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Biofilms in lab and nature: a molecular geneticist's voyage to microbial ecology

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Summary. This article reviews the latest findings on how extracellular signaling controls cell fate determination during the process of biofilm formation by *Bacillus subtilis* in the artificial setting of the laboratory. To complement molecular genetic approaches, surface-associated communities in settings as diverse as the pitcher plant *Sarracenia purpurea* and the human lung were investigated. The study of the pitcher plant revealed that the presence or absence of a mosquito larva in the pitcher plant controlled bacterial diversity in the ecosystem inside the pitcher plant. Through the analysis of the respiratory tract microbiota of humans suffering from cystic fibrosis (CF) a correlation between lung function and bacterial community diversity was found. Those that had lungs in good condition had also more diverse communities, whereas patients harboring *Pseudomonas aeruginosa*—the predominant CF pathogen—in their lungs had less diverse communities. Further studies focused on interspecies and intraspecies relationships at the molecular level in search for signaling molecules that would promote biofilm formation. Two molecules were found that induced biofilm formation in *B. subtilis*: nystatin—released by other species—and surfactin—released by *B. subtilis* itself. This is a role not previously known for two molecules that were known for other activities—nystatin as an antifungal and surfactin as a surfactant. In addition, surfactin was found to also trigger cannibalism under starvation. This could be a strategy to maintain the population because the cells destroyed serve as nutrients for the rest. The path that led the author to the study of microbial biofilms is also described. [**Int Microbiol** 2010; 13(1):1-7]

Keywords: *Bacillus subtilis* · *Pseudomonas aeruginosa* · *Sarracenia purpurea* · biofilms · nystatin · surfactin

Introduction

Populations of surface-associated bacteria are commonly referred to as biofilms [7]. In most natural settings bacteria are found predominantly in biofilms, yet for many years studies of bacterial physiology focused primarily on the planktonic state. The widespread recognition that biofilms impact myriad environments, from water pipes to indwelling devices in hospital patients, led to an increased interest in investigating the molecular mechanisms underlying the formation and maintenance of these communities. From the diversity of biofilm formation strategies thus far described emerges much knowledge that allows us to formulate a general hypothesis for this phenomenon: "Biofilm formation is a developmental process in which bacteria undergo a regulated lifestyle switch from a nomadic unicellular state to a sedentary multicellular state where subsequent growth results in structured communities and cellular differentiation." [10,15,22] In this article I present our latest findings on how extracellular signaling controls cell fate determination during the process of biofilm formation by *Bacillus subtilis* in the artificial setting of the laboratory. But what do such findings tell us about biofilms in natural settings? To comple-

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ment our molecular genetic approaches we have also investigated surface-associated communities in settings as diverse as the pitcher plant *Sarracenia purpurea* [19] and the human lung [5]. I discuss these results in the context of how one may begin to meld knowledge gleaned from both types of approaches to better understand molecular regulatory mechanisms underlying microbial ecology. But I begin with a brief description of the path that led me here.

Ce qui est vrai pour le colibacille est vrai pour l'éléphant

As far back as I can remember, I have had a lifelong fascination with genes and the molecular mechanisms that underlie their replication and expression. In my early training during the late 1960s and 1970s my guiding lights were Salvador Luria, Max Delbrück, Jacques Monod, François Jacob, Francis Crick, Sidney Brenner, et al. I fell in love with *Escherichia coli* and Monod's wonderful summation: *Ce qui est vrai pour le colibacille est vrai pour l'éléphant* (what is true for the 'colibacillus' is true for the elephant). I was and remain, totally taken by the universal features underlying all of life and how genetics, the analysis of mutants, can reveal so much to us. So early on, during my graduate training I focused my attention on how a DNA molecule can become two and I as went on to postdoctoral training this evolved to studying the processes regulating the growth of a cell [8,9]. Growth curves held a unique fascination, particularly that great unexplored region marked by the cessation of growth. We began dissecting this by obtaining mutants, called it all "life after log", people listened, and much was learned about the remarkable differences between growing and non-growing cells [21]. And then, we let our cultures of *E. coli* incubate a little bit too long, observed the onset of death and were inevitably drawn by the eternal philosophical question: "What is death?" And what we found through genetic analyses was rather extraordinary. Death allowed new life; we were witnessing evolution in real time [27]. Underlying the usually observed death phase was a dynamic world of dying and growing bacteria. There were constant population takeovers such that pre-existing fitter bacterial mutants grew as the original population met its demise. Evolutionary cheating we would call it later on [24], but first we thought of this as a mutant's expression of a "growth advantage in stationary phase", or GASP, and we knew the bacteria were GASPing for life in stationary phase [26]. At least some people listened and liked what they heard. Some even seemed to think such results might be of relevance to microbial ecology. In fact, in

1992 I was invited to speak at the 6th International Symposium of Microbial Ecology held in Barcelona and chaired by Ricardo Guerrero. Some other senior members of our discipline whose identity I do not know must also have thought well of our early results and probably wrote nice letters for I was awarded tenure a couple of years later.

The epiphany of the fish tank

The years that followed represented for me a dramatic turn of direction in my research. One might ascribe the change to some sort of "post-tenure depression"; I refer to it as the "epiphany of the fish tank" now. This is an anecdote that I have told several times in public presentations so it seems fitting to finally put it in writing here. After all, it may deserve some explanation since William (Bill) Costerton has alluded to it in his writings without entering into much detail [2]:

> In a recent newspaper article in Boston, Roberto Kolter recounted to a breathless reporter how he had discovered biofilms by watching a cloudy film develop on the front glass plate of his tropical aquarium. But several decades before Roberto had gotten depressed, and found much too much time on his hands, dentists had surveyed plaque in people's mouths and sanitary engineers had carefully followed the accretion of slimy films on surfaces exposed to wastewater. These intrepid pioneers had taken the plaque or the slime, placed it under simple microscopes, and found that they were completely composed of bacterial cells, separated by very large amounts of amorphous matrix material that dampened Brownian movement.

Microbial life on surfaces, for decades studied by Bill Costerton and other intrepid pioneers of the biofilm field, had been long ignored by most microbial physiologists and molecular geneticists, myself included. However, things changed for me in 1994 when, noticing my depressed state, members of my laboratory gave me a fish tank in a effort to draw me out of the blues. As I sat locked-up in the office staring at the tank, I realized that by studying shaken cultures of *E. coli* I had been barking up the wrong tree. The water in the fish tank remained crystal clear, it was on the surfaces where most microbial activity was occurring. I came out with a passion to understand life on the surface and, thanks in great part to George O'Toole, then a postdoc in the laboratory, we made some quick inroads. Our attention was now on the surface-adhered bacteria and no longer on the cells growing in suspension. The key for us was to be able to apply a genetic approach to study biofilms so we developed a high-throughput method to screen for mutants unable to form biofilms. In this way we contributed to a better understanding of the molecular processes underlying the formation of biofilms for a number of species, and many others used this method [16–18]. Alain
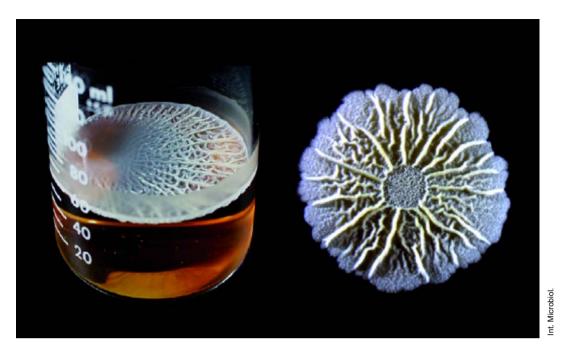
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Fig. 1. Highly structured biofilms formed by *Bacillus subtilis* strain NCIB 3610.

Filloux, now at Imperial College in London, has commented in his lectures that: "If O'Toole and Kolter had charged one dollar for every time someone has stained a microtiter dish for biofilms, they would never again have to write a grant proposal." (He referred to the methodology we described in [17]) Quickly, the field realized how idiosyncratic biofilm formation was for each strain, each environmental condition. The universality of biofilm formation was made manifest by a remarkable diversity of pathways. Yet, with all of the success, our initial attempts mostly helped dissect the first stages of development-initial surface attachment and colonization.

From molecular biology to cell biology

We began to make inroads into the later stages of biofilm development as a consequence of a remarkable collaboration. My good friend and colleague Rich Losick and I began to discuss working together on biofilms. Rich is widely known for his elegant and numerous studies on the molecular mechanism leading to the formation of dormant endospores by *Bacillus subtilis*, a non-growing but developing cell, very different from *E. coli*. He is a molecular geneticist extraordinaire that, like me, longed for doing some field work. So for a while we went on collecting samples of *B. subtilis* from many and distant locations on Earth. It was through this collection of samples that we began to realize that the biofilms made by the laboratory strains of *B. subtilis* were largely

unstructured when compared to those made by wild isolates. We blamed the lack of robustness of the laboratory strains on "domestication" and opted to work with a wild strain of *B. subtilis* known as NCIB 3610 which forms robust biofilms with complex architecture be their floating pellicles at the surface of standing liquid cultures or colonies on agar plates (Fig. 1). We were fortunate in this and in having Eduardo González and Steve Branda get the collaboration to a running start [1].

Bacillus subtilis makes spectacular looking biofilms in the form of floating pellicles in standing liquid culture and colonies on plates [3]. Most importantly for us, we could obtain mutants defective in the process and were soon able to characterize the genes involved in producing the extracellular matrix. This was so quick because we were working with one of the most studied and therefore best understood organisms of the planet. The formation of several distinct cell types, e.g., dormant spores, swimming cells and competent cells, is understood in great molecular detail for *B. subtilis*. This allowed us to quickly focus our inquiries on the spatial and temporal organization of different cell types in a growing biofilm. This is the work that Hera Vlamakis and Claudio Aguilar chose to pursue in their research [23]. They focused on when and where the three cell types could be found. In order to follow the cell types, they constructed fusions of cell-type-specific promoters to genes encoding different colors of fluorescent proteins. In this way they could follow cells that were expressing genes involved in motility, extra-
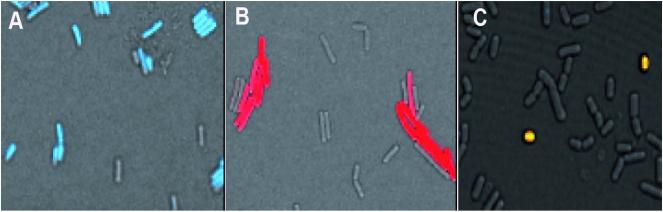
Fig. 2. Identification of individual cell types in a mixed population due to the fusion of cell-type-specific genes encoding different colors of fluorescent proteins: (**A**) motility; (**B**) matrix; (**C**) sporulation.

cellular matrix production and sporulation (Fig. 2). Their first key finding was that the different cell types formed dynamic populations in the developing biofilms and, importantly, these different cell types co-existed in the biofilm, very much as different cell types co-exist in multicellular organisms. By using flow cytometry, Vlamakis and Aguilar were able to follow the dynamic nature of cell type switching [23]. However, what was perhaps most striking was their ability to locate the different cell populations within the biofilm. When they froze biofilms made by strains harboring one or more cell-type specific reporters, and cut them in thin sections, they could observe where different cell types were localized (Fig. 3A). By doing these thin sectioning on strains harboring two different cell-type-specific reporters, the images obtained showed the striking spatial organization of the co-existing cell types (Fig. 3B). Note that swimmer cells are predominantly found in the bottom and edge of the biofilm, where they are probably exploiting their motility to search for nutrients where they are most available. Matrix producers are found throughout as they are needed to make matrix in the entire biofilm. Finally, sporulating cells are predominantly found on the top of the biofilm, in aerial projections reminiscent of fruiting bodies.

I would be a microbial ecologist

It is indeed wonderful what we can learn from the study of such a model biofilm by simply analyzing mutants and some molecular biology, biochemistry and cell biology. In the back of my mind, however, was the constant reminder that these colonies and pellicles were completely artificial biofilms seen in no natural setting on Earth. I had always wanted to study biofilms in natural settings to complement the pure cul-

ture analyses that I have just described. But it had always been clear to me that in order to do so I needed to ask very different questions, approach the subject differently, after a complete change in mindset—almost *tabula rasa*. I certainly was not going to make mutants in natural, multi-species biofilms. Where could I go for inspiration? For me, the inspiration to study biofilms in nature came from E.O. Wilson, who, in ending his autobiography, writes a paragraph familiar to many microbiologists [25]:

> If I could do it all over again, and relive my vision in the twenty-first century, I would be a microbial ecologist. Ten billion bacteria live in a gram of ordinary soil, a mere pinch held between thumb and forefinger. They represent thousands of species, almost none of which are known to science. Into that world I would go with the aid of modern microscopy and molecular analysis. I would cut my way through clonal forests sprawled across grains of sand, travel in an imagined submarine through drops of water proportionately the size of lakes, and track predators and prey in order to discover new life ways and alien food webs. All this, and I need venture no farther than ten paces outside my laboratory building. The jaguars, ants and the orchids would still occupy distant forests in all their splendor, but now they would be joined by an even stranger and vastly more complex living world virtually without end.

One does not need to wonder what led this remarkable naturalist and ecologist to feel that way. There is little doubt that he himself was inspired by those giants than transformed our world view over the last few decades. I am referring to Carl Woese and Norman Pace. They lay the foundation for the different world view that so attracts E.O. Wilson and so attracts all of us. I was motivated by Wilson's classic paper with MacArthur from 1963 wherein they set forth the theory of island biogeography [14]. But I was most inspired by Wilson's description of his efforts to study "new" islands in attempts to test many of the predictions his biogeography theory. I was thrilled reading his descriptions of spraying
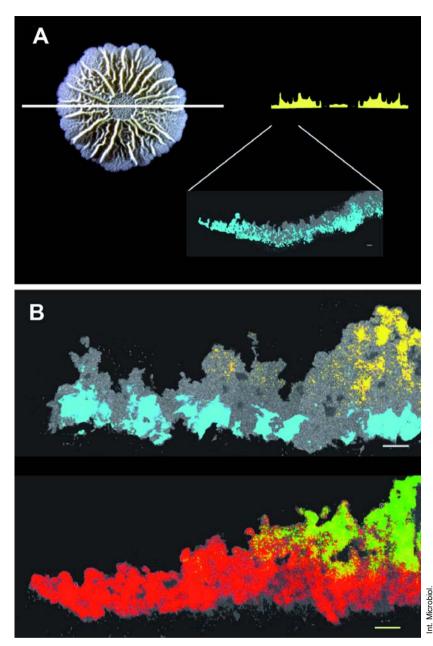
Fig. 3. Thin sectioning of a biofilm to localize cell types. (**A**) Graphic of thin sectioning. The white line across the biofilm represent the transect across which the colony was sliced. (**B**) Motile cells (blue) and sporulating cells (yellow) at the top panel; matrix producers (red) and sporulating cells (green) at the bottom panel. Bars represent 50 µm.

whole islands with pesticides and holding on to mangroves for dear life in the middle of a hurricane. So, I needed to find biofilms in islands, new islands. To some extent, I believe I have found two very interesting sets of biofilms in islands. These are two chapters of microbial island biogeography that may give us hints of new ways to approach the study of biofilms in natural settings.

Two microbial islands

The first chapter comes out of a wonderful collaboration with Anne Pringle at Harvard. Anne is a fungal ecologist and together we recruited Celeste Peterson, fresh out of Tom Silhavy's lab at Princeton and an *E. coli* geneticist herself, to tackle questions of natural biofilms. Celeste is truly wonderful and a brilliant scientist. I am way past any ability to display phenotypic plasticity... Truth is, as much as I would love it, I am not going to be able to become an ecologist. But Celeste became one, and a good one indeed. She very quickly became deeply steeped in ecology under Anne's guidance. This allowed us to hone in on our islands, the carnivorous pitcher plants *Sarracenia purpurea* (see photo on the center of the cove of this issue).

Long the subject of study by ecologists, *S. purpurea*'s biofilm communities had not been investigated thoroughly.

We initially tested the unopened pitchers and found them to harbor no bacteria inside. Thus, each time one of these pitchers opens up, it is the birth of a new island, and we can study its biogeography in one short season. Then you can understand why E.O. Wilson would want to be a microbial ecologist! The initial results of this collaboration can be summed up by stating that we found that the presence or absence of the keystone predator (a mosquito larva) in the pitcher plant controls bacterial diversity in this ecosystem [19].

The second chapter is the description of a very different microbial island and this is the work that Vanja Klepac-Ceraj and Katherine Lemon carried out in my laboratory. The island is the respiratory tract of humans with cystic fibrosis (CF). Taking advantage of a microbial microarray known as the PhyloChip, Vanja, Katherine and their collaborators analyzed the respiratory tract microbiota of 45 patients [6]. The diversity they found was remarkable. In this limited number of patients, they detected 46 phyla and 2083 taxa. Members from sixteen of these phyla were present in all patients. But it was in the subsequent ecological analyses that the key insights were obtained. There was a correlation between lung function and community diversity. Patients with the best lungs had the most diverse communities. More importantly, it appeared that community complexity greatly decreased upon the arrival of *Pseudomonas aeruginosa*, the predominant CF lung pathogen, whose presence correlates with loss of lung function.

Thus, we can begin to think of the microbial communities in CF lungs as being reduced in diversity due to the arrival of invasive species such as *P. aeruginosa.* The process has similarities to the arrival of rabbits in Australia. The key questions are intimately tied to the ecology of the habitat. What are the interactions among the species present in each of these ecosystems? For microbial ecosystems, it is my view that the interspecies interactions will be predominantly mediated by chemical signaling [20]. And to fully understand those interactions, I feel it is necessary to return to the more reductionist approaches of molecular biology.

A matter of molecules

So, to end I present a story of interspecies and intraspecies signaling that was discovered by Daniel López in my laboratory. He focused on *B. subtilis* biofilm formation and wondered whether there would be chemical signals produced by other species that could induce the synthesis of the extracellular matrix by *B. subtilis* under those conditions that otherwise do not favor biofilm formation. Indeed, he found that a number of small-molecule natural bacterial products induced

biofilm formation in *B. subtilis*. Surprisingly, one of these compounds was nystatin, the well-known antifungal. Another was a product from *B. subtilis* itself, surfactin. Even though these compounds are not related structurally, their physiological effect is the same, they cause the release of potassium from the cytoplasm. When all was said and done, the key common feature of all the molecules that induced biofilm formation was their ability to cause potassium leakage and this somehow—through a mechanism that remains mysterious—activated a membrane kinase, KinC [11]. Perhaps the most exciting aspect of these findings is that molecules well known for other properties—e.g., nystatin as an antifungal and surfactin as a surfactant—were shown to be able to act as signaling molecules. We hypothesized that the production of surfactin would be a great mechanism to induce biofilm formation. Surfactin does not induce biofilm formation acting as a surfactant but rather as a signaling molecule for quorum sensing. *Bacillus subtilis* might produce it under certain conditions that might regulate the expression of genes involved in biofilm formation.

The same subpopulation that produces the extracellular matrix that keeps cells together in biofilms can, under starvation, produce toxins to lyse a fraction of their sibling cells, which then serve as nutrients for the rest of the population. This sort of cannibalism is a means to delay sporulation and thus save the energy needed to produce the dormant cells. There is evidence that surfactin can trigger both the production of the extracellular matrix and cannibalism, and that the nutrients that the lysed cells release are mainly used to build the extracellular matrix [12].

Under other conditions, however, *B. subtilis* might not produce surfactin but sniff the presence of other microbes via their small molecule natural products. We can wonder whether this response is a form of defense or cooperation. One could argue for either, we simply do not know the answer yet. But the realization of this phenomenon has opened the door for innumerable such searches for signaling molecules. There is much exploring to be done by melding molecular microbiology and microbial ecology. It will be wonderful to continue our explorations.

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