The electrophoretic softness of the surface of *Staphylococcus epidermidis* cells grown in a liquid medium and on a solid agar

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Many *Staphylococcus epidermidis* strains possess capsule or slime layers and consequently the staphylococcal cell surface should be regarded as a soft, polyelectrolyte layer allowing electrophoretic fluid flow through a layer of fixed charges. The presence of such a soft layer decreases the energy barrier due to electrostatic repulsion in the interaction of the organisms with negatively charged substrata [Morisaki, H., Nagai, S., Ohshima, H., Ikemoto, E. & Kogure, K. (1999), *Microbiology* 145, 2797–2802] and hence plays an important role in their adhesion. In this paper, the authors compare the electrophoretic softness and amount of fixed charge in the outer cell surface layers of 20 *S. epidermidis* strains, grown in a liquid medium or on a solid agar, as determined from the dependencies of their electrophoretic mobilities upon the ionic strength of a suspending fluid. Most of the staphylococcal cell surfaces were relatively soft, with a mean cell surface softness ($1/\lambda$) for strains grown in liquid medium of 1.7 ± 0.6 nm (standard deviation over all 20 strains) which is soft by comparison with a completely bald, peptidoglycan-rich streptococcal cell surface ($1/\lambda = 0.7$ nm). When the staphylococcal strains were grown on solid agar, the cell surface softness of 17 of the 20 strains increased, sometimes by a factor of two. On average for 20 strains, the cell surface softness increased significantly ($P < 0.05$, Student's *t*-test) to 2.8 ± 1.8 nm. The amount of fixed charge in the outer cell surface layer was -28 ± 9 mM for bacteria grown in liquid medium and -24 ± 12 mM for bacteria grown on agar. A soft, highly negatively charged polyelectrolyte layer was inferred by microelectrophoresis for all the staphylococcal cell surfaces, regardless of whether staining had indicated the presence of a capsule or slime layer.

Keywords: electrophoretic mobility, staphylococci, capsules, slime

INTRODUCTION

Staphylococcus epidermidis, a coagulase-negative bacterium, is a common cause of infections associated with prosthetic devices and indwelling catheters, such as heart valves, pacemakers, orthopaedic implants, Hickman catheters, and cerebrospinal fluid shunts. It is also a frequent infectious agent in many non-biomaterial-related infections, as for example peritonitis, neonatal sepsis, and native valve endocarditis (Dankert *et al.*, 1986). An understanding of the fundamental mechanisms of microbial adhesion is imperative for effective preventive and eradication strategies against all bacterial infections (Bos *et al.*, 1999).

For *S. epidermidis* strains, two distinct extracellular entities have been identified with a potential impact on their adhesion: a capsule, which is intimately associated with the cell wall, and a slime layer, which can be removed by washing (Christensen *et al.*, 1982; Matthews *et al.*, 1991). Capsular material and slime can be regarded as soft, polyelectrolyte layers surrounding the organism. Bacterial cell surfaces most frequently carry a net negative surface charge, and negative zeta potentials are reported for physiological pH values (Jucker *et al.*, 1996), as for nearly all surfaces in nature. Staphylococci are thus expected to experience a strong electrostatic repulsion in their adhesion to substratum surfaces, which is opposite to many experimental findings (Bos *et al.*, 1999). Bacterial zeta potentials are derived by

Fig. 1. Schematic representation of the surfaces of an electrophoretically rigid and an electrophoretically soft particle. The electrokinetic potential decreases exponentially with distance from the rigid particle surface and the slip plane, determining its zeta potential, is close to the surface. The soft particle is covered by a polyelectrolyte layer with fixed negative charges, through which electrophoretic fluid flow is possible. Consequently, the slip plane remains at approximately the same position as for the rigid particle and is inside the soft layer, and the zeta potential is much more negative than the potential Ψ_0 at the outside of the soft layer.

particulate microelectrophoresis (Hiemenz, 1977; James, 1979). During particulate microelectrophoresis, bacteria are suspended in a solution of a given ionic strength and composition and the velocity (mobility) that bacteria acquire under the influence of an applied electric field is measured. For so-called rigid particles (see Fig. 1), the potential decreases exponentially with distance from the particle surface, while a thin, stagnant layer separates the movable part of the ionic double layer and the particle surface. The potential at this slip plane is the zeta potential (Lyklema, 1994). The outer layer of biological cell surfaces differs substantially from the surface of a rigid particle and is, depending upon the strains and species under consideration, covered with an ion- and fluid-penetrable layer of charged polymers, which may be associated, for instance, with bacterial fimbriae, fibrils, capsular material or a slime layer.

Consequently, during particulate microelectrophoresis, a fluid flow will develop through this charged layer. The zeta potential at the slip plane will be determined by a combination of the electrolyte charge density, as for rigid particles, and the fixed charge density of surface polymers (see Fig. 1). From Fig. 1, it can be seen that, due to the fixed charge density, the zeta potential at the slip plane of a soft particle is usually considerably more negative than the potential at its outer surface, which interacts with the environment and plays a role in adhesion.

H. Ohshima and colleagues (Ohshima, 1995; Ohshima & Kondo, 1991) have proposed a new theory to interpret the electrophoretic mobilities of soft, polyelectrolyte-covered particles as opposed to rigid particles, when measured as a function of ionic strength. This theory describes the electrophoretic mobility of soft particles, of which micro-organisms are typical examples, as originating from a weighted average of potentials over the surface charge layer and that of the volume density of fixed charges. The polyelectrolyte layer around microbial cell surfaces allows electrophoretic fluid flow, and the ease by which fluid can flow through the soft layer is expressed as a so-called electrophoretic softness, $1/\lambda$. The theory of Ohshima enables quantitative determination of the volumetric charge density and the softness ($1/\lambda$) of this polyelectrolyte layer from the dependence of the electrophoretic mobility upon ionic strength, and it has been successfully applied to describe the surfaces of human erythrocytes (Kawahata *et al.*, 1990) and rat lymphocytes (Morita *et al.*, 1991).

Recently, we studied the softness of two oral streptococcal strains, *Streptococcus salivarius* HB and HBC12, by particulate microelectrophoresis in KCl solutions of varying ionic strengths (Bos *et al.*, 1998). Electron microscopy of negatively stained organisms, and X-ray photoelectron spectroscopy, confirmed that strain HB had several classes of proteinaceous fibrils with lengths up to 178 nm on its outermost surface (Weerkamp *et al.*, 1986), while variant HBC12 had a bald, peptidoglycan-rich outer surface. The fibrillated strain HB appeared as relatively soft ($1/\lambda = 1.4$ nm) from analysis of its electrophoretic mobility according to Ohshima, while the bald variant HBC12 was hard ($1/\lambda = 0.7$ nm) because of its comparatively rigid, peptidoglycan-rich outer surface, characteristic of Gram-positive bacteria. When the electrophoretic softness and ion-penetrability of polyelectrolyte layers on bacterial cell surfaces is properly accounted for (Morisaki *et al.*, 1999; Poortinga *et al.*, 2001), the energy barrier in adhesion of negatively charged organisms to negatively charged substratum surfaces is calculated to be far lower than usually estimated. Accurate, predictive models based on the DLVO theory for bacterial deposition to substratum surfaces can then be prepared (Poortinga *et al.*, 2001).

The aim of the present study was to compare the cell surface softness and fixed charge densities as derived from microelectrophoresis of 20 *Staphylococcus epidermidis* strains grown in liquid medium or on solid blood agar.

METHODS

Bacterial strains and culture conditions. The *Staphylococcus epidermidis* strains used in this study (see Table 1) were cultured in Trypticase soy broth (TSB, Oxoid). Strains were inoculated from blood agar (Blood agar no. 2, Oxoid) into broth, grown in batch culture for 24 h at 37 °C, and this culture was used to inoculate a second culture that was grown for 16 h at 37 °C. Bacteria were harvested by centrifugation (5 min at 10000 *g*), washed twice with demineralized water and suspended in water. To prepare agar-grown cells for experiments, bacteria were grown for 24 h on blood agar at 37 °C, harvested from the plates and resuspended in water.

All staphylococci cultured, both in TSB medium and on blood agar, were examined microscopically for the presence of a capsule by India ink staining or slime layer by methylene blue staining as described by Christensen *et al.* (1982) and Matthews *et al.* (1991).

Particulate microelectrophoresis. Electrophoretic mobilities were measured at 25 °C with a Lazer Zee Meter 501 (PenKem, Bedford Hills, NY, USA) equipped with an image-analysis option for tracking and zeta sizing (Wit *et al.*, 1997). Measurements were carried out in KCl solutions of various ionic strengths (pH 6). Immediately prior to each measurement, an aliquot of the bacterial suspension was added to the appropriate KCl solution at a density of approximately 1×10^8 cells ml⁻¹. The pH of the solutions did not change upon addition of the bacterial cells. The Lazer Zee Meter was set at 150 V for determination of the electrophoretic mobilities of the bacteria.

The electrophoretic mobilities, measured as a function of ionic strength, were fitted to equation (1) using a least-squares curve-fitting routine kindly provided by Dr H. Ohshima, Tokyo, Japan:

$$\mu = (\epsilon_r \epsilon_0 / \eta) [(\Psi_0 / \kappa_m + \Psi_{\text{DON}} / \lambda) / (1 / \kappa_m + 1 / \lambda)] + (zeN / \eta \lambda^2) \quad (1)$$

in which μ is the electrophoretic mobility, ϵ_r the relative permittivity, ϵ_0 the permittivity of vacuum, η the viscosity of the solution, $1 / \kappa_m$ the Debye–Hückel length in the polymer layer, $1 / \lambda$ the softness of the polyelectrolyte layer, z the valence of charged groups in the polyelectrolyte, e the electrical unit charge, N the density of charged groups in the polyelectrolyte layer, Ψ_0 the potential at the boundary between the polyelectrolyte layer and the surrounding solution, and Ψ_{DON} the Donnan potential within the polyelectrolyte layer (Ohshima, 1995; Ohshima & Kondo, 1991). By taking $1 / \lambda$, the softness of the polyelectrolyte layer, and zeN , the density of charged groups in the polyelectrolyte layer, as parameters of the fit, both $1 / \lambda$ and zeN can be calculated from electrophoretic mobilities measured as a function of ionic strength. Note that $1 / \kappa_m$, the Debye–Hückel length, is also a function of ionic strength and ranges from 3 nm in 0.01 M to 1 nm in 0.1 M KCl (Hiemenz, 1977).

All electrophoretic mobilities and softness values reported are the mean values of three different measurements with separately cultured bacteria.

RESULTS

As an example, Fig. 2 shows the electrophoretic mobility of *S. epidermidis* ATCC 35983 as a function of ionic strength. The electrophoretic mobility of this strain levels off at an ionic strength of between 0.05 and 0.10 M, and the electrophoretic mobility at high ionic strength is more negative for bacteria grown on solid

Fig. 2. Electrophoretic mobilities in KCl solutions (pH 6) of *S. epidermidis* ATCC 35983 as a function of ionic strength. The solid lines represent the best fit to equation (1). Error bars indicate the standard deviation over three experiments with separately cultured bacteria. ●, Staphylococci grown in liquid medium; ○, staphylococci grown on a solid agar.

agar than for organisms grown in liquid medium. For rigid particles, electrophoretic mobilities are essentially zero in 0.10 M KCl (Ohshima & Kondo, 1991) and according to equation (1) the residual electrophoretic mobilities of the staphylococci at 0.10 M are a result of fixed charges in the outer cell surface layer. The relatively minor change in electrophoretic mobility over the range of ionic strengths in Fig. 2 is, in combination with the residual mobility at high ionic strength, indicative of a soft cell surface.

The cell surface softness ($1 / \lambda$) and charge density (zeN) of all 20 staphylococcal strains, as derived by fitting the dependence of the electrophoretic mobilities upon ionic strength as presented in Fig. 2 to equation (1), are compiled in Table 1. All strains were also assessed for presence of a capsule or slime layer (Table 1). Strain ATCC 35984 had a relatively hard cell surface ($1 / \lambda = 0.5$ nm) when grown in liquid medium, while the softest strain grown in liquid medium, 3399, had a cell surface softness of 2.9 nm. On average over all 20 staphylococcal strains grown in liquid medium, the cell surface softness was 1.7 ± 0.6 nm, showing that staphylococcal cell surfaces were soft. For comparison, a completely bald, peptidoglycan-rich streptococcal cell surface had $1 / \lambda = 0.7$ nm (Bos *et al.*, 1998). Upon growth on agar instead of in liquid medium, 17 out of the 20 strains become softer. For some strains, e.g. ATCC 35983, ATCC 35984, HBH 171 and HBH₂ 3, the cell surface softness more than doubled. On average over all 20 strains, the cell surface softness increased to 2.8 ± 1.8 nm, which was significant by comparison with the softness of staphylococci grown in liquid medium ($P < 0.05$, Student's *t*-test). Conclusively, a soft polyelectrolyte layer was inferred from microelectrophoresis for all staphylococcal cell surfaces. The softness of the staphylococcal cell surface does not correlate with microscopic evidence for the absence or presence of a capsule or slime layer, as can also be seen in Table 1.

The amount of fixed charge zeN in the outer cell surface layer (see Table 1) varied from -16 to -56 mM for

Table 1. Cell surface softness $1/\lambda$ and charge density zN of 20 *S. epidermidis* isolates, as derived from particulate microelectrophoresis on three separate cultures of each strain

Both isolates grown in liquid medium and those grown on solid agar were tested for the possession of a capsule or slime layer. On average, the standard deviation over the three cultures amounted to 0.5 nm and 1 mM for softness and charge density, respectively.

<i>S. epidermidis</i> strain	Liquid medium			Solid agar		
	Capsule/Slime*	$1/\lambda$ (nm)	zN (mM)	Capsule/Slime*	$1/\lambda$ (nm)	zN (mM)
169	+ / +	2.5	-16	+ / +	2.5	-17
236	+ / +	1.0	-26	+ / -	1.8	-31
242	+ / +	1.2	-32	- / +	1.3	-26
252	- / -	2.1	-27	- / -	2.4	-24
1938	ND	2.0	-23	- / -	2.6	-22
3399	+ / +	2.9	-19	+ / +	3.4	-15
11047	ND	1.6	-28	- / +	2.1	-27
26741	+ / +	1.4	-23	- / +	2.4	-18
1938A	ND	1.3	-34	- / +	1.2	-48
ATCC 35983	- / -	1.9	-28	+ / +	4.2	-11
ATCC 35984	+ / +	0.5	-56	+ / -	1.3	-38
C	ND	1.9	-22	+ / +	1.2	-50
HBH 154	- / -	1.8	-17	- / -	2.4	-12
HBH 171	+ / +	2.2	-24	+ / +	6.5	-10
HBH 276	+ / +	1.4	-40	- / +	2.6	-24
HBH 298	+ / +	1.1	-34	+ / +	1.9	-27
HBH 45	- / -	1.6	-21	+ / -	2.5	-19
HBH ₂ 169	+ / +	1.8	-33	- / +	3.2	-8
HBH ₂ 3	- / -	2.6	-24	- / -	6.6	-6
HBH ₂ 277	- / -	1.0	-35	+ / +	1.2	-36

* The first symbol indicates the possession or not of a capsule; the second symbol indicates whether or not a slime layer was observed.

ND, Not determined.

staphylococci grown in liquid medium; a similar range in amount of fixed negative charge was observed for cells grown on blood agar. On average over all strains, staphylococci grown in liquid medium had a fixed charge of -28 ± 9 mM, while for bacteria grown on a solid agar the fixed charge was -24 ± 12 mM. No relation existed between the amount of fixed charge and the cell surface softness.

DISCUSSION

Over the past decade, several authors have argued that bacterial cell surfaces should be considered much more as a gel-like layer rather than as a rigid surface. The negative cell wall charge at neutral pH of most bacterial strains results from high concentrations of anionic carboxyl, phosphate and amino groups that may associate at reduced pH to cause a positive cell wall charge. This charge is compensated in part by counterions penetrating into the gel-like layer (Van der Wal *et al.*, 1997). Van der Wal *et al.* (1997) concluded that for their collection of bacterial species (including corynebacteria, rhodococci and *Bacillus*, all organisms virtually devoid of any structural surface appendages),

most of the mobile charges were located in a hydro-dynamically stagnant layer in the gel, envisaged to form the cell surface. In the Ohshima model an electrophoretic fluid flow is thought to be possible through this gel-like, soft layer around microbial cell surfaces. The ease with which fluid can flow through this layer is expressed as an electrophoretic softness. For two streptococcal strains, Bos *et al.* (1998) showed that an electrophoretically hard strain was devoid of structural surface appendages, while an electrophoretically soft strain had surface fibrils with lengths up to 178 nm. Recently, we applied atomic-force microscopy to these two streptococcal strains and found that an electrophoretically soft, fibrillated strain is also mechanically soft when probed by the tip of an atomic force microscope (Van der Mei *et al.*, 2000), providing substantial support for the Ohshima model from independent experimentation. Moreover, this finding suggests that a layer allowing fluid flow is more open and consequently also mechanically softer than a more closed structure and thereby simplifies the rather abstract concept of 'electrophoretic softness'.

Staphylococcal cell surfaces are very different from streptococcal cell surfaces and physico-chemical models to explain staphylococcal adhesion to substratum

surfaces have generally been less successful than models of streptococcal adhesion (Bos *et al.*, 1999; Van der Mei *et al.*, 1997; Van Pelt *et al.*, 1985). This is probably due to a strong overestimation of the staphylococcal zeta potentials as derived from particulate microelectrophoresis neglecting the cell surface softness (see also Fig. 1). Note that the average staphylococcal cell surface after growth in liquid medium ($1/\lambda = 1.7$ nm) is softer than that of a heavily fibrillated streptococcal strain grown in liquid culture ($1/\lambda = 1.4$ nm), while staphylococci grown on a solid agar are even softer ($1/\lambda = 2.8$ nm). As another comparison, the amount of fixed outer layer charges for the proteinaceous streptococci ranged between -13 and -15 mM, while the fixed charge densities of the staphylococcal cell surface layers were between -24 and -28 mM on average, indicative of the presence of highly negatively charged polysaccharides.

Morisaki *et al.* (1999) described that *Vibrio alginolyticus* had a cell surface softness of between 5.1 nm and 6.4 nm, with highly negative zeta potentials calculated. As a consequence the interaction energy barrier between *V. alginolyticus* and negatively charged glass was highly repulsive, with an energy barrier of around 150 kT. When the cell surface softness was accounted for, surface potentials were far lower and the energy barrier disappeared completely. Analogously, Poortinga *et al.* (2001) failed to obtain theoretical deposition rates for staphylococci to glass in a parallel-plate flow chamber by solving the convective-diffusion for mass transport. Theoretically obtained deposition rates did not correspond with the high experimentally observed deposition rates, but a perfect match was calculated when the staphylococcal cell surface softness and ion-penetrability were taken into account.

The present study shows that staphylococcal cell surfaces are relatively soft, and that their softness increases when the organisms are grown on a solid agar. However, there appears to be no relation between the physico-chemical softness measured in this study and previous reports on the possession of a capsule or slime layer by the strains. This is consistent with previous attempts to relate physico-chemical cell surface properties of the staphylococci like charge, chemical composition or hydrophobicity (Van der Mei *et al.*, 1997) with encapsulation or slime layer. The most likely explanation for this is still the hypothesis that all staphylococci are indeed encapsulated, with an effect on the physico-chemistry of their cell surfaces, but that in many cases the traditional India ink staining methods (Matthews *et al.*, 1991) to visualize capsules are inadequate to detect thin capsules, which may potentially have a thickness in the nanometer range.

Summarizing, we have demonstrated that the outermost layers on *S. epidermidis* cell surfaces are electrophoretically soft with a high density of fixed, negative charge, especially when compared with streptococcal cell surfaces. The staphylococcal cell surface softness increases after growth on a solid agar rather than in a

liquid culture, although the difference in nutrient medium employed may contribute to this conclusion. Accounting for the softness of staphylococcal cell surfaces allows us to deal adequately with electrostatic interactions in the adhesion of the cells to negatively charged substratum surfaces.

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