Formation of concentric rings in bacterial colonies

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Foreword

> "...at the heart of everything there is a question, not an answer. When we peer down into the deepest recesses of matter or at the farthest edge of the universe, we see, finally, our own puzzled faces looking back at us."
>
> John Horgan, *The end of science*

This is the story of a failure. A failure to solve a very simple puzzle. The question was not high physics, philosophy or molecular biology; it was -it is- rather plain, very down to earth, about something you can touch and see and smell so easily as the delicious fermented beans it produces: *Why the devil do these minute creatures called B. subtilis dance making rings?* This is all the puzzle. It began to bother me two and a half years ago. I searched -probably clumsily enough-, I failed in the quest. I didn't find it, the answer keeps eluding me.

So what is it going to be all this paper about? Well, the amazing thing is how very fruitful this failure has turned out to be. Not only mesmerizing, which it is for the sheer beauty of the colonies that *B. subtilis* creates. Not merely stimulating, which is something any worthy research is supposed to be. These colonies have kept questioning me with such a stubbornness that I have been compelled to open many windows in search of explanations. It's been like going hiking into wild regions where our goddess science has never set foot upon. No surprise we got lost! But it's been fun, and challenging. The experience to perceive or think differently is more important than the knowledge gained. *Je ne regrette rien.*

Then you get back into the traffic jam of polluted scientific motorways. I wonder how to explain the journey. And foresee the objections: *Almost no mathematical formulation!*- true. *Nothing of biochemistry!*- I agree. *Poor experimental data!* -yes I wasn't smart enough. *This is not physics!* -some say. *Nor is it biology!* -the others reply. *And you are still fool enough to have some disagreements with your own co-workers?* Well, I do not mean to be right. Sometimes you take a different perspective for the frivolous sake of watching some new questions sprout. *Oh, no, you shouldn't write this sham! Come on... not a single original idea!* - indeed.

This paper is a mere account, a dull description of the journey. Not much understanding has come out of it and I am sure you will find it disappointing. We shall agree on this. Then, why should I like the reader to join me in my troubles? I will say it again: because of the questions. I believe that this simple puzzle may touch some fundamental problems that are yet to be solved: morphogenesis, collective behaviour of living beings, the cycles of life. And it does it from an experimental perspective -not from the frenzy of computer models nor from mathematical towers of ivory. This is indeed a mess, but a real one. Life as it is: wonderfully sticky, smelly and ...ordered, chaotic? Never mind, just get a touch of it, dare to put your nose into the Petri dish!

Tokyo, March 1998.

Guidelines

In this Master Thesis I present a study on the formation of concentric ring pattterns in bacterial colonies. The thesis is organized as follows.

The introduction in **Chapter 1** seeks to explain the motivations of this research, while putting it in a general context of scientific endeveour. I argue that study of bacterial colonies is relevant to some fundamental questions of several disciplines of science since can give us insights into: (i) pattern formation, (ii) collective behaviour of living beings, (iii) morphogenesis as a dynamical process and (iv) the experimental foundations of the sciences of complexity.

Chapter 2 presents the morphological diversity of *Bacillus subtilis* colonies when varying environmental conditions. First I describe the bacterial species and its types of motility, then the experimental procedures, to proceed later to review previous works that had studied and eventually understood why under particular conditions particular patterns arise. The chapter ends with a comparison between patterns produced by different bacteria.

Chapter 3 displays the experimental characterization of the concentric ring patterns which constitutes the core of this research. To begin with, the growth dynamics is described. Then a study on the length of bacteria shows evidence of a cell differentiation process. A macroscopic characterization of the pattern follows -its main result is the period of the ring cycle is practically constant. Finally we report on two points inoculation and re-inoculation experiments.

Chapter 4 compares the ring pattern in *B. subtilis* with rings obtained in other systems: Liesegang rings, concentric rings in electrodeposition and those formed by the species *P. mirabilis*. Later it reviews and discusses models of bacterial colonies, from reaction-diffusion models based on nutrient depletion, to cellular automata that assume chemical communication between bacteria and new kinetic models based on age-syncrhonization.

Chapter 5 presents a summary on the main conclusions of the research, followed by a discussion on the soundness of motivations and assumptions pointed out in the introduction.

In the **Epilogue** I indulge myself in an essay that looks into the possible coherence between scientific studies on diversity (in a broad sense) and the threats that ecological, cultural diversity is facing in contemporary society.

Chapter 1

Introduction: the scope of this research

Why on earth should you, physicists, study bacterial colonies? I thought this was biology! - friends and strangers often tell me. When this happens I confess that I doubt for a few seconds, then move the hands as to begin a discourse, shut the mouth, let the hands drop, mumble unintelligibly, sip a little of wine -if possible-, and after all, I go humble and, well, I realize that I quite like the question.

Yes, I like the question for several reasons. First of all I like the question because it is indeed a challenge to try to understand and convey why you do this or that kind of research -my experience says that sadly enough many of us, scientists, are interested in little puzzles but do not have solid foundations on why we study them. Second, I like the question because it may seem naive but it responds to certain view of science that is quite common: science as cumulative endeavor that progresses by means of specialized, narrow and rigid disciplines. And I am glad to have the opportunity to debate against this. But above all, I like the question because it has beautiful answers.

Again: *Why do we physicists study bacterial colonies?*. In this introduction I shall argue that we study them because:

1. **Bacterial colony patterns are very similar to non-living system patterns**, such as lightnings or snowflakes, that physics is supposed to explain.

2. **Bacterial colonies are a collective phenomenon**, a sort of multicellular organism, and statistical physics has been very successful at explaining how collective phenomena, such as liquids, emerge out of the interactions of many elements, such as molecules.

3. As a loose multicellular organism with a given temporal and spatial structure (pattern formation) and different kind of cells (differentiation), **bacterial colonies can give us insight of how morphogenesis works**, *i.e*, how multicellular organisms are formed.

4. A *scientific revolution* in the form of a new way to grasp the principles of complex systems was claimed some ten years ago. However, it had -and has-

a very frail experimental basis. **Bacterial colonies offer a testing ground for the so-called sciences of complexity**.

Let me elaborate a bit on each of the four points.

1. Pattern formation

The aim of the study of pattern formation is to understand why objects and phenomena display particular shapes. It was found that in some given conditions of growth, bacterial colonies produce patterns that resemble extraordinarily to patterns of non-living systems such as snowflakes, lightnings, and many others [Vicsek92]. Then it turned out that the similarity in the patterns responded indeed to an intrinsic similarity in the mechanism that leads to the formation of bacterial colonies [Matsushita90]. In other words: sometimes, the growth of bacterial colonies is not very different to the growth of snowflakes [Ben-Jacob96]. In slightly different conditions, though, the growth of the colonies may also depend on some characteristics that non-living systems do not have, such as, let's say, reproduction or chemotaxis (chemical communication) [Budrene95].

From the perspective of pattern formation it has been interesting to realize that very simple growth mechanisms can apply to systems with many degrees of freedom (a bacterium has a bigger span of behaviors than a particle). But now it is also exceedingly interesting to see how from these extra degrees of freedom that bacteria possess, a larger diversity of patterns arises.

2. Collective behaviour of living beings

A number of microbiologists have recently been claiming that bacteria can not be understood taking only the the perspective of the individual bacterium, but it has to be viewed as a member of a community [Shapiro95b, Dworkin97]:

> "The strictly Cartesian, reductionist strategy that has characterized most of twentieth century science in general and microbiology in particular has been immensely successful in generating a detailed understanding of the workings of the cell, It has faltered, however, when confronted with the problem of producing integrated explanations of the workings of the multicellular organism or of the interactions within a multimember population. Apropos, we have been taught that the microbe is a unicellular organism, but we have not been sufficiently taught that the microbe is also, almost always, a member of a community of organism, both micro- and macro."
>
> Martin Dworkin, *Multiculturism versus the single microbe*

If we want to have a look at a community of a large number of elements that interactuate among themselves and with the environment, we know from the study of condensed matter that statistical physics can be an extremely useful tool. Just as in liquids, solids or gases only a few of the atomic characteristics of are relevant to the collective behavior, our working hypothesis will be that only a few relevant features of the bacterial cell will affect macroscopic colonies. However these relevant features interact in a non-additive way, what means that the bacterial colony can not be regarded as a mere sum of its elements. What arises out of a collectivity of bacteria is a new phenomenon in itself: the bacterial colony.

This kind of approach is not exclusive of bacteria. Collective behaviour of living beings can be observed in many natural systems: a collectivity of trees makes a forest; a collectivity of fishes makes a school of fishes; a collectivity of birds makes a flock. Since in nature bacteria, trees, fishes and and (some) birds are seldom found alone, a reallistic description of them needs to deal with the collectivity. And statistical physics may be good at this.

3. Morphogenesis: what DNA cannot explain

One of the "deep" questions of life that science has not solved is morphogenesis. It is not understood how from the fertilized egg a multicellular organism is formed: how and why several kind of cells appear (differentiation) and how these cells know where they have to place themselves, do some particular tasks and eventually die at a chosen moment.

After some decades of unprecedented success, molecular biology is being given nowadays the star role in the kingdom of biology. Darwin's theory of evolution has been re-written to pay absolute tribute to DNA [Dawkins95], and an enormous effort is being put in the sequencing of many living beings. In such a state of mind of the scientific community, one can come to devoutly believe that the processes involved in morphogenesis are somehow encoded in the DNA. But absolute believes look often funny from historical perspective:

> "It used to be believed that inside every human germ cell there is a little homonculus, a miniature human being complete in every detail, that simply grows in the womb into a human infant. And the same was assumed for all other species. It was thought that if you could see the miniature adult in sufficient detail, you would know what type of organism to expect from the egg of that particular species. The idea of genetic program in an egg that specifies all the details of the organism by the information it contains, is another version of this story. According to the program concept, everything we need to know to understand how an egg develops into an adult organism is written in its DNA. This is the old trap of assuming that whatever is created must result from the action of a creator whose word is written down in some way."
>
> Brian Goodwin, *How the leopard changed its spots.*

What Brian Goodwin and other biologists argue is that morphogenesis is ruled by physico-chemical processes whose nonlinear dynamics is robust. These processes still depend dramatically on genetic information and environmental conditions, but their rules are far from absolute. They propose that during morphogenesis, shapes develop and cells differentiate as the forming organism as a whole goes through a succession of attractors of the its own dynamical space [Kaneko94]. Thus, the simple (and not so simple) mechanisms of pattern formation found for non-living systems and bacterial colonies also play a major role in morphogenesis multicellular organism, from the (relatively) simple slime moulds, to flies and leopards [Devreotes89, Koch94, Nüsslein96]. This is relevant for the understanding of evolution, too, since complex organs -like the eye- can badly be explained by spontaneous mutations of DNA. Besides being favoured by natural selection, complex organs first appeared because they were possible attractors of developmental dynamics.

As bacterial colonies are ruled by these kind of processes, and at the same time susceptible of easy genetic manipulation, understanding their formation can constitute a first step towards the comprehension of morphogenesis.

4. An experimental playground for complexity

The discussion on complexity has been one of the pastimes of the decade. Some got enthralled by it, some got strangely angry, some got disappointed, some got plainly deceived... I guess most of us enjoyed it -I mean the pastime. However, complexity itself, whatever the poor word means, was badly abused, both for good and for bad [Waldrop92, Horgan95]. One of the main problems of complexity studies is that they are almost always based on computer simulations and have a very fragile experimental support.

As it happens, bacterial colonies seem to be a complex system. I hope we shall agree on this. We have little elements (bacteria) that are difficult to describe, (you need more than 4 millions DNA base pairs if you go the genetic way), but whose interactions with each other and with the environment give rise to highly diverse but limited, ordered macroscopic structures. Thus we can argue that they offer a suitable ground both for relatively simple, reproducible experiments and for the development of challenging theoretical models. This line has been followed, I would say with remarkable success by some groups [Ben-Jacob94b, Woodward95, Matsushita98b].

* * *

So, these are the reasons why we began studying bacteria as physicists, yes. But, to be honest, having experienced that bacteria do not mind being cultivated and studied by a physicist, I have come not to worry at all whether it makes sense being a physicist -and not a medical doctor- studying bacteria. The quest for knowledge goes far beyond these petty classifications. I believe that each of the four points mentioned (pattern formation, collective behavior of living beings, morphogenesis and complexity) has enough interest by itself as to appeal to any curious man or woman, independently of his/her background. And I shall later argue in the epilogue (pag. 85) that these topics reflect some social concern with diversity that goes with our historical period.

Bacteria as multicellular organisms

Bacteria began to be understood the last twenty years of the XIX century, by Robert Koch. Koch set the basis of modern bacteriology by isolating species of bacteria and creating **pure** cultures (cultures with only one kind of bacteria). This allowed to discrimate between the pathologies of different bacteria -which had most extraodinary benefits in medecine- and led to study them as unicellular organisms with some given properties, without caring on the interactions between them or with the environment (see [Dworkin97]).

It was not been until the 80's that the realisation by several groups that "bacterial colonies are highly organized, differentiated structures made it clear that assumptions about the single-cell nature of bacterial life needed to be reexamined" [Shapiro97]. Significantly, the multicellular behaviour of bacteria was undertaken in parallel by biologists, medical doctors and physicists. Shapiro studied how gene expression in Escherichia coli colonies displays unexpected symmetries [Shapiro97]. Belas, how the specices *Proteus mirabilis* formed ring patterns that are related to a periodic change in the bacterial cell morphology and physiology [Belas97]. Matsushita first and later Vicsek and Ben-Jacob, how environmental conditions determine the shape of the bacterial colony [Fujikawa89, Ben-Jacob92]. Budrene showed how spotted patterns are formed by aggregation of bacteria [Budrene91]. (See [Shapiro95b] for a review). Therefore, the field is new but it has already foundations.

Here a study of bacterial colonies on agar surface from the point of view of pattern formation will be presented. **Our aim was to explain why under certain conditions, the colonies grow periodically producing a pattern of concentric rings.** Mark that this the same as asking how periodic collective motion is organized, or as we shall see, why bacteria display different morphological and physiological characteristics at different stages of colony growth. The research has been a failure in the sense that the mechanism producing these periodicities has not been found or understood. We have, nevertheless, characterized experimentally the pattern, what will be of use to compare with models aimed at reproducing this morphology.

Chapter 2

Pattern formation in bacterial colonies: an overview

In this chapter we shall quickly overview the research on colonies of *Bacillus subtilis* (*B. subtilis* that has been developed from the point of view of Pattern Formation by Matsushita's group (for a thorough review see [Matsushita97]). Later, we shall compare these results with experiments carried by other group with the same and other bacteria.

2.1 A bacterium called *B. subtilis*

The research that will be presented has been mainly done on the wild strain OG-01 of the bacterium *B. subtilis* (rod-shaped, $\sim 0.7\ \mu m$ of diameter and $2 - 10\ \mu m$ of length), which has been extensively studied for more than 40 years and is considered to be an exemplar of Gram-positive bacteria[1]. Its relevance in research has been emphasized recently by the publication of its complete genome sequence by F. Kunst *et al.* [Kunst97].

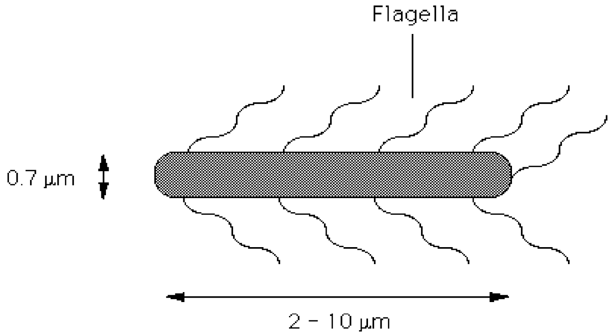

Figure 2.1: Schematic drawing a *B. subtilis* cell.

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[1] Bacteria can be either Gram-positive and Gram-negative depending on the composition of their cell wall. These main two groups display different characteristics.

A brief introduction to *B. subtilis*

B. subtilis is an aerobic, endospore-forming, rod-shaped bacterium commonly found in soil, water sources and in association with plants. B. subtilis and its close relatives are an important source of industrial enzymes (such as amylases and proteases), and much of the commercial interest in these bacteria arises from their capacity to secrete theses enzymes at gram per litre concentrations (...). B. subtilis (natto) is also used in the production of Natto, a traditional Japanese dish of fermented soya beans[a] *(...). Under conditions nutritional starvation, B. subtilis stops growing and initiates responses to restore growth by increasing metabolic diversity. These responses include the induction of motility and chemotaxis (...). If these responses fail to re-establish growth, the cells are induced to form chemically, irradiation- and desiccation-resistant endospores.*

From reference [Kunst97].

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[a] Delicious and very healthy, just try it!

To address the problem of colony formation we are a priori concerned on the reproduction, sporulation and motility of *B. subtilis.* The cell cycle of a bacterium mainly consists of DNA replication plus the process of cell division, called *septation.* Typically, the life span of a generation of *B. subtilis* is 0.43 hours [Neidhardt90]. Sporulation process is thought to take about 20 hours in the environmental conditions of our experiments.

The motility of *B. subtilis*, as of other many bacteria, is due to a set of flagella [Berg93, Neidhardt90]. When grown in liquid media, it is known that these flagella rotate around their axis clockwise and counterclockwise alternatively. During the counterclockwise rotation, flagella coordinate to produce a synchronous bundle that pushes the body steadily forward; the bacterium is said to *run.* For the clockwise rotation the bundle comes apart and the flagella turn independently, moving the body this way and that in a highly erratic manner, what results in a random re-orientation of the body; the cell is said to *tumble.* The alternation of these two modes results in the three-dimensional random walk executed by the cells. In the presence of chemical gradients bacteria can modulate the duration of the *run* mode; they will lengthen or shorten the duration of the *run* mode depending on whether they are swimming up or against the gradient. The random walk becomes thus a *biased random walk* and bacteria are capable of moving effectively towards or against chemicals. In general, this kind of movement of bacteria in liquid media is called *swimming.*

Unfortunately, for bacteria on solid and semi-solid media the movement is not understood yet to such extent. Translocation of bacteria on agar happens in a number of manners that do not yield themselves to easy explanation or classification. Therefore I will just try to describe our experimental observations on the wild strain of *B. subtilis.*

In fact, *B. subtilis*bacteria can not move on a thoroughly dry agar surface. So as to be able to move, they need a thin layer of liquid on top of the surface that they create themselves. It is also known that *B. subtilis* secretes a surfactant (a chemical aimed at reducing surface tension of water) that makes movement easier. A third characteristic is that, despite bacteria can move easily inside the colony, they face difficulties to go into regions of the surface that have not been colonized yet. This means that in most cases (except for very low concentrations of agar) the interface (the perimeter) of the colony is well-defined, *i.e.*, there is a clear line separating the inside and the outside.

We have observed two main kinds of surface translocation:

(i) Swimming: On semi-solid agar, each bacterium can move independently tumbling apparently at random on the surface. As this movement looks very similar to the one observed in liquid media we shall refer to it as *swimming*. Nevertheless, it should be pointed out that this kind of 2-dimensional swimming often differs from the simple random walk explained before. When agar is soft and cell density low, it was checked that inside the colony bacteria do indeed behave as brownian particles [Wakita94]. But when cell densities are not so low (as is the case for intermediate agar concentrations), temporal grouping of individual bacteria is often observed. This means that each bacterium still moves freely and randomly, but now it also *flows* within a group, as though bacteria were enhancing each other's motility. This observation is particularly clear in the interface between the inside and the outside of the colony, where the difficulty for bacteria to access non-colonized surface is often overcome by means of collective "pushing".

(ii) Gliding: On very hard solid agar, the wild strain of *B. subtilis* becomes immotile. Cells often reproduce without breaking the cell walls of the new daughter cells, what results in the formation of bundles or *spaghetti* of bacteria. Eventually the colony becomes so packed that surface translocation is simply produced by the swelling due to reproduction. Fagella do not play any role -experiments using mutants that had no flagella testified to this.

I would like to stress that this is a tentative and hardly objective description of our observations. Terminology used in literature for migration of bacteria on agar surfaces is often confusing.[2]

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[2] Some researchers call *swarming* to any kind of active, flagella-propulsed migration. I have followed the classification criterium that distinguishes between at least two kinds of active migration: (i) *swimming*, as described above, and (ii) *swarming*, a kind of migration typical for example of *P. mirabilis*. In the case of swarming, the movement is produced through the action of flagella, but now instead of a random back and forth, it follows swiftly the long axis of the cell. It is a highly cooperative motion. Thus defined, we can say that *B. subtilis* almost does not present *swarming*.

However, in some conditions (e.g., high cell density) *B. subtilis* colonies display remarkable degree of interaction between bacteria, some kind of *collective flow*. I really wonder how this regime should be called and characterized -I quite don't like referring to it plainly as *swimming* because it does not seem to be *only* a random walk. And pretending that is somehow similar to *swarming* may as well be too adventurous -since individual bacteria move erraticly. This *collective flow* of *random walkers* may constitute an interesting problem to be addressed from an statistical physics perspective.

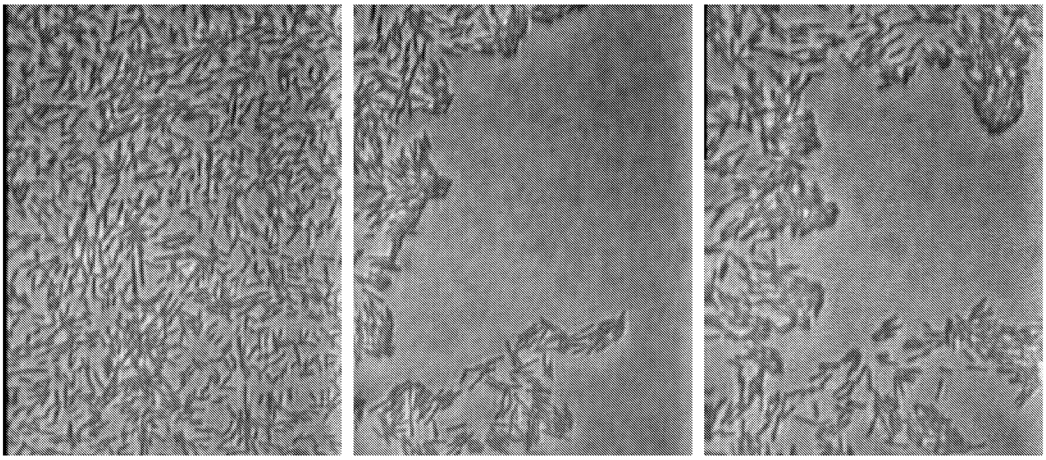

Figure 2.2: Swimming of *B. subtilis* on rich semi-solid agar ($C_a = 6\ g/l$, $C_n = 20\ g/l$). The width of a picture corresponds to $\sim 30\ \mu m$. (Left) Inside colony, performing a random walk. (Center) At the interface of the colony, forming temporal groupings to be able to overcome the difficulties to access new agar. (Right) The same interface 90 seconds later.

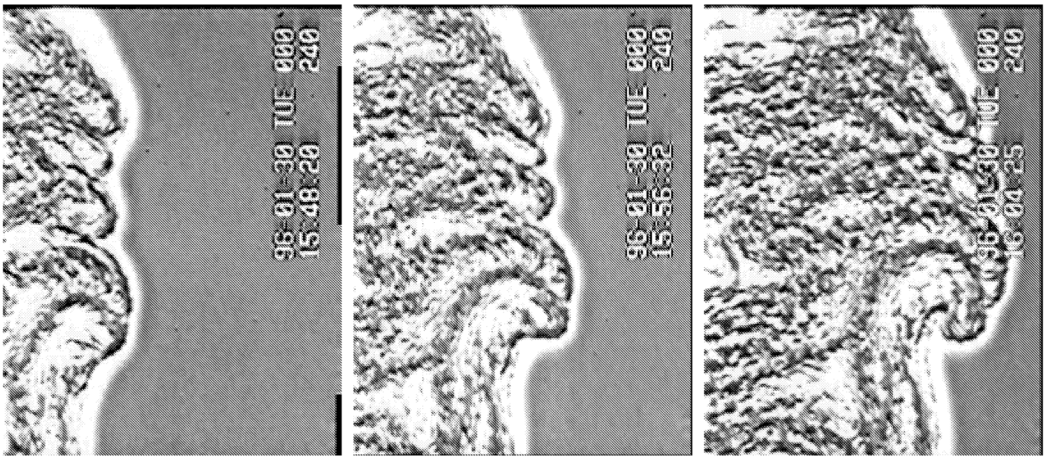

Figure 2.3: Gliding of *B. subtilis* on rich solid agar ($C_a = 10\ g/l$, $C_n = 10 g/l$). From left to right the front is shown at 8 minutes interval. The width of a picture corresponds to $\sim 0.3\ mm$.

2.2 Experimental procedures in bacterial culture

Bacillus subtilis colonies are grown in agar plates following simple specified procedures that are standard for bacterial cultures. A solution containing 5 g/l of sodium chloride ($NaCl$), 5 g/l of potassium phosphate dibasic (K_2HPO_4), and a specified amount of the nutrient Bacto-Peptone[3] is prepared using distilled water, and then adjusted to pH 7.1 by adding some drops of hydrochloric acid (HCl). The solution is then mixed with a specified amount of Bacto-Agar.[4] The mixture is autoclaved at $121°C$ for 15 minutes and then 20 ml of the solution are poured into a plastic Petri dish of 88 mm of inner diameter. After solidification at room temperature for 60 minutes, the agar plates are put to dry at $50°C$ for 90 minutes. The thickness of the agar plate thus prepared is about 3 mm.

On the other hand, a suspension of bacteria on a minimal media solution (H_2O, 20 g/l of NH_4Cl, 40 g/l of KH_2PO_4 and 48 g/l of Na_2HPO_4) is prepared, its optical density (OD) adjusted to 0.5. This was found to correspond to a bacterial density of around $10^7 cells/ml$.[5] After a drop of 3 ml of the bacterial suspension is inoculated in the center of the agar plate, the plates are placed and kept inside an incubator at $35°C$ and 90% of humidity for a specified period of time until the colony grows (see figure 2.4).

To obtain reproducible patterns the protocol has to be respected precisely, even for parameters -such as thickness of plates- that are irrelevant when doing standard analysis of bacterial cultures. In our experiments **the only parameters that are systematically changed are agar and nutrient concentrations** of the plate. Their variation causes dramatic effects in the development of patterns. Whenever anything else is varied it will be specified.

In the case of *B. subtilis* and in particular for regions C and E, the experiment didn't yield to quantitative reproducibility whenever the following parameters were changed:

Humidity of incubator. *B. subtilis* motility is very sensitive to the amount of water on the surface of agar. Environmental humidity plays an important role in regulating it [Mendelson96].

Drying time of agar plates. Agar plates are semi-solid media composed mainly of water. This water evaporates rather quickly what causes the motility of bacteria to decrease.

Thickness of agar plate. Given the ratio 3 $mm/88$ mm between thickness and diameter of agar plates, one can consider them quasi-two-dimensional to most effects. In practice, though, there seem to be some tricks hidden in the system and the patterns change with depth. Two might be the reasons: (i) Thinner

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[3] Peptone is a *ready cooked* powder containing sugars, aminoacids and vitamines and produced by Difco Laboratories, Detroit, MI, USA.

[4] Agar is a crude commercial product made from marina algae that contains many kinds of unidentified substances. It seems that even properties depend on the manufacturer [Matsuyama95]

[5] Put it rigorously we should say that we measured 8×10^6 colony forming unities (CFU) for each ml of 0.5 OD suspension.

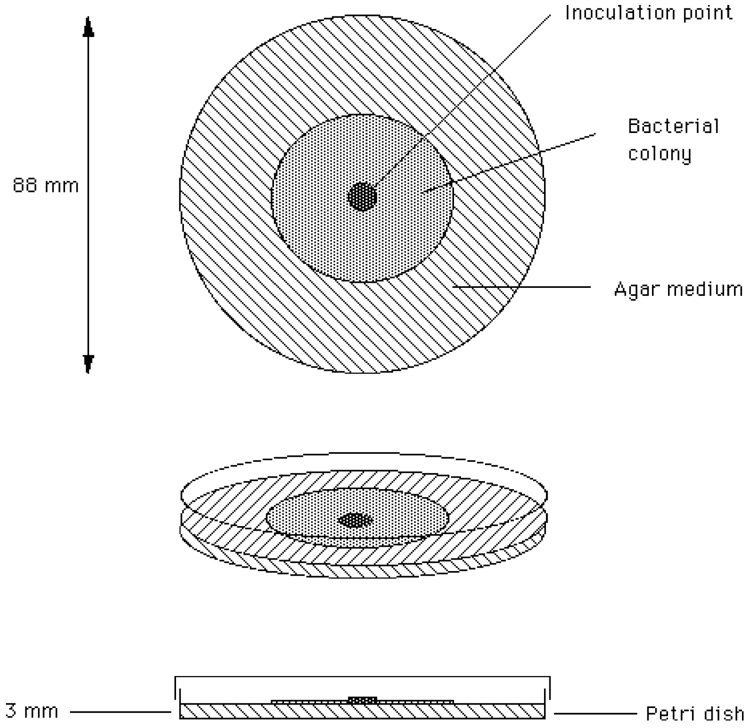

Figure 2.4: Schematic drawing of an agar plate, an inoculum and an expanding bacterial colony.

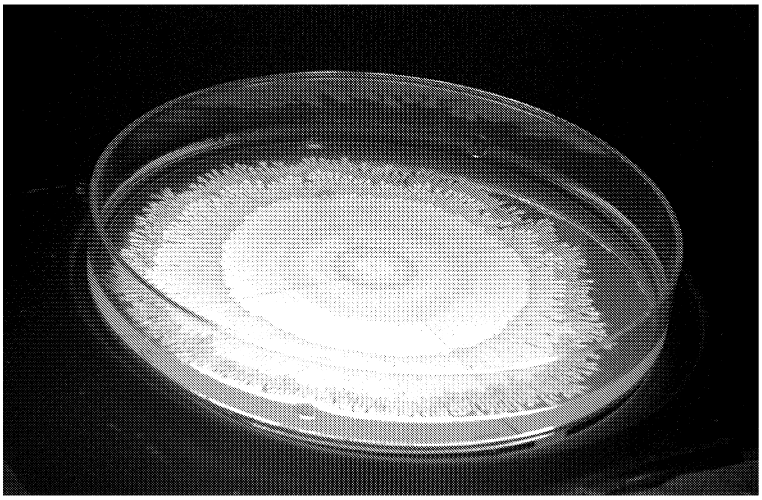

Figure 2.5: Agar plate with an expanding bacterial colony.

plates suffer the same surface evaporation but have a lower contents of water, what means that they dry faster. Thus bacteria are less motile in thinner plates. (ii) Dilution of waste or chemotactic chemicals secreted by bacteria (no experimental evidence). One has also to be careful that the Petri dishes are not even slightly tilted during solidification -so that depth is uniform.

Age of bacterial suspension. Taking profit of *B. subtilis* capability to sporulate, the best method is to prepare a suspension from spores. Spores can survive long time in minimal liquid media. This allows to use the same suspension for at least one month without change in colony development. Contrarily, it was found that very fresh (hours old) suspensions with *B. subtilis* in non-spore state formed less reproducible patterns.[6]

Observation and measurement of the macroscopic characteristics of patterns are performed taking photographs of the agar plates or recording the whole growth with a video camera. In this case, the agar plate is placed inside a transparent incubator and the images are recorded from outside by means of a CCD camera and a time-lapse video system. Microscopic observations are performed by means of an inverted microscope and recorded on video tapes using a CCD camera and a time-lapse video system.

2.3 Morphological diversity in *B. subtilis* colonies

Figure 2.6 displays the patterns produced by colonies of *B. subtilis* when grown on agar surfaces for different environmental conditions. The parameters that are changed are agar concentration (C_a) and nutrient concentration (C_n). The amount of agar determines the hardness of the plate. The more agar there is, the harder the plate becomes and the more difficult it is for bacteria to move on it. In figure 2.6 the inverse of agar concentration ($1/C_a$) is plot on the x-axis, solid, hard media corresponding to regions on the left, while softer semi-solid media correspond to regions on the right. The other parameter that is changed, nutrient concentration, is plot on the y-axis using a logarithmic form. Media on the top of the diagram are nutritiously rich while media on the bottom are poor.

Changing only these two parameters, C_a and C_n, a wealth of quasi 2-dimensional patterns is found. The patterns are reproducible, *i.e.*, given C_a and C_n colonies always grow with the same morphology, and a mere visual -naive- inspection allows to classify them into some main morphologies. If each morphology is observed over a range of growth conditions, we can define a *morphological diagram* (fig. 2.6) analogous to the *phase diagram* that is common in equilibrium thermodynamics (as, let's say, the one characterizing the solid, liquid and gas states of matter). This kind of description in terms of morphological diagram is standard in pattern formation of non-living systems [Vicsek92]. As a matter of fact, some of the morphologies

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[6] This result contrasts with of the report by Rauprich *et al.* [Rauprich96] that *P. mirabilis* colonies grown of older inocula a had less coherent initial growth than those with fresher inocula. The difference of behavior might simply be due to the fact that *P. mirabilis* can not produce spores.

displayed by *B. subtilis* colonies resemble amazingly to patterns obtained in non-living systems.

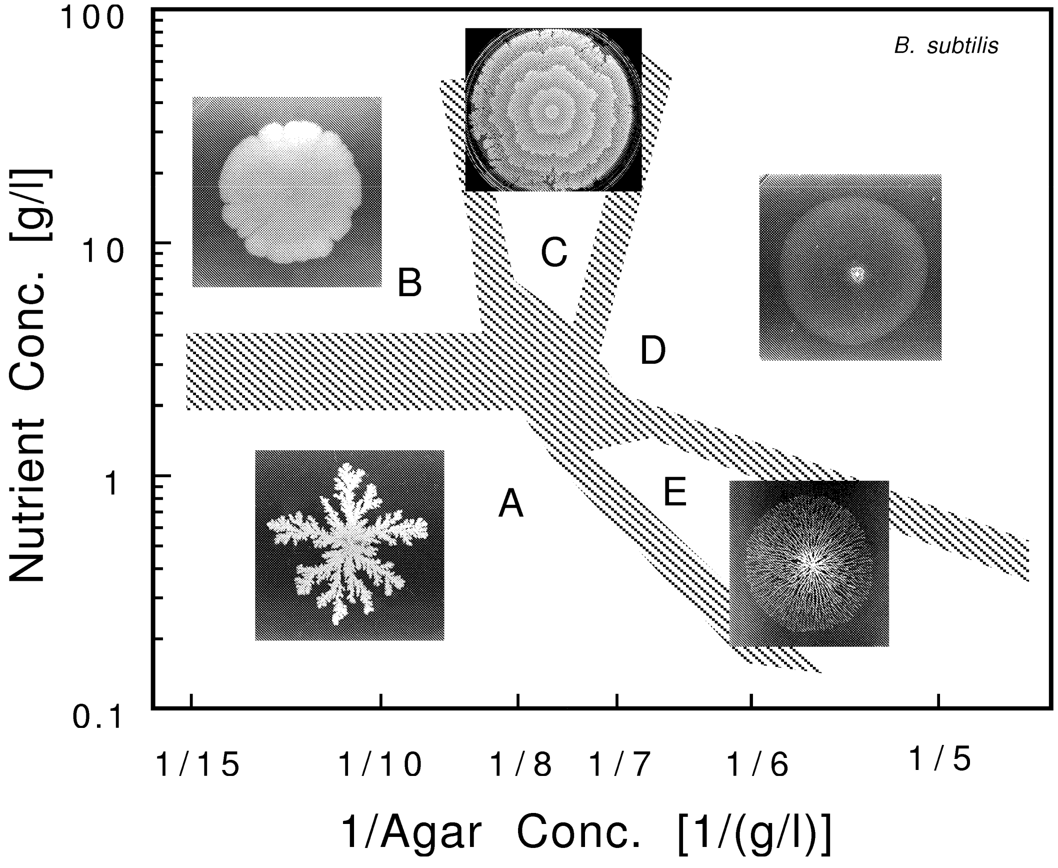

Figure 2.6: Morphological diagram of *B. subtilis* colony patterns obtained when varying hardness of agar surface and nutrient concentration. X-axis represents the inverse of agar concentrations. Left side corresponds to hard solid media and right side to soft semi-solid media. Y-axis represents the logarithm of nutrient concentration, the bottom of the diagram corresponding to poor media and the top to rich media. Colony patterns are classified into five main morphologies: DLA-like colony (region A), dense disk with rough interface (region B), concentric ring pattern (region C), homogeneous fast spreading disk (region D) and dense branching morphology (DBM) (region E). Striped areas represent transitional, not well-defined morphologies.

Region	Agar Plate	Nutrients	Time	Motility	Main Characteristics	Main Mechanism
A	Solid	Poor	20-30 days	Gliding	Self-similar branching	Diffusion-Limited Growth
B	Solid	Rich	5-7 days	Gliding	Dense rough disk	Reaction-Limited Growth
C	Semi-solid	Rich	2 days	Swimming	Periodic growth, rings	Unknown
D	Semi-solid	Rich	1 day	Swimming	Homogeneous disk	Active Bacterial Diffusion
E	Semi-solid	Poor	2 days	Swimming	Dense Branching	Unknown

Table 2.1: Classification of morphologies found in *B. subtilis* colonies

The morphology selection principle

The existence of a morphology diagram implies the existence of a morphology selection principle and vice versa. (...) In general, if more than one morphology is a possible solution, only the fastest-growing morphology is non-linearly stable and will be observed, that is, selected. [This] selection principle implies that the average velocity is an appropriate response function for describing the growth processes and hence should be correlated with the geometrical character of the growth. In other words, for each regime (essential shape) in the morphology diagram, there is a characteristic functional dependence of the velocity of the growth parameters. At the boundaries between the regimes there is either discontinuity in the velocity (first-order-like transition) or in its slope (second-order-like transition).

From reference [Ben-Jacob97].

The morphological diagram of *B. subtilis* is classified into five regions, from A to E, as displayed in figure 2.6 and table 2.1. A main division in the morphological diagram can be done separating the regions where bacteria are immotile and the colonies grow by means of *gliding* (regions A and B), and the regions where bacteria can move individually and the colony expands by means of swimming (regions C, D and E). In the following subsections we shall describe briefly each morphology and its growth dynamics.

2.3.1 Region A: DLA-like pattern

Figure 2.7 shows a pattern obtained in region A, for high agar and low nutrient concentrations. The colony needs around 30 days to expand all over the agar plates. It makes a very ramified structure that turns out to be fractal, with a fractal dimension $D \sim 1.71$. The explanation to such a fascinating pattern is amazingly simple. As said before, agar plates in region A have a hard surface due to the high agar concentration. As the agar is very hard bacteria cannot move actively, *i.e.*, they can only glide, and have to "wait" for the nutrients to diffuse through the plate and reach the colony. Being nutrient concentration very low, the characteristic time of nutrients diffusion τ_{nut} is much longer than the time τ_{bac} required for bacteria to septate (reproduce) and expand the colony. In such a case it is always the slowest process the one that governs the pattern growth. Because bacteria cannot reproduce without nutrients, and without reproduction there is no expansion of the colony, the colony growth is limited by the diffusion of the nutrients. This can be put mathematically in an extremely elegant form as a Laplace equation for nutrient concentration $n = n(\vec{r})$: $\nabla^2 n = 0$ (see section 4.2).

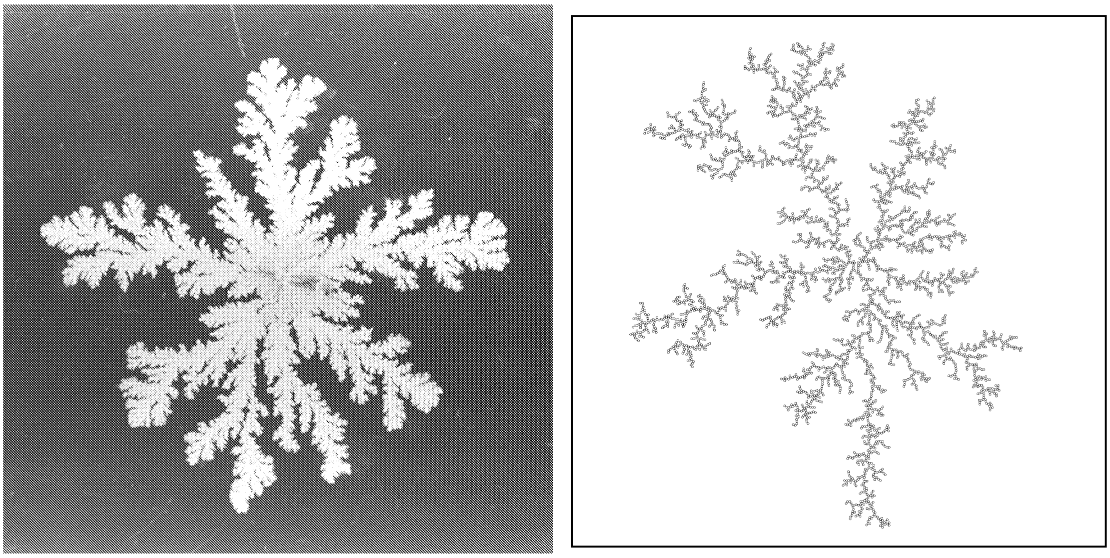

Figure 2.7: (Left) Example of DLA-like colonies of *B. subtilis* ($C_n = 10\ g/l$, $C_n = 1g/l$). The colonies need 1 month to grow up to this size ($\sim 5\ cm$ of diameter). (Right) Example of a two-dimensional DLA cluster, obtained by a computer simulation off-lattice with 4000 particles. Note the similarity between the two patterns. Courtesy of O. Moriyama.

The understanding of diffusion-limited growth was one of the main success of the whole field of (non-living) pattern formation in the 1980's. Many systems happen to show this kind of patterns: electrodeposition (deposits of metals leaves in a chemical reaction driven by an electric potential, [Matsushita84]), viscous fingering (fingers that appear when blowing a viscous liquid or gas on another liquid or gas kept between two layers of glass, [Ben-Jacob90]), crystal growth and dielectric breakdown among others (see [Vicsek92] for details and more examples). This is because the growth of all these patterns is governed by a Laplace equation. Those involving aggregation can be easily and intuitively understood by means of a computer model called *Diffusion-Limited Aggregation*, nowadays universally known as DLA.

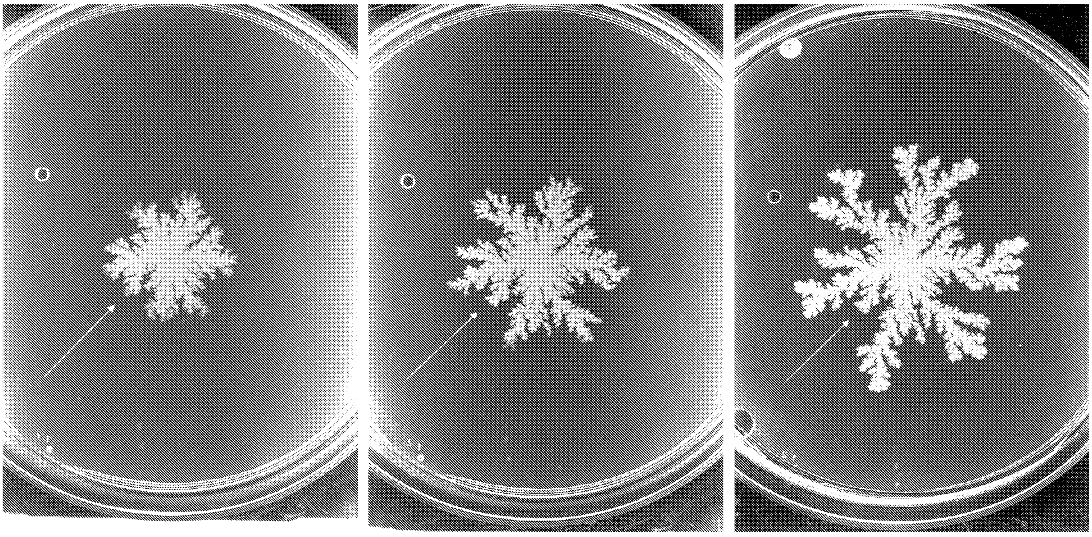

Figure 2.8: DLA-like pattern at different stages of growth: 7 days after inoculation (left), 12 days (middle), 31 days (right). $C_a = 10\ g/l$, $C_n = 1\ g/l$. The so-called *screening effect* can be observed in the branch pointed by the arrow. Although this branch could develop in the beginning, when other neighbouring branches became longer, the branch stopped completely its growth.

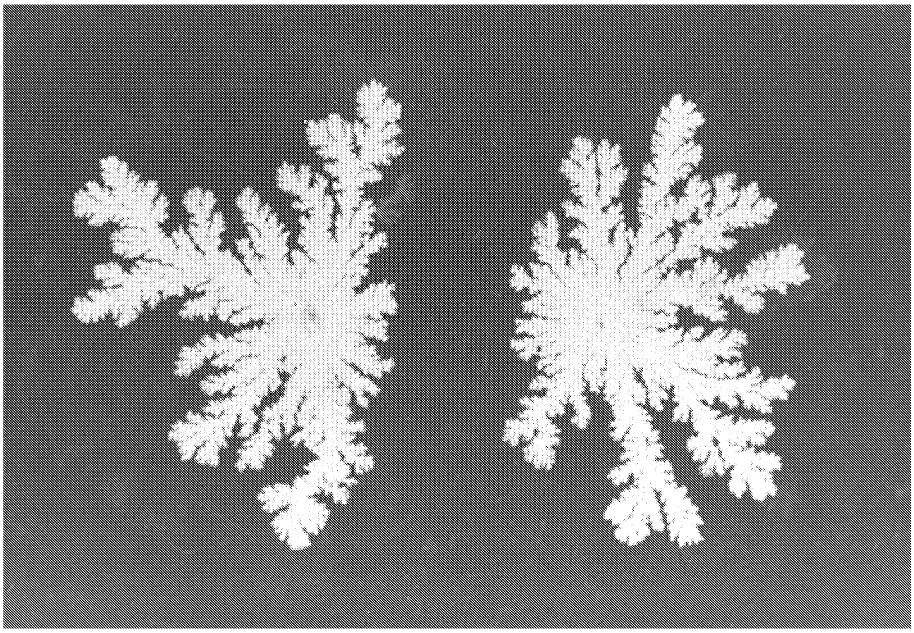

Figure 2.9: Two neighbouring colonies inoculated simultaneously at the same agar plate. $C_a = 10\ g/l$, $C_n = 1\ g/l$. The pattern resembles those of electric repulsion. In both phenomena Laplace equation governs the dynamics.

Diffusion-Limited Aggregation, DLA

This model is a simple one, and the growth rule is as follows: Initially, a seed particle is placed at the origin. A brownian particle of the same size is then released far away from the origin. When the brownian particle reaches the seed particle, it sticks to the seed, forming a two-particle cluster. Another brownian particle is again launched randomly but far away from the origin. When it collides with the cluster, it stops there and becomes a a member of a now three-particle cluster. (...) This aggregation process of brownian particles, one at a time, is repeated for as long as time and money allow, ultimately creating a large grouping called a DLA cluster.

From reference [Matsushita97].

DLA explains why the colonies in region A develop branches: if one part of the colony grows randomly a little bit outwards, a brownian particle that *walks* through the system has a higher probability to stick there and a lower probability to stick to the parts that don't stand out. This little difference of probabilities enhances the growth of the perturbation: the parts that stand out tend to grow more and more and those not standing out tend to grow less and less until they stop completely. This produces first the branching and then competition between branches characteristic of DLA-like patterns (see figure 2.8) that is known as the *screening effect.* It can as well be observed experimentally in the repulsion between colonies grown on the same agar plate (Figure 2.9). These and other observations [Matsushita90], have confirmed that in region A the growth of the colony is indeed limited by the diffusion of nutrients.

2.3.2 Region B: Dense disk with rough interface

As nutrient concentration is increased while keeping agar concentration at high levels, the branches of the the colony grow thicker and thicker, until they eventually fuse and form the dense disk that is characteristic of region B (figure 2.10). Growth is much faster than in region A; the colony spreads all over the Petri dish in 5-7 days. However, observation in the microscope reveals that bacteria can not move actively or *swim* -the agar medium is too solid. As in region A, the expansion is due to the *gliding* of bundles of bacteria on the surface and this *gliding* is caused by the pressure that builds up inside the colony as bacteria reproduce (see figure 2.3). The colony has a roughly round shape that keeps swelling slowly, at constant speed.

If in region A the growth is governed by nutrient diffusion, in region B the growth is limited by the time of reproduction of bacteria. In this sense we say that the system is *reaction* limited -understanding reproduction as the *reaction* of bacteria to the intake of nutrients.[7] Indeed, now there are so many nutrients that the time τ_{nut} that a bacterium has to wait to “receive” nutrients is much shorter

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[7] As everybody can guess the terms of diffusion-limited and reaction-limited growth are borrowed from pattern formation of non-living systems.

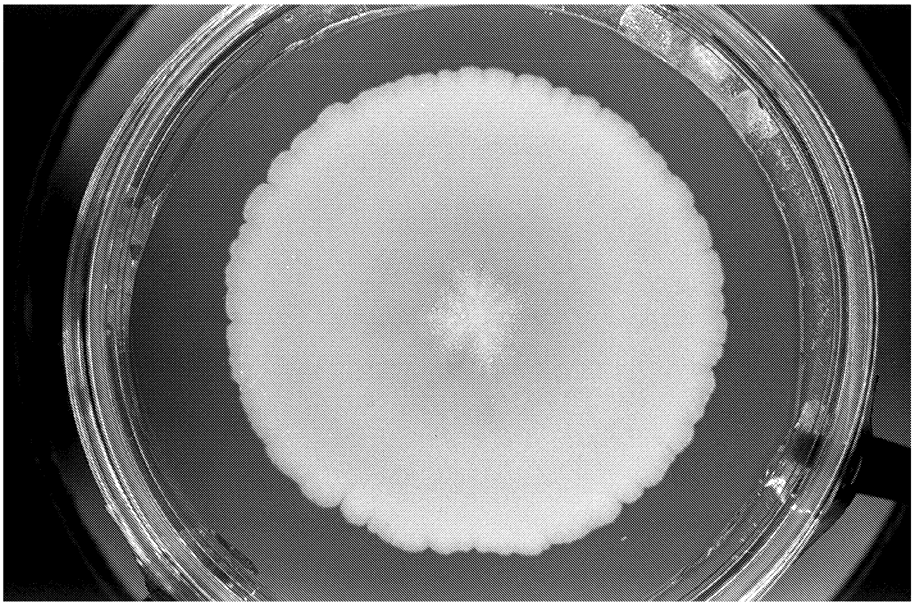

Figure 2.10: Dense disk morphology, obtained in region B after 7 days of incubation. $C_a = 8.5\ g/l$, $C_n = 20\ g/l$. The interface of the colony is rough both at the macroscopic and microscopic level.

that the time τ_{bac} that it actually needs to reproduce itself into two new bacteria ($\tau_{nut} \ll \tau_{bac}$). Again, the slowest process is the one that governs the growth.

The crossover from patterns in regions A to patterns in region B can also be explained as the competition these two times, τ_{nut} and τ_{bac}. Let's see it in detail. As we have explained before, for very low nutrient concentrations DLA-like colonies are formed. Gradients of nutrients extend over long distances and this results in patterns having branches of all sizes (the power-law distribution characteristic of fractals). When some more nutrients are added, gradients become steeper, diffusion is faster and for short length scales ($l \sim l_0$), time of nutrient diffusion becomes smaller that of bacterial reproduction ($\tau_{nut} < \tau_{bac}$). This means that for the smallest scales ($l < l_0$), there is no *screening effect*, *i.e.*, no branching, and the colony grows more or less homogeneously. As we add more and more nutrients this lower cutoff of branch size l_0 becomes bigger as can be seen in the fattening of the branches displayed in figure 2.11, until eventually the patterns become completely round. Such a symmetric pattern is easy to understand. A priori, a round pattern is what you would expect when the probability to grow is the same in all directions.

In the microscopic level another interesting phenomenon is observed. Bacteria form bundles that when *gliding* on agar create a very rough interface. It has been found that this interface is self-affine within the range $1 - 100\ \mu m$ with a roughness exponent $\alpha \simeq 1.78$ [Wakita97]. This is telling us that there are long range correlations.

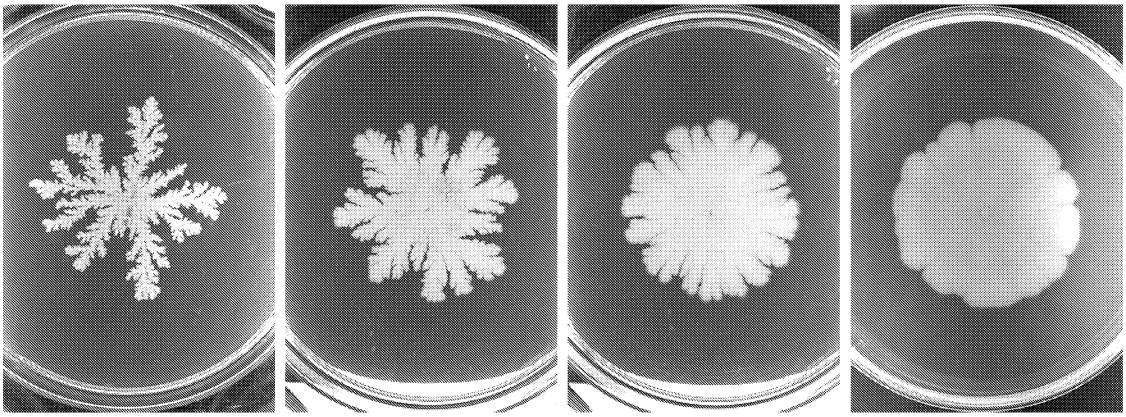

Figure 2.11: Morphological crossover from DLA-like (region A) to dense disk colonies (region B) at increasing nutrient concentrations. All the patterns showed have an agar concentration around C_n~10 g/l, while the nutrient concentration is increased from left to right from $C_n = 1$ g/l to 3 g/l, then 4.5 g/l and finally 8 g/l. The minimum width of the branches increases progressively as more nutrients are added to the agar plate until the pattern becomes completely round. (C_n from left to right: 10 g/l, 8 g/l, 9 g/l and 11 g/l.)

2.3.3 Region D: Homogeneous fast spreading disk

Now let's move to semi-solid, nutritiously rich media (low agar, high nutrient concentrations). A disk of homogeneous low cell density is formed a few hours after inoculation. The colony spreads rapidly all over the agar plate, covering it all in less than 8 hours (see figure 2.12). It looks like a (2-D) balloon that inflates steadily. The envelope of the pattern is nicely rounded and cell density is low (just one layer and not fully packed) and very homogeneous.

Since the surface of agar is soft, in region D bacteria can move individually, dancing back and forth on the agar, *i.e.*, *swimming* as explained in section 2.1 and shown in figure 2.2. For not so high nutrient concentration ($C_n < 10$ g/l), cell density is lower and corresponds exactly to a random walk [Wakita94]. For higher nutrient concentrations, though, cell density increases (still within the same layer) and the length of bacteria becomes a bit longer. Under these conditions, formation of groups and collective motion of bacteria is observed to some degree.

As in region B, the nutrient concentration is so high that nutrient diffusion does not play a relevant role. As in that case, too, it is the *reaction* (response) of bacteria to the environment what governs the kind of growth and determines the pattern. The difference is that now the response of bacteria is not only reproduction, but also includes active movement. The dynamics can be grossly explained as a random walk of bacteria, but while a random walk of particles spreads spatially as the square root of time ($x \sim t^{1/2}$), now the reproduction of bacteria makes possible that the expansion be much faster (experimentally one finds $x \sim t$ or even faster rates of expansion). This can be written down mathematically (or computationally) into naive but successful models that include bacteria diffusion, their reproduction plus

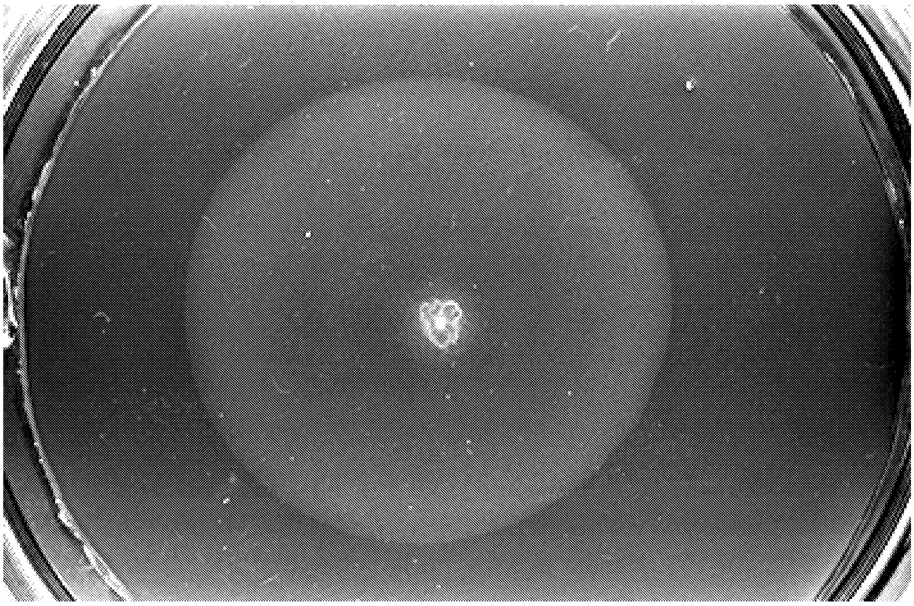

Figure 2.12: Colony growing as a homogeneous fast spreading disk (region D), 13 hours after inoculation. $C_a = 6\ g/l$, $C_n = 20\ g/l$. The colony can be hardly seen because it is made of only one layer of sparsely distributed bacteria.

a term that accounts for the limited space and nutrients that they can use, in what is known as a *population dynamics* approach [Murray89]. We shall discuss this in detail in section 4.2.

2.3.4 Region E: Dense Branching Morphology

For very low nutrient concentrations and intermediate agar concentrations, the colony develops in densely branched patterns reminiscent of the so-called *dense branching morphology* (DBM) found in non-living patterns [Ben-Jacob90, Vicsek92] (see Figure 2.13). These branches, unlike those in region A display a characteristic width and characteristic gap between them. The ration branch width/gap has been found to be constant, equal to unity over the whole region. Branch length distribution is exponential, which means that tip splitting is random. Growth is quite fast, it covers the Petri Dish in less than 24 hours, and the velocity depends linearly on nutrient concentration. Microscopic observation reveals that bacteria can move individually (swimming), but are only active in the very tip of each branch producing a dynamic grouping that resembles a finger-nail (Figure 2.13). Bacteria behind this *finger-nail* present much less activity.

The mechanism generating this pattern has not been understood yet. In spite of this, the fact the for non-living phenomena such as viscous fingerings, electric breakdown or crystal growth, the transition from open fractal-like patterns to dense branching morphologies is also observed, hints that the pattern can be explained with simple physical principles. It seems clear that the dynamics is related to the depletion of nutrients, but unlike DLA-like pattern, now bacterial motility also plays an important role. The discussion lies on what governs this motility. Since bacteria are *swimming*, their movement is thought as a diffusion or its discrete equivalent, a random-walk. Some researchers have proposed a non-linear diffusion that depends on nutrient and cell density [Kawasaki97, Kitsunezaki97], or a switch-off in motility and reproduction for low nutrient concentrations and low cell density [Matsushita98b]. Others claim that repellent chemotaxis between bacteria should also be included in the explanation (but have not brought to the moment experimental evidence of the chemotactic chemical driving the system) [Ben-Jacob94b]. On the other hand, it is also known that *B. subtilis* secretes a surfactant to enhance its motily. Experiments with surfactant-less mutants produce branched patterns that do no maintain the branch/gap width ratio [Wakita96]. A recent study on the geometrical characteristics of the experimental patterns of DBM will provide a framework to see to which extent the proposed models are realistic [Wakita98]. A more detailed discussion on the models will be presented on section 4.2.

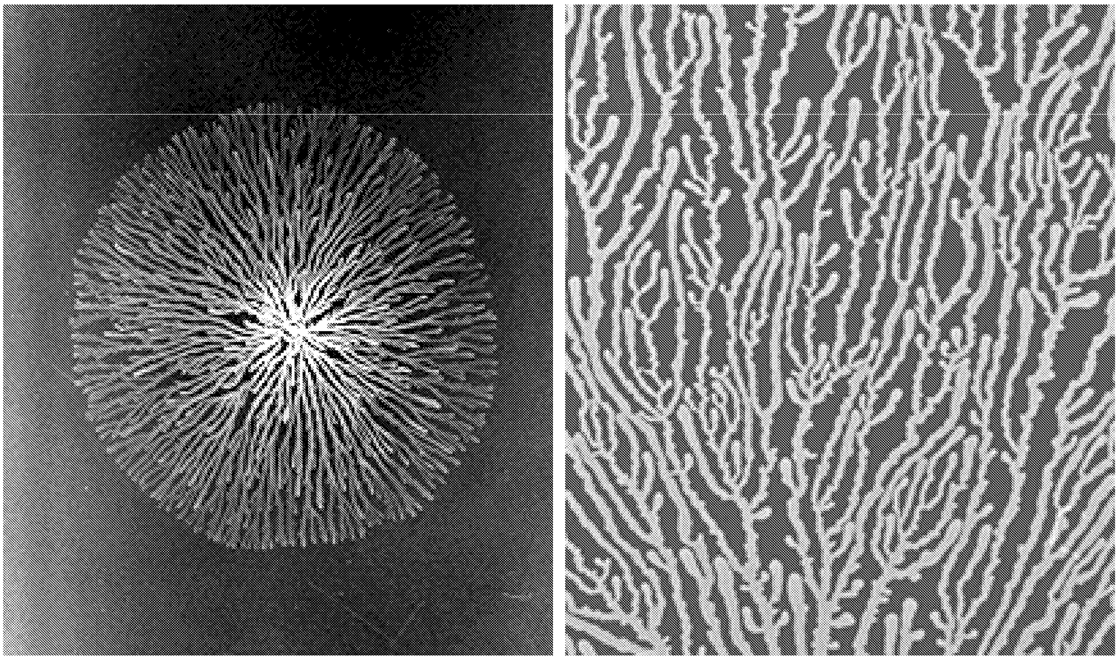

Figure 2.13: (Left) Dense branching morphology (DBM) of a *B. subtilis* colony 25 hours after inoculation. $C_a = 5\ g/l$, $C_n = 0.5\ g/l$. Diameter $\sim 4\ cm$. Despite the branching, the envelop of the colony remains very circular. (Right) Detail of the branches of a DBM pattern. Branches have a very well defined characteristic width (from a gaussian distribution) and a characteristic length (from an exponential distribution). It can be seen that many branches stop growing because of the presence of others. Collision and fusion of branches is very rare. The width of the picture corresponds to $\sim$5 *mm*.

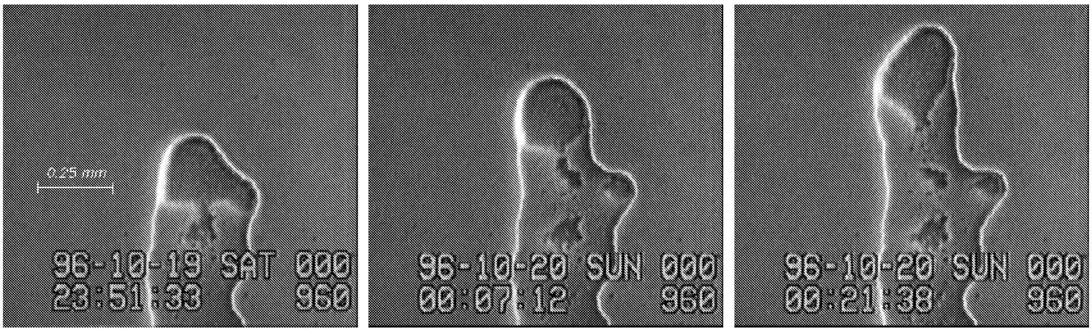

Figure 2.14: Advance of a DBM branch tip by means of a its *finger-nail* structure. This finger-nail is dynamical, it keeps growing towards the outside of the tip while it vanishes inside the branch. This structure is thought to show areas the of higher cell density and activity. Pictures are ~1 *mm* wide. Time interval between them is around 15 minutes.

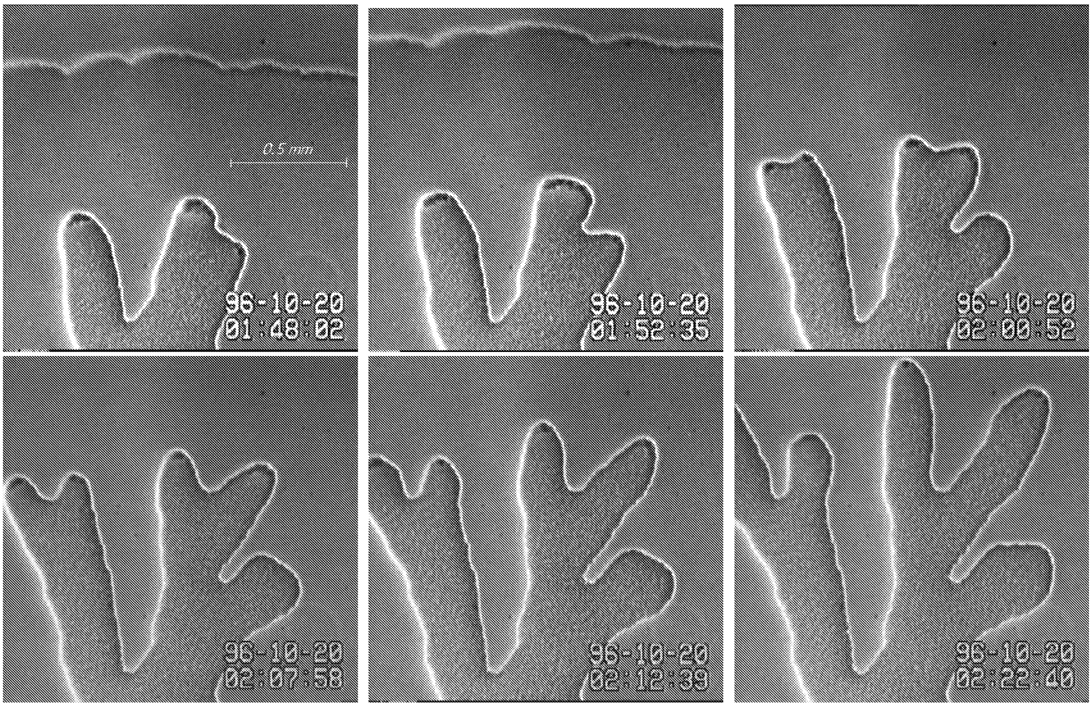

Figure 2.15: Sequence of a DBM tip splitting. It is thought that the tip splits when facing irregularities of the agar surface that make advance difficult. This has been proved to happen randomly (since the distribution of branch lengths is exponential). The flat interface in front of the tip (1st and 2nd pictures) is thought to be the liquid medium secreted by bacteria and/or extracted from the agar plate on which bacteria swim. Pictures are ~1.5 *mm* wide.

2.3.5 Region C: Concentric ring pattern

If the agar medium is softened a little more than in region B by decreasing the agar concentration, but leaving the nutrient contents high, concentric ring colonies are formed (Figure 2.16). The appearance of these rings is due to the periodic growth dynamics that colonies display under these particular conditions: bacteria move actively for about 2-3 hours (colony expands), and then they suddenly stop for 2-3 hours while performing cell division (colony does not grow but cell density increases); this cycle repeats several times. It is not understood why bacteria display such a periodic motile/immotile behaviour.

The research on this pattern constitutes the core of this investigation. Its further description, characterization and tentative explanation(s) shall be presented in chapters 3 and 4.

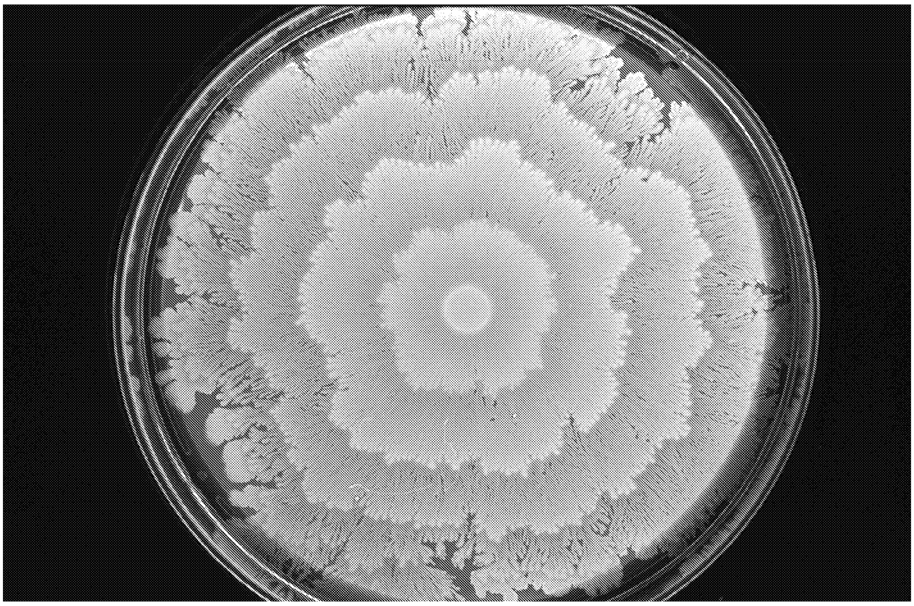

Figure 2.16: Concentric ring pattern obtained after 35 hours of incubation. Each ring corresponds to cycle of growth. $C_a = 7.7\ g/l$, $C_n = 25\ g/l$. Agar plate 1.5 mm thick.

2.4 Do other bacteria fit into *B. subtilis* paradigm?

After reviewing the present state of knowledge on bacterial colony formation for a *particular* strain of a *particular* bacterial species under the *particular* conditions chosen by some *particular* researchers, one wonders whether we are dealing indeed with a particular case that has a very *particular* -and limited- interest, or on the contrary we have found the *universal* paradigm for bacterial colony formation. As it happens, in nature there are not only many bacteria but many strains of each bacterial species:

Bacterial strains

Every bacterial isolate has its individual character. Even though they have the same species name (e.g., Bacillus subtilis), their characteristics are never identical one to the other. So, we usually give a strain name to each isolate to distinguish it from others and keep a record of the isolation source (e.g., sputum of a patient suffering from bronchitis). A bacterium without such a strain name is outside the realm of experimental science. The real bacterium with the strain name always has its own specific properties. (...) Bacteria are small and have simple anatomical structures. As a result, they are often thought of as simple organisms that are easy to manipulate and have a straightforward life style that is easy to elucidate. We do not think so. Their life strategy must be sophisticated, as they are creatures that have thrived throughout an extremely long evolutionary history (3.5 billion years). Their behavior in various environments is made quite sensitive and variable due to their response to biological or physico-chemical factors (mostly not identified).

From reference [Matsuyama95].

After reading this paragraph one might think that through the study of our strain of *B. subtilis*, we have only come to understand an extremely narrow scope of bacterial reality. However, the same T. Matsuyama -a bacteriologist- that so strongly warns us about the very individuality and irreducibility of bacterial strains and species, has published some of the more enlightening articles on the (let's say) *universality* of some of the patterns produced by bacterial colonies, as we shall see. In his studies of pattern formation with several strains and species of bacterial colonies, he showed that, in spite of the diversity of the fundamental units (the bacterial cell), many bacterial colonies produce some of the main morphologies that we have found for *B. subtilis*.

We shall begin by checking if other strains of *B. subtilis* display similar colonies. The group of research of E. Ben-Jacob (composed, as our group, by physicists with a pattern formation background) provide the example of *B. subtilis* strain 168 [Ben-Jacob92, Ben-Jacob94b, Ben-Jacob97]. We shall restrict our attention to what this group calls the "$\mathcal{T}$ morphotype" of this strain.[8]

B. subtilis 168 has a stronger motility than the wild OG-01 strain we use. The main effect of this higher motility is a shift of the morphological diagram towards harder surface media (*i.e.*, it shifts x-axis of figure 2.6 towards the right). In their study the same five morphologies are obtained. The most noticeable differences are that: (i) *B. subtilis* 168 does not develop any pattern by means of *gliding*, what makes growth faster. (ii) In adverse environmental conditions *B. subtilis* 168 grows DLA-like patterns as expected, but in even poorer nutritional conditions, it develops a

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[8] Ben-Jacob's group had claimed that several phenotypes sprout from a single strain of *B. subtilis* 168. Some researchers pointed out that these other phenotypes might be contaminations from of other bacteria, such as *B. circulans*. The controversy is far from settled -but of the utmost relevance for research on mutations.

form of dense branch morphology that is reported to be due to chemotactic signalling (the wild OG-01 strain can not produce colonies in these conditions). In spite of these differences, the comparison of the patterns produced by the two strains is reassuring.

Comparison with colonies formed by other bacteria on agar media is not so straightforward. As said before, T. Matsuyama has experimented with a variety of bacteria on a variety of agar media (hundreds of parameters can be changed: temperature of incubation, kind of nutrients, salts, etc.) [Matsuyama95]. He found a wealth of colony patterns but many of them can be grossly classified within the five pattern obtained for OG-01 *B. subtilis*. A compact round form (the dense disk of region B of *B. subtilis*), seems to be the most familiar morphology for a bacterial colony. It is the usual form under which many bacteria are cultivated in medical or biological laboratories, e.g., *Escherichia coli* -one of the main *tools* of genetic engineering. Branching patterns are also often observed in stressing conditions. Patterns resembling DLA and specially dense branching morphologies have been found for several species. Concentric ring patterns have been known for *Proteus mirabilis* for more than a century (see references in [Rauprich96]).[9] Fast spreading disk colonies (with active motion of bacteria) have been found for *Escherichia coli* and *Salmonella typhimurium* [Harshey94].

Other bacterial colonies also develop patterns that are not produced by *B. subtilis* OG-01. To cite a few, there are bacterial colonies that develop a chiral morphology, in which the branches consist of twisted branches all with the same handedness (each branches spirals around the center) [Ben-Jacob95]. Others in which branches wander capriciously over the agar plates without respecting any symmetry [Matsuyama95]. For very soft semi-solid agar some bacterial colonies form patterns that keep changing their shape for a long transient time (spatiotemporal patterns) until a final pattern is formed [Shimada95]. In some cases it has been proved that this final stadium is reached by means of chemotactic aggregation [Budrene95, Woodward95]. Finally, some other bacteria have a very complex (should I say *clever*?) behavior whose study surpasses the possibilities of our pattern formation approach -at least in the present stage of research [Shapiro88, Losick97].

In conclusion, bacterial colony formation on agar surfaces is far from universal and the framework provided by the study of *B. subtilis* OG-01 colony pattern is too limited to account for the diversity of bacterial colony patterns observed in nature. However, I think that the framework that has been constructed for the morphological study of *B. subtilis* colonies can be applied to many bacterial colonies. Although there have not been many systematic studies of bacterial colonies from a pattern formation perspective, a remarkable number of other bacterial colonies have been found to share some of the features of *B. subtilis* colonies. Many bacterial strains reproduce one or two of the patterns of the *B. subtilis* morphological diagram: rich, hard media give rise to round colonies; poor, hard media produce branched patterns; soft agar allows fast, diffusion-like expansion; etcetera. In this sense we can speak of a *B. subtilis* paradigm, this is, we can take *B. subtilis* colonies as an exemplar

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[9] One of the questions that this research sought to answer is whether ring patterns of *B. subtilis* and *P. mirabilis* are produced by the same mechanism. This shall be discussed in section 4.1.3.

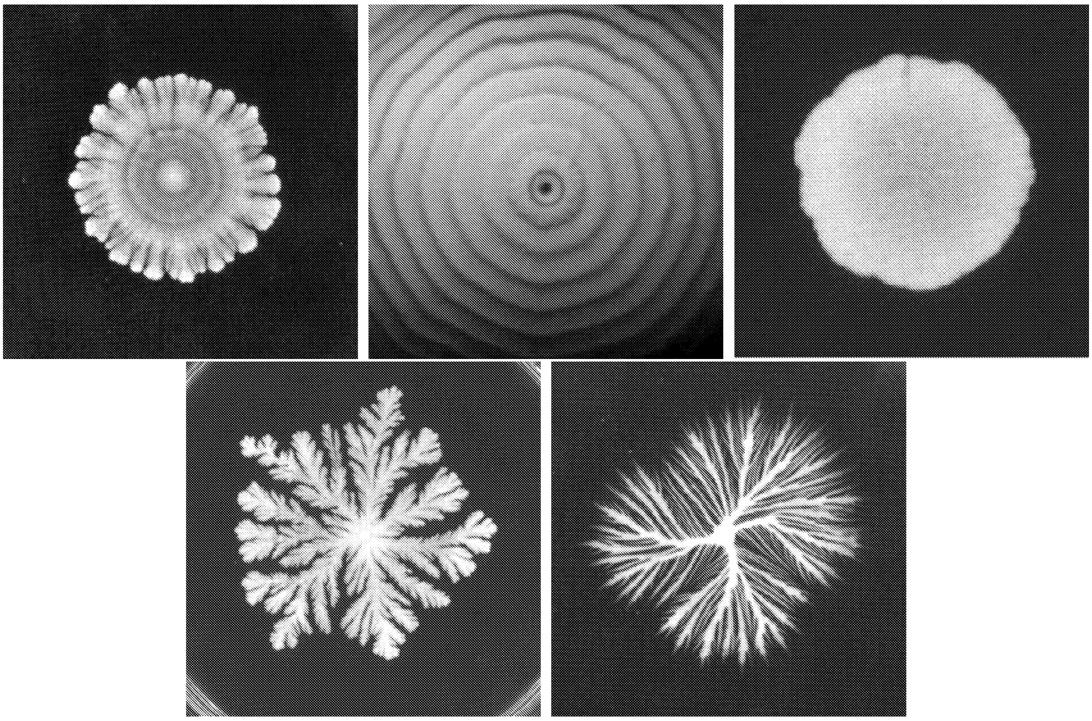

Figure 2.17: The same patterns obtained with *B. subtilis* colonies have also been observed in other bacteria. (Top left) Dense disk made by a *Serratia marcescens* colony. (Top middle) *Proteus mirabilis* concentric ring pattern. (Top right) Round disk formed by a *Micrococcus luteus* colony. (Bottom left) *Salmonella anatum* DLA-like pattern. (Bottom right) Dense branching produced by a *Serratia marcescens* colony. Pictures taken from reference [Matsuyama95] except *P. mirabilis*, courtesy of H. Itoh.

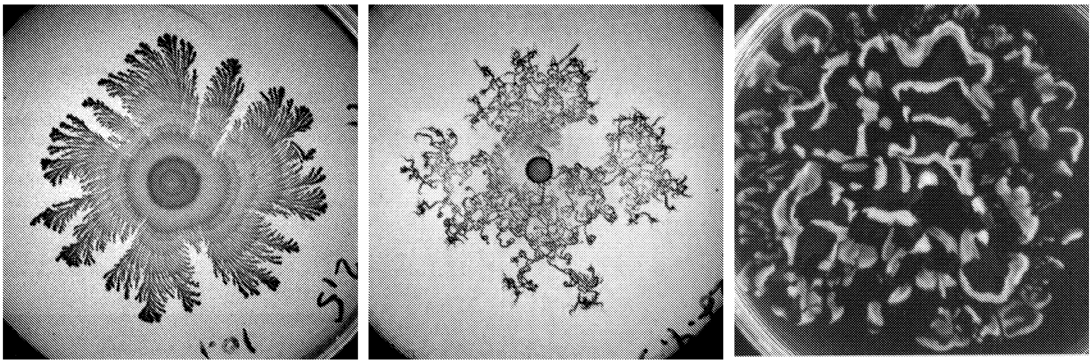

Figure 2.18: Some patterns different from those obtained for strain OG-01 of *B. subtilis*. (Left) Slightly twisted branched morphology of *B. subtilis* of the tip-splitting phase from [Ben-Jacob94b]. (Center) Chiral phase of a *B. subtilis* colony from [Ben-Jacob94b]. (Right) Highly disordered pattern of *Salmonella typhimurium* colony (flagella-less mutant). From [Matsuyama95].

case of bacterial colony formation on solid and semi-solid agar[10]. It does not provide thorough answers or predictions, but it illustrates how a good number of bacterial colonies grow.

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[10] In liquid or semi-liquid media patterns are different, though.

Chapter 3

Experiments on ring patterns of B. subtilis **colonies**

In this chapter I will explain in detail the experiments that we have performed on ring patterns of *B. subtilis*. The chapter is organized as follows. In section 3.1 the growth dynamics is described. Next, 3.2, we study the bacterial cell during the different phases of colony formation. Sections 3.3 and 3.4 present a macroscopic quantitative characterization of the ring pattern in terms of its *cycle duration* and *ring width*. Section 3.5 explains in which range of nutrient and agar concentrations the concentric ring pattern can be found and what transitional morphologies look like. Section 3.6 is a description of other experimental trials. The chapter ends with a summary of the experimental results.

3.1 Description of growth dynamics

Following the experimental procedures explained in section 2.2 with agar and nutrient concentrations within the range of region C ($C_a \sim 7.0 - 8.5\ g/l$, $C_n > 10\ g/l$, see figure 2.6), bacterial colonies forming concentric ring patterns are obtained. The temporal evolution of the colony is shown in figure 3.1, as a sequence of pictures from a time-lapse video recording. Figure 3.2 displays how radius of the colony expands on time. Figure 3.3 displays the pattern that typically remains once all the agar plate has been colonized.

We discerned three distinct phases during colony formation.

(i) Lag Phase:

We call *lag-phase* the time between inoculation of the bacteria on agar and the beginning of active movement of bacteria. Figure 3.4 shows a complete temporal sequence of this phase. Since the moment that the inoculum is dropped on the agar surface, bacteria start reproducing but do not, in most cases, break completely the cell wall between *daughter* cells. This results in the appearance of bundles of bacteria, as it can be seen in figure 3.4 (b). Cell density increases with time but

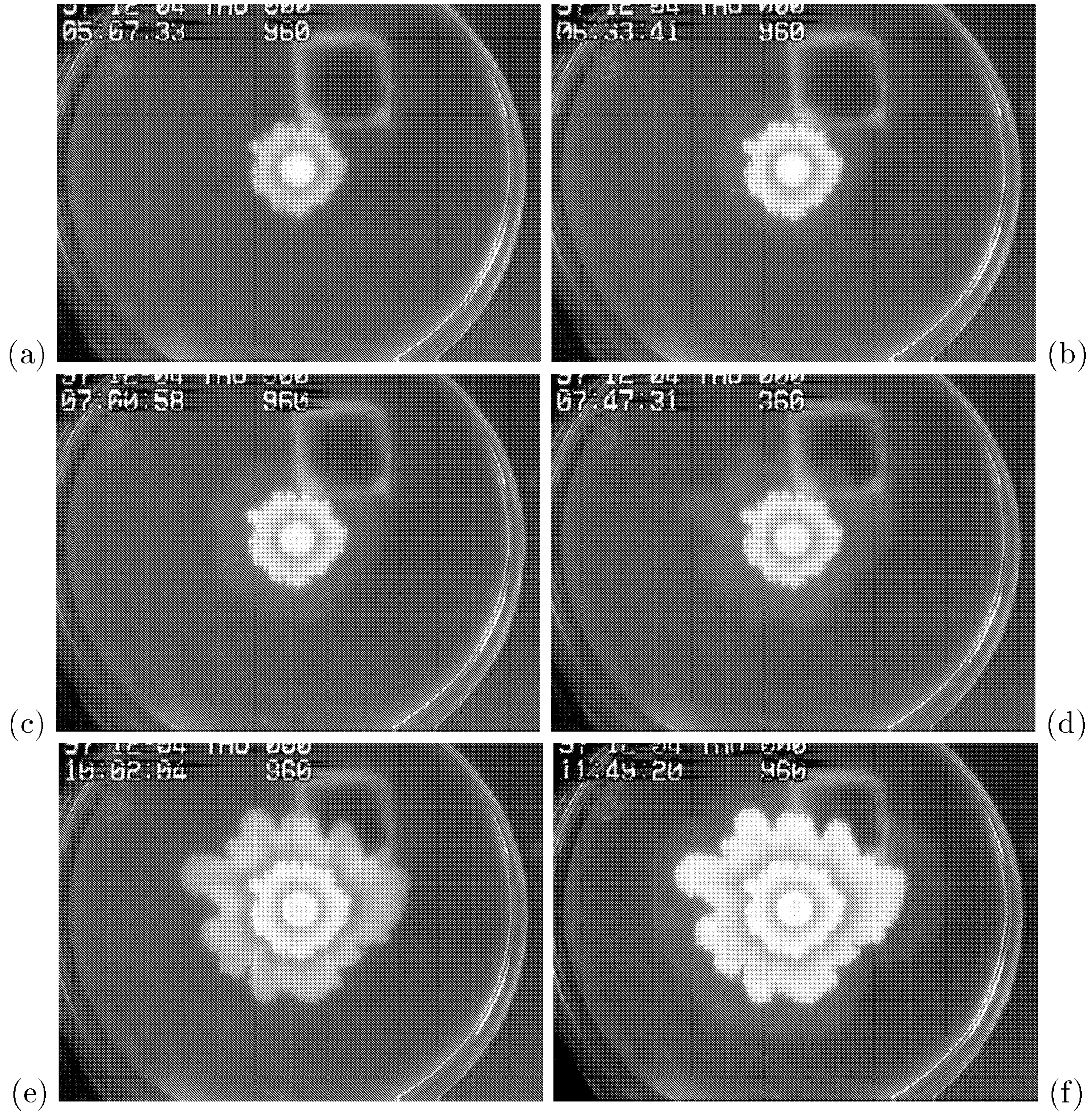

Figure 3.1: Macroscopic sequence of a ring pattern growth displaying the formation of the 2nd ring. **(a)** End of 1st consolidation phase. Let's set time to (0 hours). **(b)** Middle of 2nd growth phase (1.5 hours). The colony is expanding by means of a thin layer of bacteria that is hardly visible. **(c)** End of 2nd growth phase (2 hours). Comparing with (b) one can appreciate that the envelope of the colony grows rapidly. **(d)** Beginning of 2nd consolidation phase (2.5 hours). Growth has stopped and density begins to increase. **(e)** End of 2nd consolidation phase (5 hours). The 2nd ring becomes very dense. Comparing with (d), one can see that the colony is better defined but has not changed its shape. A whole cycle has been completed since (a). **(f)** Middle of 3rd growth phase (6.5 hours). A new ring develops.

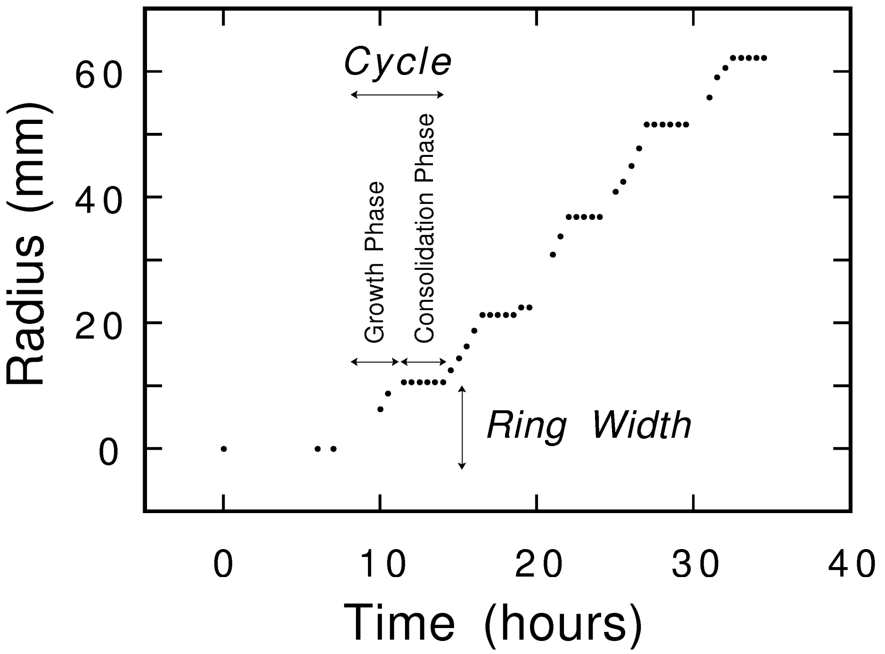

Figure 3.2: Radius of a region C colony as a function of time. Data were measured from a time-lapse video recording on a 146 *mm* diameter Petri dish, $C_a = 7.6\ gl$, $C_n = 20\ gl$. The graph allows us to characterize the pattern with the macroscopic quantities that determine the stair-like shape: *cycle duration*, *growth phase*, *consolidation phase* and *ring width*.

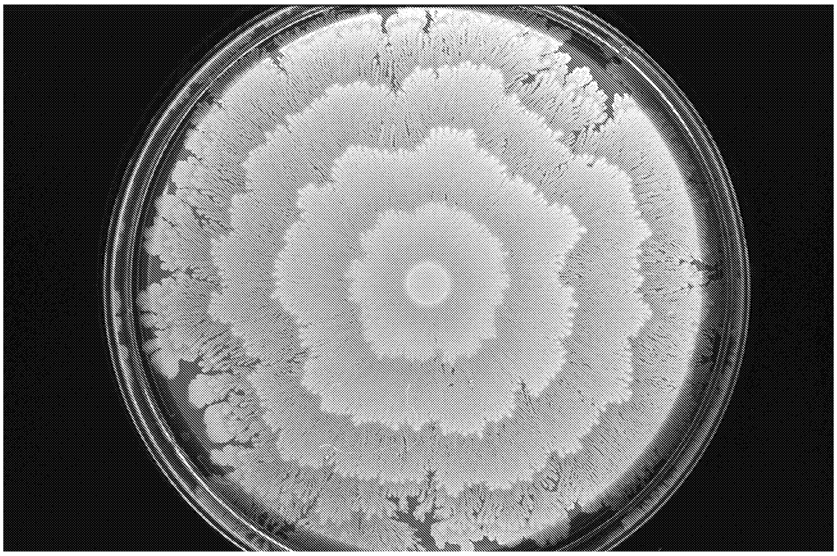

Figure 3.3: Concentric ring pattern that remains once all the agar plate has been colonized. $C_a = 7.7\ gl$, $C_n = 25\ gl$. Compare the spacing between rings of this picture with the temporal evolution of radius displayed in figure 3.2

bacteria do not move actively[1]. Eventually, though, very slow surface translocation begins in the form of *gliding*, *i.e.*, apparently due to the pressure that builds up inside the colony as density becomes very high (see figure 3.4 (c)-(d)). At this stage, cell density is so high that we cannot see clearly anymore what is happening inside the colony. We can observe nevertheless some individual bacteria that are not anymore linked to their *brothers* and perform a back and forth movement among bundles of immotile bacteria. The number of individual cells seems to increase progressively, starting a kind of spasmodic flow that finally breaks the envelope of the colony and forms thin branches of one only layer of motile, individual bacteria. This marks the end of the lag phase.

(ii) Growth Phase:

[2] This phase is characterized by a fast expansion of the colony by means of thin branches of bacteria with low cell density -unilayered- but populated by very active bacteria. The advance of these branches is displayed in figure 3.5. The appearance of these branches is sudden, as if it were triggered by the overcoming of some threshold in surface tension, density or some other parameter. The sharp discontinuity can be appreciated in the precise microscopic measurement of radius of colony versus time presented in figure 3.6. In contrast with branches of the DBM pattern of region E, where only bacteria in the branch tips are active (see figure 2.14), in region C bacteria are very active all along the branch, what allows for lateral growth, collision and eventual fusion of neighboring branches. The better the conditions for growth (higher nutrient concentrations, soft agar) the more the branches fuse together. The envelope of the colony grows with a velocity ranging between $2-20$ $mm/hour$ ($0.5-5$ $\mu m/s$), that is surprisingly constant all along the growth phase (see figure 3.6). After 2.5-3 hours of colony expansion, branches stop suddenly.

(iii) Consolidation Phase:

This phase is characterized by an increase of cell density on the regions that have been colonized in the previous growth phase. Figure 3.7 presents a temporal sequence of the consolidation phase. Branches are stopped, bacteria can not move actively but septate repeatedly forming bundles just like in the end of the lag phase. If the colony advances a little bit (no more than 1 mm) it is also thanks to *gliding*. About 2.5 hours after this monotonous increase of cell density, some kind of precursor activity can be observed inside the colony. Individual bacteria pushing back and forth appear again, first in the most inner regions (within the area colonized during last growth) and progressively in outer regions. When this "wave" of activity reaches the interface of the colony, branches sprout suddenly again. This marks the beginning of a new growth phase.

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[1] It is known that in these first stages of colony growth the population increases exponentially, which is something one can expect from a sequence of septations.

[2] Many groups call this phase *swarming* in the case of the ring patterns generated by *P. mirabilis*, in accordance to the kind of movement displayed by this bacterial species [Rauprich96]. From a macroscopic perspective we can refer to it plainly as *growth*.

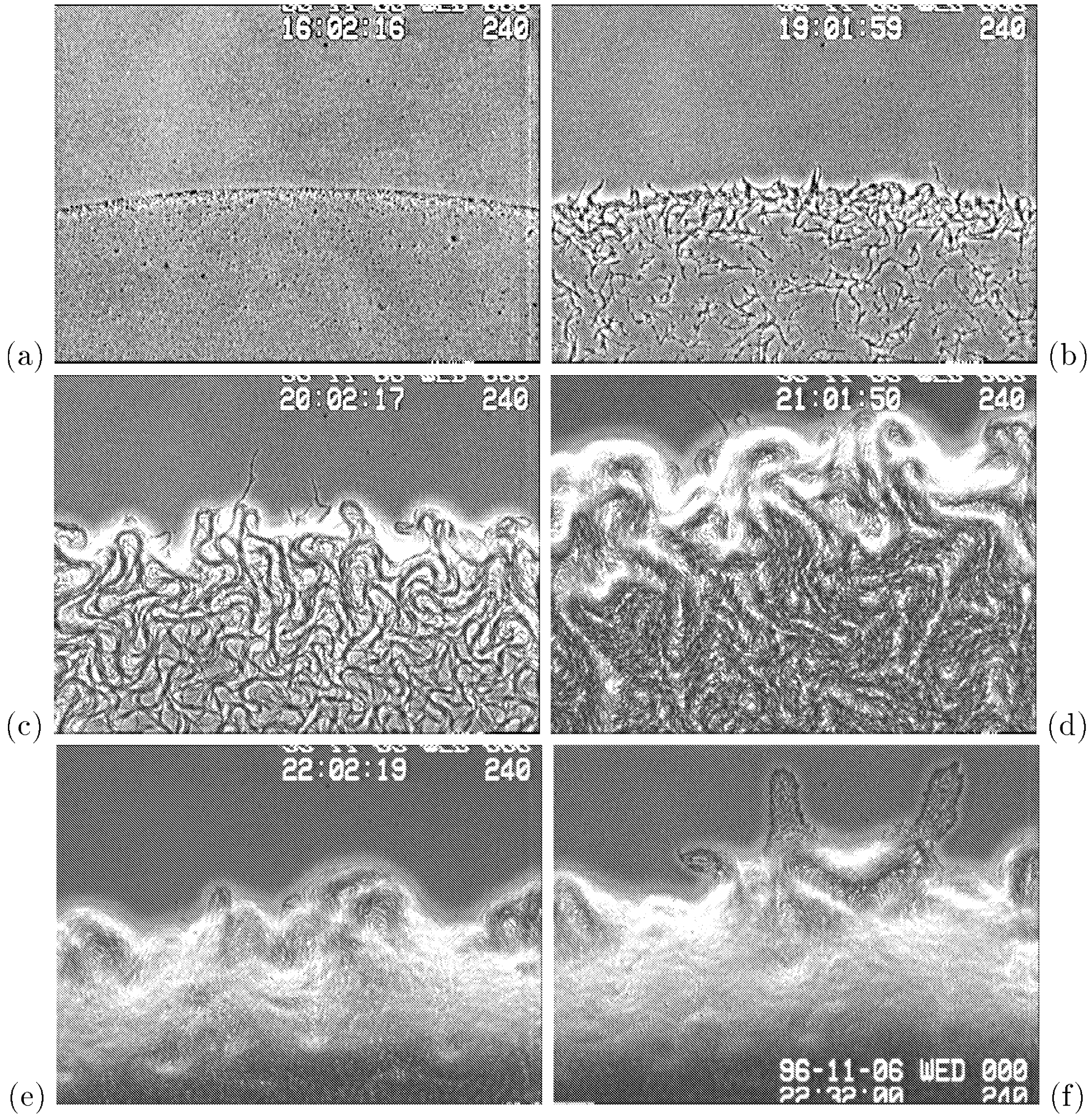

Figure 3.4: Microscopic sequence of a lag phase, *i.e.*, since inoculation until the rapid expansion begins. Each picture is 0.8 *mm* wide. **(a)** 0 hours. Just after inoculation. Beginning of lag phase. The perimeter of the inoculum can be seen as a line made of many point-like bacteria. **(b)** 3 hours. Density begins to increase visibly. Elongation of bacteria due to successive septations without complete breaking of the cell wall. **(c)** 4 hours. Bacteria make bundles. Colony interface begins to expand very slowly by effect of pressure that built up as cell density increased. **(d)** 5 hours. Slow expansion of the colony by means of *gliding*. **(e)** 6 hours. Density has kept increasing. Inside the colony some cells are observed moving individually and back and forth. **(f)** 5.5 hours. These individually motile bacteria break the envelope of the colony by forming branches that advance quickly over the agar surface. End of lag phase.

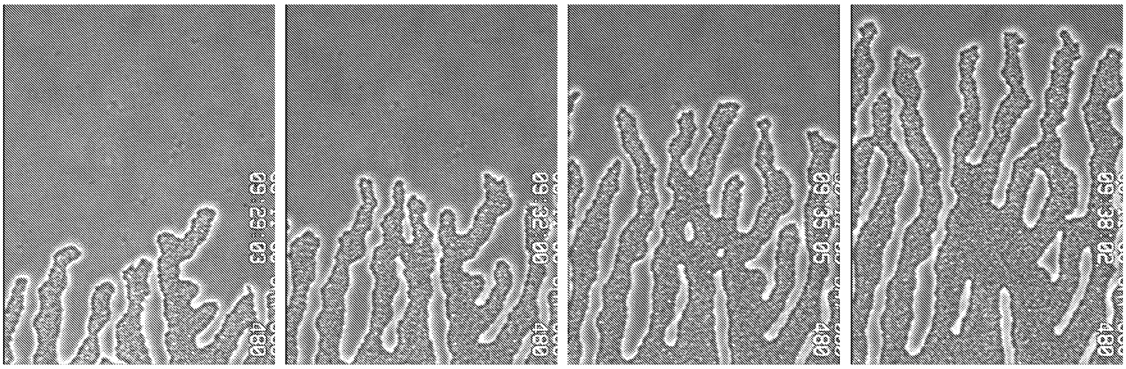

Figure 3.5: Sequence of branch expansion during growth phase. Width of a picture corresponds to 1.3 *mm*. Interval between images is 3 minutes. The sequence shows how branches collide and fuse a little behind the front of the colony. The fact that all branches advance quite coherently allows to define the envelope of the colony from a macroscopic point of view. No finger nail structure is found; bacteria are very active both at the tip and the inside of the branches.

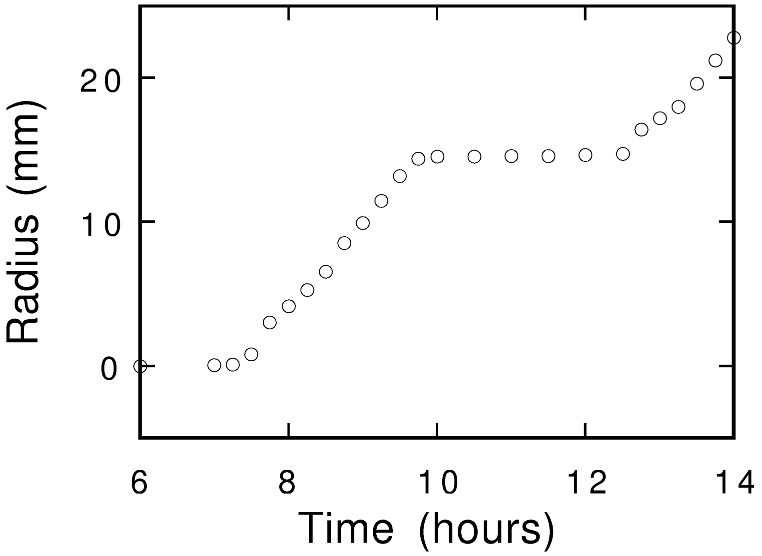

Figure 3.6: Radius of the colony versus time for a concentric ring pattern. Data were measured to ±0.1 *mm* accuracy from a microscopic time-lapse video recording (as shown in figure 3.5). The graph displays the evolution of the radius of the colony from the end of the 1st consolidation phase to the beginning of the 2nd growth phase. It should be noted the almost absolute constancy of velocity within a phase and its sharp discontinuity when phase changes.

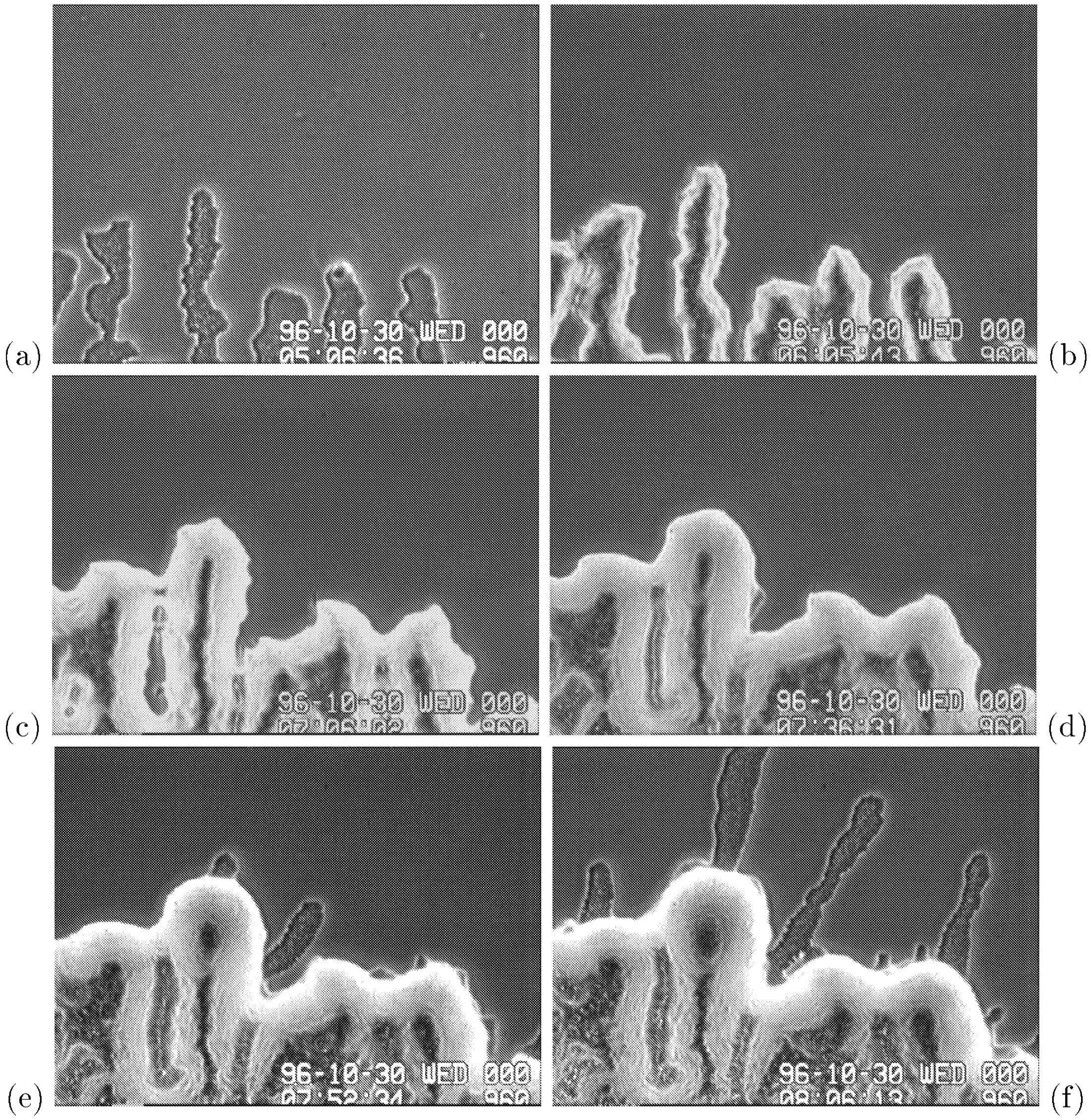

Figure 3.7: Macroscopic sequence of a consolidation phase, *i.e.* since colony expansion stops until it is resumed again. Each picture is 2.0 *mm* wide. **(a)** Let's set time to 0 hours. Branches that have been growing for about 2.5 hours suddenly stop. At this stage branches are formed by one only layer of bacteria. **(b)** 1 hour. Density increase suddenly but the colony does not expand at all. At a finer scale it can be observed that bacteria make bundles (as seen in figure 3.4). **(c)** 2 hours. Density keeps increasing dramatically what makes neighboring branches fuse. **(d)** 2.5 hours. Spasmodic movements of individual bacteria are observed coming from inside the colony and propagating towards the interface of the colony. Now most of the space between branches behind the envelope has been covered by this *flow*. **(e)** 2.75 hours. The *flow* of individually motile bacteria breaks the envelope of the colony and makes branches. **(f)** 3 hours. The colony expands by means of these branches. A new growth phase has begun.

The formation of the colony can be succinctly summed up as a lag phase plus periodic cycles made of a growth phase and consolidation phase. The concentric rings that are seen macroscopically, appear in the circular bands where the colony stops during the consolidation phase. Each ring corresponds to a cycle. Figure 3.2 shows this in a graphic way. We shall characterize the ring pattern in terms of the simple macroscopic quantities defined in this graph. *Cycle duration* stands for the time between the beginning of a *growth phase* and the beginning of the following *growth phase*. When referring to *growth phase* or *consolidation phase* we shall often just mean their respective temporal durations. And finally *ring width* stands for the increment in the radius of the colony during one cycle, which is equivalent to the distance between the new ring and the former.

Successive cycles seem to be very similar and one could expect, in principle, the pattern to have a very regular geometry. In practice, environmental conditions keep changing during the growth of the colony and successive rings do not always look exactly the same. In general, as time runs over several cycles, the ring width decreases and the interface becomes rougher -as though it became more and more difficult for individual bacteria to move actively- until the active growth completely stops, *i.e.*, until the colony falls into a permanent consolidation phase that only allows a very slow expansion of the colony by means of *gliding*. We do not know the reason of this progressive worsening of environmental conditions. It might be due to the drying of the agar surface (*B. subtilis* motility is extremely sensitive to humidity changes [Mendelson96]) or to the accumulation of metabolic by-products that inhibit growth. Since spatial and temporal periodicity of the cycle is nicely preserved for experiments within the best growth conditions (in the middle of region C), we shall assume that the properties of a cycle (cycle duration, ring width, etc.) are constant for given environmental conditions and do not depend on the cycle ordinal (this should be more carefully checked in future experiments).

A last thing to be mentioned about the growth dynamics is that sometimes, inside the colony, there are waves of cell density propagating from the center towards the perimeter. In microscopic observations up to four successive density propagations have been seen. They are thought to correspond to the expansion of a new layer all over the colony (the first expansion during a growth phase consisted of only one layer of bacteria). This phenomenon is more often observed in good conditions of growth (low agar, high nutrient concentrations).

3.2 Cell differentiation in *B. subtilis*

[3] The fact that individual bacteria can move during the growth phase but can not move during the consolidation phase, prompted us to investigate whether bacterial cells change their physiological and morphological characteristics during a ring cycle (as it was known to be the case for the bacteria *Proteus mirabilis* [Belas97]).

The investigation was performed the following way. Agar plates ($C_a = 8\ g/l$,

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[3] The research described in this section was undertaken under the guidance of T. Matsuyama in the School of Medicine of Niigata University and the help of his graduate student Y. Takagi who has performed a similar study for *Proteus mirabilis* ring patterns.

$C_n = 20\ g/l$) were prepared and inoculated as usual. Once the colony had grown, bacteria were picked, coloured with the standard gram-stain, deposited on glass slide and observed under the microscope. The images were displayed on a screen, where the length and width of bacteria could be measured up to a $\pm 0.2\ \mu m$ accuracy by means of an analysis image software.

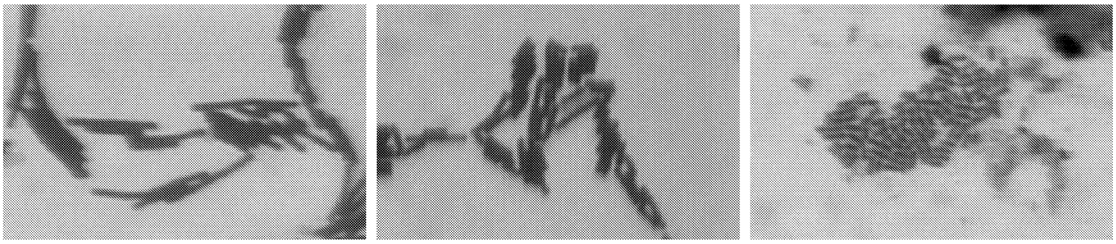

Figure 3.8: Photographs of *B. subtilis* picked up at different stages since the beginning of a consolidation phase, gram-stained, and put on a glass slide. Despite bacteria make bundles and are tight to each other during the consolidation phase, their actual length decreases. (Left) Beginning of consolidation phase (Middle) Three hours later (Right) Five hours later. Each picture is about $40\mu m$ wide.

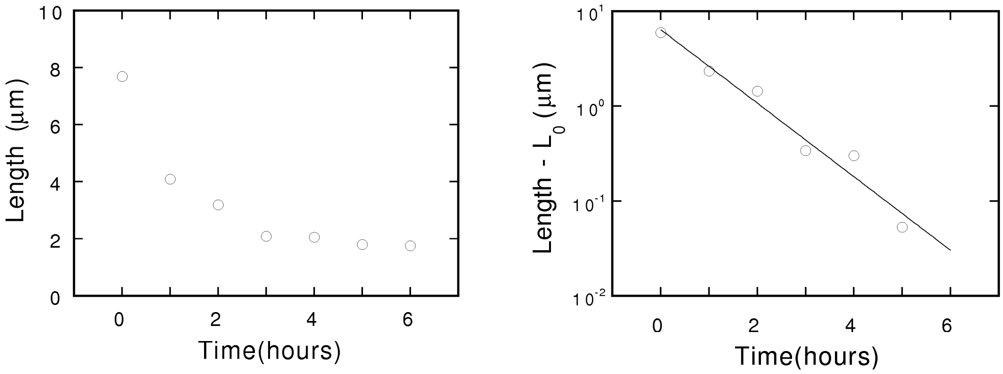

Figure 3.9: Mean length of bacteria versus time for a fixed point of the colony in linear (left) and logarithmic (right) scales. Data were taken from the distributions showed in figure 3.11). The time is set to zero for the beginning of a consolidation time. The decrease in length is robustly exponential. The main change in length happens during the first three hours -approximately the time that a consolidation time lasts.

First, we analyzed how for a given fixed point of the colony, the length and width of bacteria change as time increases. This was done by picking cells from the front of a colony that had just finished a growth phase (*i.e.*, that had just stopped expanding). Then the colony was put back into the incubator and the picking of some bacteria was repeated every successive hour. Secondly, we sampled cells of different regions from a growing colony that had already developed several rings. For each sample, 100 cells were measured.

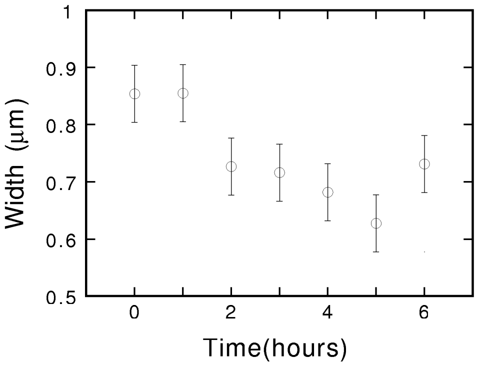

Figure 3.10: Mean width of bacteria versus time for a given point of the colony. Mean width is found to decrease in the beginning of the consolidation time.

Figure 3.11 shows how the distributions of bacterial length evolves in time for a fixed spot of the colony. The first measurement, taken when the spot was at the outer front of a colony that had just begun a consolidation phase, presented many long cells. The mean length is around 8 μm and the distribution is wide. One hour later, the distribution shifts dramatically towards shorter cells, and the peak of the distribution is found for the $2-3$ μm interval. After two hours, the amount of long cells is even further reduced. After 3 hours all cells seem to be rather short, about 2 μm long. For later times it is difficult to appreciate any difference in length in natural scales. Figure 3.9 (left), expresses this decay in terms of the mean length of bacteria. Now, in figure 3.9 (right) we have plotted the same data again in a logarithmic scale after subtracting to the cell length the minimum mean length L_0. The straight line tells us that this decay of length is exponential. The result is surprisingly robust. On the other hand, the statistical study also shows that width of cells decreases with time (see figure 3.10), though much less than cell length.

Figure 3.12 displays the distribution of cell lengths at different spots of a pattern for a given time. The colony is in the middle of a consolidation phase, around two hours after the 2nd growth phase stopped. The two graphs at the top of the figure show the distributions of lengths of the last grown ring (the 2nd). In either the inner and outer part of this ring, cells are still long within a wide, sparse distribution. The two graphs in the middle of the figure correspond to the previously stopped ring, where the cells have settled to the minimum length, already. The same should be said for the center of the colony, the spot were the inoculum was deposited.

Finally, we measured the length of cells for an expanding front, *i.e.* for the outer part a colony that is going through a growth phase (figure 3.13). In this case cells are actively swimming on the agar. The distribution is very similar to that found for a spot of a ring that had just begun a consolidation phase (see figure 3.11.

When summing up the data presented above, one can form the following overall picture of how cells change their length as the colony grows:

1. First, during a growth phase cell bodies are relatively long, with a mean length around $4-8$ μm, a mean width around $0.8-1.0$ μm, and wide distributions

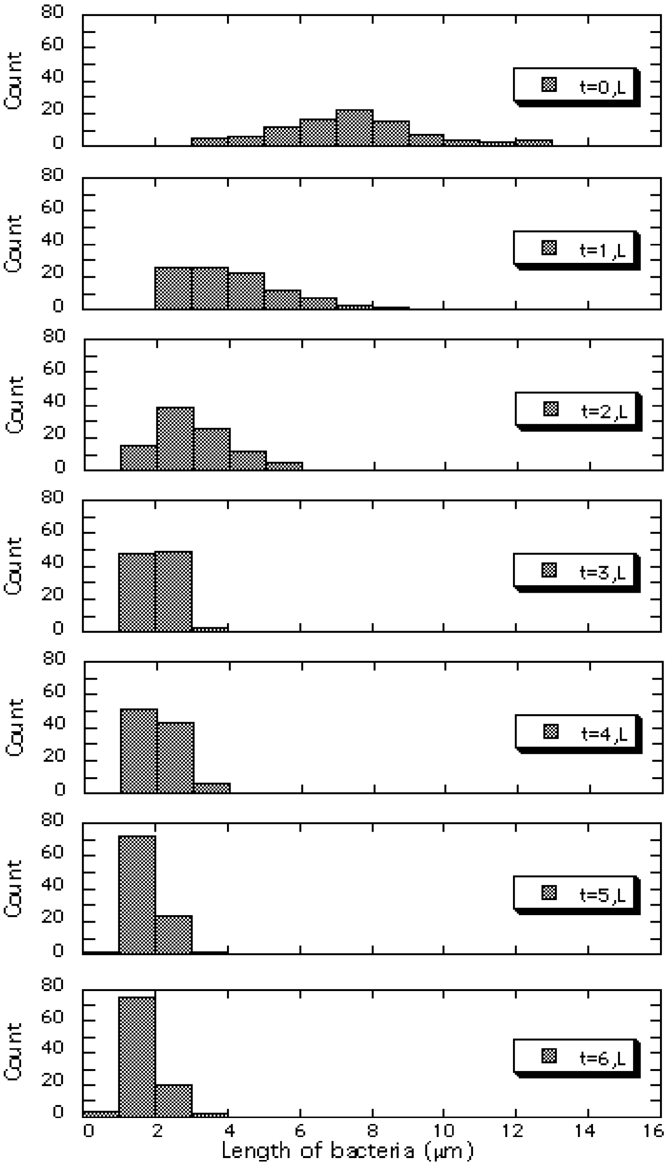

Figure 3.11: Distribution of length of bacteria vs. time at a fixed point. Bacteria were picked up from the expanding front of a colony that had just begun a consolidation phase. Every successive hour, 100 cells were sampled and measured -as shown from top to bottom in the figure. At the beginning of the consolidation phase, a wide distribution centered around 8 μm, is found. This distribution shrinks sharply in about 3 hours to become centered around 2.0 μm.

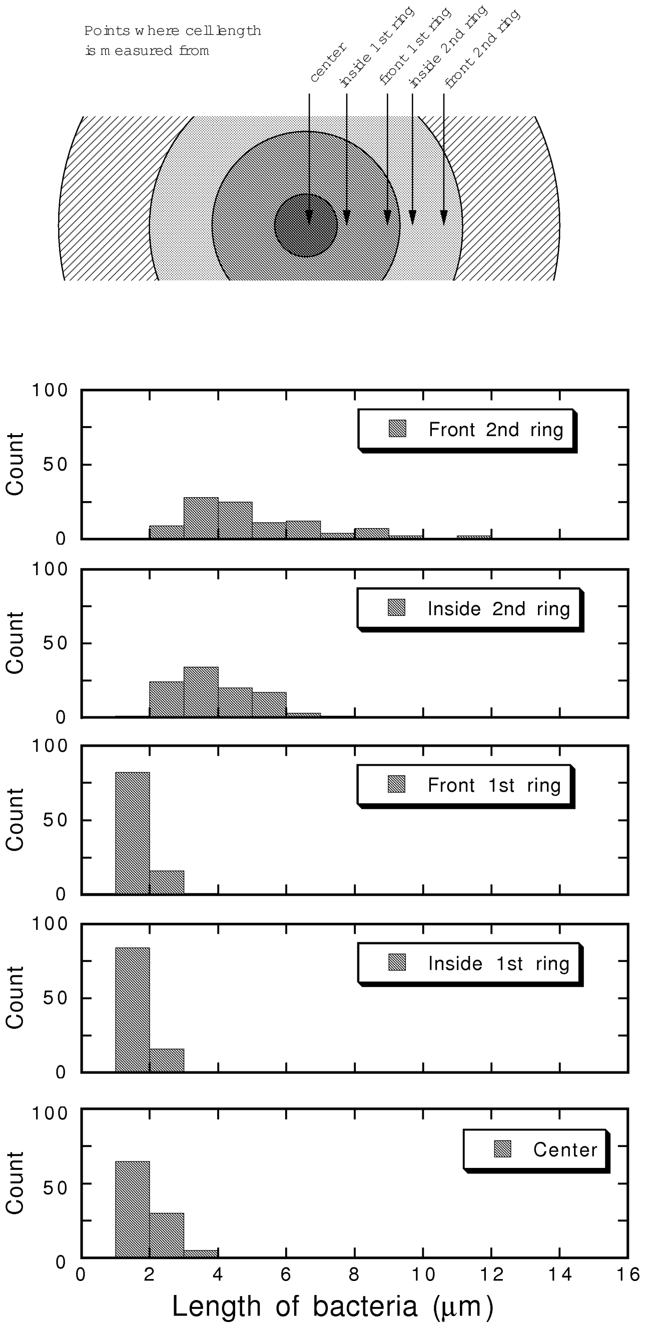

Figure 3.12: Cell length distributions at different points of the colony. Bacteria were picked up about 2 hours after the 2nd consolidation phase had finished. Radius of their position increases from bottom (center=inoculation point) to top (front 2nd ring = colony perimeter). It is found that cells in areas that have been colonized for more than one cycle (~5 *hours*), have settled in a short cell distribution ($1.5 - 2.0$ μm long). Contrarily, areas recently colonized have much longer cells and a much wider distribution of cells.

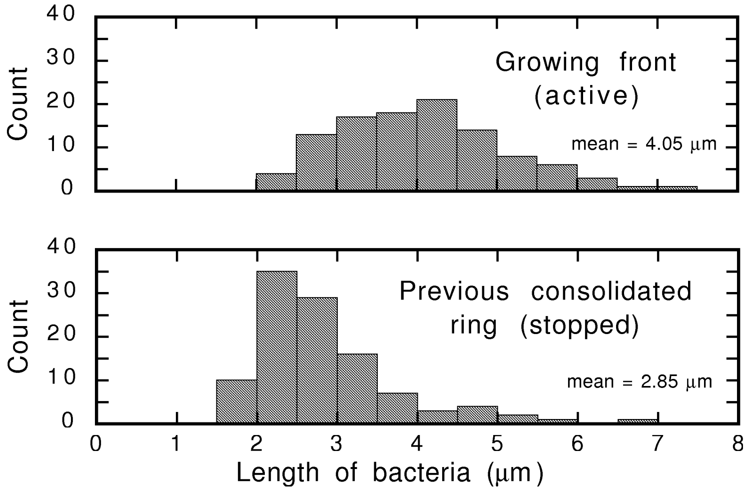

Figure 3.13: Cell length distribution during the beginning of a growth phase from a growing front (motile bacteria) in comparison with the distribution in the outer part of the previous consolidated ring (immotile). Bacteria with motility are longer and have a wider distributions.

for both quantities In such a case bacteria can move around active and individually.

2. When consolidation phase begins, cells lose their motility and density in a given spot of the colony increases. In these conditions, the average body cell becomes shorter and thinner, until it reaches a size of 1.9. μm of length and 0.7 μm of width.

To simplify: colony expansion (growth phase) is marked by the presence of long cells, colony consolidation by their gradual substitution by short cells. Data suggest that *B. subtilis* colonies have two main kind of cells: long-motile cells, and short-immotile cells. The development of several kind of cells is known as a differentiation process. To our knowledge, this cell shortening-lengthening process had not been experimentally described previously as a differentiation in the case of *B. subtilis*, although the change of motility had been observed and modelled [Ben-Jacob94b]. It was also known that in in fast growth conditions, septation did not occur as usual. Neidhart reported it this way: "In *B. subtilis*, the time from inception of a septum to its use is much longer, 138 minutes at $30C°$. Interestingly , this time is independent of growth rate. In fast growing cells, septum formation initiates repeatedly before the original cells separate, thus producing cells with three, four, seven or even more septa." [Neidhardt90]. This means that in *B. subtilis* the time needed for a whole septation (reproduction by splitting into two parts) process to occur is independent of growth conditions. In spite of this, under good growth conditions, cell can begin a second (and third) septation without having finished the former, what results in the lengthening of the cell. All this is very well in agreement with the data presented above and might be taken as its explanation from the perspective of the individual cell.

The fact that long cells (allegedly going through several septations at the same time) present physiological and morphological characteristics different than those shorter (besides multi-septation, a markedly higher motility) suggests that this lengthening is indeed a manifestation of a cell differentiation process. We shall discuss in section 4.1.3 its resemblance with the differentiation process of *Proteus mirabilis*. Cell differentiation has also been observed in *Escherichia coli* and *Salmonella Thyphymurium* [Harshey94]. It might turn out to be a common feature of bacteria moving on semi-solid agar surfaces.

On the other hand, the exponential decay of mean length of cell as time increases during the consolidation phase can be explained in two ways: (i) Assuming that is due to the septation of long cells into a short cell at a constant rate. (ii) Assuming that it is due to the increase of population of short cells (increases of population in non-saturated colonies are known to be exponential[4]. In fact it can be argued that both processes happen and the discussion is only one which one dominates the shift of distribution that is observed in figure 3.11. As experimental observations show a sharp increase in cell density during the consolidation phase (see figure 3.7), it seems reasonable to infer that it is the increase of population of short cells what determines the exponential form in the decrease in the averaged cell length.

3.3 Determination of cycle duration

Figure 3.14 displays how the duration of a cycle depends on nutrient and agar concentrations. The experiments were performed as follows. For each given set of agar and nutrient concentrations C_a and C_n, 4 Petri dishes were prepared. Agar plates were the deposited in the incubator ($35°C$, 90

The data presented here correspond to the growth of the first ring. Since agar plates were taken regularly out of the incubator to perform the measurements, growth conditions slowly deteriorated. The first cycle was the least affected and its data are considered to be the most reliable. Nevertheless, the cycle duration during this experiments was around 1.5 hours longer than usual -as one could expect from the temperature decrease that the sample suffered when taken out of the incubator. It has been later checked in video recordings over several cycles inside an incubator that the cycle duration does not depend on cycle ordinal.

The mean duration of a cycle was of 6.5 ± 0.1 hours, while growth and consolidation phases accounted for half this time. **The results indicate that the duration of a cycle does not depend significantly on neither nutrient no agar concentration.** The same can be said for the duration of growth phase and consolidation phase. One can also appreciate a slight tendency of cycle duration to shorten when agar concentration increases. The effect of increasing agar concentration, what hardens the surface, is more manifest in the duration of growth phase, which decreases up to a 25%. The increase of consolidation phase (10%) falls within

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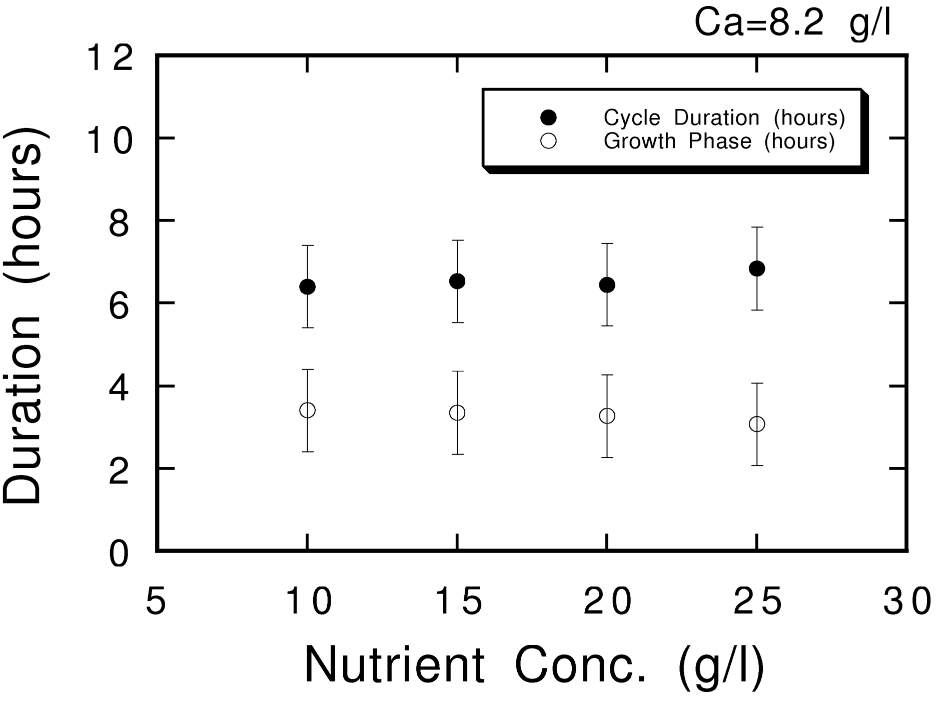
[4] If we had absolutely regular reproduction by division of the cell into two, the population would increase as 2^n.



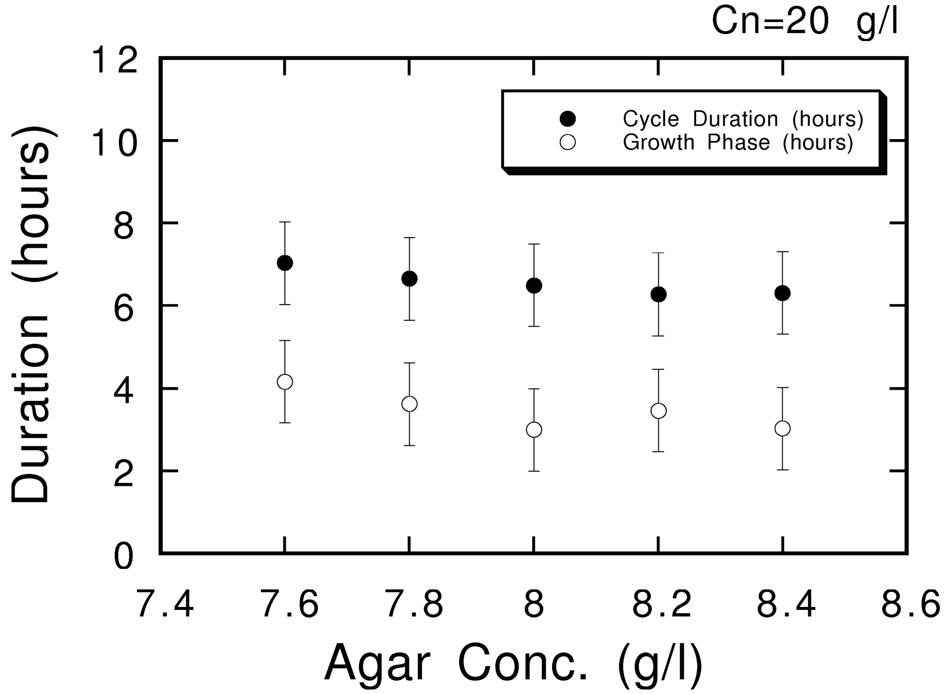


Figure 3.14: Cycle duration (•) and growth phase (∘) versus nutrient and agar concentrations. Each point corresponds to an average over 4 axis x 4 colonies. Neither cycle duration nor growth phase depend on nutrient concentration. Both quantities decrease slightly as agar concentration is increased.

the error bar. In general duration of consolidation phase has less fluctuations than growth phase.

3.4 Determination of ring width

If cycle duration was found to be very constant even when changing the environmental conditions of the plate (except temperature), the size and shapes of the rings displayed by the colonies vary substantially. The ring width is greatly affected by small changes in drying conditions or humidity. Even for plates prepared with the same conditions, the fluctuations of ring size are remarkable.

On the other hand, it was found that the ring width depends on the thickness of the plate: shallow plates (1.5 mm thick) can produce small rings (up to $3-4$ mm wide), but thick plates (3.0 mm thick and more) only display relatively big rings (10 mm wide) or can not produce the ring pattern at all. It is not understood why the thickness of the plate causes such effect. Whatever the mechanism, as it was experimentally convenient to use thin plates so as to measure ring width over a wide range of sizes, we performed the measurements that follow on agar plates with 1.5 mm of thickness, half the thickness of standard experiments (this meant pouring 10 ml into the Petri dish instead of the habitual 20 ml).

Figure 3.15 shows the effect of change of nutrients concentration on the patterns. As it shall be explained in section 3.5, for $C_n \leq 10$ g/l, instead of a proper ring pattern, transitional morphologies appear. For nutrient concentrations just over this threshold (as in figure 3.15a, $C_n = 12$ g/l), the ring pattern can be perfectly distinguished, though in a very branched form. The thin branches that compose the pattern do not cover all the surface. Adding just a little bit more of nutrient ($C_n = 15$ g/l, fig. 3.15b) the colonies come to grow all over the accessible surface, despite a branching canvas is still visible. For higher nutrient concentrations the branches merge and the ultimate colony is nicely round-shaped and dense ($C_n = 25$ g/l, fig. 3.15c). Finally, for very high nutrient concentrations ring size becomes smaller and the perimeter of the ring more irregular ($C_n = 50$ g/l, fig. 3.15d). Despite cell density in the final pattern was not measured, it was seen (even smelt) to increase with nutrients concentration.

Figure 3.16 displays the quantitative dependence of ring width on nutrient concentration. Each point corresponds to the average over 2 axis x 6 (or 9) samples ($C_a = 7.6, 7.7$ g/l). For the first ring it can be seen that ring width has the following behavior:

(i) It increases sharply with nutrient concentration at very low concentrations of nutrients.

(ii) It saturates in a central range of nutrient concentrations.

(iii) It decreases again for very high nutrient concentrations.

As in the second and successive rings the central saturated range is very wide, the **ring width can be considered constant to most effects when varying**

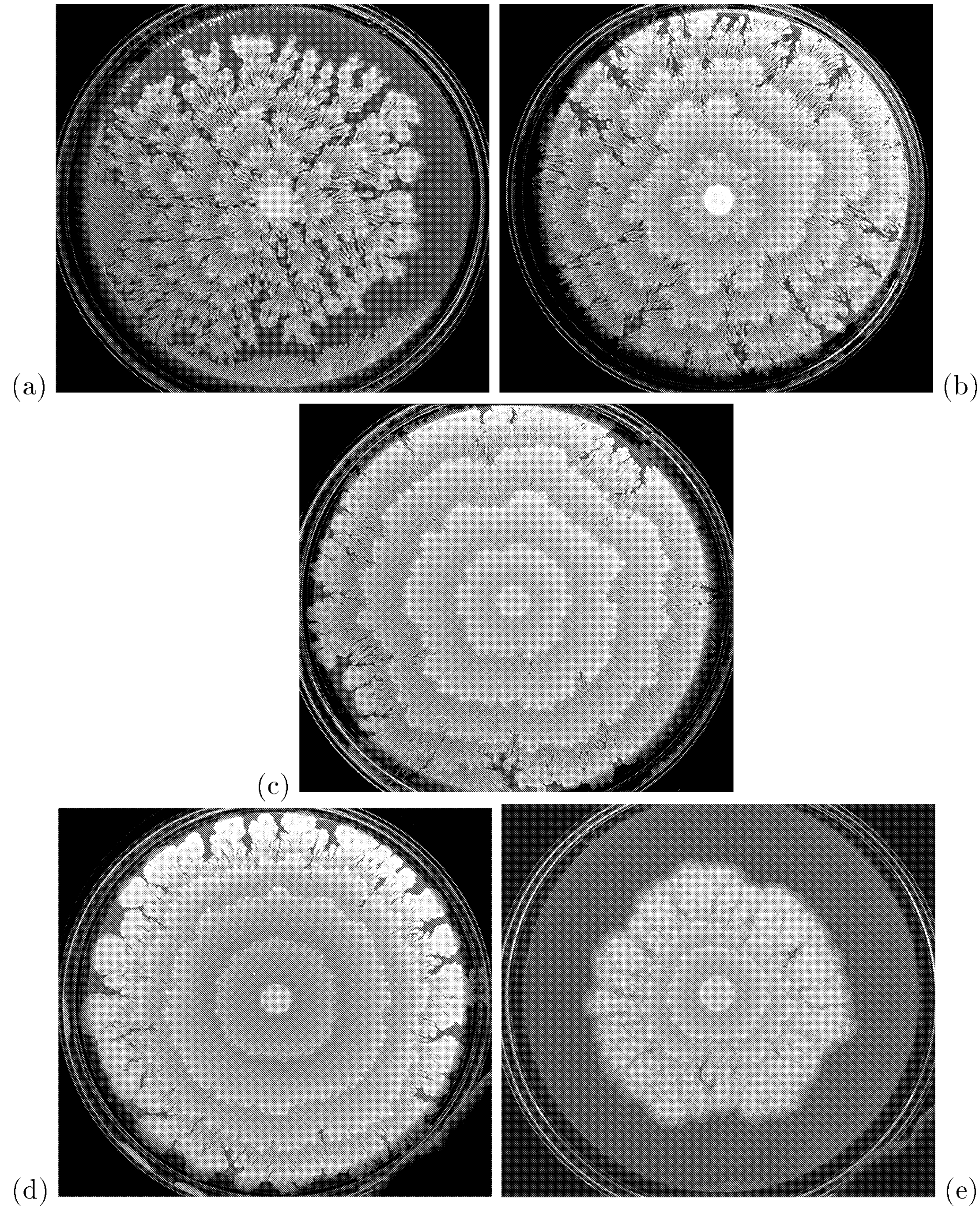


Figure 3.15: Ring patterns at increasing nutrient concentration. Agar concentration is fixed at $C_a = 7.7\ g/l$.**(a)** Nutrient concentration $C_n = 12\ g/l$, the lowest level of nutrient that can develop rings. Very branched patterns develop. **(b)** $C_n = 15\ g/l$, branches are still evident, but now they cover almost all the surface, leaving no void spaces. **(c)** $C_n = 25\ g/l$ and, **(d)** $C_n = 30 g/l$. Cell density is higher, branches are almost completely fused with each other. **(e)** $C_n = 50\ g/l$, cell density keeps increasing, but ring width decreases. Growth seems to become more difficult.

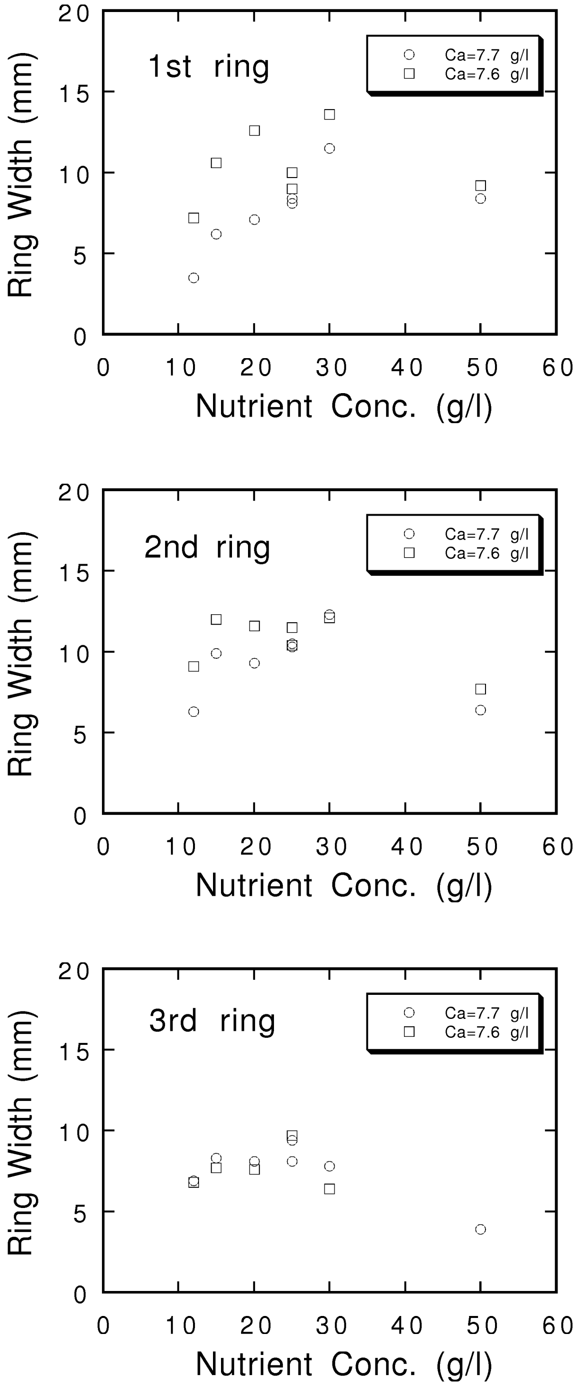


Figure 3.16: Ring width versus nutrient concentration. Agar concentration is fixed to $C_a = 7.6,\ 7.7\ g/l$. Each point corresponds to the averages of 2 axis x 6 (or 9) samples. Error bar is $\pm 1\ mm$. Ring width does not depend significantly on nutrient concentration for 2nd and 3rd rings over a wide range of values. However, for the 1st ring, ring width increases with nutrient concentration. In all cases, an extremely high concentration of nutrients seems to inhibit the expansion of the colony.

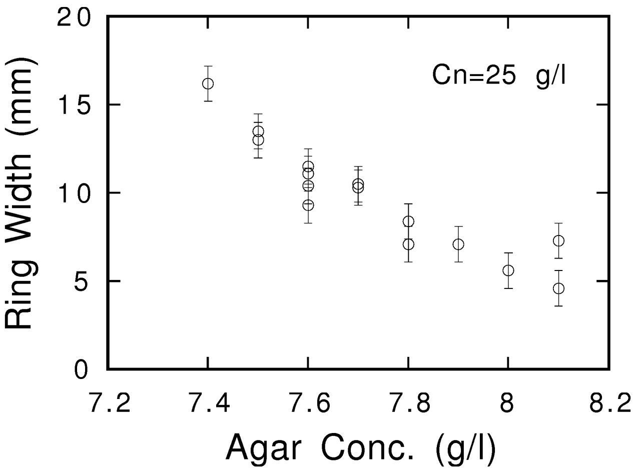


Figure 3.17: Ring width vs. agar concentration. Nutrient concentration is fixed at $C_n = 25\ g/l$. It can be appreciated the dramatic effect that a small change on agar concentration produces. A variation of less than 10% trebles the ring width.

nutrient concentration. Only in very poor or in extremely rich plates does ring width depends on nutrient concentration.

The effect of agar concentration on ring patterns is more drastic. First, let's remember that the ring patterns only appear for a very narrow range of agar concentrations. A change of little more that $1\ g/l$ can bring the plates from region D to region B without anybody noticing that a morphology has been "jumped" (see the morphological diagram in figure 2.6). This is the reason why the ring pattern in *B. subtilis* was not discovered until very recently [Fujikawa92]. Figure 3.18 shows colonies grown at different agar concentrations while keeping nutrient concentrations fairly high. Two phenomena can be remarked at mere visual inspection:

(i) At increasing agar concentrations the pattern becomes more irregular and branched.

(ii) Ring width shortens as agar concentration is increased.

Figure 3.17 presents this second observation in a quantitative way. Each point of data corresponds to 2 axis x 6 (or 9) samples ($C_n = 25g/l$). As media are rich, there is no much difference between results for the first and second rings, just a change in the slope of the graph. Third and further rings are supposed to give the same kind of dependence but in practice can not be measured. This is because for soft agar, patterns are so wide that the third ring radius happens to be bigger than the Petri dish; while for high agar concentrations (fig. 3.18(b)), 3rd and later rings are hardly formed as we shall see in section section 3.5. The roughly linear decrease of ring width with agar concentration doesn't indeed provide any further insight on the dynamics of the systems. It is just telling us what we knew: the more agar you have in the plate, the more difficult for bacteria to move on it.

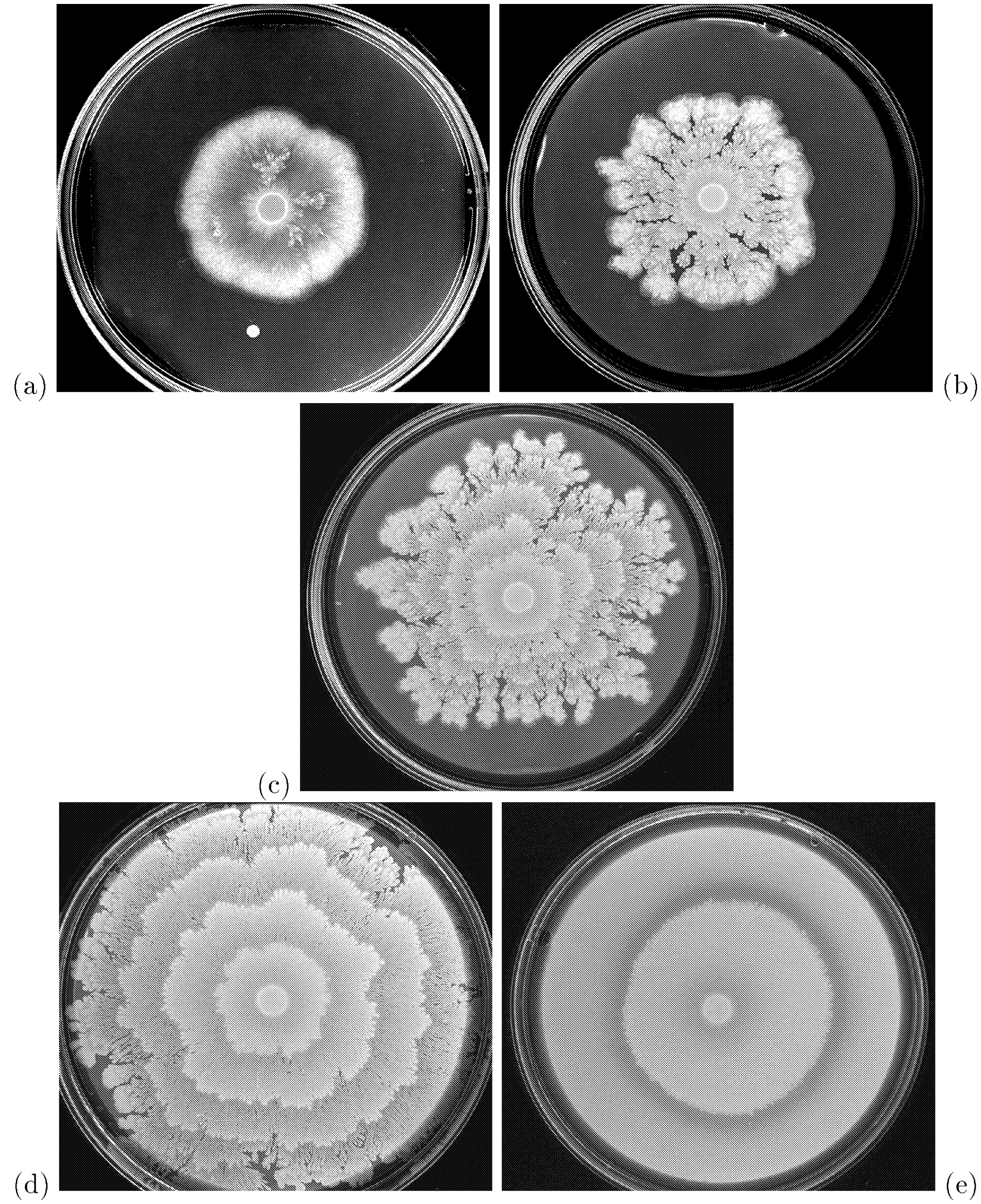


Figure 3.18: Ring patterns at decreasing agar concentrations. Nutrient concentration is fixed at $C_n = 25\ g/l$. **(a)** Agar concentration $C_a = 8.3\ g/l$. When the surface is very solid, only one tentative, often branched ring develops, which is later followed by *gliding*, the slow kind of expansion characteristic of region B. **(b)** $C_a = 8.1\ g/l$ and **(c)** $C_a = 7.9\ g/l$. As agar concentration is decreased, more rings can develop but forming irregular very branched patterns. **(d)** $C_a = 7.7\ g/l$. At intermediate agar concentrations, we find symmetric, "well-behaved" concentric rings. **(e)** $C_a = 7.4\ g/l$. As the medium becomes softer, branches tend to fuse and the rings become wider. Internal waves are often observed.

3.5 C region boundaries

B. subtilis colonies grow in the shape of clearly distinguishable concentric ring patterns for nutrient concentrations C_n such that $C_n \geq 10g/l$ and agar concentrations C_a such that $8.5\ g/l < C_a < 7.5\ g/l$ when following the usual experimental protocol as explained in section 2.2. These vague limits define region C of the morphological diagram as shown in figure 2.6. For nutrient and agar concentrations in the borderland of this region, transitional morphologies are formed. Let us examine the patterns obtained in each of these borderlands.

In the lower part of the region C, for very low agar concentrations, colony can grow in the two distinct ways:

(i) As a branched structure that looks like a crossover between DLA-like and DBM colonies, but whose branches grow periodicly by means of *swimming.*

(ii) As a dense rounded colony that grows very slowly by means of *gliding*, exactly as in region B.

These two kinds of growth can be sometimes observed in the same pattern, as in figure 3.19. This is reminiscent of a coexistence phase of a first order-phase transition, in the sense that the colony patterns change dramatically at a given set of points of the morphological diagram.

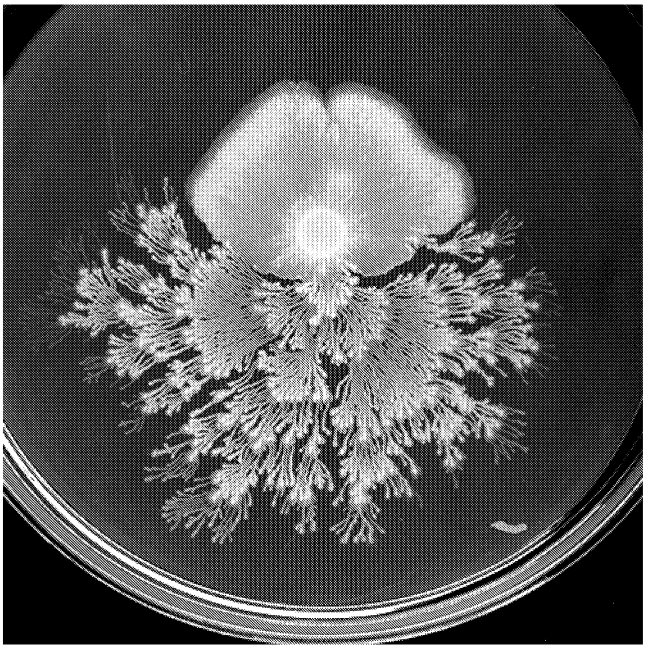


Figure 3.19: A transitional morphology between region C and regions A, B and E. Obtained for intermediate agar and poor nutrient concentrations. $C_a = 7.6g/l$, $C_n = 12g/l$. Half the pattern develops a dense disk morphology (region B), while the other half presents a ring pattern that is reminiscent of DLA-like and DBM patterns.

As opposite to this sharp transition, the borderland corresponding to high agar concentrations displays a crossover between ring pattern and dense disk morphology. As C_a is increased, it comes a moment when the colony can only develop two or three tentative and quite branched rings, to fall later into the kind of slow growth typical of region B, *i.e.*, *gliding* (see figure 3.18b). Higher agar concentrations may only allow a very narrow ring that is soon overcome by the dense disk typical of region B (see figure 3.18a).

On the other side, in the limit of low agar concentrations, the rings grow so big that the colony covers all the Petri dish during the 1st growth phase. This means that for a certain range of agar concentrations, we cannot discriminate whether we are dealing with region C (and observing the inner part of a would-be very big ring), or with region D, where the colony just expands monotonously. One might indeed wonder whether region D really exists or in just an artifact of our finite-size system -meaning that bigger agar plates would allow us to see bigger rings, *ad infinitum*. As a matter of fact, we can find bigger rings in bigger plates, but this only holds up to a certain scale. There seems to be a maximum ring width. We have have never obtained rings larger than 60 mm. Moreover, we carried out experiments using long plates ($14 \times 26\ mm^2$) were continuous expansions of the colony were found to grow at constant speed of 13 $mm/hour$ for up 10 hours.[5] This corroborates our previous assumption that region C and region D correspond to two different dynamics and morphologies.

3.6 Other observations

Some other experiments have been pursued or are still being pursued in order to gain insight into the periodic growth mechanism(s), but their results are not so clear or not so relevant. In this section I will briefly describe some of them.

3.6.1 Two inocula experiments

Experiments were performed inoculating two or more drops of suspension on the agar plate. Depending on nutrient and agar concentration and distance between the inocula two main results were found as exemplified in figure 3.20):

(i) The colonies collide and fuse to become just one oval-shaped big colony that keeps growing with the expected spatial and (allegedly) temporal periodicity.

(ii) The colonies stop their growth in the direction of collision when are close to each the other. On the other directions, though, the growth also keeps the expected periodicity.

Although the experiments where not carried out systematicly, we found out that collision of colonies as explained in (i) is often observed for good growth conditions (soft agar and high nutrient concentrations) within region C, while the non-collision

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[5] The experiments were done by taking the agar plates out of the incubator once every hour; this is why velocity was slower than usual.

in (ii) is observed for worse environment (harder agar and less nutrients). The closer the inocula, the more likelier are the colonies to fuse.

The fact that colonies can prevent collision points out that they *feel* each other somehow -most probably by means of some diffusion field (such as nutrient concentration or chemotactic signalling). But since in either the collision or the non-collision case, the shape of rings was not substantially changed, one may think that indeed diffusion fields exert an influence, but only locally. In other words: if the nutrient or chemotactic chemicals diffusion were to be the main underlying mechanism the periodic growth of the colony, one might expect the ring to show an anomaly (wider, shorter, strange rings) in the region between the two colonies. Such an anomaly is not observed for ring patterns -while it has been clearly observed, for example, in the collision of DLA-like colonies, in figure 2.9. From this one could conclude that diffusional fields play a secondary role in ring pattern formation. Unfortunately, experimental results have not been clear enough as to assert with certainty.

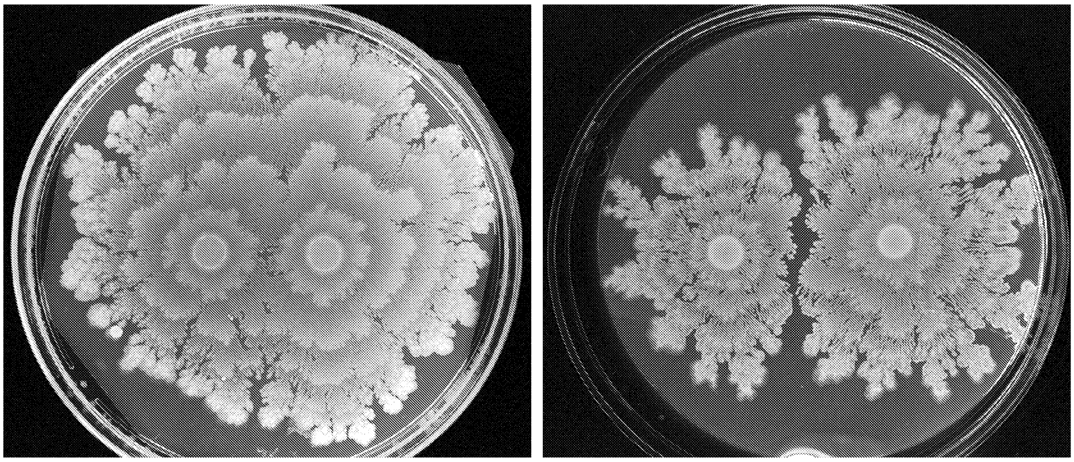

Figure 3.20: Collision of two concentric ring patterns. (Left) A case showing collision plus fusion of the colonies into a new pattern. $C_a = 7.7\ g/l$, $C_n = 25\ g/l$. (Right) A case were colonies prevent collision. $C_a = 7.8\ g/l$, $C_n = 15\ g/l$. Mark that in both experiments the spatial periodicity of rings was preserved in spite of the neighboring colony.

3.6.2 Re-inoculation experiments

In order to check if the periodicity displayed by the pattern is due to a biological clock that is intrinsic of the bacterial cell, the following experiment have been carried out. One agar plate was cultivated as usual so as to generate a ring pattern. Then at different phases of the growth bacteria from this plate would be taken and re-inoculated in a new, fresh plate. If bacteria did continue in the latter plate the same phase as in the former plate, this would mean that the cyclic growth is not determined by the environment but by a cycle intrinsic of the bacterial cell, a "biological clock".

Results showed that when the amount of bacteria transported into the new plate is low, the colony can not expand, independently of the phase of growth in which the former colony is. It requires a lag phase, as in the usual inoculation (but now shorter), before a new phase growth is begun. Thus, one concludes that the ring colony formation is only possible for cell density above a given threshold.

In further experiments, bacteria have been re-inoculated in large amounts. To do so, a big region of the agar plate is swept till a drop of bacteria is formed. This drop contains not only bacteria but also the solution of chemicals that bacteria secrete. About 3 μl of the drop are sucked with a micro-pipette and inoculated in a fresh agar plate. After doing so, an interface (supposedly of chemicals) is always observed to propagate in front of the interface of bacteria (this interface can be seen in other patterns; see for example figure 2.15).

When re-inoculating bacteria from inner parts of the previous colony, *i.e.*, bacteria that were on regions at least one cycle *old* and had a short length, a lag phase was found to be necessary before the new colony could expand. The duration of this lag phase turned out to increase for bacteria from inner parts of the former colony (which have probably begun some kind of sporulation process), but was always shorter than the typical lag phase from a suspension of spores.

Re-inoculation of large amounts of bacteria taken from the outer part of an expanding colony, resulted in the continuation of the growth phase in the new agar plate. We have thus a growth phase that is initially spent in the former plate and finished in the latter. If the total duration of this disrupted growth phase turned out to be independent of environment, one might argue that the periodicity is caused by a mechanism intrinsic to the bacterial cell and unrelated to the environment, a "biological clock". Unfortunately is has not been possible to measure such a duration accurately yet.

3.7 A summary of experimental results

phase	duration	interface velocity	motility	cell length	cell density
lag phase	9.2 ± 0.2 *hours*	$0.0 - 0.2$ *mm/hour*	gliding	No data	High
growth phase	2.3 ± 0.2 *hours*	$2 - 20$ *mm/hour*	swimming	$4 - 12$ μm	Low
consol. phase	2.8 ± 0.2 *hours*	$0.0 - 0.2$ *mm/hour*	gliding	$1.5 - 2.0$ μm	High

Table 3.1: Characteristics of ring pattern cycles. Duration and velocity taken from time-lapse recordings inside a transparent incubator. Other data, as specified in preceding sections.

This research has found the concentric ring patterns formed by *B. subtilis* colonies to have the following characteristics:

1. Formation of concentric rings is due to the **cyclic dynamics** of the colony growth. The cycle is composed of a **growth phase** characterized by a fast expansion of the colony, plus a **consolidation phase** during which the diameter of the colony is constant but cell density increases sharply. Growth and phase durations amount to approximately half the cycle each.

2. Bacterial cells are motile and long ($4 - 12\ \mu m$) during growth phase, but become immotile and progressively shorter during the consolidation phase until they reach a minimum length of $1.5 - 2.0\ \mu m$. Thus, the cyclic dynamics is associated with a **cell differentiation process**.

3. **Cycle duration does not depend significantly on neither agar or nutrient concentrations.** At higher agar concentrations growth phase becomes slightly shorter what is mainly compensated by a longer consolidation phase.

4. **Ring width decreases dramatically as agar concentration is increased. It is not significantly affected though, by nutrient concentration** over a wide range of concentrations.

The experimental study has allowed to characterize the ring patterns of both at a microscopic and a macroscopic level. A clear picture of the mechanism(s) beneath the periodicity is not yet apparent, though. Further interpretation of the results will be discussed later in chapter 4 along with the presentation of theoretical models.

Chapter 4

In search of an answer

Since experimental inquiry did not yield a clear explanation on the main question that bother us (the origin of the periodicity in ring patterns) we shall go for a trip into other systems and theoretical insights in search of an answer. First, we shall review some phenomena displaying concentric ring patterns (section 4.1), in the hope that by analogy we can learn something of our system. Then we shall discuss models aimed at describing bacterial colonies on agar.

4.1 Other concentric ring patterns

4.1.1 Liesegang's rings

It has been known for almost a century that in precipitation systems, periodic bands -the so-called Liesegang bands- are often observed [Henisch88, Kai95, Chopard94]. In a circular geometry these bands form a concentric ring structure, each ring or band corresponding to a multidispersed precipitation, *i.e.* to the formation of many small crystals. It is first observed that the center position x_n of the nth band is related to the time t_n of its formation through the so-called time-law $x_n \sim \sqrt{t_n}$. Second, the ratio $p_n \equiv x_n/x_{n-1}$ of the position of two consecutive bands approaches a constant value p for large enough n. Finally, the width w_n of the nth band is an increasing function of n.

There is no consensus about how to explain the appearance of Liesegang rings or bands. The so-called supersaturation theory is the most widely accepted model. The idea goes as follows: we have initially two chemical species A and B that react to produce a third species C. C represents a colloidal state which is observed in many experiments. When the local concentration of C reaches some threshold value, nucleation occurs: C particles precipitate and become D particles at rest. Now in the experiments, bands are thought to appear as species A diffuses into a gel solution that contains species B.

Graphically, the chemical reactions are:

$$\begin{aligned} A + B &\rightarrow C \\ C &\rightarrow D \end{aligned}$$

what results in the following reaction diffusion equations:

$$\begin{aligned}\frac{\partial a}{\partial t} &= D_a\nabla^2 a - R_{ab}\\ \frac{\partial b}{\partial t} &= D_b\nabla^2 b - R_{ab}\\ \frac{\partial c}{\partial t} &= D_c\nabla^2 n + R_{ab} - n_c\\ \frac{\partial d}{\partial t} &= n_c\end{aligned}$$

where a, b, c and d stand for the concentration at time t and position $\vec{r}$ of the A, B, C and D species. The term R_{ab} expresses the production of C from A, B. n_c describes the depletion of the C species resulting form the nucleation.

As we shall see in 4.2, bacterial colonies can be studied using a similar mathematical approach, although the dynamics is intrinsically different. In the formation of Liesegang bands the main reaction progresses unhindered throughout the full growth period because A and B keep diffusing. Here the rings are formed by the precipitation after nucleation of product C, which is does not affect reactants A and B. In other words, the precipitation is discountinous but the chemical reaction is not. However, in bacterial colonies ring patterns, the main process (*i.e.*, the spreading of bacterial cell density) has an oscillation in itself.

4.1.2 Electrochemical deposition

A ring morphology in electrodeposition of silver (Ag^+) in air/water interface has been reported very recently by Zeiri *et al.* [Zeiri97]. The system consists of a glass beaker with a silver wire cathode, the tip of which is positioned at the electrolyte/air interface at the cell's center. The anode is an aluminum ring along the beaker's wall. The standard electrolyte is an $0.05M$ $AgNO_3$ aqueous solution. The difference with most of previous studies of electrodeposition is that they do not use a thin cell geomettry, what allows a 3rd dimension. The parameters of the system are the ionic strength $\mathcal{I}$ and the applied voltage V. As these parameters are changed, some different morphologies are formed.

Two kinds of deposition are observed: (i) quasi-2D deposits ($20-80$ nm thick) with a smooth shiny surface and fast radial growth rate (~1 mm/s); (ii) 3D deposits ($100-200$ nm thick) with a black rough appearance and a slow radial growth rate (~0.1 mm/s). No compositional differences are found between between shiny and black deposits. For a fixed ionic strength $\mathcal{I}$, ramified structures preserving the circular envelope are found for low voltages, with shiny deposits. As voltage is increased, a concentric ring pattern is formed: narrow black 3D rings alternate with wide, thin and shiny 2D rings. The separation between rings (ring width) decreases both as the voltage or ionic strength are increased. The appearance of this bimodal structure implies an oscillatory growth mechanism that is nor fed to the system, nor measured in the current consumed by the electrolyte. The whole morphological diagram of this system is quite similar to the morphological diagram of *B. subtilis* (figure 2.6).

Zeiri *et al.* propose that the H_2 produced in electrolysis[1] is responsible to the

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[1] Water has a certain density of H^+ cations that also reacts with the cathode producing H_2.

periodic growth. The argument goes as follows. When electrolysis begins, growth is fast and shiny deposits are formed. As electrolysis proceeds, H_2 adsorbs into tiny bubbles which eventually reduce the area available for Ag^+ deposition. When this happens, the growth rate slows down and the black and rough deposit is formed. After a while, though, H_2 bubbles become so big that they detach collectively from the surface (because the stirring of one produces a domino effect). This leaves the whole interface free for Ag^+ to react; the fast growth begins again. In other words: the growth begins with a bubble free interface that expands fast (shiny deposit), but as more bubbles are adsorbed, growth becomes slow (dark deposit). At a given critical size the bubbles detach and the fast growth (shiny deposit) is resumed. It is found experimentally that high voltage V and ionic strength $\mathcal{I}$ are accompanied by strong H_2 evolution and dark deposit. I think this kind of dynamics can be classified as that of an activator-inhibitor type [Koch94].

Let me stress that the finding of these concentric ring patterns in this electrodeposition system is of the utmost relevance for our research. Not only the pattern is reminiscent, but also the periodic growth dynamics and the whole morphological diagram contain many elements in common with those of *B. subtilis* colonies.

4.1.3 Ring patterns in colonies of *Proteus mirabilis*

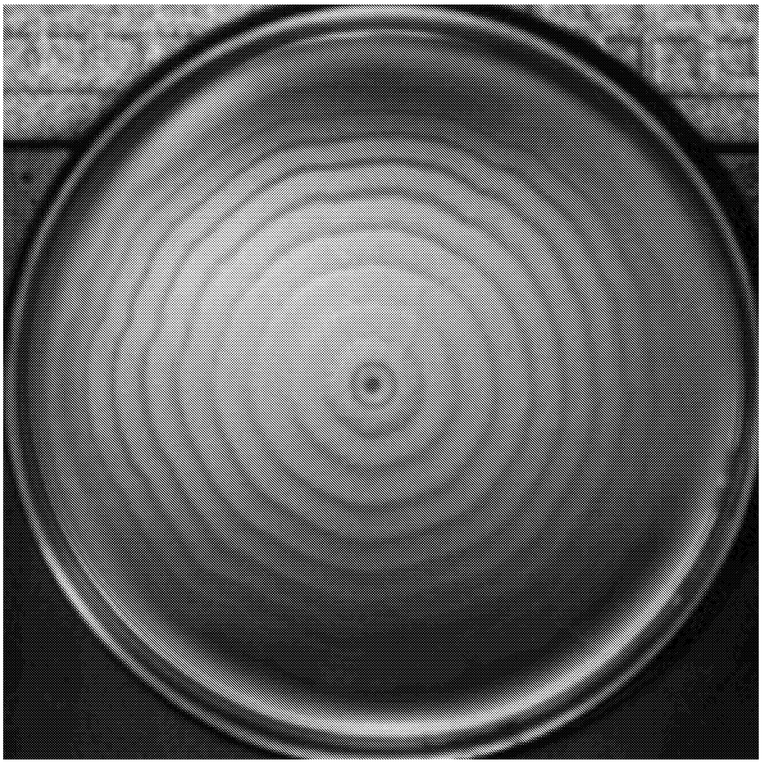

Figure 4.1: Ring pattern in *Proteus mirabilis* colonies. Courtesy of H. Itoh.

While concentric ring patterns formed by *B. subtilis* were only found recently because they occur for a very limited range of agar concentrations, concentric ring patterns produced by the bacterial species *Proteus mirabilis* have been known for more than a century [Hauser1885, Shapiro91, Belas97]. They are formed under very solid agar conditions ($C_a = 18 \sim 32 g/l$), and are much more regular and stable than those of *B. subtilis* [Nakahara96]. Moreover, they do not require such careful cultivation -for example they do not need the control in humidity that is so important for *B. subtilis* ring patterns. If in the case of *B. subtilis* the ring pattern happens to be the least probable of the morphologies, for *P. mirabilis*, ring pattern is the most usual morphology. This fact has a microbiological reason, that has been profusely investigated:

The odyssey of Proteus

> *Proteus mirabilis is a motile gram-negative bacterium, similar in morphology and physiology to other members of the family Enterobacteriaceae, such as Escherichia coli.(...) Members of the genus are found in soil, water, and the human intestinal tract. P. mirabilis was aptly named by Hauser in 1885 for the character of Homer's Odyssey who "has the power of assuming different shapes in order to escape being questioned." (...) When grown in suitable liquid media P. mirabilis exists as a* $1.5 - 2.0$ *μm motile cell with 6 to 10 peritrichous flagella. These bacteria, referred to as swimmer cells, display characteristic swimming and chemotactic behavior (...). However, a dramatic change takes place when cells grown in liquid are transferred to a nutrient medium solidified with agar. Shortly after encountering the agar surface the cells begin to elongate- the first step in the production of a morphologically and biochemically differentiated cell.*
>
> Robert Belas, from reference [Belas92].

The differentiation process of *P. mirabilis* is astonishing: with only a slight increase in cell width, it generates cells with up to $60 - 80$ *μm* body length (this is 30-40 the original size), hundreds of flagella, but the same ratio of DNA. The result of this surface-induced differentiation is a so-called swarmer cell, which has a unique ability to move over solid media by means of a type of surface translocation known as *swarming* motility (see section 2.1). As explained before (see section 2.1), *swarming* is a kind of collective migration in which the cells advance by moving swiftly on the bodies of the neighboring cells by the action of flagella. Eventually, though, swarmer cells divede and form short-immotile called *swimmers*. Thus *P. mirabilis* presents two kinds of cells: the so-called *swarmers* that are long-motile cells, and the so-called *swimmers*[2] that are short-immotile cells.

The formation of the ring pattern of *P. mirabilis* is found to evolve very much in the same way displayed by *B. subtilis* [Rauprich96].

Lag phase: After inoculation, cell density increases (probably exponentially) but

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[2] The name *swimmer* is misleading. *Swimmer* cells perform swimming (random-walk) in liquid media, but they are not motile on solid agar surface.

the envelope of the inoculum does not expand. As density reaches a threshold, rafts of swarmer cells break the envelope marking the beginning of the swarming phase.

Swarming phase: [3] During this phase the colony expands by means of very thin branches of swarmer cells that grow radially. If finishes when the advance of the colony suddenly stops.

Consolidation phase: Since the moment that colony expansion ceases, swarmer cells start dividing to become swimmer cells, while density increases exponentially. After a given density threshold is reached, new branches break out the envelope and a new swarming phase begins. The cycle repeats.

The main qualitative difference between *P. mirabilis* and *B. subtilis* ring patterns is the sort of active bacterial movement that they display. While in *P. mirabilis* is highly cooperative (swarming) in *B. subtilis* is more like a random walk (swimming). The reason for this difference may be that while *B. subtilis* motile cells are only $4 - 8$ μm long, *P. mirabilis* motile cells are usually $10 - 20$ μm long. This also may explains ring patterns in *P. mirabilis* are much more common and stable.

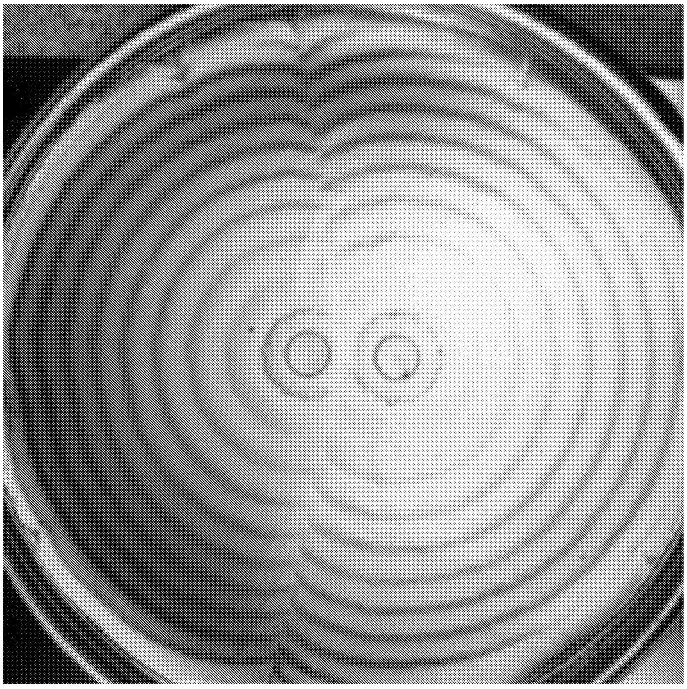

Figure 4.2: Collision of two *P. mirabilis* colonies. The colony on the left was inoculated 2 hours after the colony on the right (a delay of half a period). As it can be observed in the line separating the two colonies, the colonies grow alternatively, maintaining the difference of phase. This phenomenon can not be explained with a growth dynamics determined by a diffusion field. Courtesy of H. Itoh.

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[3] Equivalent to the growth phase defined in the study of *B. subtilis*, see section 3.1.

Apart from the type of active migration, when confronting our (rather scarce) characterization of *B. subtilis* ring patterns to the extensive characterization of *P. mirabilis* by Rauprich *et al.* [Rauprich96], there is an overwhelming qualitative agreement on the results:

i. **The periodicity of colony growth is associated with a differentiation-dedifferentiation** process between long-motile cells (in growth phase) and short-immotile cells (in consolidation phase).[4]

ii. **Cycle duration does not change significantly upon change of nutrient or agar concentration**. At higher agar concentrations, growth/swarming phase becomes longer, what is compensated by a longer consolidation phase.

iii. **Ring width decreases dramatically as agar concentration is increased.** Nutrient concentration affects the final cell density of the pattern, but hardly the pattern itself.

Neither in the case of *P. mirabilis* the mechanism generating the periodicity has been unveiled. Attempts to explain it date back to the Moltke (1929), who thought nutrient depletion caused the periodicity [Moltke29], and Lominski (1947) who proposed negative chemotaxis and supported it with some experimental observations [Lominski47]. But experimental evidence of the opposite can also be claimed: when inoculating two neighbouring colonies with a phase shift (which means that one colony begins the swarming phase when the other finishes it), no coherence of phases is seen (see figure 4.2). If the growth were determined by some diffusive field, the colliding parts of the colonies should show coherence of phase. Esipov and Shapiro [Esipov98] have recently proposed a model based on bacterial cell age and non-linear diffusion that we shall review later in section 4.2.3.

4.2 Modeling bacterial colonies

In this section I shall present some models that have been proposed to explain the formation of bacterial colonies on agar surfaces and, in particular, the appearance of concentric ring patterns in *B. subtilis* colonies. Again, I think that the interest of these models lies not only in their attempts to solve our *particular puzzle.* They illustrate in a general way how researchers of apparently unrelated disciplines deal with a wide span of problems, ranging from growth of crystals to dynamics of ecological populations.

I will try to carry on the description of the different models within one same notational and discursive framework. This can result in some -I hope minor- infidelities to the original sources. I apologize in advance. The soundness or weaknesses of each model assumptions and results will be confronted to the experimental observations presented in chapters 2 and 3. I sometimes take a rather critic viewpoint, perhaps

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[4] Experiments performed in parallel to those displayed in section 3.2, have shown that the mean length of *P. mirabilis* decays exponentially during the consolidation phase, just as in *B. subtilis* (T. Matsuyama and Y. Takagi, personal communication).

excessively critic. My admiration for the theoretical models and their proponents goes without saying. Discussion is just meant to stir sharper answers. As J. D. Murray, the very scientist who has written the *Bible* of mathematical biology[5], says with edifying humility:

> **Models in biological sciences**
>
> *If the use of a model stimulates experiments -even if the model is subsequently shown to be wrong- then it has been successful. Models can provide biological insight and be very useful in summarizing, interpreting and interpolating real data. I hope the reader will also learn that (certainly at this stage) there is usually no 'right' model: producing similar temporal or spatial patterns to those observed experimentally observed is only a first step and does not imply the model mechanism is the one which applies. Mathematical descriptions are not explanations. Mathematics can never provide the complete solution of a biological problem on its own. (...) The mathematics is dictated by the biology and not viceversa.*
>
> From the introduction of *Mathematical biology* [Murray89].

Population dynamics approach

Since bacterial colonies are made out of a vast number of individuals, we can think them as a population and undertake their study from the so-called *population dynamics approach*. First we shall formulate this approach in a continuous space and time. In general, for a population N, the rate of change can be expressed as follows:

$$\frac{dN}{dt} = migration + births - deaths \tag{4.1}$$

This is nothing else but a conservation equation for the population. As for *migration*, we know from experimental observations that bacteria perform a biased random walk when *swimming* on agar. In a continuous description this is equivalent to a non-linear diffusion, which means that the diffusivity D_b depends on the variables of the system. On the other hand, the number of "births and deaths" will also depend on some variables and parameters. The point is to decide which.

In the experiments a wealth of patterns was found when varying two parameters: agar and nutrient concentrations. We saw that less agar concentration made the movement of bacteria easier; thus we can think that agar concentration has an inverse relation with diffusivity, $D_b \sim {C_a}^{-1}$. But since C_a is fixed for a given agar plate, it can be a parameter of the model but not a variable that depends on space and time. Nutrient concentration was also found to have a major effect. In the experiments, we fixed its initial value but once bacteria were inoculated the actual

---

[5] See [Murray89]. As a modern playwright said on Shakespeare -"He wrote it all, the bastard!"-, we could complain on Murray.

nutrient concentration kept changing on time and space. We can take it as a variable while keeping its initial amount as a parameter.

Another obvious variable is the bacterial cell density itself. As described in section 3.2, bacteria of *B. subtilis* colonies can be classified into two main types: long, motile bacteria and short, immotile ones (spores are thought to be formed later from this second type). From now on we shall refer to them as active and inactive respectively, as was first proposed by Ben-Jacob [Ben-Jacob94b]. Other possible variables are the densities of the many chemicals secreted by bacteria - which are sometimes observed preceding the expansion of the colony (see figure 2.15). But for the moment being we shall keep them aside.

Let's state it clearly. We assume that the colony growth can be described using the three following variables:

$n = n(\vec{r}, t)$: Nutrient density.

$b = b(\vec{r}, t)$: Active bacteria density.

$s = s(\vec{r}, t)$: Inactive bacteria density.

Since agar plates are quasi-two-dimensional, $\vec{r}$ will be taken in two dimensions.

4.2.1 Reaction-diffusion models

The mathematical description will consist of equation 4.1 for both active and inactive bacteria plus one analogous equation accounting for the conservation of nutrients. When writing this down one find that it has the aspect of a reaction diffusion coupled equations, as they are often written for dynamical chemical reactions such as the Beloussov-Zhabotinski reaction [Koch94].

$$\frac{\partial n}{\partial t} = D_c \nabla^2 n - f(n, b, s) \tag{4.2}$$

nutrient change = diffusion - consumption

$$\frac{\partial b}{\partial t} = \nabla(D_b(n, b, s)\nabla b) + g_b(n, b) - h(n, b, s) \tag{4.3}$$

active bacteria change = diffusion + reproduction - conversion

$$\frac{\partial s}{\partial t} = g_s(n, s) + h(n, b, s) \tag{4.4}$$

inactive bacteria change = reproduction + conversion

The terms in the equations have the following meaning:

D_n : Diffusion coefficient of nutrients.

$D_b(n, b, s)$: Diffusion coeficient of active bacteria.

$f(n, b, s)$: Nutrient consumption by bacteria.

$g_b(n, b)$, $g_s(n, s)$: Reproduction of active/inactive bacteria.

$h(n, b, s)$: Conversion from active to inactive bacteria.

I have kept the dependence on all variables for all terms (except the trivial constant diffusion of nutrients) just for the sake of generality. As we shall see, the models that have actually been developed take simplifying assumptions.To begin with, they all assume that inactive bacteria can not perform reproduction, do not consume nutrients and can not become active again. These are thought to be plausible assumptions because inactive bacteria are usually in inner regions of the pattern (with less nutrients) and are never responsible for the expansion of the colony. We shall argue later that this is a major simplification -experimentally it is often observed that cell density keeps increasing in inner parts of the colony inhabited by inactive cells.

Having wiped out in a single stroke the influence of inactive bacteria s, it is easier to propose analytical forms for the functions in equations 4.2, 4.3 and 4.4.

Nutrient consumption: f(n,b)

Nutrient consumption is assumed to have the standard Michaelis-Menten kinetics (see chapter 5 of [Murray89]), this is:

$$f(n, b) = \frac{\kappa}{1 + \gamma n} nb \tag{4.5}$$

If γ is small versus nutrient concentration, then $f(n, b) \simeq \kappa nb$, which is the form most widely used (in one particular case it has been tested that γ has very limited effect [Kawasaki97]).

Reproduction: g(n,b)

It is assumed that reproduction rate is proportional to nutrient consumption rate.

$$g(n, b) = \theta f(n, b) \simeq \theta \kappa nb \tag{4.6}$$

Active - inactive conversion: h(n,b)

Conversion from active to inactive bacteria ($b \rightarrow s$) is taken to be proportional to the cell density of active bacteria. Inverse conversion ($s \rightarrow b$) is not considered.

$$h(n, b) = \mu(n, b) b \tag{4.7}$$

General framework of reaction-diffusion models

Upon imposing these functions on equations 4.2, 4.3 and 4.4, a general framework on which many reaction-diffusion models converge can be reached:

$$\frac{\partial n}{\partial t} = \nabla^2 n - nb \tag{4.8}$$

$$\frac{\partial b}{\partial t} = \nabla(D(n,b)\nabla b) + nb - \mu(n,b)b \tag{4.9}$$

$$\frac{\partial s}{\partial t} = \mu(n,b)b \tag{4.10}$$

The equations are written after suitable scaling (see [Kawasaki97] for details). As inactive bacteria s are not coupled into other equations, equation 4.10 can be overlooked. There are only two terms left to be concretely formulated: the diffusivity of active bacteria $D(n,b)$ and the form of the conversion rate term $\mu(n,b)$. For very low nutrient concentrations and small bacterial diffusivity, the equations reduce to a Laplacian for nutrients ($0 = \nabla^2 n$) that should reproduce DLA-like patterns. Unfortunately, no analytical solutions are known for the Laplacion under the boundary conditions that the system imposes, and numerical simulation require a long time.

Fisher's equation

The first proposal to use a populations dynamics approach for *B. subtilis* colonies was done by Wakita *et al.* [Wakita94]. The aim was to explain the homogeneous fast spreading disk found in region D of the morphological diagram (see subsection 2.3.3, figure 2.12), for soft agar and high nutrient concentration. Being the agar very soft, the random-walk of bacteria is a valid assumption and diffusivity of bacteria D_b can be considered to be constant. Since the amount of nutrients is high, it was taken to be constant $n = n_0$, too. As for the conversion rate, it was chosen so as to produce a logistic term, $\mu(n,b) = \mu_0 b$. This yields:

$$\frac{\partial b}{\partial t} = D\nabla^2 b + (n_0 - \mu_0 b)b \tag{4.11}$$

This is known as Fisher's equation and has been profusely studied. It is the classic and simplest case of a nonlinear reaction diffusion equation. It generates isotropic, homogeneous solutions $b = n_0/\mu$ that have stable traveling wavefronts of constant speed $v = 2(n_0 D)^{0.5}$, for a planar front (corrections due to circular symmetry are thought to be small).

Experimentally these results are difficult to check in region D because the agar plates are apparently too small as to fall into the asymptotic regime. It was shown, though, that the framework provided by this reaction diffusion equation is consistent with experimental observations [Wakita96].

Kawasaki's model

A further development was due to Kawasaki *et al.* [Kawasaki97]. In experiments it is observed that bacteria have difficulties to access regions of agar that have not been colonized yet. This can be viewed as a decrease in bacterial diffusivity for low cell density. On the other hand, it is often observed that bacteria move more actively in the outer parts of the colony (where nutrient concentrations are still high). Thus it can be assumed that diffusivity should depend on nutrient concentration. The simplest functional form of such nonlinear diffusivity is:

$$D_b(n,b) = \sigma n b \tag{4.12}$$

where $\sigma = \sigma_0(1+\Delta)$. The value of σ_0 increases as agar concentration decreases, while Δ indicates the stochastic fluctuation of random movement. As now diffusivity can describe the level of activity or inactivity of the bacteria, the term of conversion can be suppressed ($\mu(n,b) = 0$). Applying this to equations 4.8 and 4.9, one obtains:

$$\frac{\partial n}{\partial t} = \nabla^2 n - nb \tag{4.13}$$

$$\frac{\partial b}{\partial t} = D(\nabla \sigma n b \nabla b) + nb \tag{4.14}$$

The model reproduces fairly well the experimental patterns found in region E of the morphological diagram, *i.e.* dense branching morphologies (DBM) and the transition towards homogeneous fast spreading disk (see subsection 2.3.4, figure 2.13). Simulation results are presented in figure 4.3. The reasons why branches appear are the same as explained in the formation of DLA model: a part that stands a little bit our has a bigger probability to grow. In agreement with experiments performed in region E, velocity of the envelope of the colony is constant and each pattern has a characteristic branch width. The model only fails in reproducing a constant ratio between branches and gaps.

Kitsunezaki's model

Another variation of the coupled reaction diffusion equations 4.8 and 4.9 was proposed by Kitsunezaki [Kitsunezaki97]. In this case, the diffusivity is assumed to depend only on cell density, *i.e.* $D(n,b) \propto b^k$, while conversion rate is set constant, as though it was an aging process, $\mu(n,b) = \mu_0$. This results in the following equations:

$$\frac{\partial n}{\partial t} = \nabla^2 n - nb \tag{4.15}$$

$$\frac{\partial b}{\partial t} = D\nabla^2(b^{k+1}) + (n - \mu_0)b \tag{4.16}$$

It was found that for $k < 1$ the growing colony formed a straight interface regardless of the value of the other parameters D and μ. When $k > 1$, the straight interface loses stability either as D decreases or as μ increases, and a branched colony

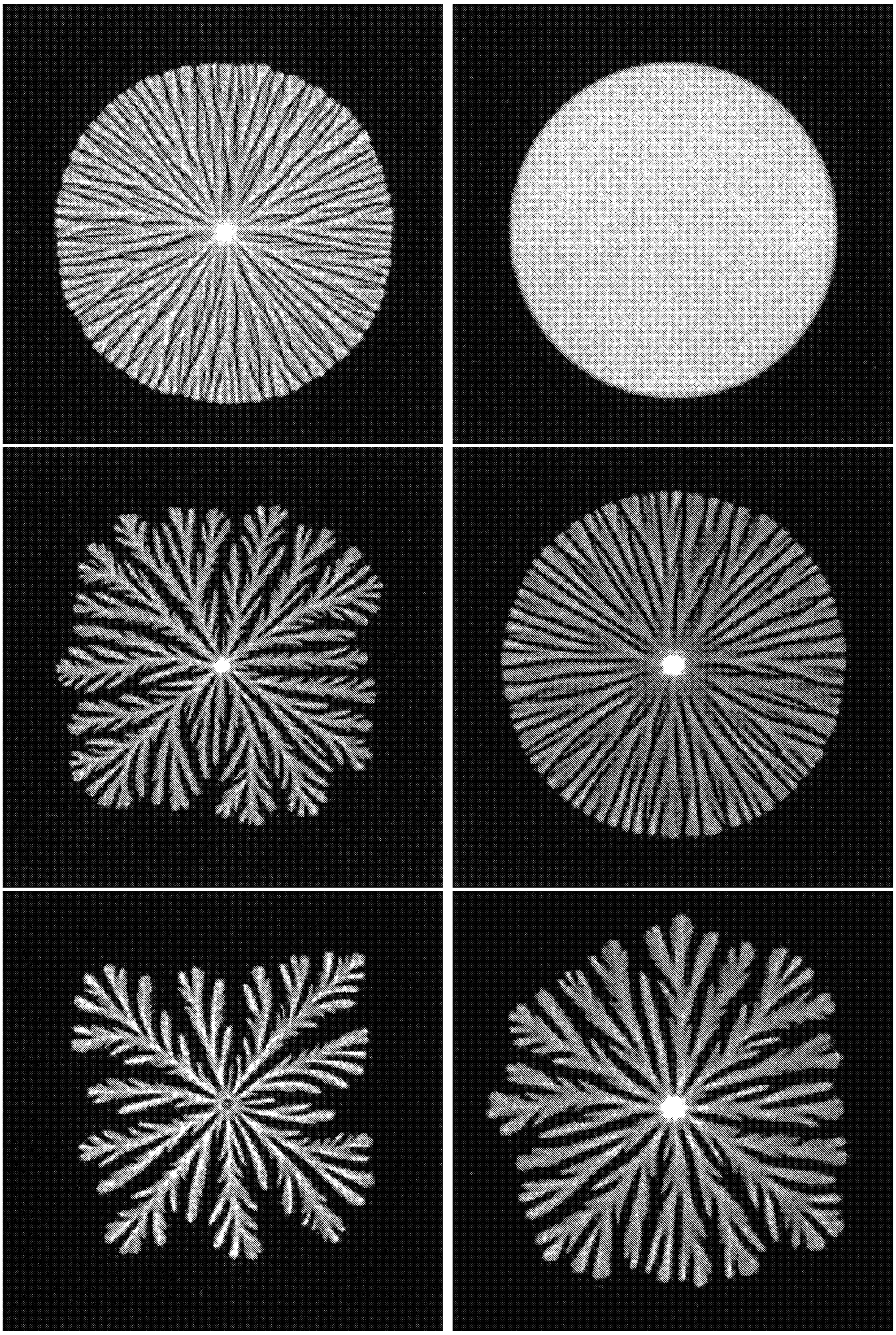

Figure 4.3: Patterns obtained with Kawasaki's model. The parameter associated with the inverse of agar concentration, σ_0, is $\sigma_0 = 1$ (column left), $\sigma_0 = 4$ (column right). Initial nutrient concentration n_0 increases from bottom to top: $n_0 = 0.35$ (bottom row), $n_0 = 0.71$ (middle row), $n = 1.07$ (top row). The tendency towards denser patterns as σ_0 and n_0 increase is in agreement with experimental observations. From reference [Kawasaki97]

is formed. For simplicity simulations were performed at $k = 1$. Again, patterns obtained are remarkably similar to the experimental dense branch morphologies. One important difference with the former models is that now the colony grows by means of of a quasi 1-D traveling wave of active bacteria. The process is somehow analog to a flame burning a paper: white paper would be the nutrients, active bacteria would be the flame and inactive bacteria the remaining ashes.

Mimura's model

Mimura's proposal, although only slightly different from the former, introduces a qualitative difference: this model is capable of producing ring patterns. As this pattern is our main concern in the present study, this model will be discussed in further detail. Diffusivity of bacteria D is taken to be constant. The conversion rate $\mu(n, b)$ is given a particular form that decreases both with cell density and nutrient concentration. This is based on the experimental observation that for low cell and nutrient concentration, bacteria can not move -are inactive. This is:

$$\mu(n, b) = \frac{\mu_0}{(1+n)(1+b)} \tag{4.17}$$

With this kind of dependence the diffusion reaction process happens to become excitable over a certain threshold of nutrient concentration.[6] The final equations after have the following form [7]:

$$\frac{\partial n}{\partial t} = \nabla^2 n - nb \tag{4.18}$$

$$\frac{\partial b}{\partial t} = D\nabla^2 b + \epsilon nb - \frac{\mu_0 b}{(1+n)(1+b)} \tag{4.19}$$

Figure 4.4 presents the patterns that are obtained varying diffusivity D and initial nutrient concentration n_0, with fixed $\mu_0 = 2400$ and $\epsilon = 20$. The patterns reproduce fairly well not only the morphologies found in regions D and E, as Kawasaki's and Kitsunezaki's models did, but also concentric ring pattern and even more branched, DLA-like patterns. The only pattern that can not be produced is the dense disk with rough surface found in region B. This can be solved with another simple assumption: a nonlinear diffusivitiy of the form $D = D_0 b$.

Let's try to understand intuitively how the model generates the concentric ring patterns. The profiles of nutrient concentration, active bacteria and inactive bacteria are shown in figure 4.6. The colony expands by means of a traveling wave of active bacteria. As these bacteria become inactive, they leave behind the final cell density that determines the shape of the pattern. According to the model, when there are few nutrients, the growth rate is limited by their diffusion -this generates tip

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[6] In the study of nonlinear dynamics, a system is said to be excitable, when two points which have very similar initial state, let's say (n_0, b_0) and $(n_0 + \delta, b_0 + \delta)$, have divergent trajectories. An infinitesimal change in the initial state means a completely different trajectory. For more details see, for example, chapter 12 of [Murray89].

[7] Equation 4.19 could as well be scaled as in the preceding models equations to avoid ϵ, but I have kept it to be able to give easily the parameters as used in simulations.

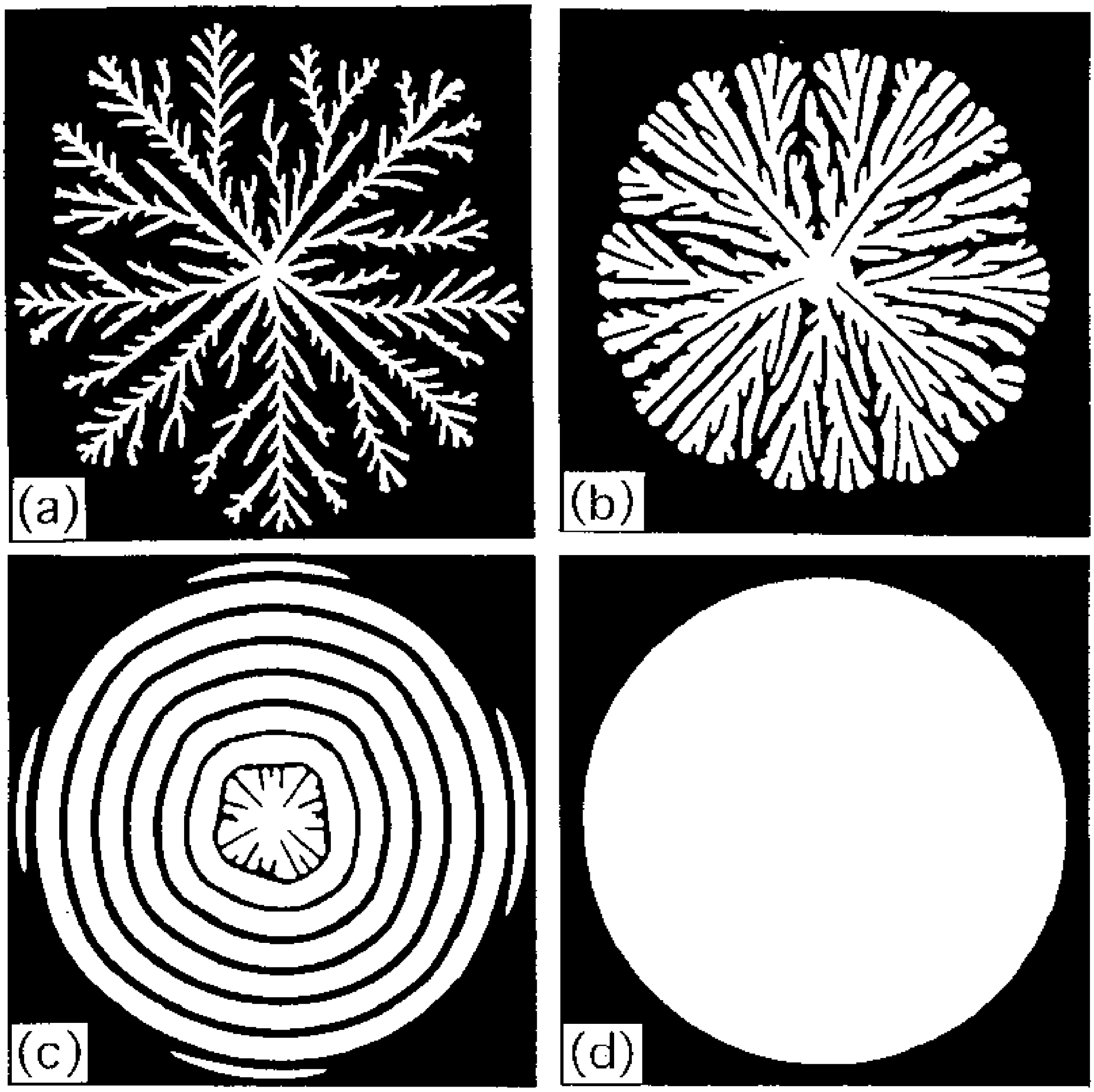

Figure 4.4: Patterns generated the total cell density, $b(\vec{r},t) + s(\vec{r},t)$, using Mimura's model. **(a)** DLA-like pattern ($D = 0.05$, $n_0 = 10.4$). **(b)** Dense branching morphology ($D = 0.12$, $n_0 = 8.45$). **(c)** Concentric ring pattern ($D = 0.05$, $n_0 = 12$) **(d)** Homogeneous disk ($D = 0.12$, $n_0 = 15$). From reference [Matsushita98b]

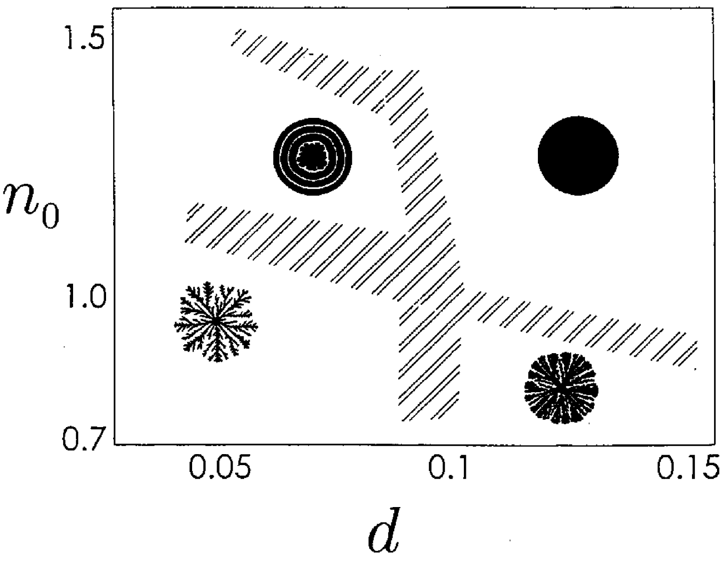

Figure 4.5: Morphological diagram of Mimura's reaction diffusion model. X-axis describes diffusivity of bacteria (broadly equivalent to the inverse of agar concentration in the Petri dish). Y-axis represents initial nutrient concentration. The diagram holds a good resemblance with the experimental morphological diagram (figure 2.6). From reference [Matsushita98b].

splitting. Contrarily, when there are many nutrients, the growth is limited by the diffusion of bacteria. See figure 4.5. Now, in a region of intermediate nutrient concentration, diffusion of nutrients and diffusion of bacteria compete:

i. First, a traveling wave of active bacteria is generated by the abundance of nutrients.

ii. Then, as active bacteria population increases, it consumes more and more nutrients. This causes a decrease in nutrient concentration that triggers the conversion of active bacteria into inactive. Population of active bacteria falls.

iii. As the (now small) traveling wave of active bacteria advances and nutrients diffuse towards the depleted areas, the population of active bacteria finds nutrients again and increases. The cycle begins again.

With this mechanism is can generate successive rings with the same spacing between peaks (same ring width), and the same cycle duration. The first impression is thus extremely encouraging. It really seems the model displays the same dynamics of the experiments. Unfortunately, it has some important pitfalls. The dynamics is not so similar as the remaining pattern may suggest.

a. **The traveling wave of active bacteria expands at a constant velocity.** The spatial and temporal periodicity of the modeled pattern arises because of

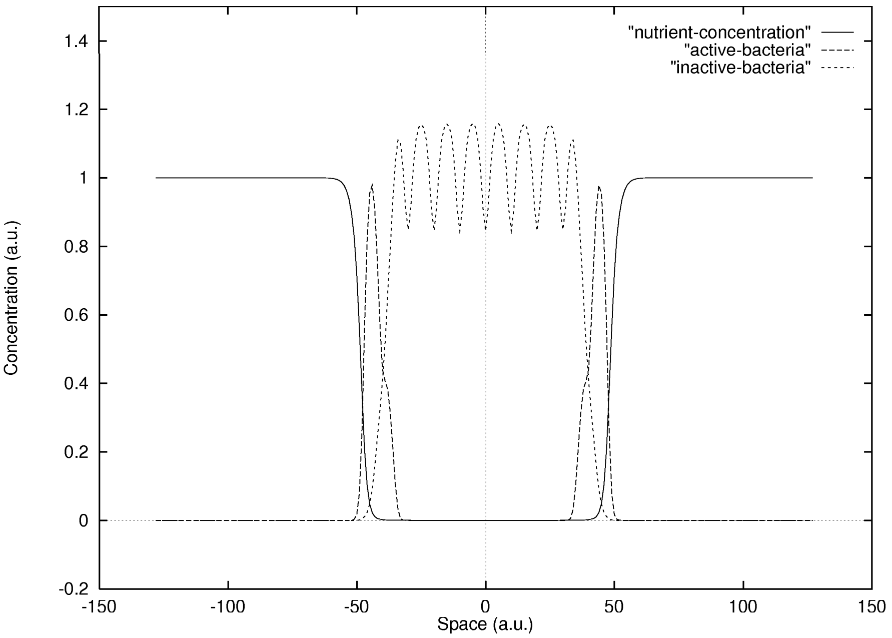

Figure 4.6: Profile of nutrient concentration, active bacteria density and inactive bacteria density in Mimura's model.

oscillations in the size of this wave. **But in the experiments the change of velocity is sharp, very discontinuous** (see figure 3.6).

b. **The duration of cycle duration depends on the parameters** of the system, particularly on the initial nutrient concentration. Cycle duration decreases with n_0 and D. **But in the experiments no significant change in cycle duration is found.**

c. **The front of active bacteria depletes completely the layer of nutrients.** This is the reason why the oscillation in the size of the traveling wave appears. However, micro and macroscopic observations revealed that inactive bacteria can reproduce several hours after the front of active bacteria has passed (see figure 3.7). This means that they have access to nutrients, i.e, **in experiments, nutrients are not completely depleted by the front**.

With these arguments in hand, I think that Mimura's model can not be taken as a satisfactory description of the formation of concentric rings in *B. subtilis* colonies. The description that the model gives to the the oscillating dynamics of the colony is not realistic.

Discussion on reaction diffusion models

As a summary of this subsection devoted to reaction diffusion models based on the description of nutrient and bacteria concentrations, I have drawn the following general conclusions:

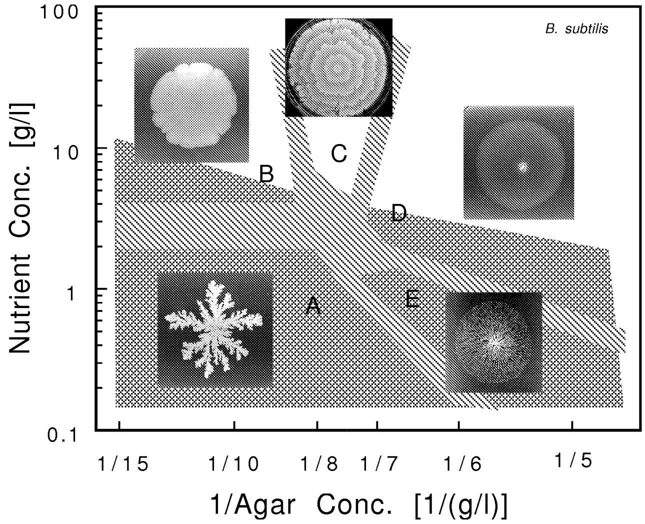

Figure 4.7: In dark, regions of the morphological diagram where reaction diffusion models based on nutrient depletion are thought to apply. They can roughly reproduce most of patterns produced by *B. subtilis* at low nutrient concentrations and the crossover towards round patterns.

a. The models provide a simple and elegant framework to carry out the theoretical study of *B. subtilis* colonies.

b. Kawasaki's, Kitsunezaki's and Mimura's models reproduce and describe roughly well dense branched patterns (regions E) and the crossover towards spreading disks (region D). It is not possible to decide which of them accounts better for experimental observations at the present stage, since we can rely in little more than mere visual comparison between experimental and theoretical patterns.

c. It seems probable, too, that all of them can obtain DLA-like patterns (region A) and the crossover towards dense disks (region B), when scanning the appropriate range of parameters (very small bacterial difussivity), as Mimura's research shown. The problem is that, particularly for DLA-like patterns, this requires a long time of computation.

d. It is my belief that with the present assumptions, the models can not describe realistically bacterial colony growth on nutritiously rich agar media. The reason is that they rely on nutrient depletion to explain the dynamics of the colony interface. Now, for high nutrient concentrations and ($C_n > 10$ g/l), and fast spreading patterns (regions C and D), the cell density keeps increasing for some hours inside the colony after the colony front has passed, what means that nutrients were not completed depleted. In these cases, nutrient concentration should be a parameter (as agar concentration is), but not a variable of the system[8]. To my understanding, this is why ring patterns produced by Mimura's model do not show a dynamics compatible with experimental observations.

I should emphasize that these conclusions are subject to debate and even within our laboratory there is no consensus on the range of applicability and the soundness of the models.

4.2.2 Communicating walkers' model

The first attempts to describe bacterial colony formation were performed in fact by means of cellular automata, rather than by using the elegant but often unfriendly partial differential equations. They are explained after the reaction-diffusion models in order to have a gradual progression on the level of biological detail that models include.

Here I will present the so-called *communicating walkers' model*, proposed by Ben-Jacob, Vicsek and co-workers. It constituted a brilliant breakthrough in the modeling of bacterial colonies [Ben-Jacob94b, Ben-Jacob97]. I think, moreover, that this proposal has a genuine interest in itself as an example of clever modelling of a complex system. It is a hybridization between a continuous approach of partial differential equation (used for nutrients) and the particle-based approach of a lattice gas (used for the bacteria). At a first stage the model includes three generic features: (1) diffusion of nutrients, (2) movement of the bacteria, (3) bacterial metabolism (food consumption, reproduction and sporulation).

1. **Diffusion of nutrients** is handled by solving the diffusion equation for the nutrient concentration $n(\vec{r}, t)$ with a diffusion coefficient D_c, on a triangular lattice.

2. **Movement of bacteria** is simulated by means of particle-like walkers, each walker representing a bunch of bacteria -not an individual cell. The walkers perform an off-lattice random walk within a boundary (in experiments the envelop is always very well defined). If a random walkers hits a segment of the boundary, a counter is increased for that segment. After being hit for N_c times, the segment subsides and the colony expands one site. N_c expresses the difficulties that bacteria have to move to non-colonized areas of the agar plate. Therefore, N_c is related to the agar concentration.

3. **Bacterial metabolism** of walker ith is described by means of an *internal energy* e_i. This energy changes as

$$\frac{de_i}{dt} = n_{cons} - e_{met} \tag{4.20}$$

   where e_{met} is the energy spent in bacterial metabolic processes, and n_{cons} is the consumption rate of nutrients. If there is enough food available, the

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[8] This is indeed the case for Fisher's equation.

walker eats at a constant rate ($n_{cons} = n_c$), or otherwise, it eats what is left ($n_{cons} = n(\vec{r}, t)$). Under good conditions, e_i increases until it reaches a threshold energy e_r, at which time the walker divides into two (reproduction). When a walker is starved for a long time interval of time, e_i drops to zero and the walker becomes inactive. This is meant to represent sporulation.[9] Equation for nutrients finally take the form:

$$\frac{\partial n}{\partial t} = D_n \nabla^2 n - \sigma_{act} n_{cons} \tag{4.21}$$

σ_{act} is the sum over active walkers for a given site.

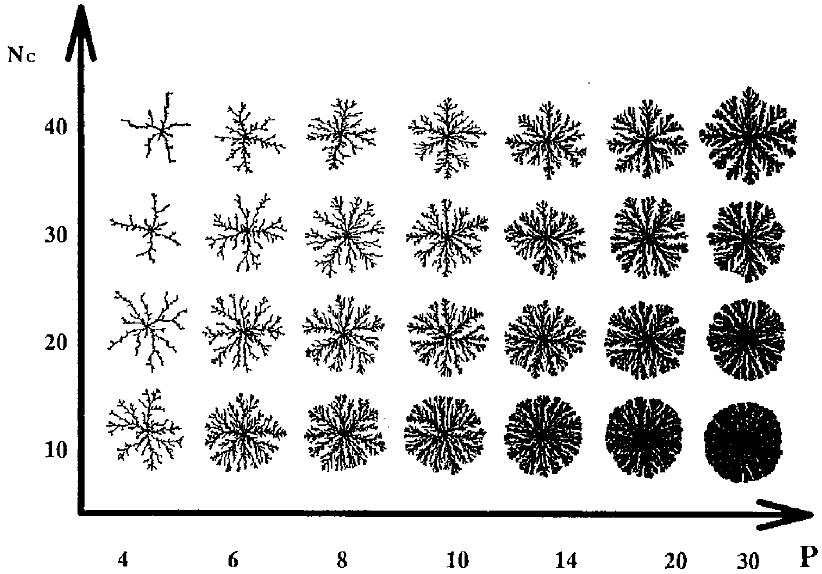

Figure 4.8: Results of the numerical simulations of the communicating walkers' model. X-axis P represents the peptone (nutrient) level. N_c is related to the agar concentration. Mind that the axis are different to Matsushita's group morphological diagram (figure 2.6). Rotate 90° counter-clockwise when comparing. From reference [Ben-Jacob97].

The results of the simulations are shown in figure 4.8. The model is broadly able to reproduce DLA-like patterns (region A) and the crossover towards dense disks (region B). However, it can not generate dense branching morphologies (DBM) at low nutrient concentrations. So as to reproduce this last pattern, Ben-Jacob introduces to his model a term accounting for chemotactic (chemically driven) communication between the bacteria. The communication consists of a "go away that I am here" message (repellent chemotaxis). Each inactive walker produces a repellent chemical $c(\vec{r}, t)$ at a fixed rate c_{prod}, and each of the active walkers decomposes the chemical

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[9] On the basis of the experimental finding of a two different bacterial cell forms (long-motile versus short-immotile), this can also be interpreted as the conversion into the immotile bacterial cell, which is possibly a step before sporulation (since it can still reproduce itself).

at a fixed rate c_{decomp}. The chemical diffuses in the medium, with a diffusion coefficient D_c. The chemorepellent decomposes spontaneously at a λ rate. The equation of the communication field has the following form:

$$\frac{\partial c}{\partial t} = D_c \nabla^2 s - \sigma_{act} c_{decomp} + \sigma_{inac} c_{prod} - \lambda c \tag{4.22}$$

where σ_{inac} is the sum over inactive walkers.

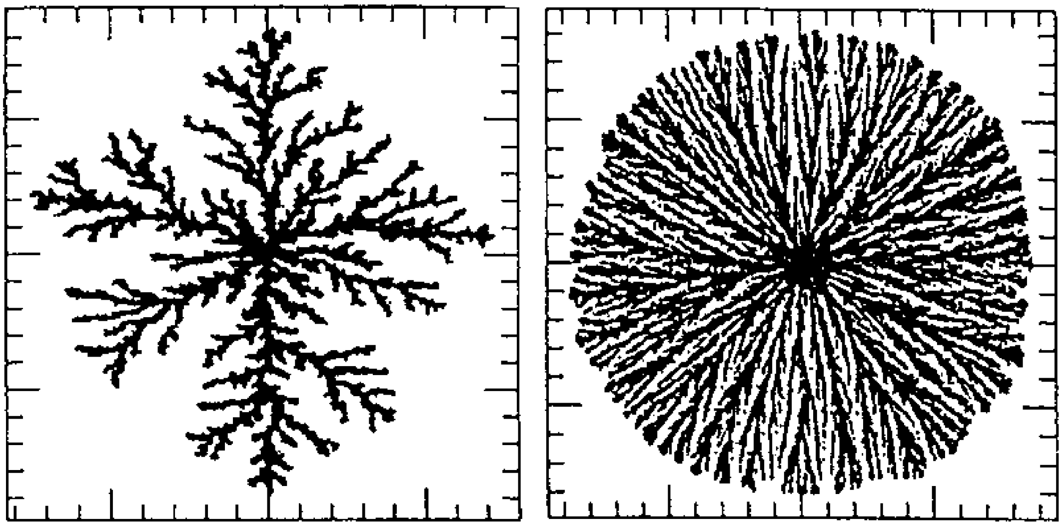

Figure 4.9: Effect of chemotaxis signalling in the communicating walkers' model. (Left) In the absence of chemotaxis. (Right) In the presence of chemotaxis. The pattern becomes denser with radial fine branches, and well defined circular envelope, in agreement with experimental observations of the dense branching morphologies (DBM). From reference [Ben-Jacob97].

In the presence of the chemorepellent, the movement of the active walkers changes from a pure random walk to a random walk with a bias to move away from high concentrations of chemorepellent. The effect of this chemotaxis can be seen in figure 4.9. The patterns produced now seem to be better in agreement with experimental patterns. When considering the dynamics, though, some contradiction arise and further chemotaxis must be added, now for the nutrient concentration. This means that active cells are also biased towards higher nutrients concentrations. See [Ben-Jacob97] for details. Despite the inclusion of at least two kinds of chemotaxis, the models have never been found to produce concentric ring patterns.

In conclusion, communicating walkers' model achievements are that:

1. In a first stage, it succeeds in reproducing some general features of bacterial colonies with simple assumptions. At this level, it is roughly equivalent to the former continuous reaction diffusion models, but computationally much faster.

2. In a second stage, it allows the inclusion of some biological details. Using a discrete bacterial dynamics, assumptions on metabolism (reproduction, chemo-

taxis, etc.) can be easily posed, then its effect on the macroscopic patterns viewed.

The main criticism it has to bear is that, as the model makes more and more assumptions on the microscopic biological mechanisms of the system, it also includes more and more parameters. And when a "clever" model has many parameters, it is always capable of reproducing whatever you ask it to reproduce -independently of its physical meaning. This can be overcome by finding experimentally the value of the parameters that the model uses -this has been partly done. However, there is no experimental evidence yet to my knowledge that real *B. subtilis* bacteria have the kind of chemotactic signalling claimed by the model (even if chemotaxis is known to exist).

In relation to the formation of concentric ring patterns, communicating walkers' model has not been reported to display any kind of periodic growth. This suprises me for two reasons: (i) this model without chemotaxis is very similar to Mimura's model but in a discrete version, (ii) the model with chemotaxis has even more degrees of freedom to generate a periodic growth. I wonder if it is not a question of finding the adequate range of parameters. It has to be said, nevertheless, that since the motility of bacteria in this model is inactivated or enhanced by difusive fields (such as nutrient or chemotactic concentrations), the periodicity would change with the parameters of the system (while in experiment periodicity is robust). We skip another model without finding an answer or even a hope.

4.2.3 Esipov's kinetic proposal

The model proposed by Esipov and Shapiro [Esipov98] is meant to reproduce the concentric ring patterns of colonies of *P. mirabilis*, which are found over a wide are of its morphological diagram, as explained in section 4.1.3. However, given the similarities between *B. subtilis* and *P. mirabilis* ring patterns, it can as well stand as a model for *B. subtilis* -with the only problem that it dispenses with other morphologies. I will maintain thus the same terminology, with the novelty that inactive bacteria are now able to reproduce.

Assumptions are quite different to those seen before and involve some biological details -I will present the very simplest version. It stands on two main ideas: (i) Active cells have a fixed life-time θ_{max} for the differentiation-dedifferentiation cycle. (ii) The collective nature of *P. mirabilis* migration can be described by means of a nonlinear (hysteretical) diffusivity of bacteria. The role of this diffusivity is to synchronize populations which would otherwise increasingly lose coherence. Nutrient depletion is not taken into account. This is because according to experimental data the movement of the colony front happens at conditions of nutrient saturation.

The model uses the following notation:

- $\rho_{act}(\vec{r},\theta,t)$: Number of active cells of given age θ at the point $\vec{r}$,time t.
- $P_{inac}(\vec{r},t)$, $P_{act}(\vec{r},t)$: Surface densities of inactive/active cells. For active cells, it is assumed that the biomass increases exponentially with age. This is equivalent to assume that bacteria keep producing biomass independently of its

motility (for inactive cells population increase is known to be exponential from experiments). This means that:

$$P_{act}(\vec{r},t) = \int_0^{min(t,\theta_{max})} d\theta \rho_{act}(\vec{r},\theta,t)e^{\theta/\tau_d} \tag{4.23}$$

where τ_d is the time constant of reproduction rate of inactive cells.

- ξ : Fraction of inactive cells per division that become active. ($0 \leq \xi \leq 1$).

The local population dynamics is written as follows. Equations 4.24, 4.25 represents the evolution of inactive, active cell populations, respectively. Mark that active cell density is for a given age and a given time, what means that $d\rho_{act}/dt$ has to be written as the sum of partial drivatives on t and θ. With this in mind, equations can be easily understood, as always, as conservation equations for the population.

$$\frac{\partial P_{inac}(t)}{\partial t} = \frac{P_{inac}(t)}{\tau_d} - \xi\frac{P_{inac}(t)}{\tau_d} + \rho_{act}(\theta_{max},t)e^{\theta_{max}/\tau_d} \tag{4.24}$$

inac change = inac reproduc − reverse convers (inac → act) + convers (act → inac)

$$\frac{\partial \rho_{act}(\theta,t)}{\partial t} + \frac{\partial \rho_{act}(\theta,t)}{\partial \theta} = \frac{\xi P_{inac}(t)}{\tau_d}\delta(\theta) - \rho_{act}(\theta,t)\delta(\theta-\theta_{max}) \tag{4.25}$$

act change = reverse convers (inac → act) − convers (act → inac)

Finally, the description of the diffusivity is made on the basis that a population threshold $P_{act,max}$ has to be reached before a collectivity of active cells can begin a growth phase. To this, an assumption of motility "inertia" is added: once the growth has begun, it can go on until the population of active cells becomes smaller than another given population threshold $P_{act,min}$. While movement is possible according to the preceding rule, diffusivity is given the following form:

$$D(P_{inac}) = D_0 \exp(-\frac{P_{inac}}{P_{inac,sat}}) \tag{4.26}$$

where the dependence on $P_{inac,sat}$ is meant to prevent active cells from moving into areas densely populated by inactive cells. Rewriting equations 4.25 with the nonlinear diffusion term ($\nabla(D\nabla\rho_{act})$ one obtains the final set of equations of the model. The authors claim that the model is intrinsically different to reaction diffusion models in PDEs "because the future evolution of the density fields is a function both of the present distribution of microscopic elements (cells) as well as the past sequence of events (their ages)." Simulations in 1 dimension have been reported to reproduce the periodic growth characteristic of ring patterns, with a period roughly stable even when varying up to 4 parameters. These and other features are commented to be in agreement with experimental measurements. According to these results, *P. mirabilis* colonies produces ring patterns because:

1. Active cells have an age limit after which they become inactive ("biological clock").
2. Nonlinear diffusivity produces, as a by-product, the synchronization of populations.

The model seems to describe reasonable well the dynamics of *P. mirabilis* and *B. subtilis* ring colonies. But I wonder to which degree it has a predictive power, since it has been introduced quite many experimental details. Does this model give us a new insight into the mechanisms that generate the periodic growth? To a certain extent is it not reproducing what it was told to do? I am not myself quite sure. I think is telling us one interesting new thing: nonlinear diffusivity can synchronize populations. But certainly it does not prove that the life-time of an active (long-motile) cell is a *biologically* fixed parameter that environment can not change. To assert this we need experimental evidence -which we failed to provide. The question keeps open.[10]

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[10] There are other models that produce ring patterns [Woodward95, Ben-Jacob95, Tsimring95]. But in these cases the dynamics is clearly different: they are meant to describe bacterial colonies where the front propagates steadily and only later bacteria behind progressively aggregate to form rings.

Chapter 5

Conclusions

5.1 The mechanism of the periodic growth? An open question

It is high time to ask ourselves again if we have solved or even come close to solve our little puzzle: *Why the devil do these little creatures dance making rings?*. The question can repeat itself in several ways. From a biological viewpoint: *Why do bacteria differentiate depending of the stage of colony growth?*. Or as collective motion: *How do bacteria coordinate their movement so as to start and stop all together?*. But we know that it is all one question, since we require only one answer. Again, now from pattern formation: *Why do the colony have a periodic growth?*.

I think the question remains open. It is still a jolly scientific challenge. The only achievement of this research has been to delimit -a little-, by means of an experimental characterization, the span of possible answers.

The following list is a tentative summary of the main points we have come to know during this study:

a. **The periodicity of the colony is linked to a cell differentiation process**. When the colony expands bacteria are long and motile. When it stops they become short and immotile. Section 3.2, [Ràfols98].

b. Although the size or final cell density changes when nutrient or agar concentration are varied, the periodicity of the colony is hardly affected by these changes in environmental conditions. **The period is robust**. Section 3.3, [Ràfols98].

c. **There is no experimental evidence that the periodicity be induced by chemotaxis**, although *B. subtilis* is known to secrete chemotactic chemicals. Section 2.1, [Kunst97].

d. **The formation of ring patterns in *P. mirabilis* colonies is very similar** to the formation of rings in *B. subtilis*. In *P. mirabilis* the differentiation process is known to dominate the dynamics, but the reasons for the differentiation to happen are also unknown. Section 4.1.3, [Rauprich96].

e. In **experiments of electrodeposition**, ring patterns with a similar dynamics, within a morphological diagram resembling that of *B. subtilis* have been found. These electrodeposition experiments show that the formation of this kind of **ring patterns can be achieved by means of a mere physicochemical inhibiting mechanism**. Section 4.1.2, [Zeiri97].

f. **Reaction-diffusion models that rely on the depletion of nutrients** to explain the periodicity, have been found to be **incompatible with experimental observations**. Section 4.2.1, [Matsushita98b].

g. **The communicating walkers' model has not been reported to display periodic growth**, in spite of being able to incorporate several kinds of chemotactic signalling. Section 4.2.2, [Ben-Jacob97].

h. **A model based on age-induced differentiation plus cell density dependent diffusivity, is compatible with present observations** but it can not explain why no periodicity is observed in other patterns of the morphological diagram. Section 4.2.3, [Esipov98].

To my understanding this is the state of the question.

If I you allow me to speculate, I would say that right now the debate should be whether:

i. Periodicity appears because of the existence of a fixed *biological clock* that regulates the cell differentiation cycle.

ii. Periodicity is due to chemotactic signalling.

iii. Periodicity is the by-product of a non-linear diffusivity that coordinates the movement of bacteria.

or some mixture of these causes. I think experimental evidence forces us to discard models based on nutrient depletion as a realistic explanation of the ring pattern. The fact that electrodeposition rings be produced by an inhibitor factor can be a hint for future attempts on modelling.

I confess a lack of ideas for experiments that can clarify "once and forever" the question. My immediate agenda is to continue experiments re-inoculating on a new plate active bacteria (as explained in section 3.6) so as to check if the growth phase is determined by an active cell life span. If so, the whole problem is caused by a "biological clock" and the only thing left to explain is how bacteria coordinate their "clocks" (in the line proposed by [Esipov98]). If not, a collective regulation is needed for the periodicity and the problem keeps being very very interesting and its implications far reaching.

5.2 End of journey

As I said before this has been the story of a failure. A failure to solve a very simple puzzle. The surprise has been how very fruitful this failure has turned out to be.

During this journey to nowhere we have first found out how much it was already understood on bacterial colonies. Our understanding is far from complete, but a robust framework has been constructed on how to deal with bacterial colonies. A number of experimental and theoretical methods have been successfully developed to cultivate, describe and model the colonies. Because it has the gift of morphological diversity, *B. subtilis* has been a good example to view many of the properties of bacterial colony formation in general. Study of bacterial colonies from a macroscopic perspective has given us some fresh knowledge on how bacteria live and behave inside the colony (reproduction, sporulation), as well as how they cooperate (chemotaxis, nonlinear diffusion). This is, the relation between this microscopic behaviour and the macroscopic shape of the bacterial colony has been roughly understood in many cases. However, the reasons why bacteria develop some particular microscopic behaviours (in terms of better adaptation, etc.) are far from being settled.

The mechanisms that generate periodic growth and concentric ring patterns remain obscure. As discussed in the previous section we have characterized macro- and microscopically the formation of the concentric rings, but for the moment being this characterization has not help us to find the clue of the puzzle.

Beyond the concrete little puzzles of everyday science, lie the more diffuse but more relevant inquiries into the nature of nature. Many puzzles fail to provide a new understanding or become just another example of what is already known. A few have the gift of providing new insight into the "deep" questions. I think bacterial colonies do. They are easy enough to experiment with and easy enough to model, and at the same time the phenomena that they display have important significances for several questions full of meaning [Shapiro95b]. In the introduction I argued that four fields or themes could benefit from the study of bacterial colonies. Let me review them again, with the findings of our little investigation in the pocket, and from a more critical look.

(i) Pattern formation: Bacterial colonies of *B. subtilis* generate patterns that are also formed in non-living systems, often with very similar dynamics[1] [Matsushita97]. We can learn from this that macroscopic structures do not reflect the complexity of the micro-elements that constitute them. Even when patterns unique of bacterial colonies are generated, they only display a few degrees of freedom more than those generated non-living elements [Ben-Jacob97, Budrene95]. Diversity of patterns depends on the nature of the interactions among micro-elements. Patterns will only be affected by the complexity of the constituting elements when this complexity can change the nature of the interactions.

(ii) Collective behaviour of living beings: Revisiting the results, perhaps more than bacteria as multicellular organims [Shapiro88], we should talk about bacteria as social or collectivist organisms. It seems certain that they communicate, cooperate, perform different functions and even change form depending on their position and "schedule" inside the colony. However, colonies as a whole do not make a coherently connected structure reminiscent of an organism. It holds true, though, that many of individual strategies of bacteria rely on the collectivity.

(iii) Morphogenesis: The conclusions drawn above for pattern formation can

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[1] The concentric ring pattern was thought to be unique for living systems, but it was recently found for electrodeposition too [Zeiri97]

give an idea of the extent to which the study of bacterial colonies can give insight into morphogenesis of truly multicellular organisms, with a much wider span of genetic and environmental determinants. In my opinion, it does give an insight, but a rather precarious one. They il.lustrate, nevertheless, that merely physico-chemical processes can produce pattern formation and (possibly) differentiation. They show both the possibilities and the limitations of the dynamical approach in mophogenesis [Kaneko97, Goodwin94].

Sciences of complexity: From the achievements of models, we can conclude that, as proposed, bacterial colonies constitute a good example on how the study complex systems can be undertaken: they allow the development of models with rather complicated rules and its experimental testing. However, two consideration should be done. First of all, the models, at the present stage, can describe the systems roughly well but don't have any predictive power (I do not know of a single phenomenon found before in models than in experiments). Second, modelling has succeed after getting *messed* into biological facts and extracting from them the few mechanisms that dominate the dynamics of the system. One can claim that some features are universal -but at best, these universal features give us some guidelines, not a down-to-earth understanding of the problem. An all-encompassing theory of complex systems seems, nowadays, sheer boasting [Bak96, Waldrop92].

I would like to finish this study with a few embarrassing confessions. As you have surely noticed I can hardly contain my enthusiasm for the topic -and this does not quite fit science, some say. The ultimate reason for this enjoyment above tedious petri dish filling, late in the morning measurements, frustrations on computer simulations, disagreements with collaborators and a thousand failed experiments, is the incredible power to generate enticing analogies that these microbial cultures possess. I have come to love the research on bacterial colonies not only for the sound scientific understanding that we can directly gain from them, or even for their naked, mesmerizing beauty, but above all for their capacity to pull from us insights or reflections into unexpected fields.

> *"Almost by definition, the physical sciences are fields characterized by conceptual elegance and analytical simplicity. So you make a virtue of that and avoid the other stuff. (...) But once you are in a partnership with biology, you give up the elegance, you give up that simplicity. You are* messy. *And from there it is so much easier to start diffusing into economics and social issues. Once you are partially immersed, you may as well start swimming."*
>
> G. Cowan in *Complexity*, by M. M. Waldrop.

And swim, we did. During this journey I was myself far from home, within a culture quite different to that in which I grew. I swam on it, I got caught in turbulences, I got caught in standstills - moments to step aside and think. Bacterial colonies gave me a pool where to stare. These creatures are closer to us than we can imagine; they are certainly closer than they are to mere particles. And as it happens, they lend themselves to easy comparison: bacterial cultures and human cultures, colonies and societies, individuals and collectivities. The pool's reflections were deeper -and wittier- than you would expect from such shallow plates. Bacterial colonies provided an experimental culture of analogies.

It was always without much foundation and even less rigorous insight that I came to discuss with *B. subtilis* on the cycles on life, the birth and death of cells [Duke96]; on how cooperation can emerge among many individuals that are expected to compete according to the Darwinian picture [Axelrod84, Nowak95, Losick97]; on genetic mutations and the mechanisms driving evolution [Shapiro95a, Ben-Jacob98]; on the fragile foundations upon which complex models are decided to apply or not to complex systems [Horgan95]; on the relation between asiatic DNA and the crash of the Hong Kong stockmarket versus the relation between DNA and *B. subtilis* collective behaviour [Mantegna95, Takayasu97]. And of many other topics that I don't have the courage to write down -being far from scientific. It's been a beautiful time: somehow sad -because we failed!-, but exceedingly fun and challenging. The experience to perceive or think differently more important than the knowledge gained. This has been the main gift of the research. In this sense I am much obliged to *Bacillus subtilis*. Well, for this and for the *Natto*, these delicious fermented beans. It's been a pleasure. If only I could have conveyed part of it.

Epilogue: Physics on diversity

To T.S.

> “Those who lived through the last decades of the century had no difficulty in understanding why such images as chaos and catastrophes should come into the mind of scientists.”
>
> Eric Hobsbawm, *Age of extremes. 1914-1991.*

In the introduction of this thesis, I presented the strict scientific motivations of the study on bacterial colonies, *i.e.*, the general scientific problems (pattern formation, collective behaviour, morphogenesis and complex systems) that may be -partially- answered when we come to understand this particular system. In this epilogue, I would like to put the research into a broader context. The question is:

Why are we interested in understanding pattern formation, collective behaviour, morphogenesis and complex systems?

In this essay I will argue that these and other topics that contemporary science is concerned with, are differents aspects of a same quest: understanding **diversity**.[2] Then I will make the point that we are now interested in diversity because we live in a world where diversity is threatened in a number of fronts. This is presented as one of the developments of a historical shift, represented by the end of the belief in progress, which is having major consequences in the conceptualization of science.

When physics studies diversity

As many people have said, these last 10-20 years have brought shift of paradigm in science, a new way to look at things. I shall briefly repeat the argument while

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[2] Let’s first find out the common meaning of **diversity** (Collins Cobuild English Dictionary, HarperCollins, London, 1995): The **diversity** of something is the fact that it contains many very different elements. *...the cultural diversity of British society.* 2. A **diversity** of things is a range of of things which are very different from each other. *Forlan’s object is to gather as great a diversity of genetic material as possible.*

It is worth confronting these definitions with another fashionable word, **complexity** (same dictionary): **Complexity** is the state of having many different parts connected or related to each other in a complicated way (Complicated: it has so many parts or aspects that is difficult to understand.) *...a diplomatic tangle of great complexity. ...the increasing complexity of modern weapon systems.*

So both words tell us abot things that contain many parts, but while diversity addresses the question of the parts being different, complexity stresses that they are related in a way that is difficult to understand.

putting the accent on the relation between the new fields of research and the question of diversity [Kuhn62, Waldrop92].

The failure of reductionism. Since the XVI century most scientists had shought to explain the world in terms of its constituents. The assumption was that if you could understand the parts, you would understand the whole. This approach is known as reductionism. Its methodology is based on analysis: the division of a physical or abstract whole into its constituent parts. This kind of reductionist, analytical science has been extremely successful in many aspects: it set the foundations of the most spectacular leap in material well-being of humanity. However it has blatantly failed to help us to understand most of the phenomena of our everyday life, from the whorls of cream in cofee to the formation of living beings. The comprehension of the parts did not bring the comprehension of the whole. This paradox was beautifully put by M. Gell-Mann, in *The quark and the jaguar* [Gell-Mann94]: scientists have come to describe remote particles but are quite lost when asked to explain how something so down-to-earth as a cat (or a jaguar) was formed or behaves.

Diversity in condensed matter *More is different*, said P. H. Anderson in the beginning of the 70's [Anderson72]. He was talking for a whole discipline, condensed matter, that is devoted to the study bulk matter (solids, liquids, semiconductors, gels, etc.). According to the reductionist approach, so as to understand bulk matter, you should first analyse its constituent parts, *i.e.* the atoms. In practice, though, the properties the bulk are mainly "created" on another hierarchical level, are produced by the interactions between the many elements. The study of condensed matter requires, more than analysis, synthesis: the process of combining objects or ideas into a complex whole. Then you can see how from systems formed with the same elements, the actual **diversity** of matter emerges: the molecules of H_2O are always the same, but they can become water, vapor or (several kinds of) ice. Moreover, the transitions between the several forms (phases) of matter occur very much in the same way independently of the system you are dealing (be it water or magnets). In other words: the fact that diversity appears does not prevent the construction of a general theoretical framework (universality).

Diversity in chaos. On the other hand, people studying dynamical systems, *i.e.* how things move, realised that even a very simple system can produce very different kinds of movements, from very regular to absolutely unpredictable [Gleick87]. This sort of problems had been known for a long time, but because of the reductionist approach only linear behaviours were considered, since linear dynamics keeps the rule that the whole is the sum of the parts. Other regimes were thought to be completely random. When scientists finally studied them in the 70's, though, they found a diversity of behaviours. Nonlinear systems are unpredictable, but within their apparent randomness different dynamics can be classified. And more than this: the transitions between the several dynamics occur in the same way. We have again diversity (now in the kinds of movement) within a universal framework.

Diversity in pattern formation. In the 80's, many researchers became interested in the shape of things [Vicsek92]. There was a new mathematical tool, fractal analysis, that allowed to put in a quantitative form the similarities between patterns that had been difficult to characterize until then. With this tool in hand it was soon realised that systems so different as fast grown crystals, lightnings, viscous fingering (a fluid that is blown into another fluid of different viscosity), or bacterial colonies, produced the same patterns.[3] The constituents are different, but the whole looks the same. Each system can produce a diversity of patterns but this diversity is displayed in the same manner for apparently different systems.

Diversity in complex systems In the 90's, the physical community was ready to undertake riskier challenges. It's been the decade of complexity [Waldrop92]; many researchers have taken the tools of statistical physics -first developed for condensed matter problems- and applied them to complex systems, *i.e.*, systems made of many (different) elements that are connected or related -whatever the elements are. The tentative applications cover all kind of disciplines (from economics to linguistics), and are particularly fruitful (or promising) in the biological and social sciences, where classic, analytical mathematics had proved of little use. These systems often present an overwhelmingly rich diversity.

These four fields of research illustrate the shift of scientific paradigm within physics[4], from the precursor activities of condensed mattern to its full expression in complex system studies. The fact that each of them has given birth to its own specialised journals underlines the degree of maturity of the shift.

For the purpose of this essay, let me stress again that these scientific disciplines appeared more as a change in attitudes and interests in the scientific community, than as the consequence of previous developments. The very simple mathematical techniques used for the first discoveries in chaos or pattern formation illustrate the point: the difficulties to overcome were mainly conceptual -a new way of thinking was required. It was only when the diversity displayed by everyday phenomena became something appealing[5] that the new disciplines appeared.

To understand diversity a synthetic approach was developed: the whole in itself has a meaning beyond its parts because it constitutes a non-additive, a non-predictable result of the interactions between the parts. The main assumption is that we can grasp the diversity of shapes, behaviours, etc. of the whole from (relatively) simple relations among many constitutive elements. This approach is (thought, claimed) to be applicable to systems of many elements that a related to each other (complex systems). It should be emphasised, though, that the interest of these systems lies not in the fact that they are complex or difficult to understand, but in the fact that they are rich in diversity and often close to our everyday life. Diversity for

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[3] It should be remarked that the formation of these patterns always occurs far from equilibrium.

[4] The list is not exhaustive. It should/could be completed with developments in other sciences. For example, the growing importance of ecology within biology or the proposal of alternatives to the Darwinian evolution such as *punctuated equilibrium* [Gould89] can as well illustrate the point.

[5] See exemplar experiments: water-vapor phase transition, chaos in a dripping faucet, pattern formation of snowflakes, fluctuations in the stockmarket.

its sake is appealing; difficulty-complexity for its sake is not.

The problems addressed by these fields had been known for many years but had not been given much attention or thought. Scientific interest was invested in other questions. Science may be objective, but the curiosity and stimuli of scientists are certainly not. Why did so many scientists change the things they were looking at by the seventies? The date fits with the end of the Golden Age of the century (1945-1970). Since then this planet has been a less stable place -not to say a turmoil [Hobsbawm94].

Diversity endangered

Among the many problems the world is facing, the menace on diversity stands as an aspect shared by many of them. Diversity is being endagered in a number of fronts. Let's quickly review the main threats that it has been posed.

Biodiversity. Ecological systems are under terrible strain. The reason is that humanity is using them either as natural resources, or for agricultural, industrial and urban purposes. This poses in danger of extinction many ecological systems as a whole and many species in particular. Extinction is a non-reversible process.

Cultural uniformation. The revolution in communication and transportation technologies of the last 50 years together with the imposition of compulsory education, have exceedingly improved the freedom of movement and acces to information. However, both communication and education systems are ruled by the most numerous/powerful cultural groups. Cultural minorities have no choice but to adapt to the dominant culture. The process might lead to a progressive uniformation as already seen in many western states.

Ideological uniformation. Since the fall of the Berlin wall (1989), capitalism and liberal democracy do not have alternatives in the economical or political arena. The ideology of the free market pervades the activities of many men and women without them (us) being conscious of having any ideology. Instead of debating ideological alternatives, we should content ourselves with the only/unique thought (*la penseé unique*) of economic liberalism.

Capitalism, which has since its birth developed in parallel with reductionist science, and technology, which is one of the outcomes of science, are the main forces driving the world towards the unification/uniformation. The economic forces rave for the "global free market". But this pushes the world towards the "global culture" and a "global ecosystem" that will look the same all over, towards a *globaritarian* system [Ramonet]. Diversity, when non-profitable in the short term, is not respected.

Physics on diversity and the historical shift

Here comes my naive, obvious proposal: among other yet not well-defined reasons, scientists have become interested in diversity because they live in a world where diversity is endangered and there is social concern with it. This is certainly an oversimplification but I think it grasps some truth. Whether they like it or not, whether consciously or not, scientists' look also reflects what society is worried about. Scientific developments can be or can seem to be independent of many socials moods -because experimental results do not change with social moods- but in the long run there is a feedback between the fields of research and the direction that societies move towards.

Let me put a classical example: the appearance of modern science (XVII and XVIII centuries) and the parallel development of capitalism and the ethics of protestantism, as presented by Weber [Weber]. He argues that the three phenomena were the expression of a single historical process. The protestant believer worked for devotion, because it was by working efficiently that he could gain salvation. In his/her personal, direct and -very importantly- rational relation with God (a historical novelty), the believer had to strive for self-improvement. Under this moral framework, both making money and developing new techniques or machines became goal by themselves. Money was not meant to be spent -it was the expression of the self-betterment. Scientific/technological advances were not meant to make life easier, but to make money more easily, to fulfill the quest of self-betterment. And since capitalist profit and scientific brilliance brought success, *i.e.* salvation, the believer could go with his religious devotion. Capitalist, scientific and protestant fanatism enhanced each other.[6]

Nowadays, the shift in scientific paradigm also coincides with a period of accelerated change, even turmoil. Economists borrow metaphors from science to point out that this is a time of *punctuated equilibrium* (meaning that we are ready for a major leap-avalanche) [Thurow96]. Historians tell us that "there are signs that we have reached a point of historic crisis" after 200-300 years of capitalism [Hobsbawm94]. Some political scientists proclaim overtly the future collapse of liberalism [Wallerstein95], while journalists denounce the mounting social faults and instabilities that the world is suffering [Ramonet]. In brief, many people argue that the times are changing, that 1989 just marked the end of the whole historical period. Capitalism is blindly developing its enormous contradictions -at social, ecological, even economical stages- to a degree that becomes unbearable, unsustainable. Ecological and social deterioration, economical fluctuations can not go beyond a critical level: when the forest is over, timber companies can not make money; when only one company survives the fall of the stockmarket, there is no free-market.

The scientific community is not indifferent to such an upheaval. It can not be. Men and women of the XIX century would be shocked to see that so many extraodinary technological advances did not bring peace and welfare to us. They believed in progress, in the betterment of humankind. Science had to play a very important role in this. But many things went wrong, technology has also brought a lot of pain and destruction. The myth of progress, upon which the scientific endeveour was constructed, is dead. This might lead to "the end of science" as it has been exhilaratingly argued by J. Horgan [Horgan97]. Instead of a funeral,

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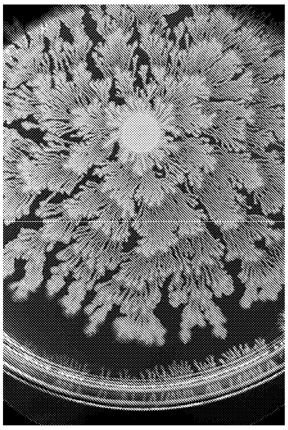
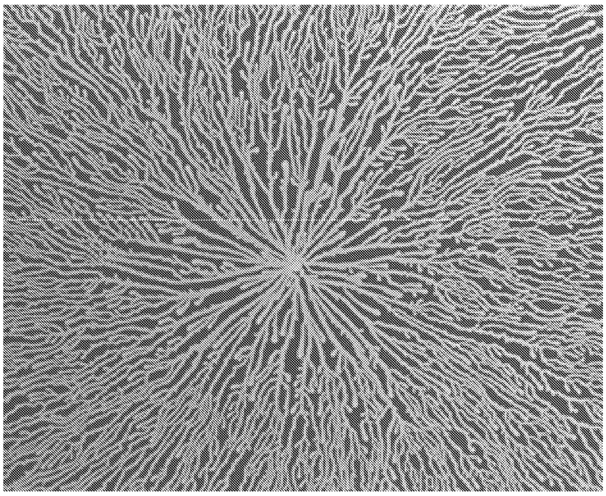
[6] Now tell me, who did Weber choose to illustrate the point? Nobody else but the most illustrious Benjamin Franklin, physicist and stateman of the United States, with a moral discourse on *time is money, credit is money, decency is credit, etc.* that is most hilarious.

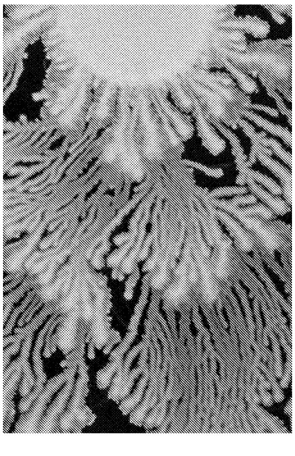
Brian Goodwin proposes a re-invention (already under course) in the form of a "science of qualities" that approaches nature not in the parts, but in the whole [Goodwin94]. This means to recognize that each hierarchical level (atoms, organs, organism, ecosystems) is produced by a *creative* process out of which diversity arises. Despite science cannot and should not teach us ethics, the perspective and the metaphors provided by this new approach can underline the respect for diversity and community, where reductionist science would underline the manipulation of the parts. This proposal illuminates the idea that behind these new scientific fields that address the question of diversity, the spirit of the times is thriving.

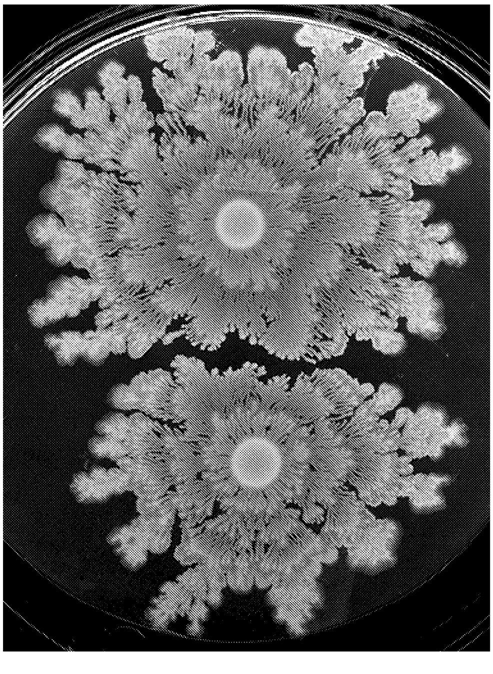
During the rise of capitalism, protestant ethics, economic profit and reductionist science enhanced each other to give birth to the modern world. At this end of millenium, the end of the belief in progress, the need to save diversity and the appearance of a science on diversity (the "science of qualities", the study on complex systems studies) can be taken as some of the expressions, responses and inducers of a major historical shift, a crisis of capitalism in its present form -some say its final fall.

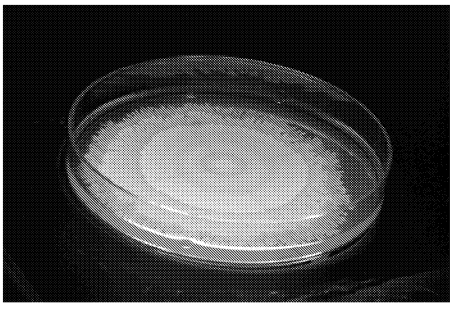
A bacterial colony art gallery











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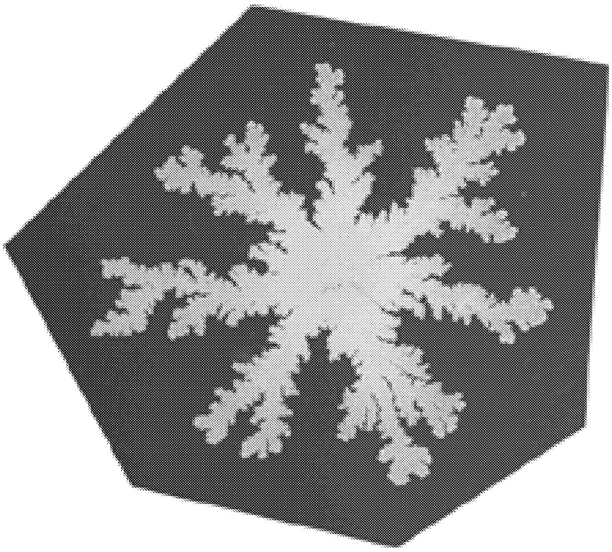

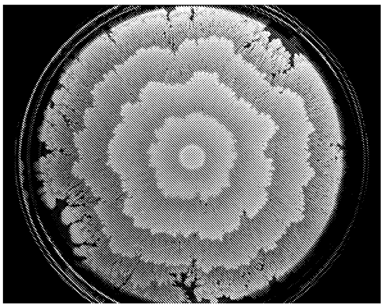

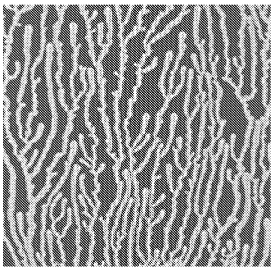

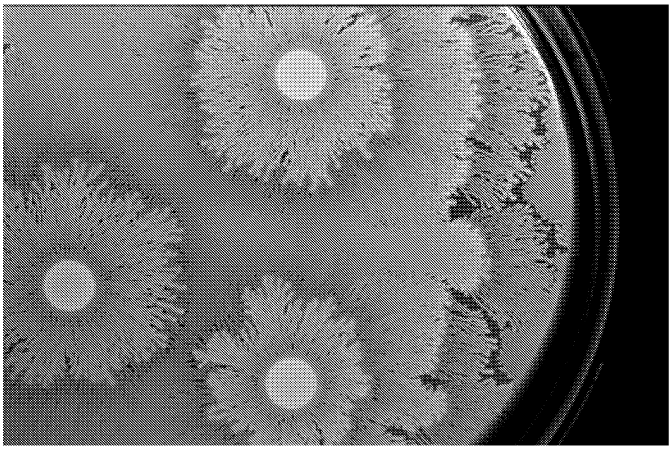

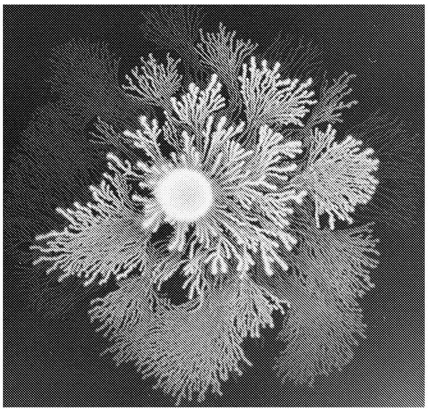

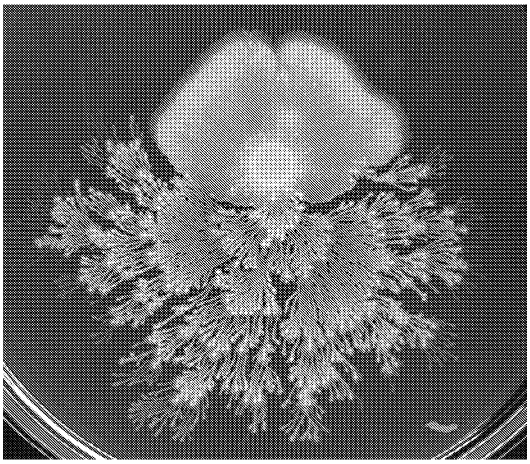

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Further reading

A thorough review on bacterial colonies has been recently published: *Bacteria as multicellular organisms*, edited by Dworkin and Shapiro. It includes most of the main topics and contributors of the discipline (as cited in [Dworkin97]). M. Matsushita presents there an extense article on *B. subtilis* colonies, as described in chapter 2.

For those willing to give a quick glimpse into the subject, I do recommend the review of Ben-Jacob on his own research [Ben-Jacob97]. Shapiro also provides an interesting overview on the whole discipline in [Shapiro95b]. Even more readable is the justification of the concept of *bacterial multicellularity* that Shapiro reported in *Scientific American* [Shapiro88].

On pattern formation in general, Vicsek's book on *Fractal growth phenomena* [Vicsek92] is probably the one that fits better to the problems posed by bacterial colonies.

As examples of breakthrough experimental reports, see the finding of fractal geometry in the *B. subtilis* [Fujikawa89, Matsushita90], or the formation of patterns by chemotaxis aggregation in *Escherichia coli* [Budrene91]. Bacterial colony modelling is best illustrated and readable in the first proposal of Ben-Jacob [Ben-Jacob94b], and in Kawasaki's and Woodward's reaction-diffusion models [Kawasaki97, Woodward95], the former nutrient-dependent, the latter driven by chemotaxis.

As for the framing of bacterial colony research within the context of morphogenesis as a dynamical system and the studies of complexity, to me the most stimulating and fruitful reading (and influence) on the topic has been *How the leopard changed its spots* by Brian Goodwin [Goodwin94].

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