Bacterial Fractal Growth in the Concentration Field of Nutrient

Hiroshi Fujikawa and Mitsugu Matsushita

Department of Microbiology, Tokyo Metropolitan Research Laboratory of Public Health, Hyakunincho, Shinjuku, Tokyo 169

Department of Physics, Chuo University, Kasuga, Bunkyo, Tokyo 112

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A *Bacillus subtilis* strain is found to grow through the diffusion-limited aggregation (DLA) process on agar plates. The organism is spotted on the agar plate containing a low concentration of peptone as a single nutrient and incubated at 35°C. The colony pattern grown on the plate surface is self-similar with the fractal dimension of 1.73±0.02. Bacterial DLA branches are shown to grow in a concentration field of nutrient from the fact that they grow predominantly in the direction of higher nutrient concentration. The colonial morphology varies with the nutrient and agar concentrations of an agar plate, including DLA, a round type, dense branching morphology (DBM), and a spreading without openings. Two neighboring colonies repulse each other in DLA and DBM types only. On an agar plate containing glycerol the colony becomes remarkably round, quite different from the DLA morphology.

fractal, diffusion-limited aggregation, bacterial growth, colony, agar plate, dense branching morphology

§1. Introduction

Studies on growing random patterns have greatly advanced due to both the introduction of fractal geometry\(^1\) and the rapid development of computer-simulation methods. Among many growth models proposed so far such as Eden\(^2,3\) and ballistic aggregation\(^4,5\) models, diffusion-limited aggregation (DLA) model\(^6\) has a potential to explain the growth mechanisms of various physicochemical random patterns.\(^2,7,8\)

Morphologies observed in living things are often very attractive. Some of them have been confirmed to be fractals such as blood vessel patterns of chick embryo,\(^9\) structure of bronchial trees,\(^10\) cerebral surface of normal human brain,\(^11\) neuronal arborization,\(^12\) and bacterial colony.\(^13\) They, however, are rather “static” analyses of the biological patterns. Given patterns cannot be fully characterized by their fractal dimensions alone. For instance, DLA and invasion percolation patterns have almost the same fractal dimension D=2.5 in three-dimensional (3-d) space, but they look quite different from each other. The observation of the dynamical behavior, therefore, is significantly needed to characterize patterns and identify more elementary growth processes.

Biological growth mechanisms seem highly complicated. We have, nevertheless, found that a bacterial colony of *Bacillus subtilis* grows predominantly through a simple physical law, that is, the DLA mechanism under a certain condition.\(^14\) It has also been shown that the strain exhibits various colonial morphologies on an agar plate.\(^14\)

In the present study we show the bacterial DLA growth and the true nature of a diffusion field of it. Also we study morphological changes of a colony with the nutrient and agar concentrations of an agar plate, the repulsion between a pair of neighboring colonies in different colonial morphologies during incubation, and local growth mechanism(s) of the organism. The present study would give an important clue to the understanding of bacterial colony growth mechanisms.
§2. Materials and Methods

2.1 Bacterial strain
The strain is isolated from food at our laboratory and identified as *B. subtilis*.\(^{14}\)

2.2 Agar plates
A solution of 5 g of sodium chloride, 5 g of potassium phosphate dibasic, and a certain amount of peptone (BACTO-PEPTONE; Difco Laboratories, Detroit, U.S.A.) as a nutrient in one liter of distilled water, adjusted at pH 7.1, is mixed with 15 g of agar (BACTO-AGAR; Difco).\(^{14}\) The mixture is heated at 121°C for 15 min. and then poured into plastic petri dishes with a diameter of 84 mm (20 ml per plate). After agar is allowed to harden, a plate is dried at 50°C for 60 min. An agar plate with the unidirectional concentration gradient of peptone is prepared as follows: A portion (5 ml) of agar prepared to contain 2 g/l of peptone is poured into a petri dish inclined at a 5° angle to a horizontal plane and allowed to harden as a slant. A second agar (15 ml) without peptone is poured into the dish positioned horizontally. After allowing the second layer of agar to harden, the plate is dried.

2.3 Inoculation and incubation
The strain is inoculated just on the plate surface at the center of an agar disc. Plates are stored in a humidified box and then incubated in a thermostatic room regulated at 35 ±1°C.

2.4 Fractal analysis
Colonies grown on agar plates are photographed clearly through transmitted light from the bottoms of the plates. The photographs are analyzed digitally by a personal computer (PC 9801VX, NEC) through an image-scanner (PC-IN503, NEC; maximum resolution of 240 × 340 pixels) to calculate the fractal dimension by the box counting method.

§3. Results

3.1 DLA pattern of bacterial colony
The organism is inoculated on the surface of agar plates containing a low concentration of peptone (1 g/l) and incubated at 35°C. The colonial morphology is flat, rough, and reticulate on the second day after inoculation. Its diameter is 5–10 mm. Then random branches emerge from the colonial front and grow on the agar surface. During incubation a screening effect is observed.\(^{14}\) The colony grown exhibits a structure which is very similar to a 2-d DLA pattern\(^{2,3}\) and those obtained from physicochemical growth experiments,\(^{7,8}\) on the twentieth day after inoculation (Fig. 1(a)). The colony pattern is found to be a self-similar fractal (Fig. 1(b)). The fractal dimension averaged over 14 samples is D = 1.73 ± 0.02, in good agreement with that of a 2-d DLA cluster.\(^{2}\)

These results clearly show that the bacterium grows through the DLA process on the agar plate.

When the microorganism is inoculated linearly on the agar surface, random clusters grow on both sides of the line, reminiscent of a "metal-forest" morphology,\(^{15}\) i.e., a diffusion-limited deposition pattern\(^{2,16}\) (Fig. 2).

3.2 Diffusion field for bacterial DLA growth

There are two possibilities for the bacterial DLA growth: (i) The bacterial colony grows through the ingestion of nutrient which may diffuse in an agar plate and attach to the colony-agar interface; (ii) Some (toxic) metabolites produced by the organism may diffuse away from the colony.\(^{7,9}\) Both cases are, if one dominates the other, equally possible to produce the same DLA patterns. The following experiments are performed to determine which one mainly contributes to the DLA-type colony formation.

When the organism is located on an agar plate which does not contain any peptone, no remarkable growth of a colony is observed. It is like a pinhead with the diameter about 2 mm 18 days after inoculation. This means that any DLA branches do not arise where no diffusion field of nutrient exists. Next, an agar plate is prepared to form the unidirectional concentration gradient (0–2 g/l) of peptone in it. The strain is inoculated at a point where the initial peptone concentration is zero. During the incubation bacterial branches grow predominantly in the direction of higher peptone concentration (Fig. 3).
Fig. 1. (a) A typical example of a bacterial DLA colony pattern on an agar plate. The bacteria is inoculated on the surface of the agar plate containing 1 g/l of peptone and then incubated at 35°C. The colony is photographed 20 days after inoculation. Its diameter is about 55 mm. (b) The estimation of the fractal dimension of the colony pattern. The pattern shown in a is analyzed to obtain a fractal dimension D using the box counting method. The abscissa R is the box size and the ordinate is the number of boxes with the size R supporting the pattern. A part of the regression line between two vertical lines is used to determine D. The pattern yields D of 1.706 in a surprisingly good linear regression line with the correlation coefficient $r = 0.9999$ over almost two orders of magnitudes.

Fig. 2. An example of a “metal-forest” morphology of the bacterial colony. The organism is inoculated linearly on the surface of an agar plate with 1 g/l of peptone and incubated at 35°C. The colony is photographed 20 days after inoculation. The height of the highest “tree” is about 30 mm.

Fig. 3. Bacterial branch development towards a place with higher peptone concentration of an agar plate. The gradient of peptone concentration (0-2 g/l, center to left of the plate) is formed in the plate. The bacteria is spotted at a point where the initial peptone concentration is zero (marked X). The colony is photographed 13 days after inoculation.

These results clearly suggest that the diffusion field of nutrient in an agar plate is essential for the growth of bacterial DLA branches.

3.3 Morphological changes of colony with nutrient and agar concentrations of agar plate

The colonial morphology varies with the
nutrient concentration in agar plates with a 15 g/l agar concentration. No remarkable colony growth is observed in the absence of peptone, as described above. As peptone concentration increases up to 1 g/l, bacterial colonies grow more largely and resemble DLA clusters more closely. At the concentrations more than 1 g/l bacterial branches become thicker (Fig. 4(a)). At still higher concentrations the colonial morphology is round and smooth, similar to petals which are fused together into one (Fig. 4(b)). A round colony is observed at much higher concentrations (Fig. 4(c)).

When the agar concentration is lower a colony grows rather radially with a branching morphology on the agar plate (Fig. 5), clearly reminiscent of a "dense branching" morphology (DBM). A colony densely covers the whole space of the plate surface when either the agar concentration is lower or the peptone concentration is higher.

These results are summarized in a diagram in terms of the nutrient and agar concentrations of an agar plate (Fig. 6).

3.4 Repulsion between two neighboring colonies

In DLA-type growth phenomena whose surrounding field satisfies a Laplace equation, any neighboring clusters do not link together.
A pair of DLA clusters grow to repulse each other in computer simulations. It, therefore, is studied for the repulsion between two neighboring colonies.

The strain is inoculated simultaneously at two points with a certain distance on the surface of an agar plate containing 1 g/l of peptone. During incubation a repulsion between a pair of bacterial colonies is observed (Fig. 7(a)): Bacterial branches facing another colony are inhibited to grow, in contrast to those facing the outer space. Consequently the two neighboring colonies never grow to join with each other. We call this the repulsion between two colonies. As the distance between two seeds become larger, the repulsive tendency between two colonies become less remarkable (data not shown). These also show the evidence that the bacterial colonies grow through the DLA process in the diffusion field of nutrient.

Two neighboring colonies do not repulse each other on agar plates with higher concentrations of peptone during incubation (Fig. 7(b)). The two colonies fuse each other.

In the case of DBM, a repulsion between a pair of neighboring colonies is found on the plate surface (Fig. 7(c)). This means that this growth pattern is influenced by the diffusion field of nutrient.

When the agar concentration is lower or the nutrient one is higher than the case of DBM, the organisms inoculated at two seeds grow fast, cover the plate surface without any openings, and fuse each other. No clear boundary

Fig. 6. A schematic phase diagram of colonial morphologies of the strain in terms of the concentrations of peptone and agar of an agar plate.

Fig. 7. Repulsion between two neighboring colonies. The organism is spotted simultaneously at two points at a distance of a, 1, b, 2, and c, 4 cm on the plate surface. The peptone concentrations of the plates are a, 1, b, 40, and c, 1 g/l. The colonies are photographed a, 20, b, 3, and c, 1 day after inoculation.
between two colonies can be observed.

3.5 Local growth mechanisms of colony formation

We believe that bacterial growth may be regulated by local growth mechanisms of an organism itself, including its surface tension, the direction of cell division, and so on. Those may play some important roles in the colony formation characteristic of the strain. In computer simulations the local growth mechanism is known to affect the appearance of a DLA pattern. Therefore the following experiment is performed aiming at the elucidation of the local growth mechanism of the strain tested.

When the plate surface is covered with a thin layer of glycerol, the colony pattern of the strain becomes remarkably round, quite different from the DLA morphology (Fig. 8). The same colonial morphology is observed on the agar plate containing 5% glycerol additionally.

§4. Discussion

The strain used in the present study is shown to grow through the DLA process at a low concentration of nutrient. This is confirmed by many experimental facts including the DLA-like colony morphology, its fractal dimension similar to that of a DLA cluster, the existence of a screening effect during the growth, and the repulsion between two neighboring colonies. In speculation based on these results, any microorganism may basically grow through diffusion-limited process of nutrient in the low limit of the concentration field.

A DLA-like colonial layer on the plate surface is flat and very thin, being about 20 μm in thickness. The layer of an agar plate is made as thin as possible, within the limit of drying up the plate during the incubation. Moreover, the fractal dimension of the colony (D = 1.73) is very similar to that of a 2-d DLA cluster. These can make us think that a colony would grow two-dimensionally on the plate surface.

Bacterial DLA-like branches (at 1 g/l of the peptone concentration) are somewhat thicker than tiose of DLA simulations. This may come from a surface tension effect of massed bacterial cells, a finite concentration of nutrient, and/or a finite thickness of an agar plate (quasi-2-d configuration). DLA clusters grow in the limit of a low concentration of Brownian particles and have no surface tension.

A colony morphology varies with sorts of agar and nutrient. A rough and round colony grows on an agar plate made of AGAR NOBLE (Difco) or SPECIAL AGAR-NOBLE (Difco) which are low ash agar for use mainly in electrophoretic and immunodiffusion techniques. In case of Ionagar No. 2 (OXOID LIMITED, London, England) a DLA-like colony is formed, similar to BACTO-AGAR. As for nutrient, when BACTO-PePTONE is replaced by BRAIN HEART INFUSION (Difco) or Trypticase Soy Broth (BBL Microbiology Systems, Cockeysville, U.S.A.), a colony has denser branching structure than a DLA-like colony. On an agar plate containing BACTO-CASITONE (Difco) as nutrient a DLA-like colony is observed.

Bacterial branches become thicker and rounder like fused petals on agar plates containing higher nutrient concentrations. The reason for this has not yet been understood. We think for the present that the DLA growth process may come accompanied by Eden-like

![Fig. 8. A round colony morphology on an agar plate covered with glycerol. The strain is inoculated in an agar plate with 1 g/l of peptone, whose surface is covered with a thin layer of glycerol. The colony is photographed 3 days after inoculation. The diameter is 30 mm.](image-url)
one in the colony formation at high nutrient concentrations. Eden growth from a single seed leads to compact objects. Our speculation is also suggested by the fact that two neighboring colonies do not repulse at high peptone concentrations during incubation (Fig. 4(b)).

In a DBM-type colony an almost constant gap distance between two neighboring branches may indicate the measure of a diffusion length of nutrient. This is supported by the fact that the outline of each colony is independent of the 2-d boundary condition, i.e., the outline of an agar disc, during incubation (Fig. 7(c)).

The reason is not understood why the colonial morphology becomes considerably round on agar plates containing glycerol. It might be due to the surface tension or another factor of glycerol at growing colonial front. This phenomenon is an interesting problem to solve.

*B. subtilis*, including our strain, generally reveals two kinds of cell morphology, vegetative cell and spore, according to its surrounding environments. A microscopic study shows that a mass of rounded cells (spores), are observed in a DLA-type colony. Rod-shaped cells (vegetative ones) are localized at growing perimeter sites of outer branches. In a DBM-type colony bacterial branches are consisted of rod-shaped organisms which are observed sparsely in them. Some of the organisms are randomly moving inside branches. Their movements are especially remarkable just under the growing heads of branches. Vegetative cells are also found in a colony spreading on the plate surface without any openings. In a round colony on a peptone-rich agar plate (Fig. 4(c)) and a glycerol-covered one (Fig. 8), spores and vegetative cells are observed in the core and the perimeter, respectively.

Bacteria represent a wide variety of morphologies. The elucidation of local growth mechanism(s) characterizing a bacterium is increasingly attractive for the study of the bacterial growth. It is also interesting to study the mutual relations and crossovers among DLA, DBM, and Eden model in the bacterial colony formation.

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