Journal of the Physical Society of Japan
Vol. 58, No. 11, November, 1989, pp. 3875–3878

Fractal Growth of *Bacillus subtilis* on Agar Plates

Hiroshi FUJIKAWA and Mitsugu MATSUSHITA†

Department of Microbiology,
Tokyo Metropolitan Research Laboratory of Public Health,
Hyakunin-cho, Shinjuku-ku, Tokyo 169
†*Department of Physics, Chuo University, Kasuga, Bunkyo-ku, Tokyo 112*

(Received August 31, 1989)

Bacteria have been shown to grow with various morphologies under different conditions on agar plates. A *Bacillus subtilis* strain was inoculated on the plate surface and incubated at 35°C. Colonies grew two-dimensionally with random branches, similar to clusters of the diffusion-limited aggregation (DLA) model. The colony patterns were analyzed and found to be self-similar with a fractal dimension of 1.716 ± 0.008, in excellent agreement with the expected value of the DLA model. Interior branches were observed to stop growing in spite of their open neighborhood during the incubation period, implying the existence of a screening effect. These results clearly suggest that the colony pattern of the organism was formed through the DLA process. Moreover, the colonies were found to grow radially with almost regular branches on agar plates with moist surfaces, reminiscent of "dense radial" morphology.

§1. Introduction

Pattern formation has fascinated mankind for many years. Recently the study of growing structures has developed greatly, mainly due to the introduction of the concept of fractal geometry[1] and computer simulations. Irreversible aggregation of small particles to form larger clusters, which can be seen in many fields including science and technology, is also an attractive topic for many investigators. In particular, much attention has focused on diffusion-limited aggregation (DLA)[2] for the understanding of this phenomenon. Many computer simulations[3] showed that patterns formed through the DLA process have open and randomly branched structures with no natural length scale, and so can be categorized into fractals. Experimentally we demonstrated that zinc metal leaves formed by electrodeposition grow in excellent agreement with the DLA process.[4] Many other experimental observations on growth related to the DLA process have been reported in physicochemical fields,[3,5] but apparently there have been none in biology so far.

In this Letter we present our successful observation that bacterial colonies are most likely to grow in accordance with the DLA process on agar plates.

§2. Materials and Methods

2.1 *Strain*

Bacterial strain No. 6 was isolated from food at our laboratory and identified as *Bacillus subtilis*.

2.2 *Agar plates*

A solution of 5 g of sodium chloride, 5 g of potassium phosphate dibasic, and a certain amount of Bactopeptone (Difco Laboratories, Detroit, U.S.A.) as a nutrient in one liter of distilled water, adjusted at pH 7.1, was mixed with 15 g of Bactoagar (Difco). The mixture was heated at 121°C for 15 min. and then poured into plastic petri dishes with a diameter of 88 mm (20 ml per plate). After being kept at room temperature overnight, the plates were dried at 50°C for about 40 min. Agar plates with moist surfaces were prepared by drying for about 10 min. under the same conditions.

2.3 *Incubation*

Strain No. 6 was inoculated just on the plate surface at the center of the disc. The plates were stored in a humidified box at 35°C.

2.4 Fractal analysis

Colonies formed on agar plates were photographed clearly through transmitted light from the bottom of the plate. The photographs were analyzed digitally through an image-scanner by a personal computer to calculate the fractal dimension by means of the box-counting method.

§3. Results

3.1 Fractal growth of Strain No. 6 on agar plates

Colonies of Strain No.6 on agar plates containing 1 g/l of peptone were observed to be very likely to grow in accordance with the DLA process, as seen in Fig. 1. On the second day after the inoculation of the organism, a colony was flat, round, a little rough, and reticulate; its diameter was about 5–10 mm. Then random dendrites generated from the front of the colony and grew on the plate surface as the incubation time passed. Interior branches, however, stopped or almost stopped growing in spite of their open neighborhood during the incubation period, suggesting the existence of a screening effect (Fig. 2). On the twentieth day after the inoculation, the colony pattern was very similar to clusters produced through the two-dimensional (2-d) DLA process. Its diameter was 50–60 mm. These colony patterns were analyzed and found to be self-similar two-dimensionally. For instance, a typical example in Fig. 1 yields the fractal dimension of 1.710 in a surprisingly good linear regression line with the correlation coefficient $r=0.9998$ over almost two orders of magnitude. The average value of the fractal dimension over 6 samples was calculated to be 1.716 ± 0.008, in excellent agreement with that

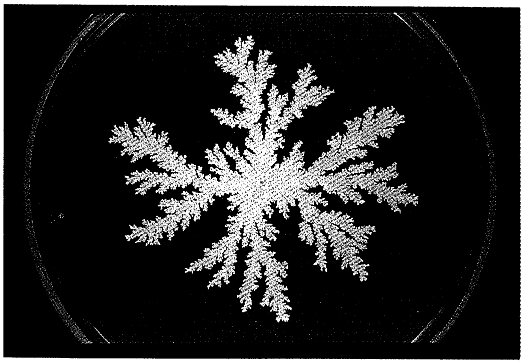
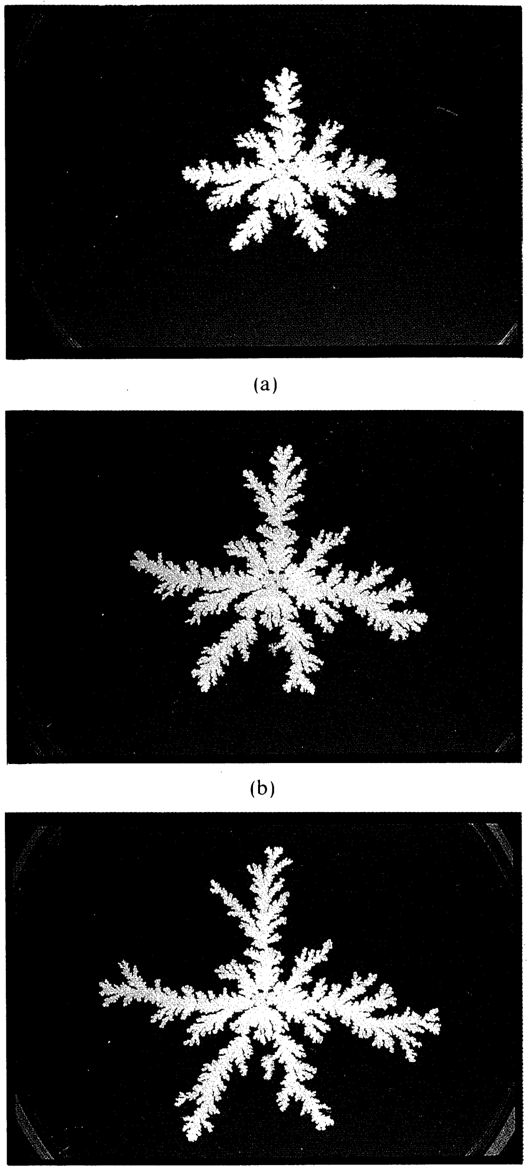


Fig. 1. An example of a DLA-like colony pattern of Strain No. 6 grown on an agar plate. The bacteria was inoculated on the surface of the agar plate containing 1 g/l of peptone and then incubated at 35°C. The colony was photographed 20 days after inoculation. The grown colony has the fractal dimension of 1.710 ± 0.016. Its diameter is about 55 mm.



(a)

(b)

(c)

Fig. 2. The growth of a DLA-like colony pattern of Strain No. 6 on an agar plate. Figures 2(a)–2(c) were photographed 8, 13, and 19 days after inoculation, respectively. Arrows show branches which were observed to stop growing afterwards.

of 2-d DLA clusters.

3.2 *Effects of nutrient and surface moisture of agar plates on the colony pattern*

Effects of nutrient, i.e., peptone, in agar plates and moisture of the plate surface on the colony pattern of the organism were investigated. The bacterial colony grew most likely in accordance with the DLA process on dried plates with a little nutrient (1 g/l of peptone), as described in the previous section. As shown in Fig. 3, colonies grew circularly and isotropically with almost straight branches with the same lengths, clearly reminiscent of the "dense radial" morphology,[6-8] on agar plates with moist surfaces. On the other hand, the colony pattern was smooth and almost round on a dried agar plate with more nutrient, i.e., 15 g/l of peptone (Fig. 4); its diameter was about 40 mm on the ninth day after inoculation. This pattern is reminiscent of that of the Eden model.[3,9] The organism spread fast and covered the entire surface of the plate overnight after the inoculation on the nutrient-rich agar plate with a moisture surface. At that time, thus, it was not possible to observe the colony front.

We summarize schematically the results described above in a diagram (Fig. 5) in terms of surface moisture and nutrient of agar plates.

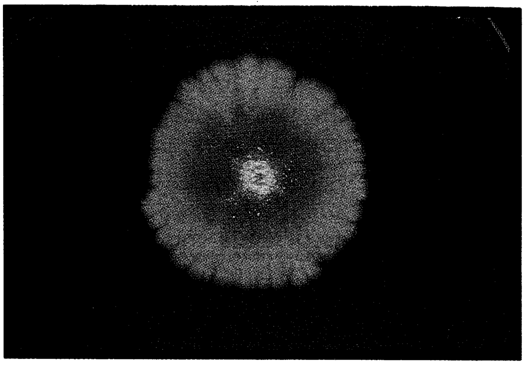


Fig. 4. An example of the colony pattern of Strain No. 6 on an agar plate containing 15 g/l of peptone. The colony was photographed 9 days after inoculation. Its diameter is about 40 mm.

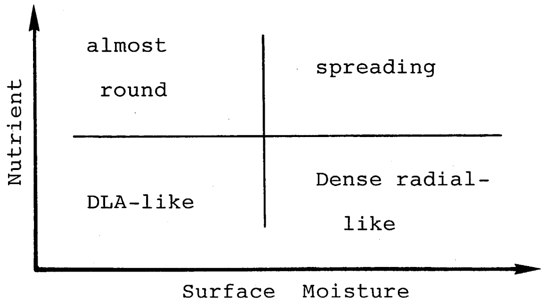


Fig. 5. A schematic phase diagram of colony patterns of Strain No. 6 in terms of surface moisture and nutrient of agar plates.

§4. Discussion

Our study showed that Strain No. 6 apparently grew in the DLA manner on agar plates which contained 1 g/l of peptone. The colony patterns of this strain seem to be very similar to 2-d DLA clusters[3] and some experimental observations related to DLA such as zinc metal leaves[4,5] and chemical dissolution of thin plaster layers.[5,10,11] The colony patterns were found to be self-similar two-dimensionally with the fractal dimension of about 1.72, which is consistent with that of 2-d DLA clusters.[3] The existence of screening effect was also observed in the colony formation. This phenomenon is analogous to that observed in zinc metal-leaf experiments[4] and inherent in DLA.[2] In our preliminary study, it was observed that dendrites of the colony grew like a metal forest[5,12] from the plate edge to the open on the plate surface. These findings clearly suggest that the colony patterns of the organism

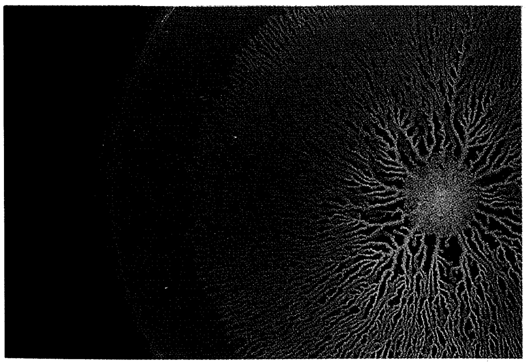


Fig. 3. An example of a dense radial-like colony pattern of Strain No. 6 on an agar plate with a moist surface. The agar contained 1 g/l of peptone. The colony was photographed one day after inoculation. Its diameter is about 65 mm.

were formed through the DLA process on agar plates. This may be the first realistic experimental report on DLA model in biology.

A bacterium grows by itself as a cellular automaton outwards on the plate surface, while clusters in DLA generally grow through the aggregation of diffusing particles. Nevertheless, it appears that the colony pattern of Strain No. 6 was formed through the DLA process. Then, in the present bacterial growth, what kind of particles do aggregate into the colony cluster (or diffuse away from it as the reversed DLA model)? The answer to this question is unknown. At present, we believe that some nutrient particles in an agar plate may randomly aggregate into a colony and/or that some metabolites generated by the organism may diffuse away from it. From the viewpoint of DLA, it is speculated that the growth rate of the colony branch is dependent on the concentration gradient of nutrients (or metabolites) in the neighboring open area in an agar plate. Hopefully, we shall report on these problems in the near future.

It was observed that branches of the grown colony were a little thicker near the center of the colony (Fig. 1), and that the colony was almost round without branches at the beginning of the incubation. Since it is difficult to explain these findings by the DLA process only, we suppose that another growth process, especially the Eden model, might also participate in the colony formation. This may be supported by the observation that the growing colonies on nutrient-rich agar plates containing 15 g/l of peptone seem to consist of very thick branches like petals, which are fused together into one (Fig. 4).

We found that other *B. subtilis* strains were also likely to grow in the DLA manner on agar plates. The bacteria is motile and grows aerobically. Thus it can grow two-dimensionally on an agar surface. It is generally known that the appearances of surface colonies of *Bacillus* strains, especially *B. subtilis* ones, vary a great deal with environmental factors.[13] These include composition of medium, temperature of incubation, and humidity as well as those resulting from changes in the environment caused by the development of the culture itself. This randomness in colony morphology for *B. subtilis* attracts us in terms of growing phenomena. Characteristically, DLA-like colonies of *B. subtilis* on agar plates are highly hydrophobic compared with colonies of other bacteria such as *Escherichia coli* and *Staphylococcus aureus*. There may be a relationship between the DLA-like pattern and surface tension of the colony. This is also a very interesting problem to be resolved in the future.

The authors wish to thank Dr. T. Matsuyama for his helpful advice and J. Terada, H. Yamada, and K. Murata for their assistance in the fractal analysis.

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