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SELF-ORGANIZING MODELS OF BACTERIAL AGGREGATION STATES

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Abstract. In this work, aggregation states of bacteria on engineered surfaces are investigated both from the experimental point of view and from the theoretical one. The starting point of this work is a series of experiments carried out on abiotic surfaces in which bacteria adhere forming self-organized patterns. To reproduce the main characteristics of the phenomenon a model based on self-organization of a group of agents has been used. The agents represent bacteria and are free to move on a given surface. On the basis of local rules they may adhere and then eventually form self-organized aggregates. Our numerical results demonstrate that few simple rules are able to explain the emergence of self-organized patterns. Depending on the parameters used, the model is able to reproduce the aggregation patterns observed under different experimental conditions and to predict the behavior of a culture of two bacterial species.

1. **Introduction.** It is now clear that cooperative behavior and self-organization are key concepts in understanding how bacterial colonies adapt to adverse environment conditions [1, 2]. Bacteria associated with plant leaves, for example, employ a range of colonization strategies resulting in the conspicuous presence of bacterial aggregates whose behavior varies in a density-dependent manner. Such cooperative interactions may occur among both homogeneous and heterogeneous populations, thus influencing the development of microbial communities [3]. It was demonstrated that the fate of solitary and aggregated cells is clearly different: the solitary cells usually represent unsuccessful colonization events that ended in their death under stressful conditions, whereas cells in aggregates are much more capable of tolerating environmental stresses, and their preferential survival promotes a highly clustered spatial distribution of bacteria on leaf surfaces. This strongly suggests that large aggregates constitute a distinct ecological niche for bacteria, allowing them to survive harsh environmental conditions [4]. Bacterial cells were not randomly distributed on the leaf surface, but occurred in a wide range of cluster sizes, ranging from single cells to more than 10^4 cells per aggregate [5]. In addition, the fate of immigrant

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bacterial cells on leaves under stressful conditions was determined as a function of the local spatial density of resident cells at their landing site. Although only a small fraction of the immigrant cells landed on established bacterial aggregates, their fate was usually strongly influenced by the presence of indigenous bacteria at the site at which they landed. Immigrants that arrived as solitary cells had about double the probability of survival when landing on cell aggregates than when landing on uncolonized areas of the leaf surface [6].

It has been demonstrated that the individuals in a bacterial colony which grow under adverse conditions cooperate and self-organize through direct and indirect, physical and chemical communication channels [1]. While simple circular structures are observed in bacterial colonies grown in the presence of excess nutrients, at low concentrations patterns revealing a self-organizing behavior appear. Furthermore, Tsimring et al. [7] showed that, in presence of oxidative stress, patterns, which do not appear under normal conditions, emerge in the structure of the bacterial colony. In this study [7], the emergence of radially aligned spots and radially oriented stripes is linked to a chemotactic behavior triggered by the oxidative stress; a model taking into account these factors is then introduced. Models based on key ideas of complex system theory have also been used to model branching growth of bacterial colonies [8], chiral growth patterns [1], and many other examples [2]. In related works [9] cellular automata models are used to model the morphology of bacterial colony growth.

Although many papers addressed the study of growth patterns, much less work has focused on the case of patterns arising when bacteria adhere on a given surface. In spite of this, microbial adhesion is a very important phenomenon. Bacteria have a strong tendency to adhere to surfaces. Once they adhere, they form a complex microbial community called a biofilm. On one hand, biofilms may pose serious problems for foods, ship hulls, historical monuments, and in the oral cavity; on the other hand biofilms may be beneficial, for instance in degradation and removal of hazardous substances in soil and natural streams, or in a bioreactor or as bioflocculants in wastewater treatment plants.

Because of these issues, considerable research efforts are currently directed towards the control of bacterial adhesion on surfaces. In particular, in this paper we study the adhesion process of *Pseudomonas aeruginosa* on PVC surfaces under different growth conditions and we show that in several cases bacteria adhere forming complex patterns.

We then show that a model based on self-organizing systems may be used to reproduce the observed behavior. Furthermore, this model is shown to be able to predict interesting properties of the biological phenomenon.

The paper is organized as follows: in Section 2 the biological background and the patterns observed when bacteria adhere on abiotic surfaces are discussed; in Section 3 the model is introduced; in Section 4 further biological patterns arising from experiments elicited by model simulations are discussed, and in Section 5 the conclusions of the paper are drawn.

2. **Biological background and bacterial adhesion patterns.**

The dynamics and kinetics of bacterial adhesion onto abiotic surfaces were investigated in static conditions. In order to analyse adhesion and biofilm formation under controlled conditions, avoiding natural effects of sedimentation, bacterial growth chambers used in these experiments were designed specifically to accommodate vertical PVC

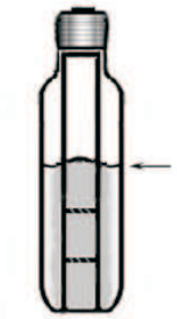


FIGURE 1. Schematic of bacterial growth chamber with vertical PVC strip; arrow indicates air-liquid interface and dashed line outlines the three square frames considered for observation of the surface area.

strips as substratum surfaces. The chambers consisted of autoclavable vials of 8.2 mm in diameter by 32 mm in length, with a bottleneck of 4.2 mm, closed by screw-caps with an air inlet. PVC strips (4x30 mm) were cleaned with 70% ethanol, dried at room temperature, then inserted down to the bottom of the vials and kept in vertical position by the bottleneck. The chambers with PVC surfaces were filled with 1 ml bacterial suspension, so that air-liquid interface was exactly at 14 mm from the bottom (about 1/2 of the height of PVC strip). Then they were incubated at 37°C. The experimental setup is schematically shown in Figure 1. After the given incubation time, each vial was opened and the PVC strip with adhered bacteria gently taken by sterile tweezers and immediately dipped into sterile water, avoiding air-liquid interface passages that could remove cells from the surface. To this aim, water was changed three times and PVC strips remained in liquid during the rinsing; then they were stained with 1% crystal violet for 15 minutes, washed, and observed by a Leica DMRE microscope endowed with motor focus controlled by Leica Qwin software. The extent of cellular distribution on PVC strips was analysed onto three surface 4x4-mm square frames, each corresponding to air-liquid interface, liquid, and bottom, respectively. Microscopic observations at 630x magnification were performed on the 3 square frames of each sample by setting the exact coordinates in Qwin software, and at least five optical fields were acquired by a Leica DC300F camera for each frame.

Pseudomonas aeruginosa ATCC 27853 was used for all experiments in bacterial adhesion dynamics. It was grown in Luria-Bertani broth (LB) with constant shaking at 37°C. An overnight LB culture was diluted in fresh broth as follows: cells were harvested by centrifugation, washed twice with phosphate buffered saline (PBS), and diluted in culture medium to an optical density at 540 nm of approximately 0.4 (Beckman DU640 spectrophotometer), corresponding to approximately $3 \div 5 \times 108$ viable cells (CFU) per ml. Previous studies had demonstrated that this optical density corresponds to the maximal adhesion in the experimental conditions adopted here. This cell suspension was transferred to bacterial growth chambers prepared as described above. The characteristics of the experiment did not permit observation of the dynamics of the phenomenon on a single surface sample. Because at each observation time the PVC strip was taken and washed, to reconstruct at

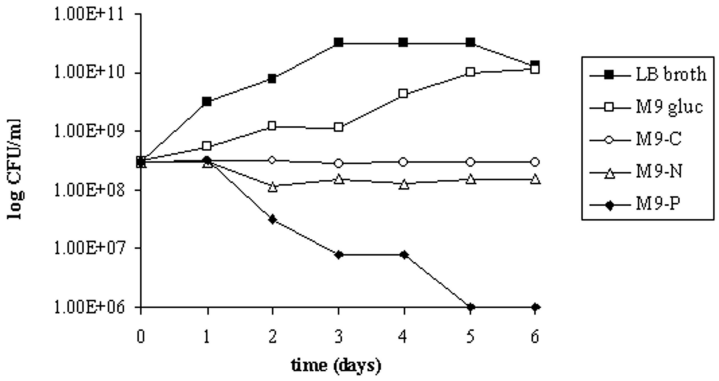


FIGURE 2. Viable cells (CFU) of *P. aeruginosa* ATCC 27853 cultivated in Luria-Bertani (LB) broth, M9 medium with glucose as carbon source (M9 gluc) and under carbon (M9-C), nitrogen (M9-N) and phosphorus (M9-P) starvation, in static condition at 37°C.

least qualitatively the evolution of bacterial adhesion, the experiment was repeated for each observation time by using several identical strips processed in an identical fashion with the same experimental environment with the same cell suspension; then the samples were taken from the chambers at given time intervals (15 min, 30 min, 45 min, 60 min, 75 min, 90 min, 105 min, 2 h, 24 h, 48 h, 72 h, 6 dd). Three replicates for each time were observed.

The experiment was repeated by varying bacterial growth conditions. As controls, cell suspensions were prepared in nutrient-rich medium, Luria Bertani (LB) broth, and also in nutrient-limited mineral media, formulated on purpose from the minimal M9 medium: M9-C, -N and -P deprived, respectively, of carbon (glucose), nitrogen (ammonia) or phosphorus (inorganic phosphate) sources. These elements are the essential building blocks for growth, so their lack (starvation) leads bacterial cells into a dormant mode, known as a stationary phase, which stops growth and protects cells against harsh conditions. In the experimental conditions adopted here (static growth chambers), both carbon-starved and nitrogen-starved *P. aeruginosa* cells stop growing and enter the stationary phase, staying viable; whereas under phosphorus starvation, regenerative capacity immediately decreases (Fig. 2). Bacterial adhesion dynamics and kinetics onto PVC surfaces were analysed under such conditions to investigate how the different effects of each nutrient limitation on bacterial growth can affect adhesion onto abiotic surfaces. Three independent experiments were carried out for each condition.

The surprising result was that, at the end of the process, under all conditions and in all three frames of each PVC strip, a pattern of bacterial adhesion was clearly visible on the surface. As expected for an aerobic microorganism, a large fraction of the total adhesion/biofilm on the PVC strip was attached within the frame at the air-liquid interface, which provided approximately one-third of the wetted surface area. Results and images reported in this work refer mainly to this frame. The main results are summarized here: both in nutrient-rich and in a mineral medium with glucose as carbon source, *P. aeruginosa* after 2 h of incubation

forms a three-dimensional biofilm, whereas in nutrient-limited conditions it forms no biofilm but still adheres to surface. In particular, under nitrogen starvation, cellular growth is not allowed but adhesion and biofilm formation take place. Under carbon starvation, cells adhere to PVC in the early stages of the experiment but, lacking energy, then detach. Also when phosphorus-starved, cells adhere at an early stage but do not form biofilm because they immediately lose viability. Interestingly, in the early adhesion stages (within 2 h) similar patterns are observed under all the conditions tested. In Figure 3 some examples of aggregational states observed on PVC after 45 minutes under different nutritional conditions are shown.

From the study of different cell suspensions, we can derive general conclusions. In many cases bacteria tend to adhere to a surface, forming a pattern. Also when a nutrient-starved cell suspension is used, the pattern forms, even if then bacteria detach from the surface and do not form a biofilm. Bacterial patterns seem to be universal, independent of growth conditions, and also of bacterial species and polymer surfaces used, as previously demonstrated [10, 11].

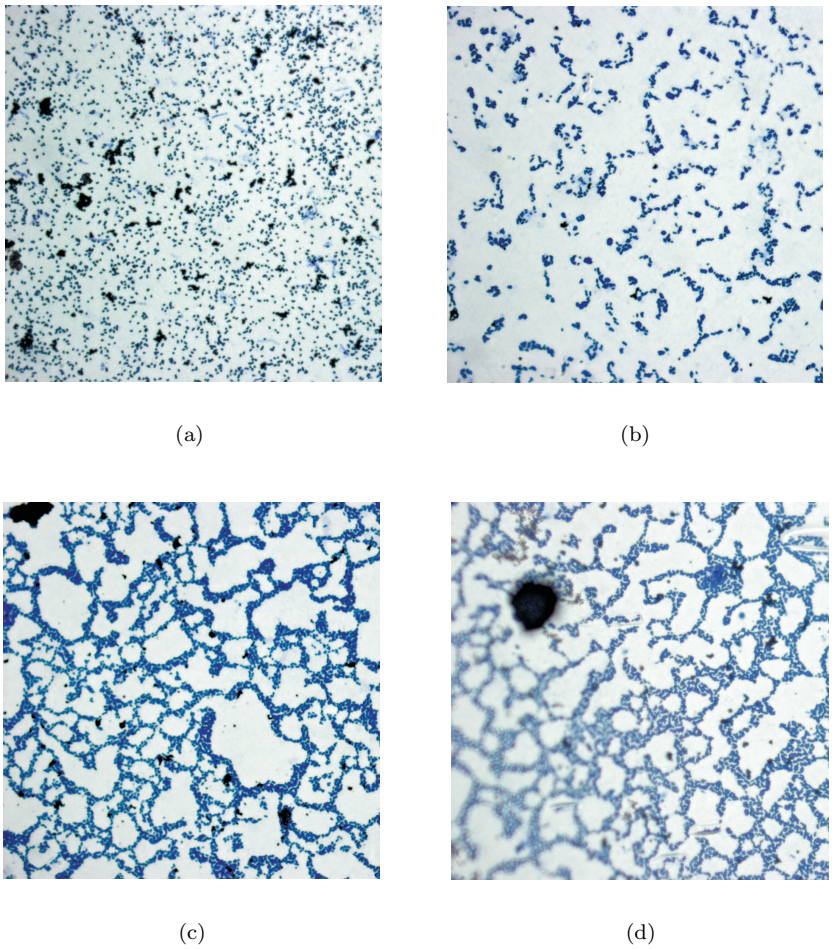
3. **Model.** Microbial adhesive interactions depend both on nonspecific long-range and on specific short-range interaction forces between microorganism and substratum [12]. These forces are physico-chemical forces such as Lifshitz-van der Waals forces, electrostatic forces, and acid-base interactions and are quite difficult to model.

Here, we plan to extract quite abstract principles from biological knowledge and the experiments, and apply them to model the phenomenon observed. In this sense, our approach is similar to the "generic modelling" introduced in [8], where the biological behavior of bacterial growth is modelled and explained by generic features and basic principles elicited from biological considerations and experimental observations. In particular, several generic models can be grouped into the category of discrete generic models [1, 13]. These models share the idea of modelling micro-organisms with discrete moving entities and to describe the time evolution of nutrients or chemicals with reaction-diffusion equations.

We focus on the surface and propose a model based on discrete entities which represent bacteria on it. Each bacterium is essentially a random walker which may adhere on the surface, i.e. stop moving and become fixed at a given position. An essential difference between the phenomenon of adhesion and that of growth should be noted. While growth occurs on a nutritive substrate, in our experimental setup nutrients diffuse in the suspension. Thus, we do not explicitly need to model nutrient diffusion on the surface.

The mechanism which leads a bacterium to adhere on a given position is instead based on the relative (local) density of the other bacteria. In fact, there are two opposite needs. On one hand, bacteria tend to stay closer and form stable structures that can then evolve into microcommunities and biofilms. On the other hand, since nutrient exchange occurs at the interface between the surface and the suspension, bacteria tend to maximize the exchange surface; i.e., to create regions in which the density of bacteria is low, and the regions are thus rich in nutrients. The access to these regions is favoured by fractal structures, like those observed in real experiments.

From these considerations, we derived the basic elements to be implemented in our model: bacterium motion and bacterium adhesion, the latter including cell-surface and cell-cell interactions (aggregation). The first is implemented taking into



(a) (b)

(c) (d)

FIGURE 3. Representative patterns of biological adhesion (after t=45 min). Cell suspensions in: (a) M9 -C; (b) LB; (c) M9; (d) M9 -N.

account a random walker model for the bacteria. The second element of the model is an abstract principle representing the dichotomy between nutrient exchange efficiency and tendency to form bacterial structures. To do this, a rule based on a test on the local density of bacteria is introduced. More specifically, for each bacterium the numbers of neighboring bacteria in three given radii are computed and if these values are greater than given thresholds, the bacterium adheres to the surface; otherwise, the actual position of the bacterium is unfavourable because the density is low. Thus the idea underlying this rule is that the condition for aggregation is that a sufficient number of bacteria on a given area should exist; otherwise, all the bacteria in that area move. We show that these very simple principles may explain pattern formation in bacterial adhesion.

In our simulations bacteria/agents are first distributed in random positions on the surface. We have observed that this distribution does not reflect the initial

distribution observed on PVC samples extracted early from the cell suspension. Thus the preliminary phase of our simulations consists of a phase in which bacteria move and aggregate in small communities. Starting from this condition we then let bacteria/agents evolve until a steady-state condition is reached.

We investigated the behavior with several different parameters and in particular with respect to the density of bacteria on the surface. Figure 4 shows several simulation frames at different times and with respect to different values of the density. For each density value the last frame shown in Figure 4 represents the steady-state condition obtained. It is interesting to note that each of these three conditions has been observed under different experimental conditions. In particular, the behavior at low density has been observed when the cell suspension is nutrient-limited for a long time, with a low initial number of bacteria, or under both conditions, while the behavior at high density is observed when the cell suspension is in the early stages of adhesion in optimal growth conditions and also in limited conditions with a sufficient number of adhered bacteria.

Another important conclusion that matches the experimental observation is that there is a threshold value of the density under which the pattern does not form. The overall conclusion is that a very important parameter for pattern formation is the number of bacteria on the surface; from an experimental point of view this parameter is connected to the cell suspension characteristics, in terms of initial number of bacterial cells and nutritional conditions in which they exist.

4. **Adhesion of two species.** After the steady-state condition is reached, we simulate what occurs if other bacteria adhere to the surface. We observed that these new bacteria do not destroy the existing pattern, but their aggregation pattern resembles the existing one. From our simulations it seems that new bacteria/agents arrange themselves on the borders of the existing structure. This conclusion derived from model simulation is very important for its possible implication. It could imply that 3D bacterial structures are built on the underlying 2D pattern.

We thus turned back to the experimental phase and verified this hypothesis by using two different bacterial species: *Staphylococcus epidermidis* (environmental isolate) and *Listeria monocytogenes* (ATCC 7644). They were cultivated in Tryptone Soya broth (TSB) at 37°C. These two bacterial species are easily distinguishable for their shape: *S. epidermidis* has a spherical shape (typical of cocci), while *L. monocytogenes* is rod-shaped. Furthermore, these two species grow with different characteristic generation times, leading to a mixed culture in which staphylococci are more numerous than Listeria, at least in the first phase of the experiment. In fact, *S. epidermidis* grows faster, and this turns to a greater probability of it to adhere on the surface. As it can be observed in Figure 5, *L. monocytogenes* tends to adhere on the borders of a structure formed mainly, and likely formerly, by staphylococci.

5. **Conclusions.** In this work, aggregational states of bacteria on abiotic surfaces have been studied. Patterns experimentally observed reveal that bacteria adhered onto the surface (PVC strip placed vertically in a dedicated growth chamber containing the cell suspension), and self-organize in a complex phenomenon to maximize the efficiency of the exchange of nutrients at the interface between surface and cell suspension. We observed pattern formation under many experimental conditions, bacterial species, and bacterial growth conditions. In particular, experiments

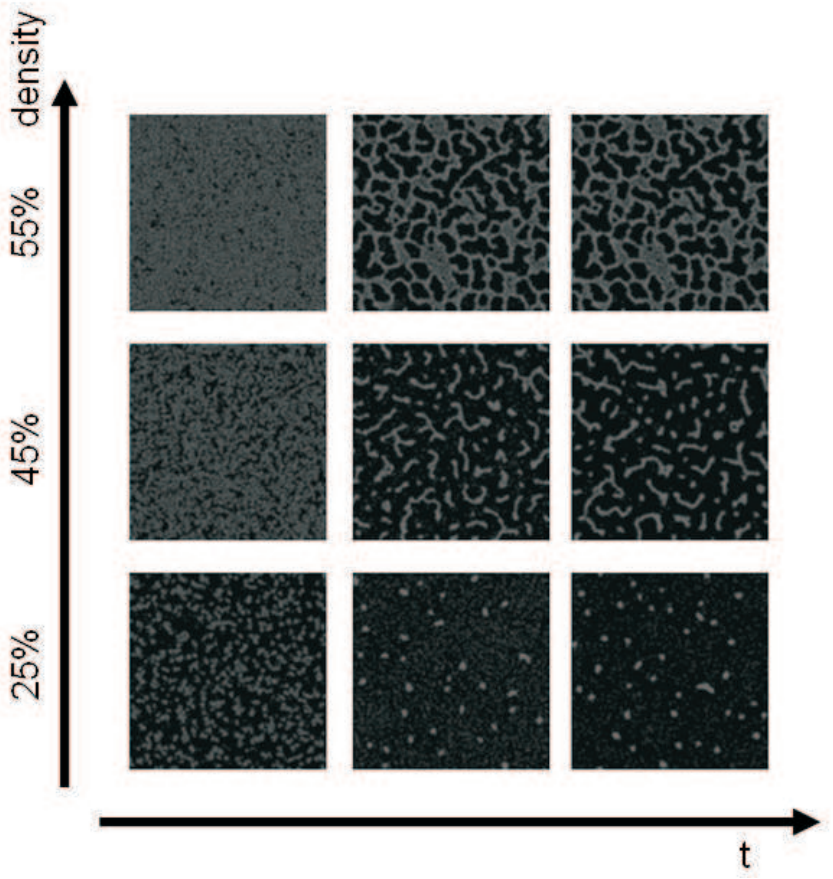


FIGURE 4. Simulation results with respect to different values of bacterial density.

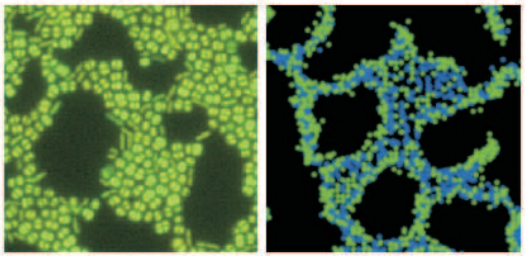


FIGURE 5. Adhesion of two species of bacteria. Comparison between experimental (left) and model (right) results.

with azote-poor cell suspension allow us to conclude that the phenomenon is due to adhesion and not bacterial growth.

To study the phenomenon, we introduced an agent-based model, where the agents qualitatively mimic the essential features of real bacteria to reproduce the

observed behavior: motion and aggregation. Model simulations matches the experimental results also in the presence of a threshold behavior (with respect to the minimum value of the density of bacteria on the surface needed to observe a self-organized structure). A further experiment, elicited by model simulations, was directed to investigate how bacteria of different species adhere to the surface. According to the prediction of the model, we observed that bacteria of one species placed on the borders of a structure mainly formed by the bacteria of the other species, which are supposed to adhere first. We retain that these conclusions can have important consequences on the formation of three-dimensional biofilms on the basis of an existing bidimensional structure.

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