Cell population migration cycle and response to environmental pH in *Bacillus subtilis*

Madoka Nakayama[1], Izumi Takagi[2], Jun-ichi Wakita[3], Wataru Shoji[4], Sohei Tasaki[5*]

[1]Institute for Liberal Arts, Institute of Science Tokyo, Chiba 272-0827, Japan

[2]Mathematical Institute, Tohoku University, Miyagi 980-8579, Japan

[3]Department of Physics, Chuo University, Tokyo 112-8551, Japan

[4]Frontier Research Institute for Interdisciplinary Sciences (FRIS), Tohoku University, Miyagi 980-8579, Japan

[5]Department of Mathematics, Faculty of Science, Hokkaido University, Hokkaido 060-0810, Japan

[*]Corresponding Author: tasaki@math.sci.hokudai.ac.jp

Running title: Cell population migration cycle in *B. subtilis*

Highlights

- We study concentric colonies of *B. subtilis* that embody the migration cycle.
- External pH changes the proportion of migration and growth phases in the cycle.
- We propose a model of cell and cell population type regulation.
- Hysteresis in the model can explain the concentric pattern formation mechanism.
- The model predicts cell population regulation in response to the environment.

Abstract

Microbial populations are ubiquitous and some form a robust, multicellular structure called biofilm that protects the cells from environmental damage. The dispersion cycle is critical for controlling the population of important target microbes (both pathogenic and beneficial). Here, we show that the hysteresis of *Bacillus subtilis* cell-type regulation with respect to auto-inducing signal strength triggers the migration cycle of the cell population. We investigate migration cycle and its dependence on environmental conditions by quantitative analysis of cyclically expanding, concentric circular colonies. Next, we construct an input/output model that controls cell types in response to environmental conditions and signal density. On the basis of this model, we propose a migration cycle model for cell populations. The proposed model will widely predict biofilm-related phenomena and provide the basis for the description of highly self-regulating multicellular systems.

Keywords

Migration cycle; Biofilm life cycle; *Bacillus subtilis*; Concentric colony; pH response

Introduction

Microbial populations form colonies and they often exist stably in a robust system called biofilm [1-8]. The structural robustness of biofilms makes it difficult to kill disease-related microorganisms [9-

11]. In addition, myriads of useful microorganisms are also being discovered, so controlling the population of target microorganisms is an urgent issue [12-14]. The process of biofilm formation is as follows: (i) bacteria attach themselves to suitable surfaces available in the environment; (ii) they proliferate while secreting extracellular polymeric substances (EPS) to form a biofilm; (iii) when the colony becomes large and the cell density increases, the outer wall is destroyed and mobile cells are released. These released cells wander around in search for a new environment suitable for growth. When they identify an environment that satisfies their growth requirements, they become non-migrating cells and colonize that environment. To summarize, these cells return to the first phase of biofilm formation as described in (i) above. This cycle of repeating a series of phases is called the life cycle of a bacterial biofilm. The concept of life cycle was originally used for individual organisms, whereas in a biofilm, it is used for a group of cells. Therefore, it involves multilevel interactions, such as information transfer within and between groups. However, the details of the biofilm life cycle generation mechanism are not well understood.

A phenomenon similar to the life cycle of biofilms is the formation of concentric circular colonies that embody the migration cycle of bacterial cell populations. (Fig. 1). *Proteus mirabilis* [15-18] and *Bacillus subtilis* [18-23] are known to form concentric colonies. During their formation, the dominant cell population for colony growth switches periodically between migrating and non-migrating states. In *B. subtilis*, the migrating cell population consists of motile cells, which can swim by bundling and rotating multiple flagella [24]. Particularly, two-dimensional collective movement, called swarming, by motile cells plays a central role in rapid space expansion during the migration phase of the life cycle [25-27]. There are three possible factors that cause the cell population to become non-migrating—without swarming motility—: (i) switching to an immotile cell type; (ii) cessation of hyperflagellation; and (iii) insufficient concentration of surface-wetting molecules, such as surfactin. In the case of this *B. subtilis* concentric colony, the non-migrating cell population is in the form of matrix-producer-like chains of proliferating cell populations, and the cells do not move at all [21]. Therefore, the major

mechanism underlying the stoppage of swarming involves the predominance of immotile cell types, most of which are matrix producers. Although the regulation of surface-wetting molecule secretion and hyperflagellation should also be involved in the stabilization of the cycle[21], we will consider this subject in a future research. Thus, here, we focus on cell type switching. A matrix producer does not move, and it proliferates while producing extracellular matrices [28]. The number of cells increases because of cell division, but in this phase, intercellular adhesion is strong and separation is rare [29-31]. Therefore, the cell group looks like a thread that continues to grow, and its growth mode is called chaining or bundling [32]. The colony not only expands two dimensionally but also increases in thickness (grows in the direction perpendicular to the medium plane). Cells called surfactin producers [33, 34] are known to coexist to assist the slow two-dimensional expansion in this case, improving sliding mobility on the medium surface [35], although it is far behind the expansion speed during the migration phase. In this way, the repetition of the two phases, the migration phase in which cells rapidly spread on the surface of the medium and the growth phase in which migration stops and the thickness increases, exactly represents the migration cycle of *B. subtilis* concentric colonies.

Although the process of concentric colony formation is known in this way, the detailed mechanism remains unclear. This is because of the lack of information on environment-dependent control patterns between different cell types, mobile and non-mobile. By focusing on *B. subtilis*, various mutually exclusive cell types have been discovered [24]. The balance between motile cells and matrix producers is particularly important for the migration cycle of the cell population. The genes related to each of these two types are mutually exclusive because of self-activation and mutual repression. When this mutual exclusivity becomes stronger, hysteresis occurs in cell-type regulation (i.e., cell-type selection depends on the history of past cell states) and a migration cycle is induced in the cell population, resulting in the formation of concentric circular colonies. Specifically, whether or not concentric colonies are formed depends on the presence or absence of hysteresis during cell-type control of the cell population and on environmental conditions. Investigating the conditions for forming concentric

89 colonies is essential for understanding the life cycle generation mechanism of cell populations.

90 However, the conditions for forming concentric colonies of *B. subtilis* have not yet been clearly
91 determined, although the need for agar concentrations of about 0.7% in solid medium and high nutrient
92 conditions is known [19, 21]. Particularly, the agar concentration is required to be in the range of 0.65–
93 0.75%. In addition, the incubation temperature is preferably 25–35°C, which is lower than the usual
94 35–37°C [19], and long-term pre-drying of the medium surface is desirable [21]. The situation seems
95 to be complicated by the effect of temperature and surface moisture other than agar and nutrient
96 concentrations. Therefore, we introduced environmental pH as a new parameter and attempted to
97 clarify the growth condition requirements of *B. subtilis*.

98 Except for concentric colonies, the dominant cell type is often defined as either a motile cell or matrix
99 producer. First, colonies formed by swarming motile cells are formed under an agar concentration of
100 0.4–0.7%. These colonies are classified into two types according to the nutrient concentration: disk-
101 like (high nutrition) [36, 37] and dense branching morphology (DBM)-like (low nutrition) [38-41]. On
102 the other hand, the slowly expanding colonies dominated by matrix producers are formed on a solid
103 medium with an agar concentration of 0.7% or more. These colonies are also classified into two types,
104 namely, Eden cluster-like (high nutrition) [42-44] and diffusion-limited-aggregation (DLA)-like (low
105 nutrition) [45-48], depending on the nutrient concentration [46, 47]. In general, the classification is
106 based on these five types. A detailed examination revealed that a small number of motile cells were
107 mixed even on the surface of a hard medium with an agar concentration of 1%, which affected
108 macroscopic colony morphology and expansion rate [49]. Particularly, a crater-like pattern in which
109 cells gather at the tip of the colony is formed by only slightly lowering the environmental pH from the
110 intracellular pH of 7.4. This is due to chemotaxis toward nutrients, which is a phenomenon that does
111 not occur during swarming [27]. On the other hand, when the agar concentration is low (0.5%), swift
112 colony expansion via swarming is observed because the energy required for migration is low due to
113 the availability of abundant water on the surface of the medium. However, under certain conditions,

114 i.e., optimum pH of 7.4, the dominant cell types become matrix producers rather than motile cells, and
115 lacework-patterned colonies may be formed [23]. Thus, other than concentric colonies, different stress
116 parameters, such as environmental pH, influence the choice between motile cells and matrix producers.
117 Here, we investigate the effect of environmental pH on cell-type selection and concentric *B. subtilis*
118 colony formation. By quantitative analysis of culture experiments, we clarify the range of
119 environmental pHs in which concentric colonies are formed, and the environmental pH dependence of
120 the temporal and spatial cycles within that range. Further, by combining the mathematical model of
121 cell-type regulation on the basis of the conventional gene regulatory network and analysis of its
122 mathematical properties, we propose a model for type regulation of cell population that changes
123 depending on the environment. Our proposed model provides a reasonable explanation for the
124 formation of concentric circular colonies and subsequent biofilm life cycle generation.

125 Results

126 Concentric colonies embody the biofilm life cycle

127 We prepared a medium with a high nutrient concentration and an intermediate agar concentration of
128 0.7% among the range of solid agar media (containing 0.2% or more agar), and inoculated *B. subtilis*
129 onto the center. When the plate was cultured at about 35°C, concentric colonies were formed on the
130 medium surface (Fig. 1). This is a simple model for biofilm life cycle generation, and its temporal and
131 spatial cycles embody that life cycle. We captured two-dimensional transmitted light images of this
132 colony using a scanner (Fig. 1(a)). The image data were converted to cell density distribution (Fig.
133 1(b)), using which a 3D structure was reconstructed (Fig. 1(c)), and the cross-section was analyzed
134 (Fig. 1(d)). By performing such quantitative analyses in a time series, not only the two-dimensional
135 cell density (colony thickness) but also the dynamics of cell proliferation and migration were analyzed
136 (Fig. 2). The boundaries of colonies were identified at each time and the depth of each terrace was
137 measured (Fig. 2(a), (b)). The concentric colonies of *B. subtilis* have distorted boundaries unlike those

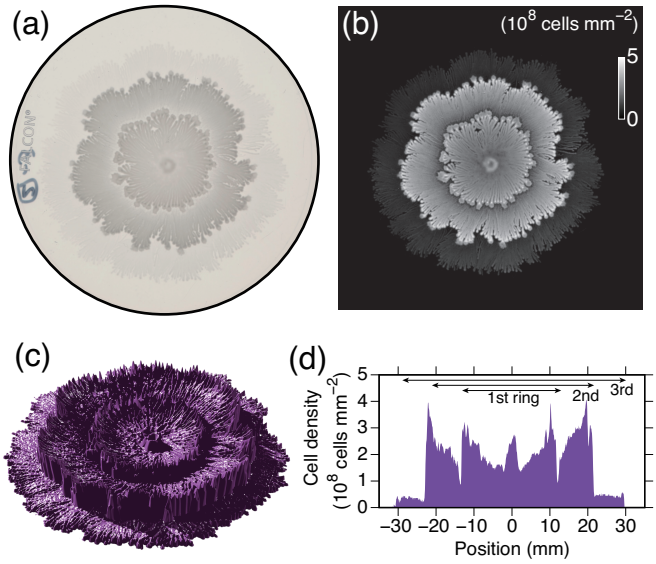

Fig. 1. Concentric ring-like colony embodies the life cycle. (a) Raw image of transmitted light obtained by a flat scanner. The center is the inoculation point. The diameter of the dish is 88 mm. (b) Two-dimensional cell density. (c) Three-dimensional reconstruction of the colony. (d) Cross-sectional view.

of *Proteus mirabilis*. Therefore, the colony radius r was defined from area A of the inner area of the colony: $r = \sqrt{A/\pi}$. Then, from the increase in the colony radius, the migration and growth phases of *B. subtilis* biofilm, and their combined life cycle were observed (Fig. 2(c)). During one period, a set of a migration phase and a growth phase, the rate of increase in total cell number is nearly constant, and when the cells enter the next cycle and become motile cells, the cell proliferation rate decreases, which may be due to the large amount of nutrients used in the transition to motile cells (Fig. 2(d)).

Microscopic observation of phase switching in concentric colonies

Using phase-contrast microscopy, we investigated how the concentric colony formation occurred, and how the cell state and its spatial distribution changed. First, the termination process of the growth

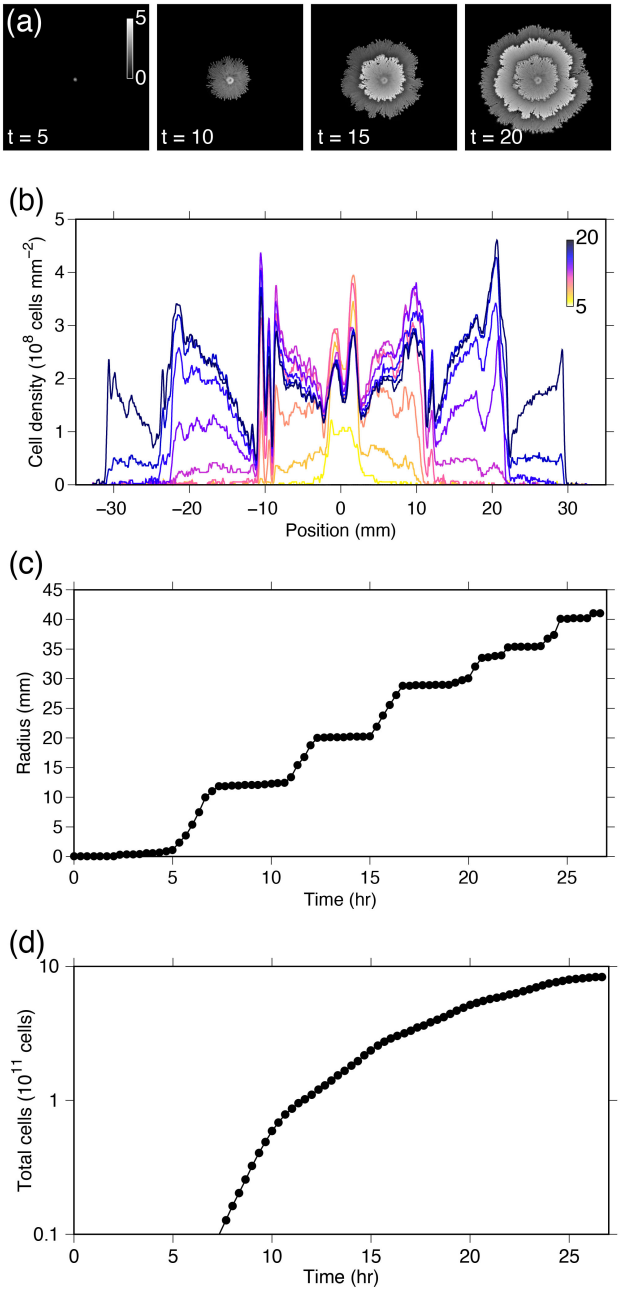

Fig. 2. Growth dynamics of concentric ring-like colony. (a) Two-dimensional cell density. Color bar, cell density (10^8 cells mm^{-2}). Incubation time, 5, 10, 15, and 20 h after inoculation. (b) Cross-section cell density. The center is the inoculation point. (c) Increasing colony radius. (d) Increasing total number of cells.

phase, in which matrix producers are dominant, begins with the appearance of motile cells just before the colony boundary (Fig. 3(a)). There, motile cells continue to grow and form ponds wherein cell

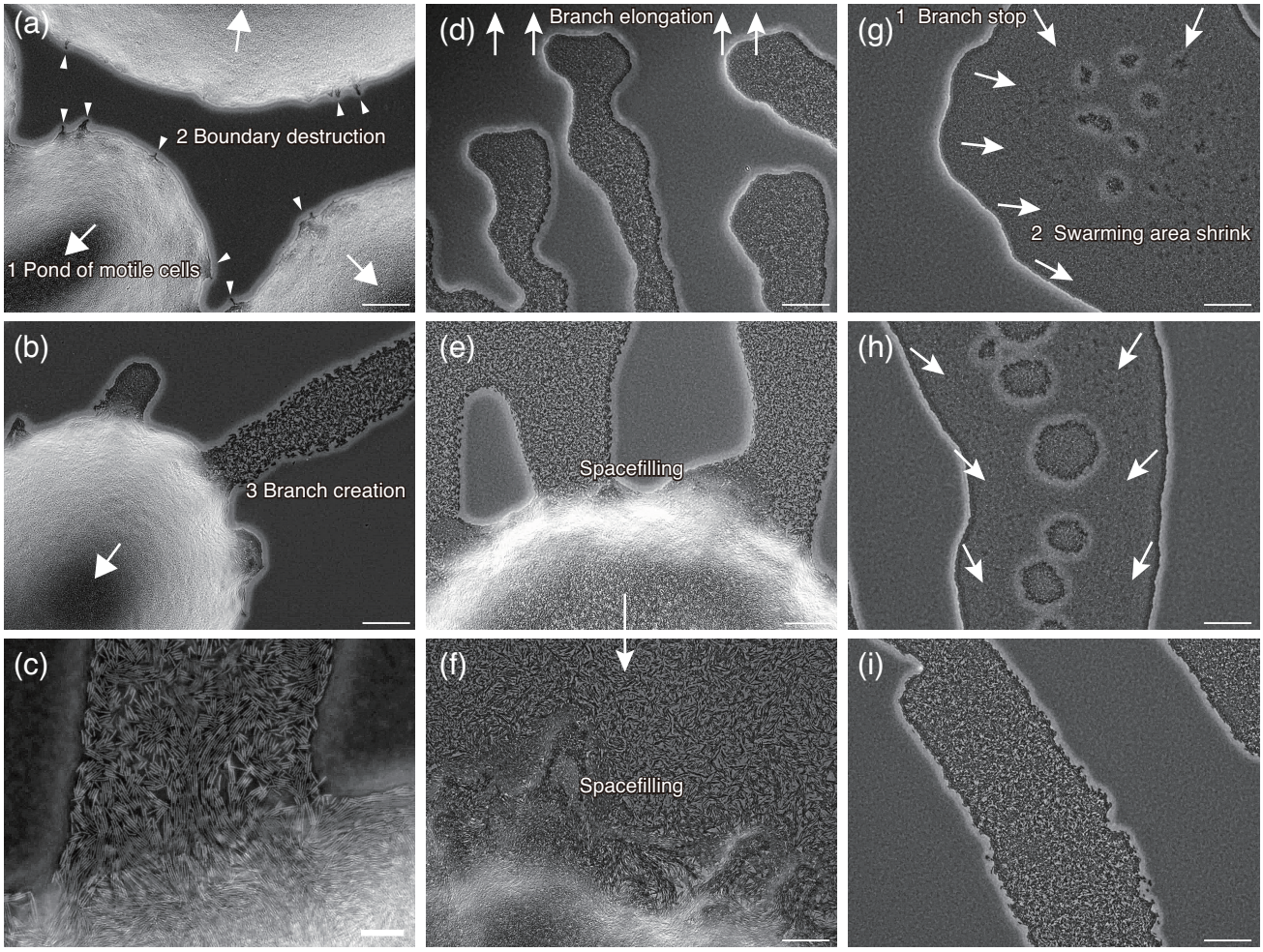

Fig. 3. Microscopic images of phase transition during colony expansion. (a)-(c) Transition from the growth phase to the migration phase. Motile cells appear slightly inside colony boundaries (large arrows in (a) and (b)). The number of motile cells increased to form bacterial ponds where cells organize to undergo swarming collective motion. The motile cells then destroy the boundary walls (small triangles in (a)). Cells flow out from some of the outermost destruction points on the colony boundaries to create rapidly expanding branches ((b) and (c)). (d)-(f) The migration phase. Numerous dense branches grow outward (d). Gradual collapse of the colony boundaries at the roots of branches, and the space around the roots is filled with the outflowing cells ((e) and (f)). (g)-(i) Transition from the migration phase to the growth phase. First, the branches stop growing (g). Then, the swarming regions composed of motile cells (small bubble-like regions in (g) and (h); whole branch region in (i) shrink from the outside to the inside of the colony ((g), (h) to (i); (g) is the outermost boundary, (i) is the innermost). Scale bars, 100 μm (except for (c)), 10 μm (c).

swarming occurs. The surrounding cells are partially separated from each other by autolysins [29, 30, 50, 51], and the pressure of proliferating motile cells is also applied, destroying many points in the colony border area.

152 Among those destroyed points, the breakage starts at points on the outer side of the colony boundary,
153 and motile cells flow out from there to form branches (Fig. 3(b), (c), Supplementary Video S1). The
154 migration phase where motile cells are dominant is shown in Fig. 3(d)-(f). In this phase, the formed
155 branches quickly expand outward because of swarming (Fig. 3(d), Supplementary Video S2). Then,
156 the number of break points near the root increases, and the gap between branches is filled with outflow
157 cells from the inside (Fig. 3(e), (f), Supplementary Videos S3, S4). The end of the migration phase
158 begins when the branches stop growing (Fig. 3(g)). Immediately thereafter, the swarming area shrinks
159 from the tip of the branch toward the inner root (Fig. 3(g)-(i)). When there is no swarming region, the
160 growth phase begins. In the growth phase, the cells do not move and proliferate, and the cell density
161 increases. When the concentration exceeds a certain threshold, motile cells reappear, and transition to
162 the migration phase occurs (Fig. 3(a)). This is one round of the life cycle of *the B. subtilis* cell
163 population.

164 Effect of environmental pH on periodically expanding colonies

165 The conditions for forming concentric colonies of *B. subtilis* have been investigated. Particularly,
166 under optimal conditions (incubation temperature around 35–37°C), concentric circular colonies are
167 formed only in an extremely narrow range of agar concentration of 0.65–0.68%, even if nutritional
168 conditions are suitable [21]. It can be inferred that other stress parameters, such as low temperature
169 [19], are necessary for the formation of concentric colonies. We investigated the effect of
170 environmental pH as a stress parameter. Conditions other than environmental pH were fixed, and the
171 agar concentration was set at an intermediate level of 0.7% (Fig. 4). First, in the neutral pH region, no
172 periodically growing colonies appeared. Matrix producers were the predominant cell type, and
173 colonies grew by increasing their volume via proliferation (Fig. 4(a), (b)). As the environmental pH
174 decreased, many, very-short migration phases appeared around pH 6.8. Under the pH condition close
175 to this transition point, the variation in space was very strong, the periodicity was not clear, and the

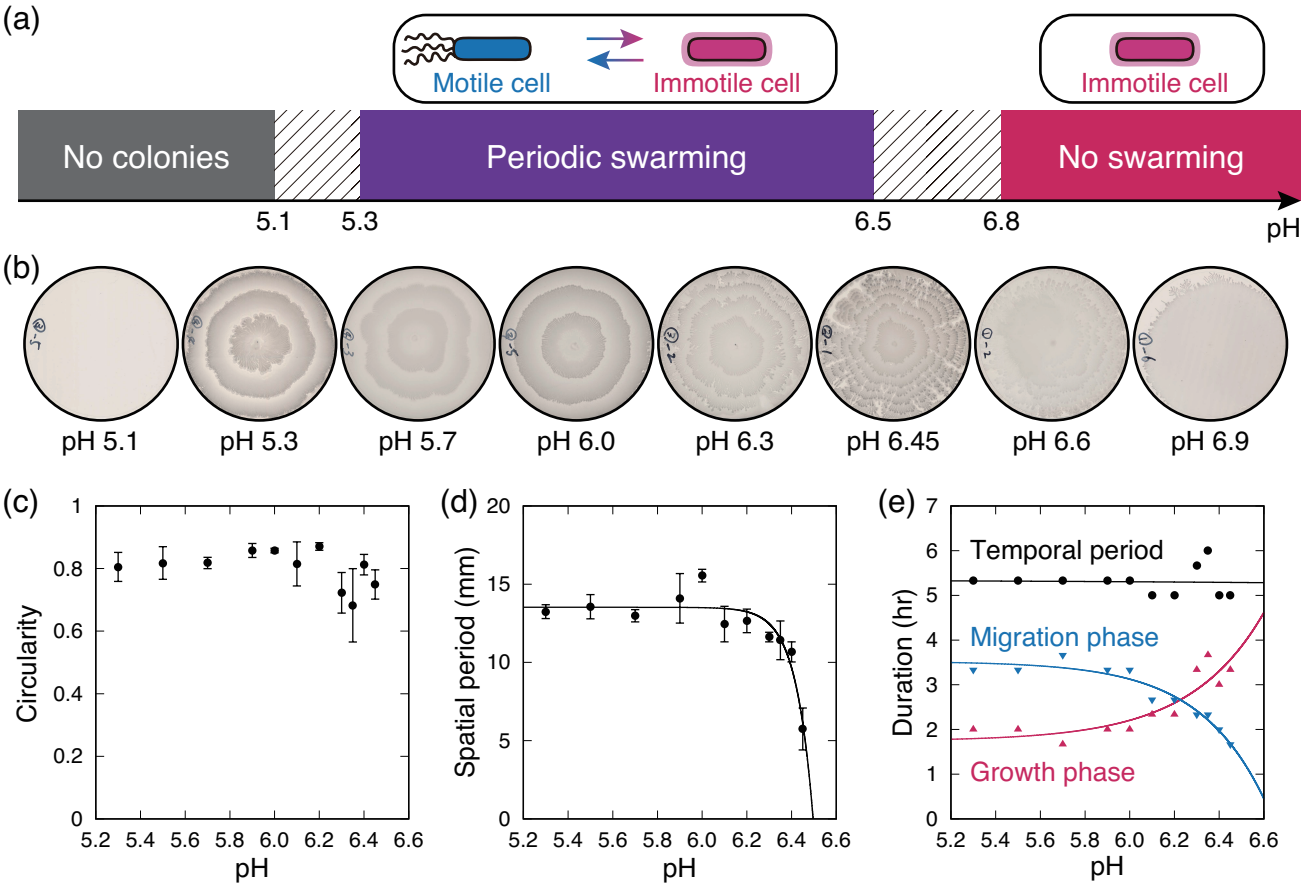

Fig. 4. Effect of environmental pH on periodically expanding colonies. (a) Environmental pH conditions for forming periodically expanding colonies. (b) Colonies under different environmental pH conditions. (c) Circularity of colony ring (defined by circularity of second ring). (d) Spatial period (width of terrace). (e) Time period: growth phase, migration phase, and total period. Data represent mean ± s.d., $n = 6$ ((c) and (d)). All fitting curves are exponential plus constant ((d) and (e)).

pattern was far from that of concentric circles. When the pH decreased below 6.5, concentric circular colonies that expand periodically were formed. When the pH was lower than 5.3, colony formation itself became unstable, and the colony formation stopped halfway or no colonies were formed. Finally, below pH 5.1, colonies were not always formed. In summary, the stable pH range for cyclic growth of colonies was 5.3–6.5.

Next, we investigated the properties of concentric colonies in this pH range. First, the circularity (4pi area divided by circumference squared) of the ring was constant, although there was some variation in the pattern transition region near the neutral pH (Fig. 4(c)). The spatial period (width of the swarm ring) seemed to increase monotonically as pH decreased (Fig. 4(d)). Specifically, in the pattern

transition region (pH 6.0–6.5), the spatial period sharply increased with decreasing pH, and then it had a substantially constant period in the range of pH 5.3–6.0.

To clarify how these differences in colony morphology were produced, we examined the periods of each growth phase and migration phase (Fig. 4(e)). We found that while the total time period was almost constant, the growth phase duration decreased as the pH decreased; conversely, the duration of the migration phase increased. Specifically, the finer spatial patterns reflected shorter migration periods, while the total length of the life cycle of the cell population seemed homeostatic.

Environment-dependent regulation of cells and cell populations

We investigated the mechanism of control of the two different states of the cell population (migration and growth phases). Cell population control should be understood on the basis of cell-level regulation in response to environmental conditions The regulation between the two cell types, motile cells and matrix producers, is well studied, particularly the switch between planktonic and biofilm states [24, 52-55]. The two cell types are designed to have mutually exclusive gene expression states. We created a simple mathematical model to investigate cellular state dynamics (Fig. 5(a)). In this model, cell states can be described according to four group variables by grouping coordinated genes and their products: (1) S: A group represented by Spo0A~P, which also includes phosphorelay of Spo0F, Spo0B, and Spo0A; (2) H: SigH; (3) A: AbrB; (4) C: A group represented by ComK, driven by the ComX-ComP-ComA pathway.

The output of this control system is the cell type. In this case, it is a matrix producer when S is high and a motile cell when S is low. Sporulation is initiated when S is continuously very high. However, predominant sporulation occurs at a nutrient-depletion stage much later than colonization.

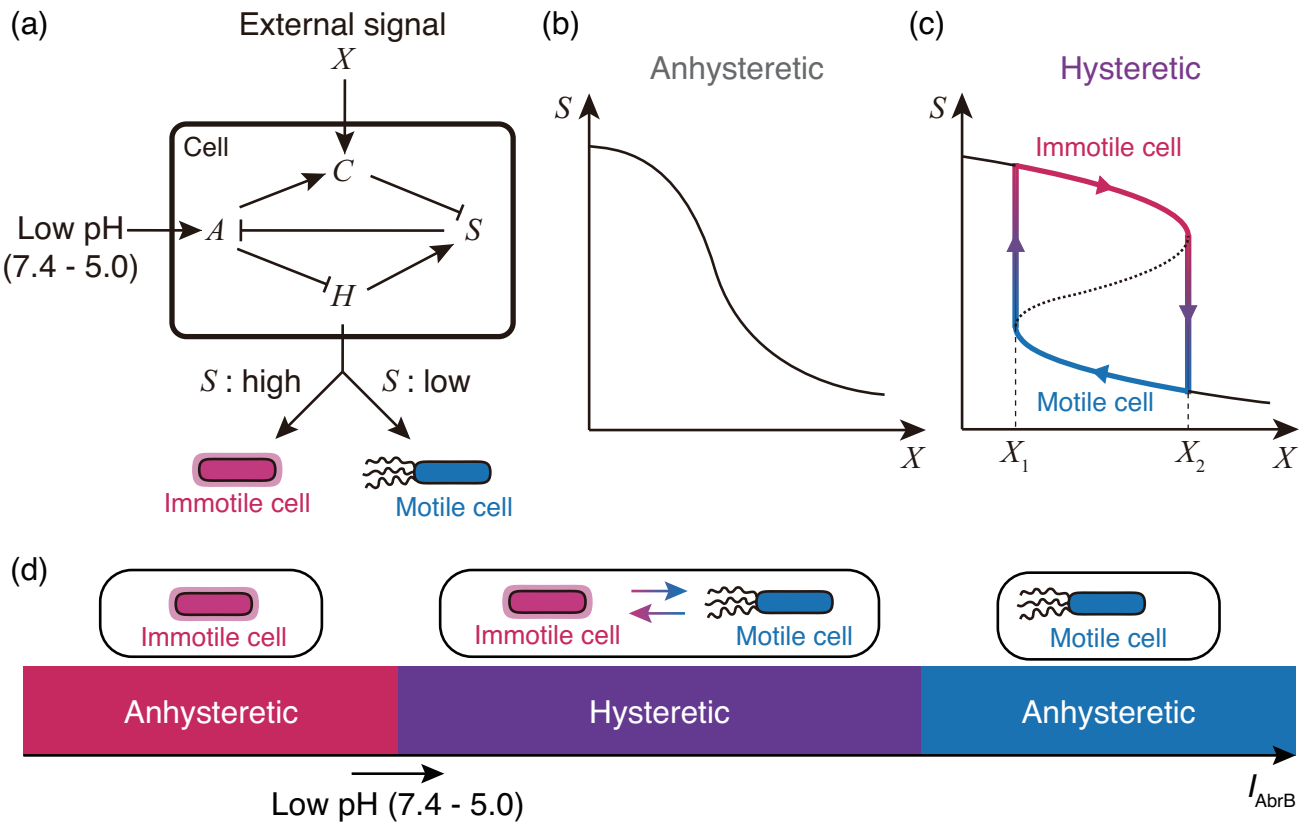

Fig. 5. A model for the response of cells and cell populations to environmental pH. (a) A model for cell-type selection for environmental pH and cell density. (b), (c) Two types of cell-type controls. Anhysteretic (b) and hysteretic (c). (d) A model for cell population-type selection. The horizontal axis (I_{AbrB}) is a parameter indicating the external activation rate of AbrB (see "Materials and Methods").

Therefore, it is not necessary to consider sporulation here. On the other hand, the inputs of the cell state control system include external environmental conditions and auto-inducing signals that represent cell density. There are various environmental conditions. For example, poor nutritional conditions may be input to activate S, but we considered the formation of periodic growth colonies, and the initial nutrient concentration was sufficiently high. Therefore, the nutritional conditions were constant and sufficient at the time of colony formation. As an auto-inducing signal related to cell-type control, a small peptide ComX is secreted by *B. subtilis* cells [56-58]. In the following, its concentration is denoted as X, which is an input from C to the cell-type regulation system [56, 57, 59]. The input mode of environmental pH is of significant interest at this stage. According to previously performed studies, H or A is the candidate input point for external pH in the current model [60, 61].

Here, we considered the most minimal and smooth dynamic model of these four variables ("Materials and Methods"). The curve of the set of equilibrium points can be divided into two types: with respect to the increase and decrease in X, the variable that represents the cell state (for example, S) is monotonic and non-monotonic (Fig. 5(b), (c)) [62]. In the latter case, the curve is an S-shaped curve with two turning points. Specifically, there are two cases where cell-type control is either not hysteretic (Fig. 5(b)) or hysteretic (Fig. 5(c)), considering the increase/decrease in the cell density signal X. The latter hysteretic control is necessary for biofilm life cycle generation and concentric colony formation. When there is hysteresis in the choice of cell state for increasing and decreasing X, a life cycle is created for the cell population as follows. Migration phase: the cell density information X at the growth front decreases as the cell population disperses in the motility state, and when it falls below a certain threshold X_1, the cell population switches to the matrix-producing state. Growth phase: as the cell population grows and matures in the matrix-producing state, X increases, and when it exceeds a certain threshold $X_2 \left(> X_1\right)$, the cell population switches to the motility state again.

AbrB is upregulated and SigH is suppressed as the environmental pH decreases in the weakly acidic region of interest [60, 61]. These two are potential candidates for inputting environmental pH information. It is not easy to determine which of these two elements, which suppress each other in the regulatory network, is upstream for inputting external pH information. According to the detailed mathematical analysis of the equilibrium state of this model, a situation in which hysteresis occurs with a decrease in environmental pH and concentric colonies are formed can be realized only when AbrB is the input point for pH (Fig. 5(a), (d); "Materials and Methods") [62] . Therefore, we proposed a model of cell-type regulation using AbrB as the input point for environmental pH information (Fig. 5(a)). On the basis of this cell-type regulation model, the generation mechanism of the life cycle of the cell population in the parameter range for hysteretic control could be explained (Fig. 5(c)), and the dependence on environmental conditions could be understood.

240 # Discussion

241 We have presented a possible mechanism of formation of *B. subtilis* concentric colonies that expand
242 periodically and embody the life cycle of the cell population. By performing culturing experiments
243 and their quantitative analysis, the dynamics of colony growth and its environmental pH dependence
244 were clarified from a multilevel perspective—from the cell level to the macroscopic morphological
245 level. We sought to understand patten formation under the assumption that the predominant cell type
246 switches between motile cells and immotile cells, such as matrix producers. Based on the accumulation
247 of molecular biology research to date, the outline of the gene regulatory network responsible for the
248 regulation between these two cell types of *B. subtilis* has been established. We proposed a model of
249 cell-type regulation by environmental conditions and cell density by combining concentric colony
250 culture experiments under different environmental pH conditions. The cell density is considered to be
251 measured by the cells themselves by the concentration of the secreted quorum-sensing factor ComX
252 [56-58]. The mathematical properties of the minimal smooth model describing the dynamics also
253 suggested the input location of environmental pH in the cell-type regulation system. The obtained
254 model should be a general control mechanism that selects an appropriate cell population structure
255 depending on the situation. Particularly, the S-shaped curve of the set of equilibrium points obtained
256 by this mathematical model analysis makes it possible to understand the hysteretic control of the cell
257 population that forms concentric colonies, and subsequently, the migration cycle generation
258 mechanism.

259 In the cell population migration cycle, phase switches are dependent on cell density. We investigated
260 the switching mechanism and found that the migration cycle was stably modulated via hysteretic
261 control of cell type. Further, the effect of environmental conditions on colony patterns can be predicted.
262 At present, however, environmental changes and cell type control were investigated via concentric
263 circle colony formation experiments under environmental pH (and only neutral to acidic) conditions.
264 In the future, we plan to elucidate how *B. subtilis* cells collectively cope with fluctuations in a large

265 range of pH, particularly via the study of the responses at high pH. Moreover, to predict the response
266 to changes in other environmental conditions, such as nutrient concentration, temperature, and oxygen
267 concentration, it is necessary to understand a wider gene regulatory network. Nevertheless, the
268 conditions for biofilm life cycle generation can be better organized as per the presence or absence of
269 hysteresis. Of note, this classification will probably be applicable to biofilm life cycle studies focusing
270 on other microbial species.

271 A morphological diagram was created for understanding the colony morphology of *B. subtilis* in the
272 1990s [36, 63], in which one of the five colonies was formed depending on the two parameters, nutrient
273 concentration and agar concentration, in the medium. These five colonies are called Eden cluster-like
274 [42-44], DLA-like [45-48, 64, 65], concentric ring-like, disk-like [36, 37], and DBM-like [38-41],
275 respectively. The concept of cell type was not established at the time, but it is now well-understood,
276 and the colony pattern can be explained as follows. The dominant cell type is immotile cells, such as
277 matrix producers, in Eden cluster-like and DLA-like colonies; disk-like and DBM-like colonies have
278 motile cells as the predominant cell type. Finally, in the concentric ring-like colony, the dominant cell
279 type switches between motile cells and immotile cells. Thus, among the five patterns, concentric
280 colonies had multiple dominant cell types, which created unsolved problems. The mechanism of
281 pattern formation was unknown, and it was not understood or reproduced by a mathematical model
282 [66]. Our proposed model for cell-type regulation has presented one possible mechanism for this
283 problem.

284 Furthermore, the concentric colonies presented uncertainties, even with respect to the formation
285 conditions, such as an extremely narrow agar concentration range [21] and the dependence on culture
286 temperature [19]. Apparently, parameters other than agar and nutrient concentrations were likely
287 involved in the formation of concentric colonies. In other words, introducing some kind of a stress
288 parameter is effective for concentric pattern formation. We obtained the conditions to stably form
289 concentric colonies by introducing environmental pH. By drawing the morphology diagram in a three-

290 parameter space, it is possible to understand the entire cell population morphology correctly and in
291 detail. By incorporating our cell-type regulation model into a multilevel morphogenesis model, we
292 expect to obtain a simulation model that accurately predicts the growth of multicellular systems with
293 non-uniform cell state distribution.

294 Robust biofilms and other complex cell populations of microorganisms are social structures involved
295 in the organization of diverse cell types. Therefore, understanding the mode of state regulation of each
296 cell should be the first key to understanding and controlling the behavior of the cell population as a
297 whole. Therefore, higher-order structures of cell populations are self-organized in a trans-hierarchical
298 manner: from gene-level and cell-level control to microscopic structures, macroscopic morphology
299 and function of cell populations, and interaction with the surrounding environment. Thus, the
300 construction of multilevel models for controlling the state of cell populations will strongly advance
301 the research on various multicellular societies.

302 Materials and Methods

303 Experimental methods

304 *B. subtilis* wild-type strain OG-01 (JCM32485) was used [46, 67]. A very small quantity of bacteria
305 (endospores) was incubated overnight (10–16 h) in 5 mL of Luria–Bertani liquid growth medium (LB
306 Broth Base (Lennox L Broth Base), Gibco BRL Life Technologies, cat. no. 12780-052) at 35°C with
307 shaking at 140 rpm. For inoculation on solid agar media, this final pellet was diluted with the buffer
308 to an OD_{600} of 0.5.

309 A solution of 86 mM NaCl and 29 mM K_2HPO_4 was prepared. As a nutrient, 20 g L^{-1} peptone (Bacto
310 Peptone, BD Biosciences, cat. no. 211677) was added to the solution, and the pH was then adjusted to
311 a designated value by adding 6 M HCl, and 0.7% agar (Bacto Agar, BD Biosciences, cat. no. 214010)
312 was added to the above liquid nutrient medium. After autoclaving at 121°C for 15 min, 20 mL of the
313 solution was poured into each plastic Petri dish. The dishes were maintained at room temperature

overnight and dried at 50°C for 90 min.

The pre-cultured bacterial suspension was inoculated using a sterilized needle at the center of the prepared solid medium surface. The dishes were incubated in a humidified box at 35°C for the designated time. Macroscopic colony-transmitted light images were obtained using a flat head scanner (GT-X980, Epson). Cell or cell cluster level morphology was observed by phase-contrast microscopy using an inverted microscope (IX83, Olympus) with a camera (DP80, Olympus).

Mathematical model of dynamic cell-type regulation.

To describe the dynamics of cell-type control (Fig. 5(a)), we consider the following minimal smooth model:

$$\begin{aligned} \frac{dS}{dt} &= \frac{c_S H}{a_S + b_S C} - d_S S \\ \frac{dH}{dt} &= \frac{c_H}{a_A + b_A A} - d_H H \\ \frac{dA}{dt} &= \frac{c_A}{a_A + b_A S} - d_A A \\ \frac{dC}{dt} &= c_C X A - d_C C \end{aligned} \tag{1}$$

where a_*, b_*, c_*, d_* $(* = S, H, A, C)$ are positive constants. There are two cases where cell-type control is non-hysteretic (Fig. 5(b)) and hysteretic (Fig. 5(c)), regarding the increase/decrease in the cell density signal *X*. We set a parameter indicating the rate at which AbrB is activated from outside the model system of cell-type regulation (equation (1); Fig. 5(a)) as follows:

$$I_A = \frac{b_H}{a_H} \times \frac{c_A / a_A}{d_A} \tag{2}$$

Similarly, we define the rate at which Spo0A~P and SigH are activated from outside the model system as

$$I_{SH} = \frac{b_A}{a_A} \times \frac{c_S / a_S}{d_S} \times \frac{c_H / a_H}{d_H} \tag{3}$$

Particularly, the external activation rate of AbrB $I_A = I_{\mathrm{AbrB}}$ is the horizontal axis in Fig. 5(d). Thus, the following can be proved [62]: There exists $I_{SH}^* > 0$ such that

1. If $I_{SH} \le I_{SH}^*$, then the steady states are anhysteretic (Fig. 5(b)).
2. If $I_{SH} > I_{SH}^*$, then there exists $1 < I_{A,L} < I_{A,U}$ such that

(i) If $0 < I_A \le I_{A,L}$, then the steady states are anhysteretic (Fig. 5(b)).

(ii) If $I_{A,L} < I_A < I_{A,U}$, then the steady states are hysteretic (Fig. 5(c)).

(iii) If $I_{A,U} \le I_A$, then the steady states are anhysteretic (Fig. 5(b)).

Case 2 above (the external activation of Spo0A~P and SigH is larger than the threshold $I_{SH} > I_{SH}^*$) shows the situation in Fig. 5(d), and it is also in agreement with the experimental results, suggesting that $I_A = I_{\mathrm{AbrB}}$ plays the role of a parameter corresponding to the environmental pH reduction. Note the mathematical feature that at least AbrB must be actively involved to reproduce the pH dependence of cell type control [62]. In the past literature, SigH or AbrB, especially SigH, was a candidate [60, 61], but at least SigH alone does not show the observed pH dependence of cell type control in the mathematical model.

CRediT authorship contribution statement

Madoka Nakayama: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Review & Editing, Visualization, Funding acquisition. **Izumi Takagi:** Formal analysis, Writing – Review & Editing, Funding acquisition. **Jun-ichi Wakita:** Formal analysis, Resources, Writing – Review & Editing. **Wataru Shoji:** Formal analysis, Writing – Review & Editing, Supervision. **Sohei Tasaki:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing – Original

Draft, Writing – Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by JSPS KAKENHI, Grant Number 23K03225 (M.N.), 23K03176 (I.T.), 19K03645 (S.T.), 23K03208 (S.T.), and MEXT KAKENHI, Grant Number 17H06327 (S.T.).

References

[1] P. Stoodley, K. Sauer, D.G. Davies, J.W. Costerton, Biofilms as Complex Differentiated Communities, Annu. Rev. Microbiol., 56 (2002) 187-209.

[2] R.M. Donlan, Biofilms: microbial life on surfaces, Emerg. Infect. Dis., 8 (2002) 881-890.

[3] L. Hall-Stoodley, J.W. Costerton, P. Stoodley, Bacterial biofilms: from the natural environment to infectious diseases, Nat. Rev. Microbiol., 2 (2004) 95-108.

[4] S.S. Branda, Å. Vik, L. Friedman, R. Kolter, Biofilms: the matrix revisited, Trends Microbiol., 13 (2005) 20-26.

[5] K. Kobayashi, M. Iwano, BslA(YuaB) forms a hydrophobic layer on the surface of *Bacillus subtilis* biofilms, Mol. Microbiol., 85 (2012) 51-66.

[6] H. Vlamakis, Y. Chai, P. Beauregard, R. Losick, R. Kolter, Sticking together: building a biofilm the *Bacillus subtilis* way, Nat. Rev. Microbiol., 11 (2013) 157-168.

[7] L. Hobley, C. Harkins, C.E. MacPhee, N.R. Stanley-Wall, Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes, FEMS Microbiol. Rev., 39 (2015) 649-669.

[8] H.-C. Flemming, J. Wingender, U. Szewzyk, P. Steinberg, S.A. Rice, S. Kjelleberg, Biofilms: an emergent form of bacterial life, Nature Reviews Microbiology, 14 (2016) 563-575.

[9] P. Gilbert, J. Das, I. Foley, Biofilm susceptibility to antimicrobials, Adv. Dent. Res., 11 (1997) 160-167.

[10] T.F. Mah, G.A. O'Toole, Mechanisms of biofilm resistance to antimicrobial agents, Trends Microbiol., 9 (2001) 34-39.

[11] C.W. Hall, T.-F. Mah, Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria, FEMS Microbiol. Rev., 41 (2017) 276-301.

[12] M. Kostakioti, M. Hadjifrangiskou, S.J. Hultgren, Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era, Cold Spring Harb. Perspect. Med., 3 (2013) a010306-a010306.

[13] A. Algburi, N. Comito, D. Kashtanov, L.M.T. Dicks, M.L. Chikindas, Control of Biofilm Formation: Antibiotics and Beyond, Appl. Environ. Microbiol., 83 (2017) e02508-02516.

[14] S. Galié, C. García-Gutiérrez, E.M. Miguélez, C.J. Villar, F. Lombó, Biofilms in the Food Industry: Health Aspects and Control Methods, Front. Microbiol., 9 (2018).

[15] K.A. Bisset, The zonation phenomenon and structure of the swarm colony in *Proteus mirabilis*, J. Med. Microbiol., 6 (1973) 429-433.

[16] O. Rauprich, M. Matsushita, C.J. Weijer, F. Siegert, S.E. Esipov, J.A. Shapiro, Periodic phenomena in *Proteus mirabilis* swarm colony development, J. Bacteriol., 178 (1996) 6525-6538.

[17] T. Matsuyama, Y. Takagi, Y. Nakagawa, H. Itoh, J. Wakita, M. Matsushita, Dynamic Aspects of the Structured Cell Population in a Swarming Colony of *Proteus mirabilis*, J. Bacteriol., 182 (2000) 385-393.

[18] Y. Yamazaki, T. Ikeda, H. Shimada, F. Hiramatsu, N. Kobayashi, J.-i. Wakita, H. Itoh, S. Kurosu, M. Nakatsuchi, T. Matsuyama, M. Matsushita, Periodic growth of bacterial colonies, Physica D: Nonlinear Phenomena, 205 (2005) 136-153.

[19] H. Fujikawa, Periodic growth of *Bacillus subtilis* colonies on agar plates, Physica A: Statistical Mechanics and its Applications, 189 (1992) 15-21.

[20] H. Itoh, J.-i. Wakita, T. Matsuyama, M. Matsushita, Periodic pattern formation of bacterial colonies, J. Phys. Soc. Japan, 68 (1999) 1436-1443.

[21] J. Wakita, H. Shimada, H. Itoh, T. Matsuyama, M. Matsushita, Periodic colony formation by bacterial species *Bacillus subtilis*, J. Phys. Soc. Japan, 70 (2001) 911-919.

[22] H. Shimada, T. Ikeda, J.-i. Wakita, H. Itoh, S. Kurosu, F. Hiramatsu, M. Nakatsuchi, Y. Yamazaki, T. Matsuyama, M. Matsushita, Dependence of local cell density on concentric ring colony formation by bacterial species *Bacillus subtilis*, J. Phys. Soc. Japan, 73 (2004) 1082-1089.

[23] S. Tasaki, M. Nakayama, W. Shoji, Morphologies of *Bacillus subtilis* communities responding to environmental variation, Dev. Growth Differ., 59 (2017) 369-378.

[24] D. López, H. Vlamakis, R. Kolter, Generation of multiple cell types in *Bacillus subtilis*, FEMS Microbiol. Rev., 33 (2009) 152-163.

[25] D.B. Kearns, R. Losick, Swarming motility in undomesticated *Bacillus subtilis*, Mol. Microbiol.,

414 49 (2003) 581-590.
415 [26] D.B. Kearns, F. Chu, R. Rudner, R. Losick, Genes governing swarming in *Bacillus subtilis* and
416 evidence for a phase variation mechanism controlling surface motility, Mol. Microbiol., 52 (2004)
417 357-369.
418 [27] D.B. Kearns, A field guide to bacterial swarming motility, Nat. Rev. Microbiol., 8 (2010) 634-
419 644.
420 [28] M. Marvasi, P.T. Visscher, L. Casillas Martinez, Exopolymeric substances (EPS) from *Bacillus*
421 *subtilis*: polymers and genes encoding their synthesis, FEMS Microbiol. Lett., 313 (2010) 1-9.
422 [29] T.J. Smith, S.A. Blackman, S.J. Foster, Autolysins of *Bacillus subtilis*: multiple enzymes with
423 multiple functions, Microbiology, 146 (2000) 249-262.
424 [30] Y. Chai, T. Norman, R. Kolter, R. Losick, An epigenetic switch governing daughter cell separation
425 in *Bacillus subtilis*, Genes Dev., 24 (2010) 754-765.
426 [31] R. Chen, S.B. Guttenplan, K.M. Blair, D.B. Kearns, Role of the sigmaD-dependent autolysins in
427 *Bacillus subtilis* population heterogeneity, J. Bacteriol., 191 (2009) 5775-5784.
428 [32] R. Honda, J.-i. Wakita, M. Katori, Self-Elongation with Sequential Folding of a Filament of
429 Bacterial Cells, J. Phys. Soc. Japan, 84 (2015) 114002.
430 [33] M.M. Nakano, R. Magnuson, A. Myers, J. Curry, A.D. Grossman, P. Zuber, *srfA* is an operon
431 required for surfactin production, competence development, and efficient sporulation in *Bacillus*
432 *subtilis*, J. Bacteriol., 173 (1991) 1770-1778.
433 [34] M.M. Nakano, L.A. Xia, P. Zuber, Transcription initiation region of the *srfA* operon, which is
434 controlled by the *comP-comA* signal transduction system in *Bacillus subtilis*, J. Bacteriol., 173 (1991)
435 5487-5493.
436 [35] J. van Gestel, H. Vlamakis, R. Kolter, From cell differentiation to cell collectives: *Bacillus subtilis*
437 uses division of labor to migrate, PLoS Biol., 13 (2015) e1002141.
438 [36] J. Wakita, K. Komatsu, A. Nakahara, T. Matsuyama, M. Matsushita, Experimental investigation
439 on the validity of population dynamics approach to bacterial colony formation, J. Phys. Soc. Japan, 63
440 (1994) 1205-1211.
441 [37] J. Wakita, H. Kuninaka, T. Matsuyama, M. Matsushita, Size distribution of bacterial cells in
442 homogeneously spreading disk-like colonies by *Bacillus subtilis*, J. Phys. Soc. Japan, 79 (2010)
443 094002.
444 [38] D. Grier, E. Ben-Jacob, R. Clarke, L.M. Sander, Morphology and microstructure in
445 electrochemical deposition of zinc, Phys. Rev. Lett., 56 (1986) 1264-1267.
446 [39] Y. Sawada, A. Dougherty, J.P. Gollub, Dendritic and fractal patterns in electrolytic metal deposits,
447 Phys. Rev. Lett., 56 (1986) 1260-1263.
448 [40] E. Ben-Jacob, P. Garik, The formation of patterns in non-equilibrium growth, Nature, 343 (1990)

523-530.
[41] J. Wakita, I. Ràfols, H. Itoh, T. Matsuyama, M. Matsushita, Experimental investigation on the formation of dense-branching-morphology-like colonies in bacteria, J. Phys. Soc. Japan, 67 (1998) 3630-3636.
[42] M. Eden, A two-dimensional growth process, in: Proceedings of the Fourth Berkeley Symposium on Mathematical Statistics and Probability, Volume 4: Contributions to Biology and Problems of Medicine, University of California Press, Berkeley, Calif., 1961, pp. 223-239.
[43] T. Vicsek, M. Cserző, V.K. Horváth, Self-affine growth of bacterial colonies, Physica A: Statistical Mechanics and its Applications, 167 (1990) 315-321.
[44] J. Wakita, H. Itoh, T. Matsuyama, M. Matsushita, Self-affinity for the growing interface of bacterial colonies, J. Phys. Soc. Japan, 66 (1997) 67-72.
[45] T.A. Witten, L.M. Sander, Diffusion-limited aggregation, a kinetic critical phenomenon, Phys. Rev. Lett., 47 (1981) 1400-1403.
[46] H. Fujikawa, M. Matsushita, Fractal growth of *Bacillus subtilis* on agar plates, J. Phys. Soc. Japan, 58 (1989) 3875-3878.
[47] M. Matsushita, H. Fujikawa, Diffusion-limited growth in bacterial colony formation, Physica A: Statistical Mechanics and its Applications, 168 (1990) 498-506.
[48] H. Fujikawa, M. Matushita, Bacterial fractal growth in the concentration field of nutrient, J. Phys. Soc. Japan, 60 (1991) 88-94.
[49] S. Tasaki, M. Nakayama, W. Shoji, Self-organization of bacterial communities against environmental pH variation: Controlled chemotactic motility arranges cell population structures in biofilms, PLoS One, 12 (2017) e0173195.
[50] S.A. Blackman, T.J. Smith, S.J. Foster, The role of autolysins during vegetative growth of *Bacillus subtilis* 168, Microbiology, 144 (1998) 73-82.
[51] W. Vollmer, B. Joris, P. Charlier, S. Foster, Bacterial peptidoglycan (murein) hydrolases, FEMS Microbiol. Rev., 32 (2008) 259-286.
[52] D.B. Kearns, R. Losick, Cell population heterogeneity during growth of *Bacillus subtilis*, Genes Dev., 19 (2005) 3083-3094.
[53] K. Kobayashi, Gradual activation of the response regulator DegU controls serial expression of genes for flagellum formation and biofilm formation in *Bacillus subtilis*, Mol. Microbiol., 66 (2007) 395-409.
[54] Y. Chai, F. Chu, R. Kolter, R. Losick, Bistability and biofilm formation in *Bacillus subtilis*, Mol. Microbiol., 67 (2008) 254-263.
[55] L.S. Cairns, L. Hobley, N.R. Stanley-Wall, Biofilm formation by *Bacillus subtilis*: new insights into regulatory strategies and assembly mechanisms, Mol. Microbiol., 93 (2014) 587-598.

[56] R. Magnuson, J. Solomon, A.D. Grossman, Biochemical and genetic characterization of a competence pheromone from *B. subtilis*, Cell, 77 (1994) 207-216.

[57] M. Ansaldi, D. Marolt, T. Stebe, I. Mandic-Mulec, D. Dubnau, Specific activation of the *Bacillus* quorum-sensing systems by isoprenylated pheromone variants, Mol. Microbiol., 44 (2002) 1561-1573.

[58] M. Okada, I. Sato, S.J. Cho, H. Iwata, T. Nishio, D. Dubnau, Y. Sakagami, Structure of the *Bacillus subtilis* quorum-sensing peptide pheromone ComX, Nat. Chem. Biol., 1 (2005) 23-24.

[59] A.D. Grossman, R. Losick, Extracellular control of spore formation in *Bacillus subtilis*, Proc. Natl. Acad. Sci. USA, 85 (1988) 4369-4373.

[60] W.M. Cosby, P. Zuber, Regulation of *Bacillus subtilis* σH (Spo0H) and AbrB in response to changes in external pH, J. Bacteriol., 179 (1997) 6778-6787.

[61] J.C. Wilks, R.D. Kitko, S.H. Cleeton, G.E. Lee, C.S. Ugwu, B.D. Jones, S.S. BonDurant, J.L. Slonczewski, Acid and base stress and transcriptomic responses in *Bacillus subtilis*, Appl. Environ. Microbiol., 75 (2009) 981-990.

[62] S. Tasaki, M. Nakayama, I. Takagi, J. Wakita, W. Shoji, Necessary and sufficient condition for hysteresis in the mathematical model of the cell type regulation of *Bacillus subtilis*, arXiv, (2020) 2009.06524.

[63] M. Ohgiwari, M. Matsushita, T. Matsuyama, Morphological changes in growth phenomena of bacterial colony patterns, J. Phys. Soc. Japan, 61 (1992) 816-822.

[64] E. Ben-Jacob, H. Shmueli, O. Shochet, A. Tenenbaum, Adaptive self-organization during growth of bacterial colonies, Physica A: Statistical Mechanics and its Applications, 187 (1992) 378-424.

[65] E. Ben-Jacob, O. Schochet, A. Tenenbaum, I. Cohen, A. Czirok, T. Vicsek, Generic modelling of cooperative growth patterns in bacterial colonies, Nature, 368 (1994) 46-49.

[66] M. Mimura, H. Sakaguchi, M. Matsushita, Reaction–diffusion modelling of bacterial colony patterns, Physica A: Statistical Mechanics and its Applications, 282 (2000) 283-303.

[67] T. Matsuyama, M. Matsushita, Comments on ‘Classification and genetic characterization of pattern-forming *Bacilli*’ *Mol Microbiol* (1998) 27: 687–703, Mol. Microbiol., 31 (1999) 1278-1279.