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Physica A 313 (2002) 609–624

PHYSICA A

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The mode transition of the bacterial colony

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Received 12 April 2002

Abstract

The colony patterns of *Bacillus circulans* on agar medium were experimentally investigated to study about the growth mode transition. From the optical microscopic observation, the bacteria distribute inside the medium, that is, the colony grows three-dimensionally (3D) in the soft agar condition ($C_A < 0.9$ wt%). On the other hand, the bacteria distribute on the surface, that is, the colony grows two-dimensionally (2D) in the hard agar condition ($C_A \geq 0.9$ wt%). It indicates that the mode transition occurs by changing the hardness of the medium. The mode transition is confirmed by the discontinuous change in the pattern diagram and in the colony growth rate, which is defined as the expanding speed of the colony. Under the softer agar condition in the range of the 2D mode ($0.9 \leq C_A \leq 1.2$ wt%), the induction time exists. Before the induction time, the colony grows in 3D mode. The length of the induction time decreases with increasing the bacterial number density. In addition, bacterial aggregation plays a key role on 2D growth. These results suggest that the increasing bacterial number density causes bacterial aggregation resulting in the mode transition. © 2002 Elsevier Science B.V. All rights reserved.

PACS: 05.—65.+b; 87.18.Ed; 87.18.Hf

Keywords: Self-organized systems; Aggregation and other collective behavior;
Spatio-temporal pattern formation; Growth mode transition; Colony growth rate

1. Introduction

During the last few decades, much attention was paid to self-assembly pattern formation in various fields from various viewpoints [1–3]. Above all, pattern formation

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in biological systems is most sophisticated and interesting. To understand the complex mechanism of such pattern formation is our ultimate goal in spite of the complexity. The pattern of populations of simple biological objects which is dominated by purely physical conditions may be a good example for the starting point. Here we will pay attention to the colony formation of bacteria, which are one of the simplest biological objects. It is known that some kinds of bacteria such as *Bacillus subtilis* (*B. subtilis*) exhibit various colony patterns on agar medium by changing the incubation condition, i.e. the agar and the nutrient concentration in the medium, the former corresponds to the hardness of the medium [4,5]. Recently, various kinds of mathematical models were proposed to understand the mechanism of colony pattern formation using reaction–diffusion equations, and many of the patterns were successfully reproduced by numerical simulations [6–10]. However, some problems still remain. First, it is reported that the colony forming rate strongly depends on the colony pattern [10,11]. But no model can reproduce the drastic change. Second, there is no model to be applied for the transition between different colony patterns. Finally, in these models the colony is assumed to be formed on the surface of the medium. However, it is reported that the bacteria distribute inside the medium in the case of the soft agar [12]. Thus, the growth mode transition is expected in the bacterial colony by changing the hardness of the agar. Observing and modeling such mode transition will be able to explain these problems. However, there are few researches about the mode transition [12].

The purpose of our study is to investigate the mechanism of the mode transition. We observe the influence of the mode transition on the colony pattern and the rate of the colony formation to propose a hypothesis of the mechanism of the mode transition.

2. Materials and methods

2.1. Bacterial strain

Bacillus circulans (*B. circulans*), which are used in this study, are rod-shaped gram-positive bacteria with flagella having the length of about 3–8 μm and the diameter of 0.75–1.0 μm (Fig. 1). The species are moderate thermophilic and aerobic bacillus, so they usually distribute in hot springs. *B. circulans* sporulate during nutritional starvation [13] and lose their activity in motion and division. *B. circulans* are known to move collectively in some conditions [14]. The aggregate of *B. circulans* slowly moves with rotation along the nutrient concentration gradient. *B. circulans* form various characteristic and complicated colony patterns, but much less researches of *B. circulans* have been reported than that of *B. subtilis* [15,16]. The reason seems to be partly due to too much complexity of *B. circulans* colony pattern.

2.2. Agarose

Bacto Agar (Difco, Detroit, MI, USA) is agarose containing ash of concentration less than 3.6 wt% and sulfate of 1.8 wt%. Agarose is a polysaccharide with alternating

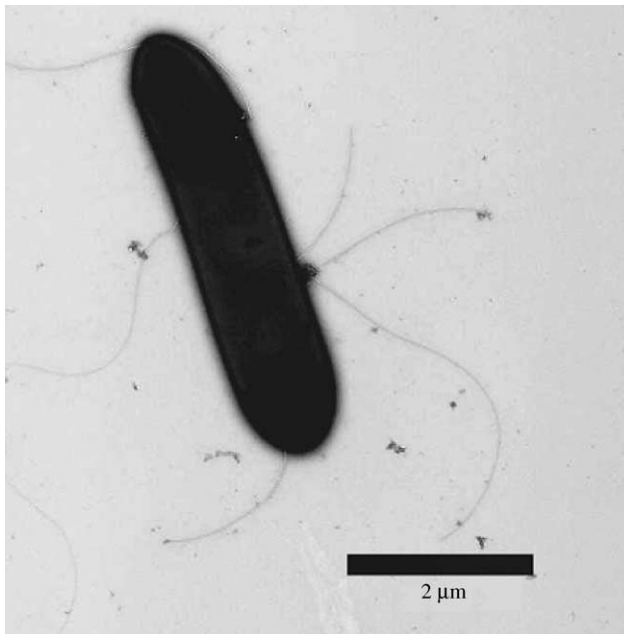


Fig. 1. The transmission electron micrograph of *B. circulans*.

copolymer of 1,4-linked 3,6-anhydro- α -L-galactose and 1,3-linked β -D-galactose and it is extracted from marine red algae. The agarose gel is stable and melts about 85°C. The agarose gels consist of thick bundles of agarose chains and large pores filled with water [17]. The average pore size is varied by the agarose concentration [18–20] and by cooling rate [20,21].

2.3. Growth culture

The culture medium on which *B. circulans* were cultured, was prepared as follows. One liter of water solutions containing 10 g sodium chloride, and the nutrient of different concentration were prepared. Two kinds of nutrition were used: Bacto Yeast Extract (YE) and Bacto Tryptonepeptone (TP). Both YE and TP are the mixture of vitamin, amino acid, minerals, and carbon sources, such as sugar and protein. The weight ratio between TP and YE is kept constant at TP/YE = 2. The total nutrient concentration C_N (TP + YE) is varied from 3 to 90 g/l. Bacto Yeast Extract and Bacto Tryptonepeptone were purchased from Difco.

To solidify the liquid culture medium, Bacto Agar was added to the nutrient solution. The concentration of the agar C_A was varied from 0.4 to 3.0 wt%. The mixture was then autoclaved for 15 min at temperature 120°C and pressure 2 atm. After the autoclave, the mixture was cooled down to 80°C in the autoclave. Then a mixture of 15 ml was poured into a circular plastic dish of diameter 86 mm, and the lid was put on the dish.

The dish was cooled down to room temperature in ambient conditions. The agarose solution gelled about 1 h after being poured.

After the gelation, the dish was turned upside down, and it was kept at room temperature for 2 days to remove excess water. The weight loss of the agar medium was 1.3–1.4 g during drying. As a result, the thickness of the agar medium was about 2 mm.

2.4. Inoculation and incubation

The bacteria were pre-incubated in the standard liquid Luria–Bertani (LB) medium for about 20 h at 37°C. After the pre-incubation, the number density of the bacteria was the order of 10^8 m l^{-3} . The bacterial suspension of 1 μl was then inoculated on the center of the agar medium by a needle (stick mate P, Ono, Japan). The diameter of the inoculated area was 3 mm. To prevent from drying, the dish with the lid was sealed with paraffin sheet and packed into the polyvinyl bag. The inoculated dish was incubated for 3–10 days at 37°C in the incubator. The photographs of the bacterial colony were regularly taken once a day by a digital camera. The area of the colony was measured from the photograph by using an image analysis software (Image Pro).

3. Results and discussion

3.1. The growth mode transition

The morphology diagram of the colony patterns shows the existence of two typical modes in Fig. 2, that is, the pattern on the soft agar, and on the hard agar. The agar concentration dependence is discussed in the case of $C_N = 15 \text{ g/l}$ unless otherwise stated.

Fig. 3 shows the cross-sectional optical micrographs of the colony in the case of the soft and the hard agar. In the case of the soft agar (Fig. 3a), the bacteria uniformly distribute in the agar medium, i.e. the colony grows in the three-dimensional (3D) mode. On the other hand, in the case of the hard agar (Fig. 3b), the bacteria distribute on the agar surface, i.e. the colony grows in the two-dimensional (2D) mode. The growth mode transition from 3D to 2D mode occurs by increasing the agar concentration. In the case of 3D mode (soft agar condition), the colony pattern named as a disk-like pattern is not affected by C_A and C_N . On the other hand, in the case of 2D mode (hard agar condition), the colony patterns are affected by C_A and C_N (Fig. 2).

Under the condition between two modes (oblique region in Fig. 2, $0.9 \leq C_A \leq 1.2 \text{ wt\%}$), the colony pattern sensitively depends on the agar condition as shown in Fig. 4. Moreover, the cooling rate of the agar during gelation process and the wettability of the agar surface also affect on the colony pattern (not shown). Strictly under the same condition, the colony almost grows to the same pattern. However, the pattern in the region is less reproducible than that of in the other region.

In this region, the colony abruptly expands during the growth process. Fig. 5 shows the time evolution of the colony pattern. The colony gradually grows for several days after the inoculation at first. In this period, the growth is almost isotropic and the

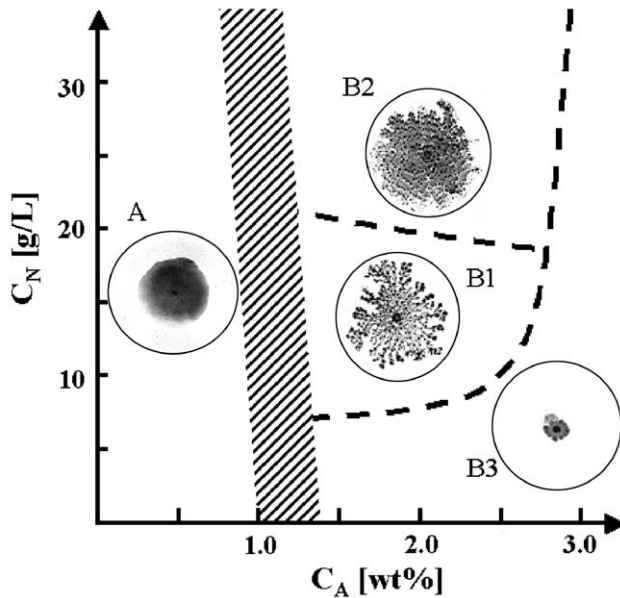


Fig. 2. The morphology diagram of *B. circulans* colony. Two typical phases A and B exist along C_A . In the phase A and B, the colony growth is three- and two-dimensional, respectively. These patterns are called disk-like (A), knotted-branching (B1), concentric-ring (B2), and Eden-like (B3) pattern, respectively. The oblique region represents the transition region and is shown in Fig. 4.

growth rate is quite low. We call the length of this period as induction time T_{ind} . After $t = T_{\text{ind}}$ ($t = 0$ at the inoculation), the colony bursts at a few points with high growth rate, although the growth rate except for burst parts is close to zero. The colony results in anisotropic pattern as seen in Fig. 5c.

The bacteria are observed inside the agar medium by using the optical microscope, before T_{ind} . However, the bacteria are observed only on the surface after T_{ind} . Before T_{ind} the colony grows in 3D mode as well as in the soft agar condition, and after T_{ind} the colony grows in 2D mode as well as in the hard agar condition. Since, this growth mode transition from 3D to 2D mode is irreversible, 2D mode is more stable than the 3D mode. The transition region is defined as the region in which the induction time exists. This region is identical to the oblique region in Fig. 2. The transition region slightly shifts to lower agar concentration condition with increasing C_N as shown in Fig. 2.

The colony growth rate is measured to characterize the mode transition. The colony growth rate is defined as the increase rate of the average radius of an expanding colony. To estimate the colony radius, the area of a colony is measured from its photograph (for the details of the estimating method of the growth rate, see Appendix A).

The time dependence of the colony growth rate is shown in Fig. 6. In the transition region, the growth rate discontinuously increases at about 130 h for $C_A = 1.0$ wt%,

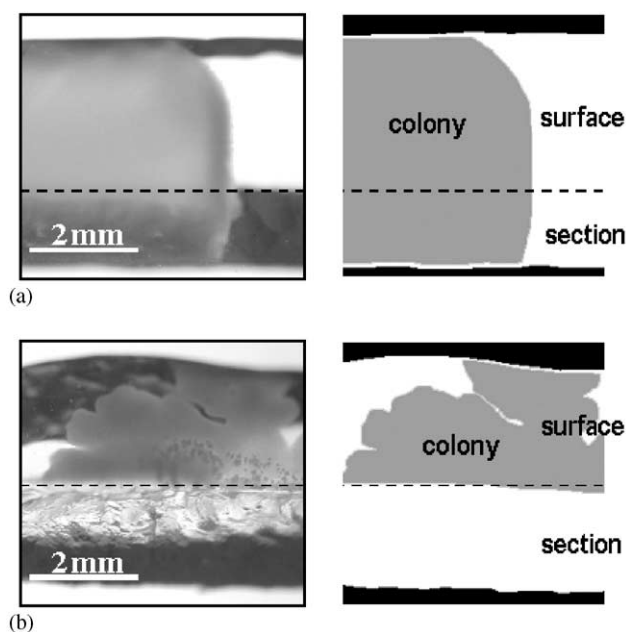


Fig. 3. The cross-sectional optical micrograph and the schematic illustration of the colony formed on soft agar ($C_A = 0.6$ wt%) (a), and on hard agar ($C_A = 1.5$ wt%) (b). The nutrient concentration is fixed at $C_N = 15$ g/l.

which corresponds to the induction time. The dependence of C_A on the growth rate is shown in Fig. 7. In the case of 3D mode, the average of the growth rate is used because the growth rate does not change with time (Fig. 6). On the other hand, in the case of 2D mode, the maximum of the growth rate is used because the growth rate decreases with time.

In the case of the soft and the hard agar conditions, the growth rate decreases with increasing C_A . The colony growth rate discontinuously increases at $C_A = 0.9$ wt%. In the transition region, two growth rates are plotted. The lower and higher values correspond to 3D growth (before T_{ind}) and 2D growth (after T_{ind}), respectively. In general, such discontinuous changes and the existence of the induction time are the typical characteristics of the phase transition.

3.2. The mechanism of the mode transition

We propose a following hypothesis as a mechanism of the mode transition. The number of large pores which bacteria can pass through decreases with increasing the agar concentration. As the number of large pores decreases, the probability that two neighboring places are linked by the passable pores decreases. The percolation theory tells that such system produces phase transition. At a threshold of agar concentration,

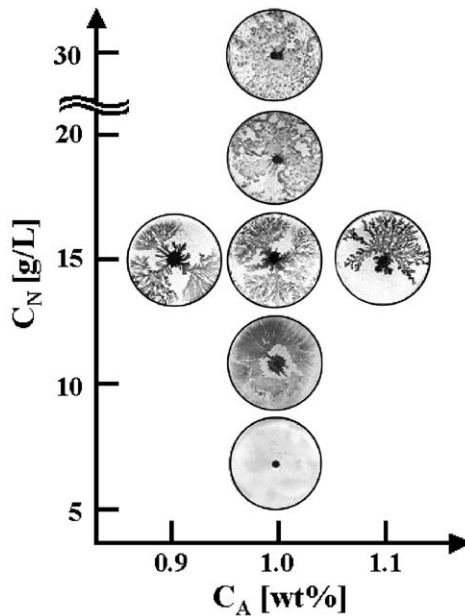


Fig. 4. The morphology diagram at the transition region. The photographs of colony patterns are taken in the final states, that is, no more significant change is observed by additional incubation.

the growth rate of 3D mode abruptly drops to zero. The hypothesis seems to explain the abrupt mode transition observed on our study.

Fig. 8 shows the microstructure of the agar in the different agar concentration conditions taken by transmission electron microscopy (TEM). The average pore size is estimated at about, $2.0\ \mu\text{m}$ (Fig. 8a, $C_A = 0.5\ \text{wt}\%$), $0.6\ \mu\text{m}$ (Fig. 8b, $C_A = 1.0\ \text{wt}\%$), and $0.3\ \mu\text{m}$ (Fig. 8c, $C_A = 1.5\ \text{wt}\%$) from TEM micrographs. Since the average pore size is almost equal to the diameter of *B. circulans* of about $1.0\ \mu\text{m}$ at the transition region, the bacteria cannot move inside the medium in the case of the hard agar.

The second characteristic in the transition region is the existence of T_{ind} . The dependence of the T_{ind} on C_A and C_N is shown in Fig. 9. T_{ind} decreases with increasing both C_A and C_N . The hypothesis can explain the dependence of T_{ind} on C_A because the mobility of bacteria inside the media decreases with increasing C_A . But the hypothesis cannot explain the dependence of T_{ind} on C_N because the gel microstructure is not significantly affected by C_N [17]. And it cannot explain the shift of the transition region to lower agar concentration with increasing C_N (Fig. 2). These results suggest the existence of another factor.

The optical micrographs of the morphology of bacteria in the case of 3D and 2D growth mode are shown in Fig. 10. The bacteria are almost isolated in the case of 3D mode (Fig. 10a), on the other hand, the bacterial aggregates are observed in the case of 2D mode (Fig. 10b). In general, at the hard agar condition, most species of bacteria cannot expand on the hard surface because the mobility of a single bacteria on the

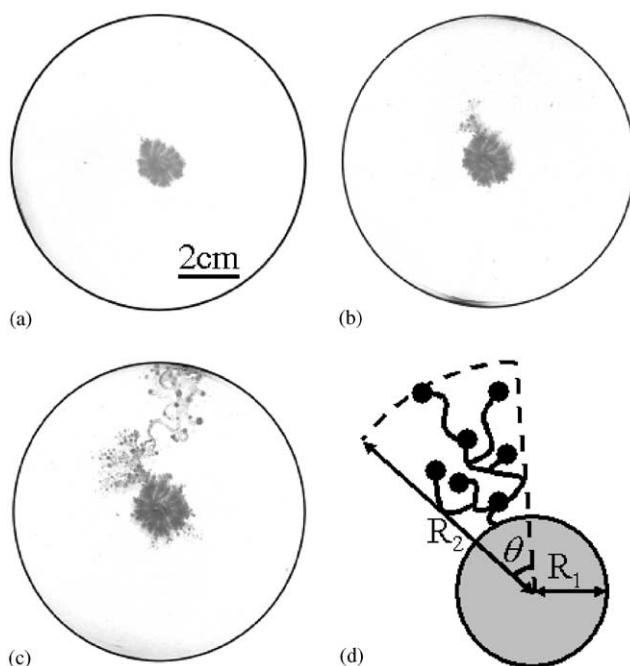


Fig. 5. The time evolution of the colony in the transition region. Each image is taken 6 (a), 7 (b), 8 (c) days after the inoculation (d). The schematic illustration for the measurement of the growth rate.

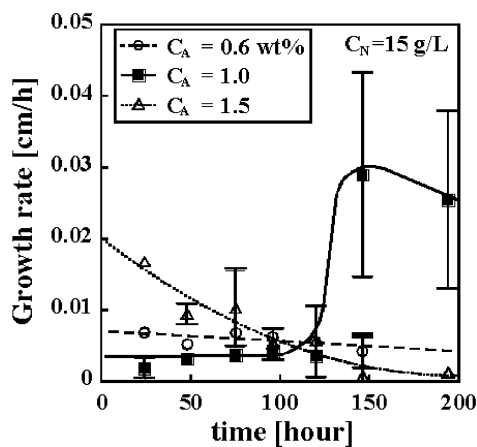


Fig. 6. The time dependence of the colony growth rate at each agar condition. Circle, square, and triangle correspond to the growth rate at the soft agar ($C_A = 0.6$ wt%), the moderate agar ($C_A = 1.0$ wt%), and the hard agar condition ($C_A = 1.5$ wt%), respectively.

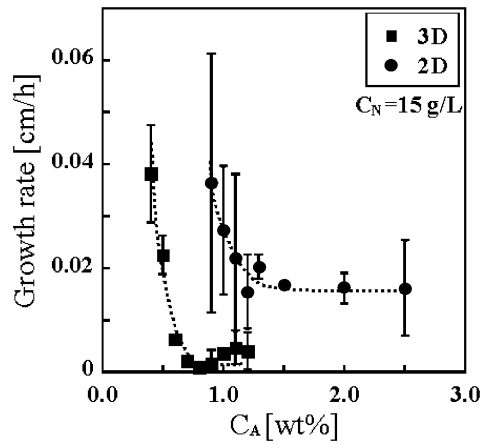


Fig. 7. The dependence of the average growth rate on the agar concentration C_A . Filled circle and filled square correspond to 2D and 3D mode, respectively.

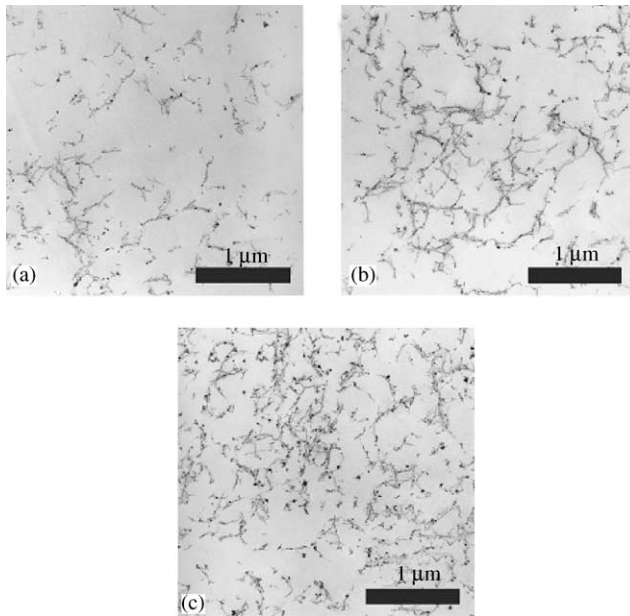


Fig. 8. TEM micrographs of the agarose gels in different C_A : (a) $C_A = 0.5$ wt%; (b) $C_A = 1.0$ wt%; and (c) $C_A = 1.5$ wt%. The black fiber corresponds to the agarose bundle. The TEM samples are prepared according to the method of Ref. [20].

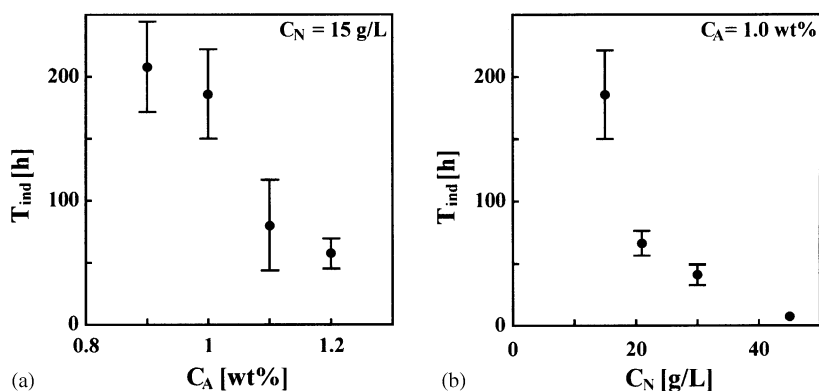


Fig. 9. The influence of C_A (a) and C_N (b) on the induction time T_{ind} .

agar surface is quite small [22]. Only some bacterial strain, such as *Proteus mirabilis* (*P. mirabilis*) [23], *B. subtilis* [7,24], and some kinds of *Paenibacillus* [12,25], form the large 2D colony on the hard agar utilizing the cooperative intercellular interaction, e.g., forming a swarmer cell, secreting lubricant fluid, and forming a bacterial aggregate. On the other hand, the collective motion of *B. circulans* is known to form a motile sub-colony [13]. According to our aforementioned experimental results and the previous study, it can be concluded that to form the aggregates is necessary for 2D growth in the case of *B. circulans*.

As another hypothesis, it is proposed that the increasing bacterial number density causes the bacterial aggregation like the phase transition from the gas phase to the liquid phase. In general, the aggregation of particles relates to its number density. In the dispersion system, e.g. colloidal suspension, nucleation takes place and the resulting aggregation is universally observed at the high number density of particles both experimentally [26] and theoretically [27]. Similarly, most bacteria coagulate above the critical coagulation concentration (CCC) in liquid culture [28]. In agarose gel culture, the aggregate should be formed in the same way. The aggregate moves around on the surface to produce 2D pattern. This hypothesis can explain both the abrupt mode transition and the mechanism of 2D growth.

To testify the hypothesis, it is necessary to show that increasing C_A and C_N cause higher bacterial number density. The dependence of C_N on the bacterial number density would be self-evident. Let us show the relation between C_A and the number density. Because the mobility of bacteria in the medium decreases with increasing C_A (Fig. 8), we investigate the relation between the bacterial mobility in the medium and the bacterial number density by mathematical modeling. In the case of 3D growth mode, because the colony pattern and the colony growth rate are not significantly affected by nutrient concentration, it is suggested that the colony growth rate in this mode is limited by the mobility of the bacteria in the agar (Fig. 11). Let us assume that the bacterial movement could be regarded as the simple diffusion of particle and use the reaction–diffusion model. The reaction–diffusion equations for the bacterial density b

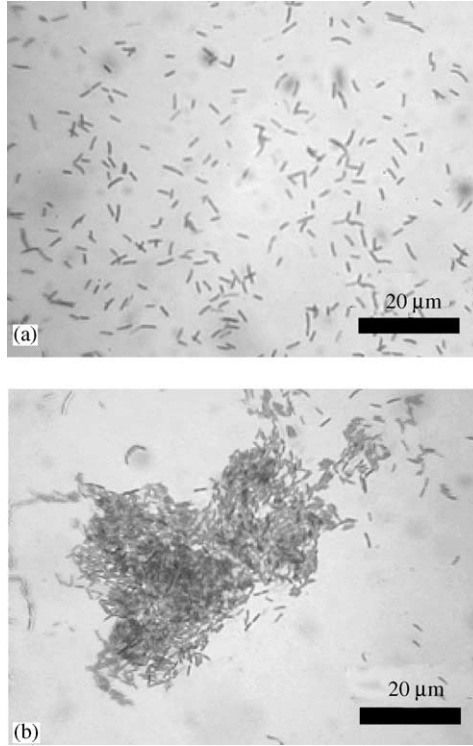


Fig. 10. The optical micrograph of bacteria at the expanding periphery of the colony of the disk-like pattern ($C_A = 0.6$ wt%, $C_N = 15$ g/l) (a) and the knotted-branching pattern ($C_A = 1.5$ wt%, $C_N = 15$ g/l) (b). The sample for the optical microscope observation is prepared as follows. The bacteria at the periphery of the colony are picked by needle and then dispersed in about 200 μ l water solution of NaCl of concentration 0.18 wt%. The dispersion was dried on slide glass and then the bacteria was stained by safranin. The aggregates of bacteria is also observed in the dispersed solution.

and the nutrient concentration n are described as

$$\frac{\partial b}{\partial t} = D_b \nabla^2 b + \frac{kn}{K_S + n} b, \quad (1)$$

$$\frac{\partial n}{\partial t} = D_n \nabla^2 n - \frac{\alpha kn}{K_S + n} b, \quad (2)$$

where D_b and D_n are the diffusion coefficient of bacteria and nutrient, respectively. Here k , K_S , and α are the reaction rate constant, Michaelis constant, and the nutrient efficiency, respectively. Second terms on the right-hand side in Eqs. (1) and (2) correspond to the reproduction of bacteria and the consumption of nutrition, respectively. To perform quantitative analysis, the values of the parameters are estimated by our experiments or quoted from the previous experimental studies (see Appendices B and C for details).

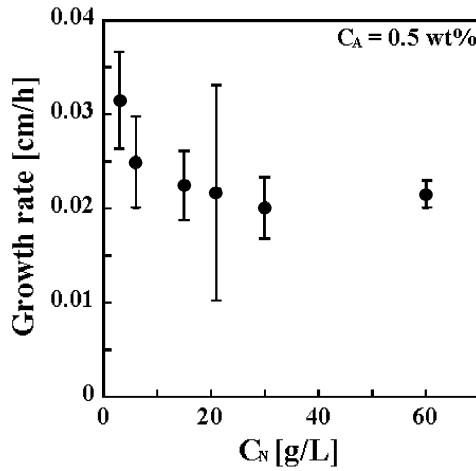


Fig. 11. The dependence of the colony growth rate on C_N at the soft agar condition. The agar concentration is fixed at $C_A = 0.5$ wt%.

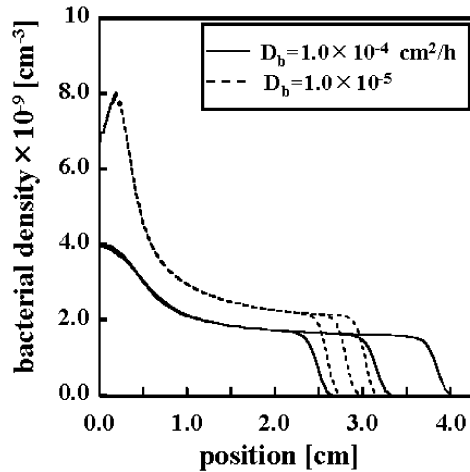


Fig. 12. The spatio-temporal distribution of the bacterial number density calculated from Eqs. (1) and (2). Solid and dashed lines represent $D_b = 1.3 \times 10^{-4}$ cm²/h (at $t = 200, 250$, and 300 h) and $D_b = 1.3 \times 10^{-5}$ cm²/h (at $t = 700, 750$, and 800 h), respectively.

The spatio-temporal bacterial number density distribution is calculated (Fig. 12). The bacterial number density distribution obeys a traveling wave solution. It is consistent with the experimental results in the point that the growth rate is constant during the growth process and that the density distribution is uniform. As diffusivity decreases, the velocity of the traveling wave decreases and the number density increases. Thus, the increase of C_A causes the larger bacterial number density due to the smaller diffusion

coefficient. These results support the second hypothesis that increasing bacterial density causes formation of the bacterial aggregate, which migrates on to the surface of the medium. The induction time would be the time until the bacterial density in the medium reaches certain critical density.

4. Conclusion

The mode transition from 3D to 2D mode by increasing the agar concentration is observed in *B. circulans* colony. The mode transition relates to the change of the colony pattern and to the discontinuous change in the colony growth rate. In the transition region, the growth mode is changed from 3D to 2D during the growth process and as a result, the colony growth rate increases discontinuously. From the dependence of the induction time on the agar and the nutrient concentration, it is suggested that the increasing bacterial density and resultant bacterial aggregation causes the mode transition.

Acknowledgements

We thank Dr. Shimada of National Institute of Infectious Diseases for providing us with the bacterial strain.

Appendix A. The method of calculating colony growth rate

In the case of the isotropic growth (except for the case after induction time in the transition state), in which the colony can be regarded as a circle, the colony growth rate dR/dt is

$$\frac{dR}{dt} = \frac{1}{\sqrt{\pi}} \frac{d}{dt}(\sqrt{A}), \quad (\text{A.1})$$

where R and A are the average radius and the area of the colony, respectively. The colony area A is measured from the photograph of the colony. In the case of the hard agar, the average radius R is estimated smaller than the apparent radius because of lower fractal dimension of the colony pattern.

In the case of the anisotropic growth, which corresponds to the case after the induction time in the transition state, the colony growth rate is calculated as

$$\frac{dR_2}{dt} = \frac{d}{dt} \left(\sqrt{\frac{2}{\theta} (A - \pi R_1^2) + R_1^2} \right), \quad (\text{A.2})$$

where R_1 , R_2 , and θ are the radius of the isotropic disk-like part formed before the induction time, the radius of the anisotropic fan-shaped part formed after the induction time, and the spreading angle of the anisotropic part, respectively (see Fig. 5d). A and θ are measured from the photograph of the colony. Since the growth rate of the isotropic part is quite low, R_1 is regarded as a constant and the average radius measured just

before the mode transition is used as R_1 . The estimated growth rate is always smaller than the actual rate because the colony pattern is not a complete sector shape and the trajectory is not straight.

Appendix B. The details of the calculating conditions

B.1. The values of the parameters used in the calculation

To perform quantitative analysis, the values of the parameters are estimated by our experiments or quoted from the previous experimental studies. The relationship between the cell division time and the nutrient concentration is fitted to the Michaelis–Menten relation using the experimental data in liquid culture. As a result, k and K_S are estimated as $k = 0.6 \text{ h}^{-1}$ and $K_S = 1.5 \text{ g/l}$, respectively. The nutrient efficiency α is in the order of 10^{-9} g . The diffusion coefficients of the nutrient molecules such as glucose and small protein are not affected by agar concentration in the range of $C_A < 7.0 \text{ g/l}$ [22]. Thus $D_n = 2.3 \times 10^{-2} \text{ cm}^2/\text{h}$ is regarded as a constant, which is the value in the water [22].

B.2. Boundary conditions and other calculating conditions

The equations are calculated numerically in one-dimensional form. The length of the space L is set in 4.3 cm, which is equal to the dish radius, and the space is cut by 700 meshes. At the initial condition, n is homogeneously distributed with $n = C_N$ and bacteria is inoculated at $x = 0$. Dirichlet boundary condition ($n|_{x=L} = C_N$) and Neumann boundary condition

$$\left(\frac{\partial n}{\partial x} \Big|_{x=0} = 0, \frac{\partial b}{\partial x} \Big|_{x=0,L} = 0 \right)$$

is used.

Appendix C. The relation between the agar concentration C_A and the bacterial diffusion coefficient on D_b

To see the dependency of C_A on D_b quantitatively, the colony growth rate of the experimental data (Fig. 7) is compared to that of the calculated results. The colony growth rate in the model is defined as the proceeding velocity of the traveling wave. The dependence of D_b on the colony growth rate is calculated as shown in Fig. 13a. Fig. 13b shows the estimated relationship between C_A and D_b . D_b decreases with increasing C_A . The diffusion coefficient of the bacteria in $C_A = 0$ is extrapolated in the order of $10^{-2} \text{ cm}^2/\text{h}$. On the other hand, the theoretical diffusion coefficient of the particle of diameter $1 \mu\text{m}$ in water is in the order of $10^{-6} \text{ cm}^2/\text{h}$ from the Stokes–Einstein relation. The difference could be due to the mobility of bacteria. From the published data [29], the diffusion coefficients of some other motile bacteria such as *E. coli* are measured experimentally, and they are in the range from 10^{-2} to

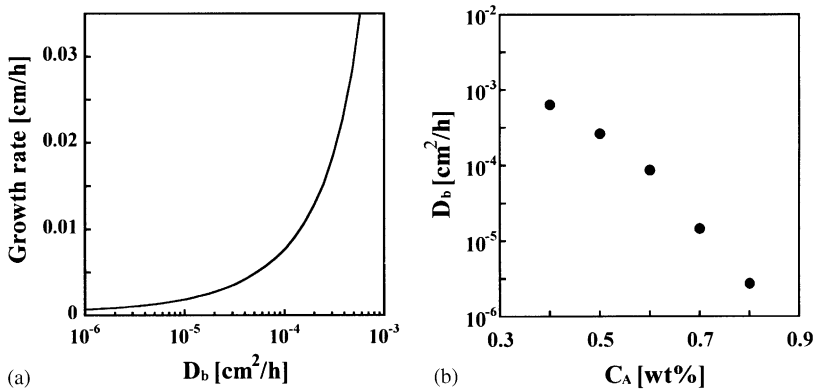


Fig. 13. (a) The dependence of the diffusion coefficient of bacteria D_b on the colony growth rate estimated from Eqs. (1) and (2). (b) The relation between C_A and D_b . Filled circle represents the data estimated by comparing the experimental data with the calculated result.

$10^{-3} \text{ cm}^2/\text{h}$ in water. Because of the good agreement with the previous study and our calculated result, it can be regarded that the calculated diffusion coefficient of bacteria in the agar medium is reliable.

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