Periodic Pattern Formation of Bacterial Colonies

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We have experimentally investigated pattern formation of colonies of bacterial species *Proteus mirabilis*, which is famous for forming concentric-ring-like colonies. The colony grows cyclically with the interface repeating an advance and a stop alternately on a surface of a solid agar medium. We distinguish three phases (initial lag phase, the following migration and consolidation phases that appear alternately) for the colony growth. When we cut a colony just behind a migrating front shortly after the migration started, the migration ended earlier and the following consolidation lasted longer. However, the following cycles were not influenced by the cut, i.e., the phases of the migration and consolidation were not affected. Global chemical signals governing the colony formation from the center were not found to exist. We also quantitatively checked phase entrainment by letting two colonies collide with each other and found that it does not take place in macroscopic scales. All these experimental results suggest that the most important factor for the migration is the cell population density.

KEYWORDS: pattern formation, colony growth, bacterial colony, *Proteus mirabilis*, concentric-ring-like pattern

§1. Introduction

Recently many investigations on pattern formation have been reported in various fields of science and technology.1–3 One of many intriguing aspects in pattern formation is that in spite of different systems there exist seemingly similar patterns. It is hoped that some universal mechanisms common to many systems exist in the formation of similar patterns. It may be very important in the statistical physics of pattern formation to search for and investigate as common mechanisms as possible. In this regard particularly interesting are periodic patterns, which are often seen in nature and in many branches of science. Famous examples are Belousov-Zhabotinsky reaction4 in chemical reaction and Liesegang band formation5 in crystal growth in gels.

Investigating pattern formation by using bacteria has many advantages: One can easily observe their behavior from microscopic events to macroscopic ones. The characteristics of bacteria are generally known from microbiological studies for some well known and familiar species such as *Escherichia coli* and *Salmonella typhimurium*. One can also use various species, strains and mutants in order to vary cell characteristics. For instance, flagellateless mutants can be used to examine the effect of cell motility on the colony formation. Thus, on the one hand, subtle biological factors are involved in the pattern formation of colonies. On the other hand, it is now known that under some conditions the growth of bacterial colonies is purely governed by physico-chemical conditions of environment such as agar and nutrient concentrations in agar plates.6–15

For the present study of concentric-ring-like pattern formation, we used the bacterial species *Proteus (P.) mirabilis* (strain PRMI). This species is famous for forming colonies of impressive concentric-ring-like pattern (Fig. 1) and its biological features are well-known.16–23 *P. mirabilis* is a flagellated rod-like bacterium. It does not produce surfactant which improves its motility on the surface of agar plates, nor changes its cell to a spore when nutrition becomes poor. The most prominent feature of this bacterium is that it has the cyclic process of cell differentiation and dedifferentiation between swimmer cell which is a normally flagellated, short cell (about 1.5–2.0 μm length) and swarmer cell which is a hyperflagellated, long cell (about 10–80 μm length) when it is cultivated on the surface of a solid medium such as agar plates. (When it is cultivated in a liquid culture, it remains the swimmer cell.) Swarmer cells have strong motility thanks to their hyperflagellation and they migrate in group by making rafts together to expand their territory. The colony grows cyclically with the interface repeating an advance with the form of a swarmer and a stop with the form of a swimmer alternately. The concentric-ring-like pattern of the colony directly corresponds to the cyclic growth. It seems that this cyclic growth of the colony has something to do with the cyclic process of the cell differentiation (becoming swarmer) and dedifferentiation (returning to swimmers).16–20 But the details are not understood yet.

We distinguished three phases (initial lag phase and the following migration and consolidation phases that appear alternately) for the colony growth. Lag phase represents the initial stage of colony growth from inoculation till the first migration. The colony itself does not expand two-dimensionally during this period, although cell division takes place actively and cell number increases inside the inoculation spot. It looks as if cells prepare their first migration. The colony then expands (migration phase) and rests (consolidation phase) alternately, forming a concentric-ring-like-pattern, as seen in Fig. 1. Each colony terrace corresponds to one swarming-
plus-consolidation cycle. It is known that the length of the swarming-plus-consolidation cycle (cycle time) is robust for variation of environmental conditions of bacteria such as agar and nutrient (glucose) concentrations in agar plates. Spatiotemporal patterns such as target or spiral patterns were also found when an incubation agar plate is soft and nutrient-rich. The colony of *P. mirabilis* was also observed to exhibit morphological diversity such as other bacterial species *Bacillus subtilis* when varying environmental conditions.

Some of the unsolved problems in the formation of *P. mirabilis* colonies are the control of swimmer cell differentiation from swimmers, swimmer cell organization into groups ("rafts") capable of moving on agar surfaces, the physical basis of multicellular motility and the origins of colony periodicities. In particular, we are interested in what the origin of periodicity in the colony growth is and the origin of spatio-temporal coherence is. In order to try to understand these questions experimentally, we mainly used physical approaches such as varying environmental conditions, cutting part of a colony during its growth and letting two colonies collide with each other.

This paper is organized as follows. In §2 we explain the experimental procedures. We then show the reexamination results in §3 for the sake of completeness and consistency. We devote §4 to presenting our new results. We finally discuss in §5 all the results and argue them from the viewpoint of modeling the colony formation.

§2. Experimental Procedures

We followed methods in previous papers for making agar plates as the substrate for inoculation and cultivation of *P. mirabilis*. The ingredients and their precise amount are described in them. The agar solution and solutions of other ingredients were autoclaved at 121 °C for 20 minutes, then all of them were mixed together at 65 °C and 25 ml of the mixture was poured into each sterilized plastic petri-dish with an inner diameter of 8.8 cm. After solidification at room temperature for 15 minutes, the agar plates were dried for 24 hours at 37 °C. The thickness of an agar plate thus prepared is about 4 mm. Our standard agar plate contains 0.4% glucose, 0.2% casamino acid, 2% agar (BACTO-AGAR, Difco) and 0.0001% nicotinic acid. Then a liquid culture with sample bacteria was prepared for inoculation. We adjusted it to the optical density (OD) of 0.5 (wave length 600 nm), which contains about $1 \times 10^6$ bacterial cells capable of forming colony per 1 μl ($1 \times 10^8$ CFU/μl). The 3 μl of the liquid culture was inoculated on the center of an agar plate surface with a micro-pipette in ordinary experiments. The plates were incubated at 32 °C for designated time. A bacterial colony grew two-dimensionally on the agar plate surface.

Macroscopic colony patterns obtained thus were photographed. The growth of colonies was recorded on video tapes by a time-lapse video (SONY, Tokyo). Microscopic and sub-macroscopic growth processes were also recorded on video tapes through a microscope (Nikon, Tokyo), stereomicroscope (Leica, Tokyo) and a CCD camera (Texas Instruments, USA).

§3. Experimentally Reexamined Results

In this section we describe reexamined results of a few experiments which had already carried out and published elsewhere. The reason to do it is as follows: First of all, we would like to show experimental results of bacterial behavior as completely and consistently as possible. Secondly, the previous results are only found in microbiology journals. Last but not least, we will discuss the physical view of bacterial behavior by using these reexamined results as well as our new ones in the last section.

3.1 Measurements of lag phase time

Lag phase time is the time interval between initial inoculation time and the beginning of the first migration period. We measured the lag phase time by varying inoculation density of cells (Fig. 2). Even if the cell density was decreased to some value, the lag phase time did not change. Below that value, however, it was found to increase monotonously. It looks as if the first migration phase starts only after the cell density reaches some critical value.

Below very low inoculation density (≤5 × 10$^{-5}$) the lag phase time seems to be saturated to a value of about 20 hr. This is, however, an apparent tendency due to the ambiguity of determining the onset time of the first migration period under the condition of such a low inoculation density.

3.2 Microscopic observations

We observed the colony growth with a stereomicroscope and an optical microscope. After inoculation on an agar plate surface the population density of bacterial cells increased gradually (lag phase). Then many branches consisting of groups of swarming cells came out suddenly from the inoculation spot (beginning of the first migration phase: Fig. 3). The branches fused together at many places during the migration phase. As the end of the migration phase (i.e., the beginning of the first consolidation phase) was approaching, the growth of
Fig. 2. Lag phase time vs inoculation density of cells. The horizontal axis is the relative inoculum density. The unity corresponds to the standard cell concentration of liquid culture (OD=0.5) for inoculation. The vertical axis is the lag phase time.

Fig. 3. Microscopic snapshot of branches coming out from an inoculation spot. The arc at the bottom is the edge of the inoculation spot. The snapshot was taken 5.8 hours after inoculation. The width of the snapshot is 2 mm.

branches was slowing down and their width was thinning gradually. Eventually branches stopped growing. Even during consolidation, cell density continued to increase with some cells moving around inside the fresh, outermost terrace of the colony. When the second migration phase started, many branches again came out from the colony front (terrace edge). This alternate process of migration and consolidation repeats again and again afterwards.

Internal waves were observed to successively advance outwards on the outermost terrace, even after the front stopped moving (Fig. 4). They were able to be observed with naked eyes, too. It turned out that each internal wave consists of a monolayer of swarming cells moving toward the front of the outermost terrace. In other words, integral layers of swarming cells pile up successively, forming the outermost terrace.

3.3 Measurements of temporal and spatial periods

Figure 5 shows time evolution of the colony interface in macroscopic scales. This indicates that the temporal period of colony growth (cycle time), $T$, which is the sum of one migration time and one consolidation time, is $4.1 \pm 0.2$ hours on average, while the migration time, $T_m$, during which a colony is expanding is $2.6 \pm 0.2$ hours and consolidation time, $T_c$, during which the colony is resting is $1.5 \pm 0.2$ hours. It is known that the cycle time is robust for the variation of concentration of agar and nutrient (glucose) in an agar plate.\textsuperscript{21} Figure 6 shows terrace width and ring radius. The width was almost constant (about 0.5 cm) when agar concentration was 2%. In contrast to the cycle time which is robust, the terrace width was found to be rather sensitive to the agar concentration: The higher the agar concentration, the smaller the width. This is understandable because the cell motility is reduced by the hardness of agar substrate.

§4. Experimental Results

4.1 Non-existence of global chemical signals

Some microorganisms are known to release chemical substances to communicate with each other or improve their environmental conditions. Examples are cyclic
AMP for a slime mold *D. discoideum* and some surfactant for a bacterium *B. subtilis*. We investigated the existence of chemical signals from the center which might dominate colony growth globally. To check this we cut radially from the center, cut the edge of the third terrace of a colony and then removed the inner part immediately after the fourth migration started. If global chemical signals spread from the center of the colony, some difference may be observed in colony growth afterwards about forming a concentric-ring-like colony between cases of cutting and no cutting. Figure 7 shows that global chemical signals does not exist because there is no difference in forming a concentric-ring-like colony, though the width of the first terrace after the cut was a little narrower (see next subsection for more details).

4.2 Relations between phase and cell density

We divided a colony into two halves by cutting the agar substrate with a scalpel and then separated the growing front from inside by cutting the edge of the third terrace of one half of the colony (right half in Fig. 8) 15 minutes after the fourth migration phase started, letting only cells constituting the migration front grow outward. Left half of the colony was kept intact for the sake of reference. Each of two divided colonies continued to grow independently. As seen in Fig. 8, however, the width of the fourth terrace (the first terrace after the separation from the inner part) in the right colony (the inner part had been separated) was smaller. In fact, the fourth migration period (the first one after the separation) ended earlier in the right colony than in the left one. However, the length of the swarming-plus-consolidation cycle (cycle time) did not seem to be affected so much by this procedure (Fig. 9). This means that the fourth consolidation period (the first one after the separation) was prolonged in the right-half colony. In the following cycles there was almost no time difference between the two colonies (Fig. 10). In order to check the dependence of the this behavior on cycle (or terrace) number and cut timing, we did the same experiments just after the first migration period started and also 45 and 90 minutes after the fourth migration started. The tendencies were found to be the same as the former case, namely, in each case the width of the first terrace after the cut was smaller, migration ended earlier, consolidation was prolonged and the cycle time was not affected by the separation.

We next measured cell population on the fourth terrace. We cut the edge of third terrace 15 minutes after the fourth migration started and let the fourth terrace grow. This is the same procedure as mentioned above. One hour after the cut we picked out ten chips of agar
substance from the edge of the third terrace toward the migrating front of the fresh fourth terrace. We then washed bacterial cells away from the surface of all ten chips together into a specific amount of cold physiological saline solution. We measured optical densities (OD) of the

4.3 Influence of perturbation to terraces

Immediately after the fourth migration period started, we cut colonies with a scalpel in the radial and azimuthal-angle directions, as seen in Figs. 7 and 11. In Fig. 7 we show how terraces grew after the radial and azimuthal cuts and removal of the inner part. The width of the first terrace (the fourth one from the central inoculation spot) is smaller and its terrace edge looks rougher, just the same tendencies as seen in Fig. 8. On the other hand, in Fig. 11 one cannot see any difference among any directions in the terrace growth. Terraces seem to grow in exactly the same way, but without any communication. The perturbation on the azimuthal-angle direction (Figs. 7 and 8), which disturbs progress of internal waves, influenced colony growth strongly. This suggests that in-
ternal waves play an important role for the macroscopic colony growth.

4.4 Non-occurrence of phase entrainment

We checked quantitatively whether phase entrainment takes place in macroscopic scales by letting two colonies collide with each other. In order to do so we inoculated sample bacteria at two points, as seen in Fig. 12, at one point first and then at the second point (1 cm apart from the first one) 45 minutes (Fig. 12(a)) and 2 hours (Fig. 12(b)) later, respectively. In both cases the two colliding colonies showed the same periodicity but remained out of phase with each other during their expansion. Figure 13 shows the result of measurement of the onset time for the corresponding migration phases of the two colliding colonies. This figure clearly indicates that the time difference is almost constant and the same as the initial one, i.e., 45 minutes for Fig. 12(a) and 2 hours for Fig. 12(b). This implies that two colonies keep their own original phases stubbornly, in spite of their successive collisions. One can, therefore, conclude that the phase entrainment does not take place in macroscopic scales in this system.

§5. Discussions

We have examined the dynamic behavior of concentrating-like colonies in bacteria *Proteus (P.)* mirabilis. According to the previous paper and our reexamination result (Fig. 2), lag phase time was found to depend on inoculation density of bacterial cells: As the cell density of inoculum was decreased below some value, the lag phase time became longer. This strongly suggests that the population density of bacterial cells must exceed some threshold value for the onset of migration.

Futhermore, when we cut a colony just behind the migrating front immediately after migration started, the first migration period after the cut ended earlier with the reduction of its terrace width, while the following first consolidation period was prolonged (Figs. 8–10). However, the first cycle time after the cut (sum of the first migration and consolidation periods after the cut) was almost unchanged and the following alternate migration and consolidation phases returned to normal cycles. The same behavior was observed when we cut a colony at any other terraces and cut timing.

Taking into consideration the fact that the successive internal waves are piled up to form an outermost terrace (Fig. 4), the shortening of the first migration period after the cut and the prolongation of the following consolidation period can be understood as follows: The migration front corresponds to the first internal wave. The cut of a colony just behind the migration front not only breaks the first internal wave there but also stops the supply of
cells from the inner part of the colony by the following successive internal waves. This results in the reduction of both the width and cell density of the newest (outermost) terrace. The shortage of cell density now induces the prolongation of the following consolidation period because it takes more time to increase cell density and start migration. And the prolongation of the consolidation phase exactly coincides with that of lag phase when decreasing the inoculum cell density (Fig. 2).

After then everything returns to normal. This scenario neither depends on which terrace is cut nor needs any chemical signal from the central part of a colony. In fact, our experiments clearly confirmed both of them.

From these experimental results and considerations one can conclude as follows: Each migration period ends (consolidation period starts) when the recruiting cell density decreases below some value. And the increase of cell density more than some value during the consolidation period is necessary for the onset of next migration.

It was also found in the present experiment that phase entrainment does not take place in macroscopic scales (Figs. 12 and 13): While two colonies are colliding, each colony stubbornly keeps its own phase. Even submacroscopic observations showed no prominent mixture of cells of two colliding colonies. One conceivable reason to this result is that the phase is mainly affected by the cell density of bacteria, namely, even if phase entrainment occurs in microscopic scales, the global colony growth is affected by the cell density there more strongly. Then a clock owned by each cell, if any, may not be necessary for the periodic growth of a colony.

A few mathematical models have been proposed to explain the pattern formation of bacterial colonies. Their main skeleton is comprised of the reaction-diffusion system of nutrient and bacteria, with the addition of some coupling terms which account for reproduction of bacteria and nutrient depletion. Mimura's model assumes that there are two types of bacterial cells: active (motile) and inactive (non-motile) cells, and the former change to the latter when nutrient becomes poor or cell density becomes low. Kawasaki's model assumes that the diffusivity of cells is linearly dependent on both nutrient concentration and cell density. Kitsunä's model assumes that the diffusivity of cells depends only on the density of active cells. Ben-Jacob's model includes both active-inactive switching and diffusivity which is indirectly dependent on the cell density, and chemotaxis effects are added. Esipov's model which was proposed to explain the growth of \emph{P. mirabilis} colonies is different from all the others and much more complicated. It involves some biological details, namely active cells have a fixed life time for the differentiation-dedifferentiation cycle ("biological clock") and the collective nature of migration can be described by means of a nonlinear (hysteresis-like) diffusivity of bacterial cells which produces, as a by-product, the synchronization of populations. The depletion of nutrient is not taken into account.

It should be noted that only two models, i.e., Mimura's and Esipov's models have succeeded in reproducing concentric-ring-like colony patterns. So far, however, we have not obtained any definite evidence for which one is more compatible with our experimental results. Still, based on our experimental results, we can put restrictions on the modeling of colony formation. The present experiments strongly suggest that the cell density is an important factor for colony growth and cell motility has two threshold values of the cell density, the higher one for the onset of migration and the lower one for the onset of consolidation. Our observations also showed that as the end of migration period approaches, the speed of an advancing terrace front decreases. This suggests that the diffusivity of bacterial cells (cell motility) depends on the cell density.

As described before, internal waves play an important role for the macroscopic colony growth. Macroscopically, isotropic, coherent and successive expansion of internal waves leads to such a regular colony pattern as seen in Fig. 1. Microscopic observations, however, indicate that cells move in groups (rafts), with no characteristic direction. This strongly suggests that the overall, global direction of expansion is determined by the (negative) gradient of cell density. This may also be an important point for the modeling of colony formation.

One may tempt to associate the robustness of cycle time with biologically inherent factors of the bacteria. However, the reproduction cycle time of bacteria, for instance, is known to be about 30 minutes, very different from cycle time of the present \emph{P. mirabilis} colony. It is difficult to understand the concentric-ring-like colony formation in terms of inherent factors of bacteria alone. Rather, simple mathematical models based on reaction-diffusion equations such as Mimura's model with some specific additions seem more promising.

Concentric-ring-like patterns are seen in various systems. In particular, under some environmental condition bacteria species \emph{Bacillus} \emph{(B.) subtilis} also forms a concentric-ring-like colony. In spite of very different species, the present \emph{P. mirabilis} and \emph{B. subtilis} form seemingly the same concentric-ring-like colony patterns. It is an interesting future problem to experimentally elucidate similarities and differences between them. We believe that there exist simple, beautiful and universal mechanisms which are independent of details of components and common to many systems.

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