The influence of ionic strength and pH on diffusion of micro-organisms with different structural surface features

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Exact knowledge of microbial diffusion coefficients is a prerequisite for the application of mass transport theories to microbial deposition data. Microbial diffusion coefficients can be calculated on the basis of cell radii using the Einstein equation. This approach, however, does not take into account the additional effects of structural cell surface features such as fibrils or a fuzzy coat. Microbial diffusion coefficients of micro-organisms in suspension can be experimentally measured using dynamic light scattering. In this paper we compare experimental microbial diffusion coefficients with those calculated from radii for a variety of microbial strains suspended in 10 mM or 40 mM potassium phosphate solutions of different pH. Furthermore, the pH dependence of the microbial diffusion coefficients is related to the pH dependence of the microbial zeta potentials in similar ionic strength solutions. Microbial diffusion coefficients ranged from 2×10^{-13} to $5 \times 10^{-13} \text{ m}^2 \text{ s}^{-1}$ and were generally higher at low pH (pH 2) than at high pH (pH 7) for strains with structural surface features. Experimental diffusion coefficients at pH 2 were approximately similar to those based on microscopic radii. This indicates that at pH 2 structural surface features are in a collapsed state, presumably due to the lack of stabilizing electrostatic repulsion between the cell surface structures as shown by the zeta potentials measured. Alternatively, a hydrodynamic radius of a micro-organism can be calculated from the experimental diffusion coefficients and the Einstein equation. Assuming that the maximal difference in hydrodynamic radius, observed over the pH range employed, represents the maximal length of structural cell surface features present, then the calculated fibrillar lengths for *Streptococcus mitis* BA and *S. salivarius* HB are in reasonable agreement with electron microscopical estimates.

Keywords: microbial diffusion coefficient, surface structure, biofilm, mass transport, dynamic light scattering

INTRODUCTION

Microbial biofilm formation is often described as a four step process (Van Loosdrecht *et al.*, 1990) consisting of (1) the transport step, (2) the initial adhesion step, (3) the attachment phase and (4) the colonization phase (see Fig. 1 for schematic representation). Whereas the initial adhesion step, the attachment phase and the colonization phase are frequently studied (Costerton *et al.*, 1986), little attention is generally given to the transport step. Yet, both from applied and fundamental points of view, the transport step is equally important since insufficient

transport can be the limiting factor in biofilm formation. Whenever the transport step is the limiting factor and when transport conditions are poorly controlled, this may lead to erroneous conclusions regarding the process of biofilm formation.

A parallel plate flow chamber (Sjollesma *et al.*, 1989a) is a most suitable device for studying initial microbial adhesion. Similarly, the modified Robbins device (Costerton *et al.*, 1986) is ideal to study the later stages of biofilm formation. In both devices the transport step is controlled by a combination of diffusion and convection. The ratio of convective transport over diffusion is

Fig. 1. Sequential steps in the colonization of a substratum surface by micro-organisms (adapted from Van Loosdrecht *et al.*, 1990).

expressed by the Peclet number (Adamczyk & Van de Ven, 1981):

$$Pe = \frac{3V_m \cdot r_h^3}{2b^2 \cdot D_\infty} \quad (1)$$

in which Pe is the Peclet number, V_m is the mean fluid velocity, r_h is the hydrodynamic microbial cell radius, b is half the depth of the device and D_∞ is the microbial diffusion coefficient.

Fig. 2 shows the velocity and concentration gradients in a parallel plate flow chamber or a Robbins device. Convective transport is solely parallel to the substratum surfaces in these systems provided flow is laminar and only diffusion creates transport towards the substratum surfaces. Under stagnant conditions, convective mass transport is absent.

Despite the obvious importance of microbial diffusion in biofilm formation, microbial diffusion coefficients are seldom measured exactly, but mostly calculated from the Einstein equation using estimates of the microbial radius from light microscopy:

$$D_\infty = \frac{kT}{6\pi\mu r_h} \quad (2)$$

in which k is the Boltzmann constant ($1.38 \times 10^{-23} \text{ J K}^{-1}$), T is the absolute temperature (295.8 K) and μ is the dynamic viscosity of the medium ($10^{-3} \text{ kg m}^{-1} \text{ s}^{-1}$).

Xia *et al.* (1989) measured microbial diffusion coefficients by dynamic light scattering for *Escherichia coli*, both live and fixed, suspended in water, and found a diffusion coefficient of $3.45 \times 10^{-13} \text{ m}^2 \text{ s}^{-1}$ for both cell types. Using this value, it can be calculated that the hydrodynamic radius of the cells would be $0.6 \text{ }\mu\text{m}$. Although this seems quite reasonable compared with phase-contrast microscopical estimates, it is unlikely that such an agreement between measured and calculated diffusion coefficients

Fig. 2. Schematically drawn aspects of mass transport in a parallel plate flow chamber or Robbins device by convection and diffusion. Note that in these systems convective transport is parallel to the surface and maximal in the middle between the surfaces, while diffusion is maximal over the so-called 'diffusion boundary layer' adjacent to the substratum surface as a result of the concentration profile in the device (adapted from Sjollem *et al.*, 1989b).

will exist in general. The hydrodynamic radius to be employed in equation (2) can probably be obtained from phase-contrast microscopy in the case of bald organisms, but structural surface features such as fibrils and fimbriae can only be visualized using special staining techniques and even then it is unknown how a long, sparsely distributed fibril contributes to the hydrodynamic radius of an organism. In addition, variations in ionic strength or pH might cause collapse or unfolding of cell surface structures, thus affecting the hydrodynamic radius of the cell and its diffusion.

The aim of this study was to determine microbial diffusion coefficients to gain an understanding of the effects of electrostatic interactions between cell surface structures on hydrodynamic cell radii and the influence of ionic strength and pH on microbial diffusion. Diffusion coefficients were measured by dynamic light scattering, and the derived hydrodynamic radii of the organisms compared with phase-contrast microscopical estimates and related to the absence or presence of structural cell surface features.

Table 1. Strains involved in this study, together with the growth medium used and our knowledge of their radius r_p (by phase-contrast microscopy) and structural surface features (by electron microscopy after negative staining)

No difference in the radius was observed by phase-contrast microscopy for micro-organisms suspended in solutions of different pH or ionic strength. Monodisperse polystyrene latex particles with a radius of 368 nm were included for comparison.

Strain	Growth medium*	r_p (nm)	Structural cell surface features
<i>S. salivarius</i> HB	Todd Hewitt broth	580	Three types of fibrils, length 178, 91 and 72 nm (Weerkamp <i>et al.</i> , 1986)
<i>S. salivarius</i> HBV5	Todd Hewitt broth	580	Two types of fibrils, length 178 and 72 nm (Weerkamp <i>et al.</i> , 1986)
<i>S. salivarius</i> HBV51	Todd Hewitt broth	580	One type of fibril, length 63 nm (Weerkamp <i>et al.</i> , 1986)
<i>S. salivarius</i> HB7	Todd Hewitt broth	580	One type of fibril, length 91 nm (Weerkamp <i>et al.</i> , 1986)
<i>S. salivarius</i> HBC12	Todd Hewitt broth	570	Bald
<i>S. mitis</i> BA	Todd Hewitt broth	620	Long fibrils, length approx. 500 nm
<i>S. thermophilus</i> B	M17 broth with saccharose added	620	Fuzzy coat
<i>Staph. epidermidis</i> HBH ₂ 169	Trypticase soy broth	630	Capsule
<i>Staph. epidermidis</i> 252	Trypticase soy broth	620	Fuzzy coat
<i>E. coli</i> 431	Brain heart infusion broth	840†	Bald
<i>A. calcoaceticus</i> RAG-1	Brain heart infusion broth	550	Various types of fibrils, length up to 180 nm (Van der Mei <i>et al.</i> , 1991)

* All media were obtained from Oxoid.

† Since *E. coli* is a rod, the effective radius is calculated by $r_e = 0.5(\text{width} \times \text{length})^{\frac{1}{2}}$.

METHODS

Bacterial strains, growth conditions and structural surface features. Bacterial strains investigated in this study comprised the oral bacterial strains *Streptococcus mitis* BA, *S. salivarius* HB and the mutant strains of HB, HB7, HBV5, HBV51 and HBC12, the dairy strain *S. thermophilus* B, the medical strains *Staphylococcus epidermidis* HBH₂169, *Staph. epidermidis* 252 and *Escherichia coli* 431, and the oil-degrading strain *Acinetobacter calcoaceticus* RAG-1. All strains were grown aerobically from a frozen stock for 24 h at 37 °C in the growth medium indicated in Table 1. These cultures were used to inoculate a second culture which was grown overnight. *A. calcoaceticus* RAG-1 was grown in a shaking incubator at 30 °C. Bacterial strains were harvested by centrifugation (5000 g, 5 min) and washed twice with demineralized water. The streptococci and staphylococci were intermittently sonicated for 20 s at 30 W with a probe sonicator (Vibra Cell model 375, Sonics and Materials Inc.) before being resuspended in 10 or 40 mM potassium phosphate solutions (the pH was adjusted to 2, 3, 4, 5 or 7 by the addition of HCl). All the strains are non-motile except for *E. coli* 431; its type of motility does not affect the measurement of a Brownian process.

In Table 1 the radii of the bacterial strains used, as determined by phase-contrast microscopy are summarized, together with our present knowledge of the structural cell surface features of the organisms as determined by electron microscopy.

Microelectrophoresis. Zeta potentials, a measure of the net surface charge on bacterial cell surfaces, were measured in 10 or 40 mM potassium phosphate solutions at appropriate pH values at room temperature with a Lazer Zee Meter 501 (PenKem), which uses scattering of incident laser light to allow detection of the bacteria at relatively low magnifications. The absolute electrophoretic mobilities can be derived directly from the

velocities of the organisms in the applied electric field, the applied voltage and the dimensions of the electrophoresis chamber (James, 1991). Electrophoretic mobilities were measured for bacteria resuspended to a concentration of about 10^7 bacteria ml^{-1} and converted into zeta potentials on the basis of the Helmholtz-Smoluchowski equation (Hiemenz, 1977). The experimental reproducibility of all zeta potentials reported was ± 2 mV (i.e. the SD over six independent readings).

Microbial diffusion coefficients and hydrodynamic radii. Dynamic light scattering measures the intensity of light scattered by suspended micro-organisms as a function of time. Intensity fluctuations are due to Brownian motion of the organisms and can be expressed in a so-called correlation function from which the diffusion coefficient of the organisms can be directly calculated (Lyklema, 1991).

Experiments were done with the various micro-organisms suspended in potassium phosphate solutions of different pH and ionic strengths to a density of approximately 5×10^6 bacteria ml^{-1} . The instrument used was a ALV-5000 (Germany) and was operated at constant temperature (295.8 K). The wavelength was 632.8 nm and the detection angle was set at 90°. Hydrodynamic radii were calculated from the measured diffusion coefficients employing equation (2). All results were obtained from three experiments using separately cultured micro-organisms.

Electron microscopy. Structural features of some bacteria involved in this study are known from previous work; the other strains were studied by transmission electron microscopy for their structural surface features. Briefly, for negative staining, washed cells were stained in 1% methylamine tungstate on carbon-coated, hydrophilic grids to detect the surface structures, as described by Handley (1990). All samples were studied in a transmission electron microscope operated at 70 kV. The structural features of the bacteria are summarized in Table 1.

Table 2. Diffusion coefficients of bacterial strains used and polystyrene latex particles in 10 mM potassium phosphate solution, measured at pH 2 and 7, by dynamic light scattering, and obtained from the Einstein equation (equation 2) and the microscopic radius (r_p from Table 1)

Note that Einstein diffusion coefficients are not influenced by pH. $\pm$ denotes SD over three different cultures.

Strain	$10^{13} \times$ Diffusion coefficient at pH 2 ($\text{m}^2 \text{s}^{-1}$)	$10^{13} \times$ Diffusion coefficient at pH 7 ($\text{m}^2 \text{s}^{-1}$)	$10^{13} \times$ Einstein diffusion coefficient ($\text{m}^2 \text{s}^{-1}$)
<i>S. salivarius</i> HB	4.00 ± 0.45	2.90 ± 0.38	4.23 ± 0.20
<i>S. salivarius</i> HBV5	4.90 ± 1.75	3.13 ± 0.44	4.06 ± 0.20
<i>S. salivarius</i> HBV51	3.49 ± 0.28	3.39 ± 0.42	3.93 ± 0.20
<i>S. salivarius</i> HB7	3.32 ± 0.90	2.88 ± 0.39	4.23 ± 0.20
<i>S. salivarius</i> HBC12	3.44 ± 0.24	3.83 ± 0.47	3.88 ± 0.20
<i>S. mitis</i> BA	3.27 ± 0.30	2.35 ± 0.54	3.54 ± 0.20
<i>S. thermophilus</i> B	4.45 ± 1.12	3.04 ± 0.41	3.66 ± 0.20
<i>Staph. epidermidis</i> HBH ₂ 169	4.81 ± 0.27	4.63 ± 0.21	3.81 ± 0.20
<i>Staph. epidermidis</i> 252	4.95 ± 0.48	3.89 ± 0.35	3.71 ± 0.20
<i>A. calcoaceticus</i> RAG-1	3.63 ± 1.88	2.99 ± 0.21	4.10 ± 0.20
<i>E. coli</i> 431	2.95 ± 0.42	3.78 ± 0.42	$2.63 \pm 0.20^*$
Latex particle	6.54 ± 0.41	6.36 ± 0.87	6.20 ± 0.20

* Calculated with the effective radius, $r_e = 0.5(\text{width} \times \text{length})^{\frac{1}{2}}$.

RESULTS

In Table 2 the diffusion coefficients measured by dynamic light scattering of the bacterial cells suspended in 10 mM potassium phosphate solution are summarized for pH 2 and pH 7. In general, a decrease in diffusion coefficient was observed with increasing pH. This effect was more pronounced with heavily fibrillated strains or strains with a fuzzy coat such as *S. salivarius* HB, *S. salivarius* HBV5, *S. mitis* BA, *S. thermophilus* B and *Staph. epidermidis* 252, than for the strains which were only sparsely fibrillated or completely bald like *S. salivarius* HBC12. For latex particles, no difference was observed between the diffusion coefficients at pH 2 and pH 7. In most cases, the Einstein diffusion coefficients, based on microscopic radii, were similar to those measured at pH 2 and different from those at pH 7. Note that this was not the case for the latex particles.

Fig. 3 shows the hydrodynamic radii derived from dynamic light scattering and equation (2) as a function of pH for four selected strains, three fibrillated and one bald. The hydrodynamic radii of *S. mitis* BA and *S. salivarius* HB increased with increasing pH in both ionic strength solutions, whereas *S. salivarius* HBC12, the bald strain, did not show any effect. A clear maximum in hydrodynamic radius was observed for *A. calcoaceticus* RAG-1 around pH 4. This maximum was more pronounced in the low (10 mM) than in the high (40 mM) ionic strength solution.

To study the effect of electrostatic interactions between cell surface structures on the hydrodynamic radius of the organisms, the zeta potentials of the four selected strains were also measured (Fig. 4) as a function of pH at both ionic strengths. The three fibrillated strains, *S. mitis* BA, *A. calcoaceticus* RAG-1 and *S. salivarius* HB all demon-

Fig. 3. Hydrodynamic radii determined by dynamic light scattering (cf. Table 2 and equation 2) for four selected strains in 10 mM (—■—) and 40 mM (---▲---) potassium phosphate solutions [for ease of comparison with the zeta potential results, the isoelectric points (IEP) are indicated as well]. Results are the mean of three separately bacterial cultures with a SD of 10%.

Fig. 4. Zeta potentials in 10 mM (—■—) and 40 mM (—▲—) potassium phosphate solutions for four selected strains as a function of pH. All zeta potentials represent means of duplicate runs with separate bacterial cultures, results of which coincided within 2 mV.

strated strongly pH-dependent zeta potentials and possessed an isoelectric point (IEP) within the pH range used, whereas the bald strain *S. salivarius* HBC12 was negatively charged over the entire pH range measured.

DISCUSSION

In this paper we studied the influence of the ionic strength and pH on the diffusion and therewith on the hydrodynamic radius of micro-organisms with different structural surface features. From Table 2 it can be concluded that, in general, the diffusion coefficients calculated with the Einstein equation on the basis of microscopic radii, more closely resemble the ones measured by dynamic light scattering at pH 2 than at pH 7. This is probably due to the fact that light microscopy detects the outer cell wall and structural surface features cannot be detected. These surface structures are presumably in a more collapsed state at pH 2 than at pH 7 because stabilizing electrostatic repulsions between surface structures are reduced at low pH, as shown by the pH dependence of the zeta potentials. Thus the organism probed by dynamic light scattering at pH 2, with collapsed surface structures, will appear bald and have a hydrodynamic radius approximating the microscopic radius.

This idea is furthermore supported by grouping the hydrodynamic radii measured at pH 2 and pH 7 for bald

Fig. 5. Hydrodynamic radii determined by dynamic light scattering for bacterial strains and polystyrene particles in 10 mM potassium phosphate solutions at pH 2 (■) and pH 7 (▨), grouped for strains without surface features, including polystyrene particles (a) and for strains with surface features (b). Results are the mean of three separate cultures with a SD of 10%.

strains, including polystyrene particles, and for strains with structural cell surface features present (Fig. 5). For strains with structural surface features, fibrils or a fuzzy coat, the hydrodynamic radius is higher at pH 7 than at pH 2, whereas for the other strains no noteworthy differences are present. Exceptions are observed, however, for both Gram-negative strains, *E. coli* 431 and *A. calcoaceticus* RAG-1, possibly because Gram-negative strains have a more fragile cell wall, more likely to be affected by an extremely acidic environment.

Many electron microscopy studies on structural cell surface features are aimed at determining the length of these structures (Handley, 1990; Weerkamp *et al.*, 1986). Often this is a tedious operation. Assuming that the maximal variation in hydrodynamic radius observed over its pH dependence represents the maximal length of surface structures present, dynamic light scattering would make an alternative method to determining their length. Analysis by dynamic light scattering, irrespective of ionic strength, gives the maximal length of cell surface structures as approximately 600, 400, 300 and 100 nm for *S. mitis* BA, *A. calcoaceticus* RAG-1, *S. salivarius* HB and *S. salivarius* HBC12, respectively. These values are quite

Table 3. Flow (δ_f) and diffusion (δ_d) boundary layer thicknesses calculated for different laminar flows in a parallel plate flow chamber (length 7.6 cm, width 3.8 cm, height 0.06 cm) and a diffusion coefficient of $3 \times 10^{-13} \text{ m}^2 \text{ s}^{-1}$ (microbial cell radius was taken as 0.6 μm)

Q , volumetric flow rate; U_0 , characteristic velocity; Pe , Peclet number; Re , Reynolds number given by:

$$\left(\frac{Q}{(w+2b) \cdot v} \right) \quad (5)$$

in which w is the width of the chamber and b the half depth of the chamber. Flow is laminar for $Re < 2000$, while the equations used to calculate δ_f and δ_d are not valid when $Re < 1$. t_c denotes the time it takes a micro-organism to pass through the chamber by convection, while t_d is the time required for a micro-organism to traverse the diffusion boundary layer by diffusion only.

$Q \text{ (cm}^3 \text{ s}^{-1}\text{)}$	$U_0 \text{ (cm s}^{-1}\text{)}$	Pe	Re	$\delta_f^* \text{ (m)}$	$\delta_d \text{ (m)}$	$t_c \text{ (s)}$	$t_d \text{ (s)}$
0.034	0.15	0.018	1	2.7×10^{-2}	1.8×10^{-4}	51	329
0.228	1.0	0.12	6	1.0×10^{-2}	0.7×10^{-4}	7.6	128
76	333	40	2000	0.06×10^{-2}	0.06×10^{-4}	0.023	6.8

* Values for δ_f exceeding the half depth of the flow chamber indicate that no constant velocity profile will develop under the conditions considered and that the velocity profile will be parabolic as shown in Fig. 2.

reasonable when compared with the data presented in Table 1, particularly when one considers that the assumption outlined above does not include any effects due to fibril density or the presence of structures with different lengths. Also, in electron microscopy studies, it is never certain that all structural features are detected. This might explain why dynamic light scattering yielded a length of 400 nm for surface structures on *A. calcoaceticus* RAG-1, while electron microscopy (Table 1) gives only 180 nm.

Not only can the present set of data be used to gain a better understanding of the interaction between cell surface structures, but also of the mass transport phenomena occurring in natural environments and in flow chamber devices, such as the parallel plate flow chamber (Sjollem et al., 1989b). For each fluid flowing over a solid surface, there is a region close to the surface where a steep velocity gradient exists, the so-called boundary layer for flow (see Fig. 2). Likewise, there is a similar region where a steep concentration gradient exists, the so-called boundary layer for diffusion. In a parallel plate flow chamber for instance, organisms enter the flow chamber mainly by convection and move towards the substratum by diffusion, sometimes sedimentation and attractive interaction forces. The thicknesses of the boundary layers for diffusion (δ_d) and flow (δ_f) are given (Levich, 1962) by:

$$\delta_d \approx \left(\frac{D_\infty}{v} \right)^{\frac{1}{3}} \cdot \delta_f \quad (3)$$

in which v is the kinematic viscosity ($10^{-6} \text{ m}^2 \text{ s}^{-1}$) and δ_f is the distance from a plate where the flow velocity becomes constant:

$$\delta_f \approx 5.2 \left(\frac{vx}{U_0} \right)^{\frac{1}{2}} \quad (4)$$

where x is the distance from the channel entrance and U_0 , the characteristic flow velocity.

Table 3 summarizes some calculated values for δ_f and δ_d

together with the corresponding Reynolds and Peclet numbers. Note that the Peclet number increases with increasing flow. The diffusion boundary layer is considerably thinner than the boundary layer for flow but nevertheless it can take an average micro-organism several seconds to minutes (see Table 3) to traverse it by diffusion only. This explains why the kinetics of microbial adhesion in the parallel plate flow chamber are slow compared to devices where convective flow is directed towards the substratum surface.

Diffusion is obviously the rate determining factor for deposition in the parallel plate flow chamber as it is much slower than convection (see Table 3). Moreover, the chances of a micro-organism reaching a substratum surface by diffusion only are rather small, and collisions between flowing particles to give them a velocity component towards the surface, sedimentation and attractive interaction forces are required for a flowing micro-organism to reach the surface. Thus the data presented here are not only of importance to understand the interaction between cell surface structures, but also for the application of existing mass transport theories to explain microbial deposition kinetics.

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