BIOFILMS

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Methods for Enzymatic Release of Microorganisms

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DEDICATION

To my grandson, Patrick Brisou

PREFACE

This monograph presents a new technique for detecting microorganisms, more specifically bacteria, that populate all the levels of the biosphere. Since the majority of these microorganisms, constructors of biofilms and agglomerates, are attached to living or inert surfaces, they are not detected by the usual investigative methods. After gaining more knowledge of the mechanisms and stages of adherence of these microbes on the host sites, which include interfaces of all kinds, we attempted to "release" them from their habitats using enzymatic means that are as nontraumatic as possible. The reasons behind this process are presented in this book, which is broken down into three parts:

Part I, which is devoted to the mechanisms of adherence, includes two chapters. Chapter 1 is devoted to membrane structures, adherence stages, and attachment organs, known by the name of adhesins. A paragraph summarizes a few concepts on genetics concerning the biosynthesis of these anchorage elements. Chapter 2 exclusively concerns the biochemical aspects of this adherence. Presented in the form of helpful reminders, this chapter reviews a few notions of biochemistry, and it is intentionally limited to the essential knowledge needed for the comprehension and justification of techniques used for release and for the understanding of the life of germs in nature.

Part II directly picks up with microbial ecology with a glance at the bacterial population of tissues as well as land and aquatic microbiocenoses in general. Numerous documents illustrate the relations maintained between microorganisms and nature, the importance of colonizations, the construction of biofilms, and the formation of aggregates.

Part III is specifically devoted to technique. What we know now about the mechanisms of adherence justifies the choice of the techniques suggested for releases using wisely chosen enzymes which sometimes act on the host surfaces and sometimes on the adherence organs. The experimental findings recorded since 1979 by us and by a few others are summarized most often in tables followed by a few remarks.

The postface comments on these results and draws some prospects for applications that suit a maximum number of situations, such as applications in nature, medicine, environmental hygiene, the foodstuffs industry, food monitoring, etc. These methods are still poorly known, but they deserve, due to the results obtained, wider acknowledgment and further testing. The possible applications are infinite.

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I would also like to mention Professor Edouard Boureau, who was so kind as to present my initial results to the Academy of Sciences (Paris) and to whom I am deeply grateful. I can naturally not mention all my colleagues, friends, and assistants who participated in my "Bacteria Hunts" in a wide range of conditions over the 65 years of my scientific career. However, as far as canvassing extreme environments is concerned, I readily thank P. Niaussat, with whom I participated in the bacterial enumeration of the extraordinary Clipperton atoll in the Pacific Ocean, and M. Courtois with whom I worked at the most extreme hypersalted lake in the world, Lake Assal, located in the Affar-Issa territory.

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On the technical level, as far as the production of this monograph is concerned, I would like to express my warm thanks to Leslie Marcus who took care of the translation, and to my nephew Jean-Pierre Gallacier for his precious help with the documentation of this work, and to my son Jean Philippe Brisou and James Cossais who took care of the illustrations.

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INTRODUCTION

INTRODUCTION

Bacteria, the first acknowledged signs of cellular life, left the print of their walls in the sediments they lived in approximately 3,600,000,000 years ago. "Life was already beginning to play a role of utmost importance in the changes of the face of the earth," wrote the French paleontologist Marcellin Boule (1863–1942). Microorganisms — bacteria, viruses, yeasts, lower fungi, protists — do indeed attach themselves very rapidly onto inert or living media, forming aggregates of an extreme complexity. As a result, a large part of the attached microbial populations elude conventional investigation techniques. To offset this capital cause of error in reading the book *Nature* in 1979, I suggested releasing these microbes from their shelters, "flushing them out", through a nontraumatic enzymatic pathway, in order to make their culture and determination easier, either by acting directly on their attachment elements or by destroying their media.

Bacteria are the only microorganisms discussed here, based on my personal experience, but it is obvious that all the general concepts presented in this book are applicable to viruses and all the other microorganisms. The reality of this adherence dominates microbial ecology, calling for a complete review of out-dated concepts. Other than a few rare exceptions, there are practically no free bacteria in nature. Many years of experience and numerous observations have made it possible to suggest a new strategy for studying environmental microbiocenoses as well as animal and plant microbisms.

As early as the 18th century, it was quickly understood that the attachment, or immobilization, of unicellular beings was related to the surfaces, volumes, and porosity of materials. "Microbists" came to the still-valid conclusion that they were the same capillary forces that retain the organic substances and germs, i.e., the food matter and the microscopic beings that feed off it in the top layers of soils (Duclaux). This knowledge has been checked again and again, and in his microbiology treaty of 1898, Duclaux[1] insists on the close bonds uniting organic matter and microbial life. Although the long survival of microbes in the environment was already acknowledged then, the physiochemical, molecular, and genetic mechanisms of this survival remained, for the most part, unknown.

This survival depends on three requirements, applicable to all living beings:

- Protection (safety)
- Food (energy supply)
- Reproduction possibilities (growth)

In the past, adsorption was acknowledged as being practically the only important factor enabling the survival of microorganisms in nature. The substrates or "interfaces" on which "biofilms" are formed were studied extensively. Although the two components of the attached/attacher (or sensor) relation cannot be dissociated, explanations based purely on physics are no longer sufficient.

The concept of life and survival will not be considered here to answer the question "why", which would lead us to the thoughts of philosphers and finalists, but the question "how", which will keep us in the field of observation, experimentation, and description.

Microbiologists do not study beings in the linear sense of the term, but in populations of billions of cells that, in the optimal conditions of a given environment are divided, on the average, every 30 minutes! In conditions such as these, the researcher only records "snapshots" and finds himself or herself, like a specialist of quantum physics, in the field of probabilities. At the heart of very complex bacterial populations, constant, beneficial, or harmful exchanges take place, which were effectively analyzed by Alexander[2] in 1971.

Genetics pursues its task enabling transformations by DNA, conjugations, phagic conversions, and transductions; plasmids and episomes go from one cell to another, but at rhythms such that it would be careless to assimilate to what we observe *in vitro* with "tamed" bacterial strains. It may be assumed that the "free" microbial populations are located at a "high-risk" level. They are in danger unless they have particular forms of resistance, such as spores resistant to aggressions. Others can adapt to prolonged fast and low maintenance rations. Such very unequally distributed aptitudes play a role in ecological valency. Although certain bacteria are extremely fragile, others, on the contrary, resist extreme environments, the study of which is especially interesting (Gould and Corry, 1980).[3]

Bacteria can enter into lethargy, or sleep, and play "Sleeping Beauty". We devoted a certain number of studies (1960–1969)[4] to these filterable, dwarf forms that we consider as "hidden forms", since they elude the usual detection techniques. Thanks to certain devices, they recover their normal form, their antigenicity, and their experimental pathogenic power. Some authors would rather consider them as "debilitated" bacteria.

Over the past 25 years, the biochemical mechanisms of adherence have been specified. Some researchers, performing a genuine return to the past, evidenced considerable differences between the behavior of bacteria *in vitro* and in nature. In 1980, Roth[5] resumed studies concerning carbohydrates serving

as a protection to bacteria living in water. Sutherland[6] confirmed the universality of the anchorage fibers that were named "Glycocalyx". Their polysaccharide nature was acknowledged. Interest was then focused on other attachment organelles which will be examined in detail under the name of "adhesins". Costerton and his colleagues[7,8] widely contributed to the spread of this fundamental knowledge. Other authors will be mentioned throughout this monograph, the goal of which is to bring together the data related to the mechanisms of bacteria attachment on a wide range of media so as to gain practical knowledge from it. The reader will learn technical means enabling the implementation of a new hunting strategy for "wild bacteria", irrespective of the environment or organism considered.

"Enzymatic Release" is the very basic foundation of this strategy. It questions a great deal of data considered as reference "standards". Released bacteria do cultivate more quickly. They become more sensitive to means of defense, and their sensitivity to antibacterial substances is increased, sometimes by 50%. This technique enables the isolation of species undetectable by conventional methods. Bacterial numerations will always be inaccurate so qualitative determinations take priority, without excluding the others. Current technology implements methods based on the amplification, by polymerized chain reactions, of nucleotidic sequences, called "PCR". This technique is guaranteed deserved success since it enables the quick detection of a microorganism, bacteria, virus, or even parasite, which are all undesirable in food, a pathological product, or even in a specific environment. Epistemologically, it is another level of observation which cannot in any case exclude the isolation, accurate identification, or possible pathogenic power of the identified microorganism, and especially its behavior in the face of the usual antagonists.

Specialists acknowledge the advantages of these methods as well as their limits. However, they will not be debated here as their discussion would lead us too far away from our subject.

The bacterial cell, or prokaryote, is the smallest known living unit. In the protoplasmic mass lies a free, unique chromosome, constantly changing in shape, with which small genetic elements are associated and sometimes attached on this chromosome: they are episomes, sometimes free in the cytoplasm and known by the name of plasmids.

There is not a membrane separating the chromosome from the cytoplasm. This chromosome is itself attached to the cytoplasmic membrane by tubular or vesiculous invaginations — mesosomes — more developed in Gram-positive bacteria.

The cell is naturally rich in ribosomes, where proteins are synthesized. In *Escherichia coli* up to 18,000 are counted. These organella, composed of 2 subunits, sometimes form veritable strings named polyosomes. Rhapidosomes, a kind of small, extremely thin stick encountered in *Pseudomonas*, a certain number of enterobacteria and Photobacterium, etc., should also be mentioned. Their function is still rather poorly known. They are interpreted as being the

remains of bacteriophages. They are attributed with lytic virtues while assimilating them to bacteriocins.

Lastly, vacuoles, sometimes rich in lipids, and various granulations should be mentioned. The focus here will only be on what directly concerns adherence, its consequences, and what leads to enzymatic release. This entire cytoplasmic mass, with its contents, is wrapped in a thin, fragile, double-layered membrane. It is all protected by a thick wall that will be studied in depth in Part I, Chapter 2. All the organs of adherence known by the name of adhesins are implanted on this wall.