et al 1990). Hydrophobicity has also been shown to vary with growth phase. The changes in cell-surface hydrophobicity occurring during growth phases reflect alterations in the concentrations of cell-surface components or their position in the cell wall from one phase to the next (Rozgonyi et al 1990; Gilbert et al 1991).

A wide variety of techniques has been employed to measure the surface hydrophobicity of the bacterial cell; a comprehensive review of their development is given by Rosenberg & Doyle (1990). The methods include contact-angle determinations (Neumann & Good 1979), adherence to hydrocarbons in two-phase systems (Rosenberg 1991), hydrophobic-interaction chromatography (Smyth et al 1978) and salt aggregation (Lindahl et al 1981). Each of these methods measures slightly different properties of the cells (Table 1) and it is, therefore, not surprising that the literature contains many contradictory observations about the hydrophobic behaviour of microorganisms. Consequently it is preferable to use a range of tests to give a more complete picture of the surface properties of the bacterial cell rather than relying on the information gained from one assay alone.

A measure of surface hydrophobicity can also be gained from determination of cell surface tension (which is inversely proportional to hydrophobicity). This can be determined by several techniques including contact-angle measurement and sedimentation behaviour (Smith et al 1986). In the sedimentation technique developed to measure the surface tension of particles (Vargha-Butler et al 1985a) particles are suspended in a range of combinations of polar and non-polar liquids which cover an appropriate range of surface tensions and the characteristics of the sediment formed either under gravity or by centrifugation are assessed (Rodgers et al 1994; Shafi et al 1995).

The free energy of cohesion, ΔF^{COH} between interacting particles is expressed as $\Delta F^{COH} = -2\gamma_{pl}$

Table 1. Comparison of the cell-surface hydrophobicity of three strains of *Staphylococcus epidermidis* grown under different conditions and determined by four different methods.

Strain	Growth conditions	Water contact angle (°)	BATH test (% cells adsorbed)	Hydrophobic interaction assay, (% cells adsorbed)	Concn salt (±0.2 M) in salt aggregation test
900	Nutrient broth-air	17 (3)	4 (1)	22 (9)	1·2
901	Nutrient broth-air	32 (9)	8 (4)	59 (6)	1·2
904	Nutrient broth-air	16·8 (4)	54 (12)	35 (5)	2·8
900	Nutrient broth-CO_2	20 (5)	7 (7·6)	25 (7)	1·0
901	Nutrient broth-CO_2	35 (10)	8·4 (4)	53 (14)	1·0
904	Nutrient broth-CO_2	28 (10)	55 (18)	36 (6)	3·2

Values in parentheses are standard deviations; n = 10, 5 and 6 for contact angle measurements, BATH test and hydrophobic-interaction assay, respectively.

where γ_{pl} is the particle–liquid interfacial tension. Because γ_{pl} is always positive or zero, ΔF^{COH} is either negative or zero, implying there will always be an attraction between like particles suspended in a liquid. The limiting case is when there is no van der Waals interaction and $\gamma_{pl} = 0$, i.e. when the surface tension of the suspending liquid is equal to the particle surface tension. If there is agglomeration as a result of van der Waals interaction, this should cease when ΔF^{COH} approaches zero. Irregularly shaped agglomerated particles will not pack so tightly as single particles when they settle so that at the limiting case the sedimentation volume will be minimal. If no agglomeration occurs in suspension van der Waals interactions will cause the particles to pack more tightly when sedimented than those where no interactions exist at the limiting case (i.e. at $\Delta F^{COH} = 0$), therefore sedimentation volume will be maximal (Vargha-Butler et al 1985b, 1989).

The aim of the work presented in this paper was to explore various previously reported methodologies for determining cell-surface hydrophobicity and surface tension and to correlate these results with those obtained from a novel method based on direct image analysis of cell–cell interactions.

Materials and Methods

Microorganisms

The test microorganisms used were clinical isolates of *Staphylococcus epidermidis* (designated strains 900, 901 and 904) isolated from patients suffering from peritonitis and attending the Renal Unit, The City Hospital, Nottingham, UK. These strains were identified by use of API Staph (API, Basingstoke, UK) and were chosen for their different hydrophobicities (Denyer et al 1993).

Media

Nutrient broth No. 2 and nutrient agar No. 1 (Oxoid, Basingstoke, UK) were sterilized by autoclaving at 121°C for 15 min.

Maintenance of Staphylococcus epidermidis

Stocks of cells were kept at −70°C in nutrient broth supplemented with 10% v/v glycerol. Before use frozen stocks were thawed and grown in nutrient broth (100 mL) and their identity confirmed by use of API Staph and Gram staining. Contact angles were determined for individual strains to check for any significant changes in surface characteristics. These cultures were maintained as working cultures on nutrient agar slopes at 4°C for 1 month.

Experimental growth conditions

A separate nutrient agar slope was used to inoculate each 10-mL starter culture, by creating a suspension of cells from the slope in phosphate buffered saline (PBS) at a concentration of 2×10^8 colony-forming units mL^{-1} and using 0·2 mL to inoculate 10 mL nutrient broth no. 2. These were then incubated statically for 16 h at 37°C in air or 5% CO_2. Samples (1 mL) of the starter culture were used to inoculate 100 mL of the medium, contained in a 250 mL flask, which was then incubated under the same conditions before experimentation.

Determination of bacterial cell-surface hydrophobicity

The hydrophobicity of strains 900, 901 and 904 grown under various conditions was determined by four different methods.

Contact-angle measurement. The interfacial tension between bacteria and a fluid medium is often determined by contact-angle measurement by use of the sessile drop technique in which the contact angle formed by drops of liquid deposited on a layer of cellular material is measured.

Preparation of bacterial lawns. Broth cultures of *S. epidermidis* 900, 901, and 904 were prepared as described above, grown in air or 5%CO_2–95% air, and harvested after 24 h by centrifugation (5000 $g \times 15$ min). The cells were washed three times in triple distilled water (5000 $g \times 10$ min) and suspended to a concentration of 10^{11} colony-forming units mL^{-1} in 100 mL distilled water. Cellulose nitrate membranes (47 mm diameter, 0·45 μm pore size) were placed in a membrane-filter unit (Sartorius, UK) and washed with distilled water (200 mL) by filtration under vacuum. The bacterial suspensions were then filtered on to the membranes under vacuum giving a coverage of $5{\cdot}7 \times 10^9$ colony-forming units mm^{-2}. The filters were removed from the filter unit and placed, until required, in Petri dishes containing moistened filter paper, to prevent them from drying. Two lawns were prepared for each strain each time the experiment was performed.

Measurement of contact angles. The filters were removed from the Petri dishes, placed on up-turned Petri dishes and left for 1 h. The contact angles of both sides of a 2-μL drop of distilled water for each lawn was measured at 20°C, by projection of a

magnified image of the drop on to a screen on which the angle was traced. The two values for each drop and both lawns were averaged and recorded. The contact angle was measured in the same way, every 10 min, until it no longer changed with drying time. This constant value was recorded as the contact angle.

Hydrophobic-interaction assay. Hydrophobic-interaction chromatography of bacterial cells is based on the hydrophobic interaction between non-polar residues on a gel bed, usually octyl or phenyl sepharose, and non-polar regions on the bacterial surface. This has been adapted to form the basis of the hydrophobic interaction assay (HIA) (Denyer et al 1993) in which a bacterial suspension is mixed with a gel suspension and the extent of bacterial adhesion to the gel measured by optical density change.

Problems were encountered with the most hydrophobic strain 901 in that adsorption of the bacterial cells by the gel particles, which presumably resulted in larger agglomerates, caused the gel to pack loosely on settling, making it harder to remove the supernatant for measurement without disturbing the gel. This was overcome by centrifuging all the samples and controls for 2 min at 100 *g*, a speed which was shown not to cause settling of cells in suspension (i.e. control suspensions showed no change in optical density after centrifugation).

Broth cultures of *S. epidermidis* 900, 901, and 904 were prepared as described above, grown in air and 5%CO_2–95% air and harvested after 24 h by centrifugation (5000 $g \times 15$ min) and washed three times in PBSS (PBS buffer pH 7·2, containing 2 M NaCl), with centrifugation (5000 $g \times 10$ min) after each wash, before being resuspended in PBSS to a concentration of 10^8 colony-forming units mL^{-1}. Phenyl sepharose gel CL-4B (Sigma) was weighed, washed with PBSS and resuspended to a concentration of 30% w/v in PBSS. Gel suspension (1 mL) was added to each test cell suspension (1 mL) in glass test tubes. Controls were set up by adding PBSS (0·7 mL, equivalent to the volume of suspending fluid in the 30% gel suspension) to the cells (1 mL). All the tubes were vortex-mixed for 30 s and left to stand at room temperature for the gel to settle (or were centrifuged at 100 *g* for 2 min) before measurement of the absorbance (420 nm) of the supernatant liquids of the test and control suspensions. The results were expressed as percentage adhesion calculated as a percentage decrease in absorbance of test compared with control absorbance.

BATH test. Rosenberg (1991) has described a simple method for measuring the bacterial cell-surface hydrophobicity in which washed aqueous suspensions of microbial cells are vortex-mixed in the presence of a liquid hydrocarbon. During mixing the hydrocarbon layer is dispersed into droplets and microorganisms accumulate at the interface, depending on their hydrophobicity. With non-adherent cells the droplets quickly coalesce and the turbidity of the bacterial suspensions in the aqueous layer does not change. If the bacteria adhere and coat the droplets they form an emulsion and the turbidity of the aqueous layer decreases. The percentage of adhering cells is indicative of the hydrophobicity of the cells.

Method. Broth cultures of *S. epidermidis* 900, 901, and 904 were prepared as described above, grown in air and 5% CO_2–95% air, harvested by centrifugation after 24 h (5000 $g \times 15$ min) and washed in PUM (phosphate urea magnesium) buffer (pH 7·1; K_2HPO_4 7·26 g L^{-1}; $MgSO_4.7H_2O$ 0·2 g L^{-1}; urea 1·8 g L^{-1}; 5000 $g \times 10$ min). The cells were resuspended in PUM buffer to an initial concentration of 10^8 colony-forming units mL^{-1}. Hexadecane (2 mL) was added to each sample (1·5 mL) in glass test tubes, and the mixture held at room temperature for 10 min, vortex-mixed for 30 s and left to stand at room temperature for the hexadecane to separate. The absorbance (420 nm) of the aqueous layer before and after separation was measured and the results recorded as the percentage decrease in absorbance of the aqueous phase after treatment in comparison with the initial absorbance.

Salt-aggregation test. The salt-aggregation test (SAT) is based on the precipitation of cells from suspension by addition of increasing concentrations of ammonium sulphate to cause bacterial aggregation. Aggregation is thought to arise as a result of reduction of repulsive charges and the subsequent dominance of hydrophobic interactions at the outer surface layer of the bacteria.

Method. Broth cultures of *S. epidermidis* 900, 901, and 904 were prepared as described above, grown in air and 5%CO_2-95% air, harvested after 24 h by centrifugation (5000 $g \times 15$ min) and washed three times in 0·002 M Na_2HPO_4, with centrifugation (5000 $g \times 10$ min) after each wash. The cells were resuspended in phosphate solution (Na_2HPO_4, 0·002 M, pH 7·2) to a final concentration of 10^9 colony-forming units mL^{-1}. A series of ammonium sulphate solutions from 0·2 to 4·0 M, in 0·2 M increments, was prepared in phosphate solu-

tion. Cell suspension (50 μL) and each ammonium sulphate solution (50 μL) were placed in turn on a glass slide, mixed, and gently rocked for 2 min. The SAT value was recorded as the lowest concentration of ammonium sulphate that caused aggregation in 2 min when the solution was viewed against a darkened background.

Estimation of cell surface tension. Surface tension is inversely proportional to hydrophobicity and can therefore be used to rank the three strains of *S. epidermidis*. The advantage of methods for estimation of surface tension is that they give quantitative results in the same units which are therefore directly comparable. The surface tension of strains 900 and 901 was estimated by use of an established method (contact angle) and also using a new method based on cell aggregation behaviour.

From contact angles. Contact angle values determined for strains 900 and 901 grown in broth (5%CO_2–95% air), using the method described above, were converted into surface tension values using conversion tables (Neumann et al 1980) derived from the equation of state.

Image analysis. Broth cultures of *S. epidermidis* 900 and 901 were prepared as described above, grown in 5%CO_2–95% air, and 10 mL was divided between ten Eppendorf centrifuge tubes. The cells were spun to a pellet (5000 $g \times 5$ min), washed three times in HPLC-grade water, and the pellets were blotted dry with filter paper. Propanol solutions were freshly prepared in HPLC-grade water and their surface tension measured by use of a Du Nouy tensionometer. The cells were then resuspended to a concentration of 10^9 colony-forming units mL^{-1} in these solutions. Three drops from each tube were placed on microscope slides and 10 frames per drop were examined under a bright-field microscope (Laborlux, Leitz) linked via a COHU high-performance charge-coupled device (CCD) camera to a Perceptive Instruments image analyser. The frames were chosen randomly but were well spread over the slide. The data were recorded as a mean ratio of particle count against percentage surface area coverage, to give a measure of particle size.

Reproducibility of the technique. A 24-h broth culture of strain 900 (1 mL) was placed in each of three Eppendorf centrifuge tubes, harvested (5000 $g \times 5$ min), washed (5000 $g \times 5$ min) and resuspended in 0·9% (v/v) propanol. Three drops of each suspension were placed on to glass slides and examined by image analysis as described above. When the 10 values for each of the three drops from each tube were compared with each other by analysis of variance the results from one tube were significantly different (tube 1, $P = 0{\cdot}000$; tube 2, $P = 0{\cdot}115$; tube 3, $P = 0{\cdot}222$). When all 30 values for each tube were used as one data set there was no significant difference between the tubes ($P = 0{\cdot}325$) and it was, therefore, decided to measure at least three drops for each sample, and that there was no requirement for duplicate tubes.

Results and Discussion

Surface hydrophobicity

Four tests, contact angle determination, HIA, BATH test and SAT, were employed in this study to determine cell-surface hydrophobicity under different growth conditions. The results from these methods are listed in Table 1. By use of a two-sample unmatched *t*-test the contact angles determined for strain 901 were found to be significantly greater than those for strains 900 (CO_2 $P < 0001$; air $P = 0{\cdot}034$) and 904 (CO_2 $P = 0{\cdot}0014$; air $P = 0{\cdot}0087$). In air there was no significant difference between strains 900 and 904 ($P = 0{\cdot}7$) but for strain 904 grown in CO_2 the contact angles were significantly greater ($P = 0{\cdot}028$).

When hydrophobicity was determined by HIA, in CO_2 and air, the percentage adsorption of strain 901 was found to be significantly greater than that of strain 900 (CO_2 $P = 0{\cdot}009$, air $P = 0{\cdot}049$) and 904 (CO_2 $P = 0{\cdot}042$, air $P = 0{\cdot}035$), which were not significantly different from each other (CO_2 $P = 0{\cdot}16$, air $P = 0{\cdot}83$). Analysis of variance showed that results in CO_2 and air for all three strains were not significantly different (strains, 900 $P = 0{\cdot}317$; 901 $P = 0{\cdot}450$; 904 $P = 0{\cdot}489$). Denyer et al (1993) have previously shown that contact-angle measurements and HIA yield similar results, in agreement with these results. Both contact angle and HIA rank strain 901 as the most hydrophobic, strain 900 as the most hydrophilic and strain 904 as intermediate. In the SAT test the smaller the SAT value the more hydrophobic the cells and by use of this method strains 901 and 900 are shown to be the most hydrophobic and strain 904 the most hydrophilic. When hydrophobicity was determined by the BATH test, in CO_2 and air the percentage adsorption of strain 904 was found to be significantly greater than that of strain 900 (CO_2 $P = 0{\cdot}042$, air $P = 0{\cdot}001$) and strain 901 (CO_2 $P = 0{\cdot}049$, air $P = 0{\cdot}012$), the results for strains 901 and 902 were not considered different (CO_2 $P = 0{\cdot}59$, air $P = 0{\cdot}21$). For all three strains

the results from growth in broth seemed independent of atmospheric conditions (strains 900 $P = 0{\cdot}43$; 901 $P = 0{\cdot}89$; 904 $P = 0{\cdot}24$). The BATH test ranks strain 904 as the most hydrophobic and strain 900 the least, a result which is not in accord with the other results. Vanhaeke & Pijck (1988) have shown that hexadecane does not damage the integrity of microbial cells during the BATH test; it might, however, alter the surface of the bacteria in some way thus changing the hydrophobic structures. In all tests except the BATH test there was little difference between the results for both sets of growth conditions.

If the term hydrophobicity, as determined by the above mentioned tests, is meaningful, good correlation between the results of the four tests would be expected. The probable reason for the discrepancies observed is that the methodologies probe different components of the cell surface. For instance the small molecular-weight hydrocarbons used in the BATH test would be expected to penetrate the surface of the bacterial wall to a greater extent than high molecular-weight polymers such as phenylsepharose. In the SAT test aggregation is thought to occur as a result of reduction of repulsive charges and the subsequent dominance of hydrophobic interactions at the bacterial cell surface. Contact-angle measurements give a result dependent upon the summation of polar and nonpolar interactions between water and the surface of the bacterial cells. In addition, all these assays are sensitive to bacterial cell density, ionic strength and pH, a point which was highlighted by Jones et al (1996).

Contact-angle measurements, although the most time-consuming, were easy to perform and gave consistent results. The most important factor influencing the results is that contact angles are substantially affected by the cell-drying procedure (Mege et al 1984). Although the effect of drying on the cell surface is not known, Absolom (1988) showed that the contact angle remains stable for about 30 min, after which it starts to change again because of conformational and structural changes and possible denaturation of the specimen. Initially the contact angle is low owing to interaction with surface water, but as the bacterial lawn dries the water droplet comes into contact with the cell surface at which point the angle remains stable and is a measure of the surface hydrophobicity of the cell.

The HIA is simple and quick to perform, but it is very sensitive to pH and must be carefully buffered. The nature of the calculation of percentage adsorption relies on there being exactly 30% gel in the suspension but the gel had a tendency to adhere to the glass tube in which the suspension was prepared. This might contribute to the poor reproducibility of the assay, although the reliability of the results could be increased by performing many replicates.

The BATH test is performed at room temperature which is close to the solidification point (16°C) of *n*-hexadecane. This might have an effect on hydrocarbon viscosity and thus droplet size. The greater the droplet size the smaller the surface available for adhesion and, therefore, the smaller the percentage adhesion, creating an apparent fall in hydrophobicity. For this reason the assay was not performed if room temperature was below 20°C, and replicates were performed on different days. Busscher et al (1995) showed that at the pH of PUM buffer (7·2) hexadecane has a negative charge; this was shown by Van der Mei et al (1995) to reduce adhesion. Maximum adhesion occurred at acidic pH, under which conditions hexadecane has no charge. On this basis Van der Mei et al (1995) dismissed the BATH test as a hydrophobic assay.

The main problems with the SAT test are that in the absence of a dark background and hand lens visualization of the bacterial aggregates is poor, and even with the use of these the presence/absence of aggregation at the SAT value is still very subjective.

Despite these disadvantages the HIA, BATH and SAT assays have the advantage that they can be performed with equipment found in virtually every microbiology laboratory; they can, however, be used for cell comparisons only as they do not give absolute values for hydrophobicity.

Surface tension

Surface tension is inversely proportional to hydrophobicity but has the advantage that, regardless of the method used, the results generated are quantitative values with the same units and are therefore directly comparable from one method to another.

To enable comparison with results from other methods, results from contact-angle determinations were taken and, using the equation of state conversion tables of Neumann et al (1980), an estimate of the surface tension of the strains of *S. epidermidis* (grown in broth in 5% CO_2–95% air), were made assuming a water surface tension of $72\,mN\,m^{-1}$ at 20°C. The equation of state enables determination of the surface tension of a low-energy solid from a single contact angle formed by a liquid of known surface tension which is chemically inert relative to the solid. Smith et al (1986) showed that the equation of state, when used in

conjunction with contact angle data, yields surface tension values which agree with those obtained from other techniques. The surface tensions of *S. epidermidis* strains 900, 901 and 904 grown in nutrient broth in 5% CO_2 are approximately 68, 61 and 65 mN m^{-1} respectively. Observations of *S. epidermidis* 900 suspended in a range of propanol solutions and viewed by phase-contrast microscopy showed that at extremes of the range of propanol concentrations many of the cells aggregated into groups of 10–50 with the rest remaining as single cells. As the surface tension of the solution approached that of the cells the number and size of the aggregates decreased until at 66·8 mN m^{-1} single cells predominated (Table 2).

Microscopical aggregation behaviour could more appropriately be quantified by use of an image-analyser system. Here, the field of view would be assessed for percentage area coverage (to give the total number of cells present) and number of objects. For a given number of cells, the lower the number of objects the larger the aggregate size. As the surface tension of the solution approaches that of the cells the amount of aggregation should decrease and the count/coverage ratio will increase to a maximum.

The results from this approach for strains 900 and 901 can be seen in Figure 1. Maxima are obtained at surface tensions of 67 and 61 mN m^{-1}, respectively. It can be seen that there is close agreement between these results and those obtained from contact angles via the equation of state. The main advantage of this novel method is that the number of cells required is at least 100-fold less than that needed for contact-angle determinations.

Table 2. Qualitative observations of the effect of propanol concentration on the aggregation behaviour of *Staphylococcus epidermidis*.

Amount of propanol (%)	Surface tension (mN m^{-1})	Description of aggregation
0·1	69·7	Clumps of 10–20 cells
0·2	69·3	Clumps of 4–10 cells
0·3	68·2	Clumps of 4–10 cells
0·4	67·8	Clumps of 4–10 cells
0·5	66·8	Mostly single cells
0·6	65·4	Single cells and clumps of 5–10 cells
0·7	65·0	Clumps of 5–10 cells
0·8	64·2	Clumps of 10–20 cells
0·9	63·9	Clumps of 10–20 cells
1·0	63·4	Some clumps of 50 cells

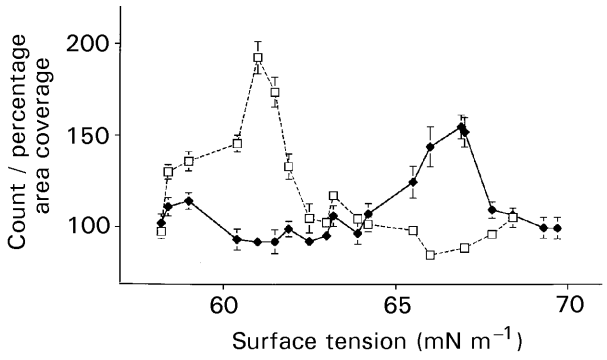

Figure 1. Effect of surface tension on the aggregation behaviour of *Staphylococcus epidermidis* strains 900 (◆) and 901 (□), as determined by image analysis. Data are means ± standard error of the mean.

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