INFLUENCE OF ION STRENGTH AND VIABILITY IN BACTERIAL SELF-ASSEMBLY PATTERN ON ABIOTIC SURFACE: PRELIMINARY MODELS

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1 Introduction

Bacterial colonization is a very complex process influenced by several factors such as material surface, characteristics of cell surface and environmental condition. Colonization starts by adhesion of single cells or cell aggregates at the surface; if the conditions are favorable, attached cells will grow, divide and develop micro-colonies.

Adhesion process consists in two phases: the first step is reversible, and non specific, governed by physical and chemical interactions between planktonic cells and abiotic surface. The second step is characterized by expression of specific binding proteins that make process practically irreversible. When freely suspended bacteria approach a solid surface in an aqueous medium, long-range forces, mainly Brownian movements, Van der Waals forces, acid-base interactions and electrostatic forces, i.e DLVO (Derjaguin-Landau-Verwey-Overbeek) forces, come to play at first. The DLVO theory [1] has been used to describe the net interaction between a cell and a flat surface as a balance between two additive factors, one resulting from Van der Waals interactions, generally attractive, and repulsive interactions from the overlap between the electrical double layer of the cell and the substratum, due to the negative charge of both cells and abiotic surfaces. The electrostatic forces are considered the most important, being markedly influenced by the surface potential of both cell and surface or the chemical properties of the solution, i.e. ionic concentration and valency of ions.

The DLVO theory assumes a hypothetical smooth surface; bacterial cells, however, have surface structures, such as pili, fimbriae, flagella, lipopolysaccharides (LPS), polysaccharides and/or proteins. Such surface structures/molecules are not accounted for in the DLVO theory, but are often described in terms of their contribution to the overall cellular properties, e.g. cell surface hydrophobicity (CSH), charge or energy. Different structures on the same cell give their individual contributions to the net cell character in a more or less predictable way [2,3]. For these reasons the DVLO theory is now obsolete, and nowadays reference is made to the Oshima model, a novel mobility theory, the so-called ”soft particle electrophoresis theory”, because much care has to be taken in the interpretation of mobility data of biological cells whose surface organization is much complex than that of an inert particle [4]. This model assumes a charged ion-penetrable layer of finite thickness around a core particle, while the conventional one has no surface structure. In according with soft particle theory, when charged polyelectrolytes, as cells, are immersed in an electrolyte solution, a macroscopic potential difference is established between the polyelectrolyte interior and the external electrolyte solution. The distribution of electrolyte ions between these two regions has often been treated by a two-phase model, or the macroscopic Donnan model [5], where the potential of the polyelectrolyte interior phase, measured relative to the external solution phase, is called the Donnan potential.

It is particularly complex to describe the phenomenon of bacterial adhesion on abiotic surfaces with an analytical model that should account for the multiple and interacting factors depending on bacteria, substratum, medium characteristics and so on.

Our previous studies [6,7] demonstrated that self-organization plays a fundamental role in the emergence of cell patterns during adhesion on polymer surfaces. The emergence of a pattern in the structure of the adhered bacteria is the result of a self-assembly process ruled by local laws of interactions, well modelled by CA [6]. Cellular self-assembly on chemically well defined solid surfaces was demonstrated to be prompted by controlled dewetting processes that determine capillary forces responsible for the formation of ordered well defined cellular patterns [7].

It is necessary found a model which considers the dynamics of the process taking into account bacteria as ”active” soft particles, thus involving the two most important factors influencing the outer region, i.e. the ion

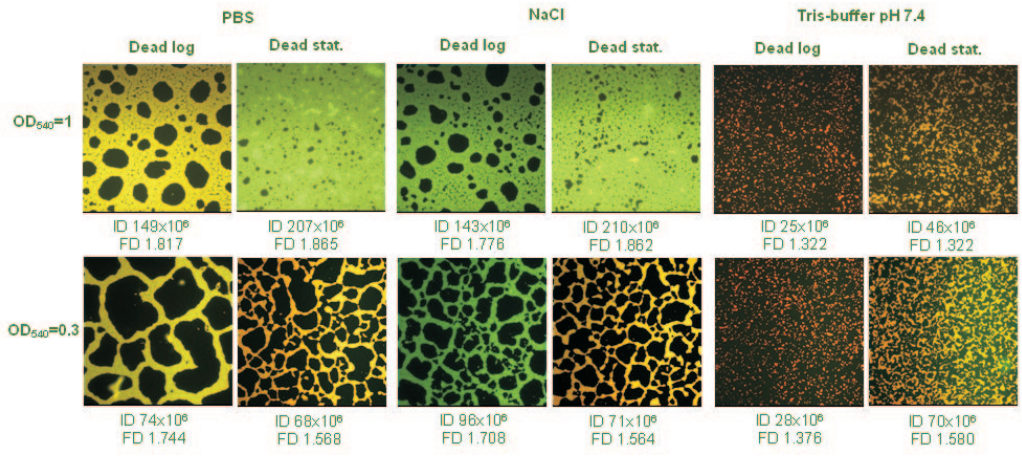

Figure 1. Dead exponential and stationary cells in the different suspending solutions after 2h.

strength of medium and, above all, the capacity of viable bacteria to actively change their own ion penetrable layer.

In this work, preliminary models on the influence of ionic strength and viability in bacterial self-assembly patterning on abiotic surface are introduced. To this aim, experiments were carried out on *Staphylococcus epidermidis* ATCC 12228 surface colonization of an abiotic surface (i.e., boro-silicate glass). In this strain the *ica* operon was completely absent [8], thus cells do not produce the polysaccharide intercellular adhesin involved in cell-to-cell interactions occurring during biofilm development; moreover, in addition to *ica* operon, the AaP (accumulation-associated protein) is an extra-cellular protein also essential for bacterial accumulation in forming biofilm. ATCC 12228 strain carries the *aap* gene [8], however it was demonstrated that it was not expressed in the active extra-cellular form and that amounts of Aap from membrane as well as whole-cell extract proteins were lower when compared to those of a biofilm-forming invasive strain [9]. It was proposed that the full-length molecule of Aap is represented on the cell surface as fibrillar appendages localized in a tuft structure [10]. It is likely that the full-length not proteolytically cleaved Aap is expressed on cell surface of the biofilm-negative strain ATCC 12228, as hydrophobic fibrils anyway contributing to the overall cell surface properties.

It was reasonable to use *S. epidermidis* ATCC 12228 in order to investigate the influence of the parameters of interest on single cells, accounting only for the physico-chemical properties, in particular electrostatic interaction forces so important in the first phases of adhesion and regulating the self-assembly process of bacterial cells on abiotic surface.

2 Materials and methods

The bacteria ability to actively change their own "state of soft particles" was analysed in cells both living and killed by 4% formaldehyde for 1 hour. In addition, in order to include the possible changes in cell surface thermodynamics accompanying physiological state changes, as widely observed [11-14], both alive and dead cells were harvested by exponential ($OD_{540} = 0.8$) and stationary ($OD_{540} = 1.8$) growth phases.

In all the experiments, different suspending solutions were used, to the aim to control ion strength, as well as the single ion type contribution; in particular, ultrapure H_2O and Tris Buffer pH 7.4 were used as control media lacking any ion, TRIS-HCl buffer pH 6.6 as medium with no ion contribution except protons, saline solution (NaCl 0.9%) as medium with only ionic contribution, and phosphate buffered saline (PBS) as balanced ionic buffer.

Harvested cells were washed with the relative buffer, then resuspended in each medium to OD_{540}=1 (5×10^8 cells/ml) or $OD_{540} = 0.3$ (1×10^8 cells/ml), in order to investigate the influence of cell density on self-organization process.

The different capacity to adhere onto surface was highlighted by dewetting force that, causing detachment of bacteria weakly adhered, was considered as an indirect measure of adhesion strength. The bacteria adhered onto surface were stained with the fluorochrome acridine orange, observed by a Leica DMRE epifluorescence microscope, and at least five optical fields per condition were acquired by a Leica DC300F Camera and Leica Qwin software. At fixed time intervals, from 30 minutes to 2 hours, a quantitative evaluation of cells adhering onto the different conditions was performed using the Scion Image software in terms of integrated density (I.D.).

On the other hand, a suitable measure to assess self-assembled pattern organization was used from the theory of fractals, the Hausdorff dimension (FD), computed by using the box counting method (Image J software).

In order to model the phenomena experimentally observed, we developed an approach similar to the dis-

crete generic models [15], where the biological behavior of bacterial growth is explained by generic features and basic principles elicited from biological considerations and experimental observations. Bacterial cells were modeled with discrete moving entities. The simulator software NetLOGO was used to design this model. Each bacterium was modelled as a random walker which may adhere on the surface, i.e. stop moving and become fixed at a given position. In the simulator, agents represent bacteria that move in a bi-dimensional grid of patch space. Any patch is a square cell where agents can born, die or move.

Such an agent-based model can be viewed as a generalization of a cellular automaton model, in which the agents move in the space and therefore interact with a time-variant neighborhood [16]. Bacterial position was indicated with x_i, and with v_i its velocity. We assumed that the module of the agent speed was constant and that the heading was a random variable chosen in the interval $[-\pi, \pi]$ with uniform distribution. At each time instant, the position of each not adhered agent was thus updated by taking into account the following equations:

$$\begin{aligned} \mathbf{x}_i(t + \Delta t_M) &= \mathbf{x}_i(t) + \mathbf{v}_i(t)\Delta t_M \\ \theta_i(t + \Delta t_M) &= \xi_i \ , \end{aligned} \tag{1}$$

where $\mathbf{v}_i(t) = ve^{j\theta_i(t)}$ and $\xi_i \in [-\pi, \pi]$.

A sufficient number of bacteria on a given area was needed for aggregation, otherwise all the bacteria in that area moved. The second part of the model focused on the density-dependent rule for bacterial adhesion. For each time instant, each agent of the system was sequentially processed. For each of them, the density of agents in a given neighborhood of it was indicated with ρ, where the neighborhood is defined as those agents whose position, at the given time, is within a given radius r of the given agent. In particular, for each agent and each time instant, three different radii were taken into account, and, thus, three different values of ρ (ρ_1, ρ_2, and ρ_3). We, then, compared ρ_1, ρ_2, and ρ_3 with fixed thresholds and, if these values were greater than the given thresholds, the bacterium adhered to the surface; otherwise all the bacteria in the given radius moved according the equations (1).

3 Results and discussion

In our experimental setup, dead cells represented "passive" soft-particles influenced only by the growth phase they come from, and the medium where they are suspended during adhesion, because they are not able to further modify their own surface potential (Fig.1). Stationary phase cells showed a significant higher adhesion than exponential, independently from the medium used, confirming changes in physico-chemical surface properties accompanying the two physiological

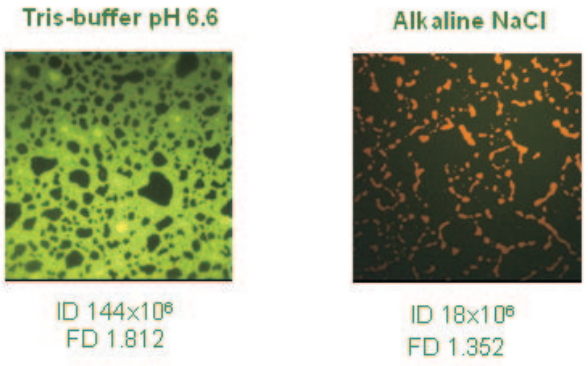

Figure 2. Dead stationary cells in the different suspending solutions at OD=1 after 2h.

states, as widely observed [11-14]. Experiments carried out at $OD_{540} = 0.3$ and $OD_{540} = 1$ demonstrated the influence of cellular density on the self-assembly dynamics, as showed by the emergence of fractal patterns at low cell number whereas surface was completely covered at the higher cell concentration. In H_2O, no cell adhesion was observed, likely for both ion lack and osmotic stress. When incubated in Tris buffer pH 7.4, an isosmotic medium with no ion contribution, cells showed a poor adhesion and were not self-organized, whereas in both saline buffers - NaCl 0.9% and PBS - the highest ID values were recorded and the fractal dimension was that typical of a self-assembled pattern (FD=1.6-1.8). The essential role of ions in adhesion and self-organization was further confirmed by results obtained in Tris-HCl pH 6.6, where both ID and FD values were the same of those obtained in PBS/NaCl, demonstrating that protons are sufficient to support the two processes (Fig. 2a). In contrast, in alkaline saline solution poor adhesion (ID=18×10^6) and no self-assembly were observed, suggesting that anion predominance prevents the two cellular events (Fig. 2b).

Results about living bacteria showed that, independently from the growth phase they come from and on the medium they are suspended, the number of adhered cells is significantly lower than dead cells (Fig. 3). This seems directly related to cell capacity to modify their own ion penetrable layer, by means of their metabolic activities and in particular the regulation of ion fluxes. This behaviour could be related with different magnitude of net cationic fluxes (such as H^+, K^+, NH_4^+), as previously demonstrated for exponential and stationary phase cells, with a substantial decrease in net efflux through the plasma membrane - less permeable to protons -, as bacterial cells progressed from the exponential to stationary stage of their growth [17]. In addition, it is known that bacterial adhesion to surface is accompanied by a change in electric potential of the substratum, due to a charge transfer from bacteria to surface [18].

In this paper we show that alive bacteria indeed act as

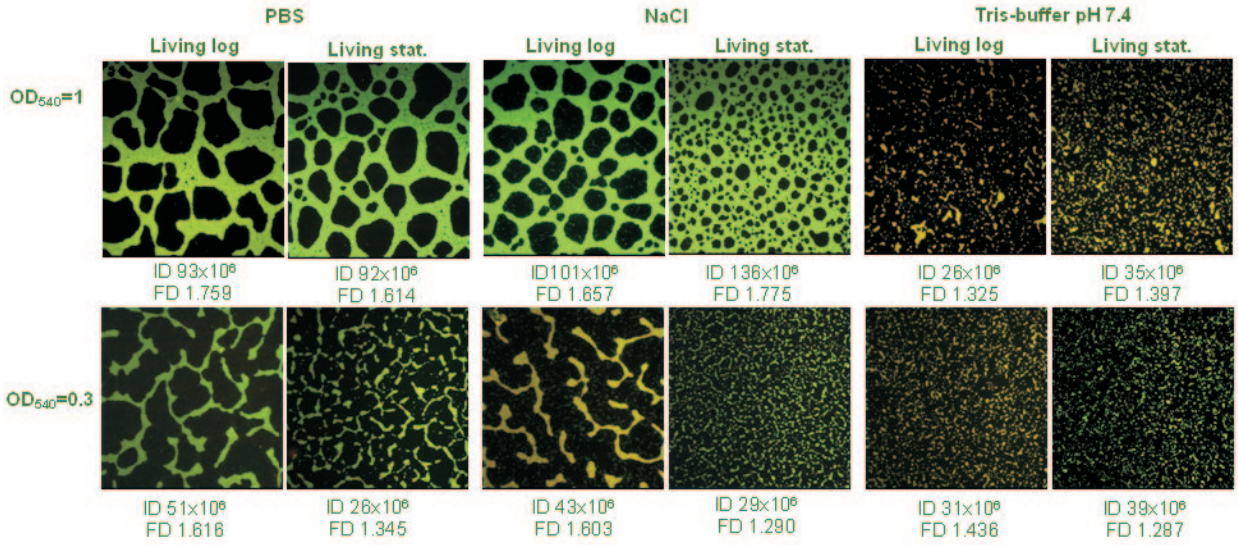

Figure 3. Living exponential and stationary cells in the different suspending solutions after 2h.

"active" soft particles able to change their surface potential as well as the substratum charge, thus modifying the electrostatic interactions ruling the first phases of adhesion and the self-assembly process of bacterial cells on abiotic surface. The overall experimental data demonstrated that bacterial cell adhesion and self-assembly on abiotic surface are affected not only by the ionic strength of the suspending solution but especially by cell viability, both influencing the bacterial surface charge, changing the ionisation of the different chemical groups on cell wall, as well as the contact area between adhering bacteria and substratum.

The simulation by the model developed confirmed the observed behaviour, as regards several different parameters, in particular bacterial density and mobility on the surface. Indeed, the mechanism which leads a bacterium to adhere on a given position was based on the relative (local) density of the other bacteria. Thus the idea underlying this rule is that the condition for self-organization is that a sufficient number of bacteria on a given area should exist; otherwise, all the bacteria in that area move. In the preliminary phase of our simulations bacteria move and aggregate in small communities, then, starting from this condition, bacteria/agents were let evolve until a steady-state condition was reached. The simple rules of local density and mobility were sufficient to explain behaviour of dead cells in saline buffers (Fig.4).

Model simulation showed that a minimal number of bacteria (density 68%) was needed in order to cover all surface, confirming experimental data (OD 1). In this contest, initial mobility played an important role only at low density, as when cell density was high it did not further influence disposition. This behaviour could be explained by higher cohesion cell-to-cell forces at high cell density.

However, local cell density and initial mobility were not sufficient to model behaviour of alive bacteria. To this aim, we have introduced another parameter, coefficient-of-mobility, that represents the entity of bacterial movement during model evolution. In particular, in equations (1), $v' = \mu v$ was introduced, where μ was the coefficient-of-mobility. This coefficient-of-mobility was related to cell capacity to modify their own ion penetrable layer, by means of regulation of ion fluxes, thus modifying the electrostatic interactions with neighbour cells as well as the abiotic surface. Indeed, as coefficient-of-mobility increased, agents did not cover all the surface, not even at high density, as consistent with experimental results, when living bacteria formed fractal patterns, likely due to prevailing repulsive force (Fig. 5).

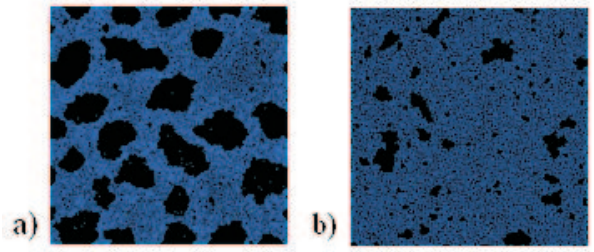

Figure 4. Simulations of dead exponential (a) and stationary (b) cells in saline buffers after 2h, setting density to 65% and 68%, respectively.

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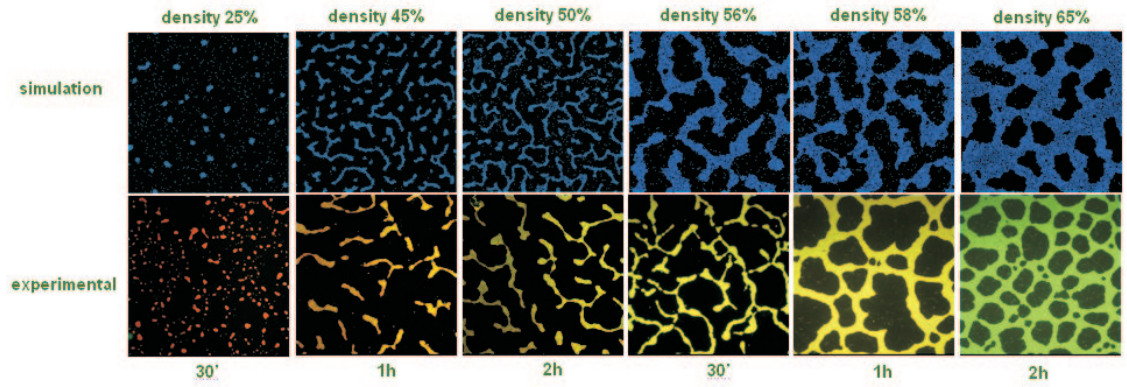

Figure 5. Simulation frames for different values of density, with a coefficient-of-mobility of 7-8, in comparison to experimental dynamics at different times.

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