Life cycle of *Bacillus subtilis* population: biofilm life cycle generation and response to environmental pH

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Running title: Life cycle of *B. subtilis* population

Abstract

Microbial populations are ubiquitous and some form a robust, multicellular structure called a biofilm that protects the cells from environmental damage. The biofilm life 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 leads to the life cycle of the cell population. We investigate life cycle generation 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 life cycle generation 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.

Introduction

Microbial populations form colonies and they often exist stably with a robust system called biofilms[1-8]. Its structural robustness makes it difficult to kill disease-related microorganisms[9-11]. In addition, a myriad of useful microorganisms are also being discovered, so controlling the population of target microorganisms is an urgent issue[12-14]. The outline 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 inside increases, the outer wall is destroyed and mobile cells are released. These released cells wander around in search of a new environment suitable for growth. When they identify an environment that satisfies their growth requirements, they become non-migrating cells and attach to 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 the life cycle

46 of a biofilm is produced by a group. Therefore, it involves multilevel interactions such as information
47 transfer within and between groups; However, the details of the biofilm life cycle generation
48 mechanism are not well understood.

49 The simplest model of bacterial biofilm life cycle generation is concentric circular colonies formed
50 by a single species of bacteria (Fig. 1). *Proteus mirabilis*[15-18] and *Bacillus subtilis*[18-23] are known to
51 form concentric colonies. During their formation, the dominant cell type for colony growth switches
52 periodically between migrating and non-migrating states. In case of *B. subtilis*, the migrating state is
53 motile cells, and they can swim by bundling and rotating multiple flagella[24]. Particularly, two-
54 dimensional collective movement called swarming by motile cells plays a central role in rapid space
55 expansion during the migration phase of the life cycle[25-27]. Most of the cells in the non-migrating state
56 are called matrix producers, which do not move and proliferate while producing extracellular
57 matrices[28]. The number of cells increases because of cell division, but in this phase, intercellular
58 adhesion is strong and separation is rare[29, 30]. Therefore, the cell group looks like a thread that continues
59 to grow, and its growth mode is called chaining or bundling. The colony not only expands two-
60 dimensionally but also increases in thickness (grows in the direction perpendicular to the medium
61 plane). Cells called surfactin producers[31, 32] are known to coexist to assist the slow two-dimensional
62 expansion in this case, improving sliding mobility on the medium surface[33], although it is far behind
63 the expansion speed during the migration phase. In this way, the repetition of the two phases, the
64 migration phase in which cells rapidly spread on the surface of the medium and the growth phase in
65 which migration stops and the thickness increases, is exactly the essence of the life cycle of biofilms.

66 Although the outline of concentric colony formation is known in this way, the details of its mechanism
67 remain unclear. This is because of the lack of information on environment-dependent control patterns
68 between different cell types, mobile and non-mobile. By focusing on *B. subtilis*, various mutually
69 exclusive cell types have been known[24]. Among them, the control between motile cells and matrix
70 producers is particularly important for the life cycle of the cell population. The genes that characterize

71 each of these two types are designed to be mutually exclusive because of self-activation and mutual
72 repression. When this mutual exclusivity becomes stronger, hysteresis occurs in cell-type regulation
73 and a life cycle is generated in the cell population, resulting in the formation of concentric circular
74 colonies. Specifically, whether or not concentric colonies are formed depends on the presence or
75 absence of hysteresis in cell-type control of the cell population, and it depends on environmental
76 conditions. Investigating the conditions for forming concentric colonies is essential for understanding
77 the life cycle generation mechanism of cell populations.

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

86 Except for concentric colonies, the dominant cell type is often defined as either a motile cell or matrix
87 producer. First, colonies formed by swarming motile cells are formed in an agar concentration of 0.4–
88 0.7%. It is classified into two types according to the nutrient concentration: disk-like (high nutrition)[34,
89 35] and dense branching morphology (DBM)-like (low nutrition)[36-39]. On the other hand, the slowly
90 expanding colonies dominated by matrix producers are formed on a solid medium with an agar
91 concentration of 0.7% or more. These colonies are also classified into two types, namely, Eden cluster-
92 like (high nutrition)[40-42] and diffusion-limited-aggregation (DLA)-like (low nutrition)[43-46], depending
93 on the nutrient concentration[44, 45]. In general, the classification is based on these five patterns. A
94 detailed examination revealed that a small number of motile cells were mixed even on the surface of
95 a hard medium with an agar concentration of 1%, which affected macroscopic colony morphology and

96 expansion rate[47]. Particularly, a crater-like pattern in which cells gather at the tip of the colony is
97 formed by only slightly lowering the environmental pH from the intracellular pH of pH 7.4. This is
98 due to chemotaxis toward nutrients, which is a phenomenon that does not occur during swarming[27].
99 Chemotaxis is also observed in concentric colonies. Specifically, in the inner region after the end of
100 the migration phase, slow swimming migration of the cell population occurs because of chemotaxis
101 from the inside to the outside, and as a result, the ring tip becomes longer. The mechanism of this
102 phenomenon is the same as that for crater-like pattern formation. On the other hand, when the agar
103 concentration is low (0.5%), swift colony expansion via swarming is observed because the energy
104 required for migration is low due to the availability of abundant water on the surface of the medium.
105 However, only under certain conditions, the optimum pH of 7.4, the dominant cell type becomes matrix
106 producers rather than motile cells, and lacework-patterned colonies may be formed[23]. Thus, other than
107 concentric colonies, different stress parameters such as environmental pH alter the choice between
108 motile cells and matrix producers.

109 Here we investigate the effect of environmental pH on cell-type selection and concentric *B. subtilis*
110 colony formation. By quantitative analysis of culture experiments, we clarify the range of
111 environmental pH in which concentric colonies are formed, and the environmental pH dependence of
112 the temporal and spatial cycles within that range. Then, by combining the mathematical model of cell-
113 type regulation on the basis of the conventional gene regulatory network and analysis of its
114 mathematical properties, we propose a model of type regulation of cell population that changes
115 depending on the environment. Our proposed model provides a reasonable mechanism for the
116 formation of concentric circular colonies and subsequent biofilm life cycle generation.

117 Results

118 Concentric colonies embody the biofilm life cycle

119 We prepared a medium with a high nutrient concentration and an intermediate agar concentration of

120 0.7% among the range of solid agar media (containing 0.2% or more agar), and inoculated the center
121 with *B. subtilis*. When the plate was cultured at about 35°C, concentric colonies were formed on the
122 medium surface (Fig. 1). This is a simple model for biofilm life cycle generation, and its temporal and
123 spatial cycles embody that life cycle. We captured two-dimensional transmitted light images of this
124 colony using a scanner (Fig. 1a). The image data were converted to cell density distribution (Fig. 1b)
125 using which, a 3D structure was reconstructed (Fig. 1c) and the cross-section was analyzed (Fig. 1d).
126 By performing such quantitative analyses in a time series, not only the two-dimensional cell density
127 (colony thickness) but also the dynamics of cell proliferation and migration were analyzed (Fig. 2).
128 The boundaries of colonies were identified at each time and the depth of each terrace was measured
129 (Fig. 2a, b). The concentric colonies of *B. subtilis* have distorted boundaries unlike those of *Proteus*
130 *mirabilis*. Therefore, the colony radius r was defined from area A of the inner area of the colony:
131 $r = \sqrt{A/\pi}$. Then, from the increase in the colony radius, the migration and growth phases of *B. subtilis*
132 biofilm, and their combined life cycle were observed (Fig. 2c).

133 Microscopic observation of phase switching in concentric colonies

134 Using phase-contrast microscopy, we investigated how the concentric colony formation phase
135 transition occurred, and how the cell state and its spatial distribution changed. First, the termination
136 process of the growth phase, in which matrix producers are dominant, begins with the appearance of
137 motile cells just before the colony boundary (Fig. 3a). There, motile cells continue to grow and form
138 ponds wherein cell swarming occurs. The surrounding cells are partially separated from each other by
139 autolysins[29, 30, 48, 49], and the pressure of proliferating motile cells is also applied, destroying many
140 points in the colony border area.

141 Among those destroyed points, the breakage starts at points on the outer side of the colony boundary,
142 and motile cells flow out from there to form branches (Fig. 3b, c). Here is the migration phase where
143 motile cells are dominant (Fig. 3d-f). In this phase, the formed branches quickly expand outward

144 because of swarming (Fig. 3d). Then, the number of break points near the root increases, and the gap
145 between branches is filled with outflow cells from the inside (Fig. 3e, f). The end of the migration
146 phase begins when the branches stop growing (Fig. 3g). Immediately thereafter, the swarming area
147 shrinks from the tip of the branch toward the inner root (Fig. 3g-i). When there is no swarming region,
148 the growth phase begins. In the growth phase, the cells do not move and proliferate, and the cell density
149 increases. The cell density is considered to be measured by the cells themselves by the concentration
150 of the secreted quorum-sensing factor ComX[50-52]. When the concentration exceeds a certain threshold,
151 motile cells reappear and transition to the migration phase occurs (Fig. 3a). This is one round of the
152 life cycle of *the B. subtilis* cell population.

153 Effect of environmental pH on periodically expanding colonies

154 The conditions for forming concentric colonies of *B. subtilis* have been investigated. Particularly,
155 under optimal conditions (incubation temperature around 35–37°C), concentric circular colonies are
156 formed only in an extremely narrow range of agar concentration of 0.65–0.68% even if nutritional
157 conditions are suitable[21]. It can be inferred that other stress parameters such as low temperature[19] are
158 necessary for the formation of concentric colonies. We investigated the effect of environmental pH as
159 a stress parameter. Conditions other than environmental pH were optimal, and the agar concentration
160 was set at an intermediate level of 0.7% (Fig. 4). First, in the neutral pH region, no periodically growing
161 colonies appeared. Matrix producers are the predominant cell type, and colonies grew by increasing
162 volume by proliferation (Fig. 4a, b). As the environmental pH decreased, many, very-short migration
163 phases appeared around pH 6.8. Under the pH condition close to this transition point, the variation in
164 space was very strong, the periodicity was not clear, and the pattern was far from that of concentric
165 circles. When the pH decreased below 6.5, concentric circular colonies that expand periodically were
166 formed. If the pH was lower than 5.3, colony formation itself became unstable, and the colony
167 formation stopped halfway or no colonies were formed. Finally, below pH 5.1, colonies were not

168 always formed. In summary, the stable formation pH range of cyclic growth colonies is 5.3–6.5.

169 Next, we investigated the properties of concentric colonies in this pH range. First, the circularity of
170 the ring was constant, although there was some variation in the pattern transition region near the neutral
171 pH (Fig. 4c). The spatial period (width of the swarm ring) seemed to increase monotonically as pH
172 decreased (Fig. 4d). Specifically, in the pattern transition region (pH 6.0–6.5), the spatial period
173 sharply increased with decreasing pH, and then it had a substantially constant period in the range of
174 pH 5.3–6.0.

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

180 Environment-dependent regulation of cells and cell populations

181 We investigated the mechanism of control between the two different states of the cell population
182 (migration and growth phases). Cell population control should be understood on the basis of cell-level
183 regulation in response to environmental information. The regulation between two cell types, motile
184 cells, and matrix producers is well studied, particularly the switch between planktonic and biofilm
185 states[24, 53-56]. The two cell types are designed as mutually exclusive gene expression states. We created
186 a simple mathematical model to investigate cellular state dynamics (Fig. 5a). In this model, cell states
187 can be described according to four group variables by grouping coordinated genes and their products:
188 (1) S : A group represented by Spo0A~P, which also includes phosphorelay of Spo0F, Spo0B, and
189 Spo0A; (2) H : SigH; (3) A : AbrB; (4) C : A group represented by ComK, driven by the ComX-
190 ComP-ComA pathway.

191 The output of this control system is the cell type. In this case, it is a matrix producer when S is

192 high and a motile cell when S is low. Sporulation is initiated when S is continuously very high.
193 However, predominant sporulation occurs at a nutrient-depletion stage much later than colonization.
194 Therefore, it is not necessary to consider sporulation here. On the other hand, the inputs of the cell
195 state control system include external environmental conditions and auto-inducing signals that represent
196 cell density. There are various environmental conditions. For example, poor nutritional conditions may
197 be input to activate S, but we considered the formation of periodic growth colonies, and the initial
198 nutrient concentration was sufficiently high. Therefore, the nutritional conditions were constant and
199 sufficient at the time of colony formation. As an auto-inducing signal related to cell-type control, a
200 small peptide ComX is secreted by *B. subtilis* cells[50-52]. In the following equation, its concentration is
201 denoted as X, which is an input from C to the cell-type regulation system[50, 51, 57]. The input mode of
202 environmental pH is of significant interest at this stage. According to previously performed studies,
203 H or A is the candidate input point for external pH in the current model[58, 59].
204 Here we considered the most minimal and smooth dynamic model of these four variables
205 ("Methods"). The curve of the set of equilibrium points can be divided into two types: with respect to
206 the increase and decrease in X, the variable that represents the cell state (for example, S) is
207 monotonic and non-monotonic (Fig. 5b, c)[60]. In the latter case, the curve is an S-shaped curve with
208 two turning points. Specifically, there are two cases where cell-type control is either not hysteretic (Fig.
209 5b) or hysteretic (Fig. 5c), considering the increase/decrease in the cell density signal X. The latter
210 hysteretic control is necessary for biofilm life cycle generation or concentric colony formation. When
211 there is hysteresis in the choice of cell state for increasing and decreasing X, a life cycle is created
212 for the cell population as follows. Migration phase: The cell density information X at the growth
213 front of the cell population decreases as it disperses in the motility state, and switches to the matrix-
214 producing state when it falls below a certain threshold X_1; Growth phase: As the cell population grows
215 and matures in the matrix-producing state, X increases, and when it exceeds a certain threshold
216 $X_2 \left(> X_1\right)$, it switches to the motility state again.

217 AbrB is upregulated and SigH is suppressed as the environmental pH decreases in the weakly acidic
218 region of interest[58, 59]. These two are potential candidates for inputting environmental pH information.
219 It is not easy to determine which of these two elements that suppress each other in the regulatory
220 network is upstream for inputting external pH information. According to the detailed mathematical
221 analysis of the equilibrium state of this model, a situation in which hysteresis occurs with a decrease
222 in environmental pH and concentric colonies are formed can be realized only when AbrB is the input
223 point for pH (Fig. 5a, d; "Methods")[60]. Therefore, we proposed a model of cell-type regulation using
224 AbrB as the input point for environmental pH information (Fig. 5a). On the basis of this cell-type
225 regulation model, the generation mechanism of the life cycle of the cell population in the parameter
226 range that performs hysteretic control could be explained (Fig. 5c), and the dependence on
227 environmental conditions could be understood.

228 **Discussion**

229 We have shown the mechanism of formation of *B. subtilis* concentric colonies that expand periodically
230 and embody the life cycle of the cell population. By performing culturing experiments and their
231 quantitative analysis, the dynamics of colony growth and its environmental pH dependence were
232 clarified from a multilevel perspective from the cell level to the macroscopic morphological level. The
233 mechanism of cyclic stagnation and expansion is that the predominant cell type switches between
234 motile cells and matrix producers. Based on the accumulation of molecular biology research to date,
235 the outline of the gene regulatory network responsible for the regulation between these two cell types
236 of *B. subtilis* has been established. We proposed a model of cell-type regulation for environmental
237 conditions and cell density by combining concentric colony culture experiments under different
238 environmental pH conditions. The mathematical properties of the minimal smooth model that
239 describes the dynamics also suggested the input location of environmental pH in the cell-type
240 regulation system. The obtained model should be a general control mechanism that selects an

241 appropriate cell population structure depending on the situation. Particularly, the S-shaped curve of
242 the set of equilibrium points obtained by this mathematical model analysis makes it possible to
243 understand the hysteretic control of the cell population that forms concentric colonies, and
244 subsequently, the biofilm life cycle generation mechanism.

245 In the biofilm life cycle, the phase-switches are dependent on cell density. We elucidated the
246 switching mechanism and found that the life cycle was stably modulated via hysteretic control of cell
247 type. Also, the effect of environmental conditions can be predicted. At present, however,
248 environmental changes and cell type control were investigated via concentric circle colony formation
249 experiments under environmental pH (and only neutral to acidic) conditions. In the future, we plan to
250 elucidate how *B. subtilis* cells collectively cope with fluctuations in a large range of pH, particularly
251 via the study of the responses at high pH. Moreover, in order to predict the response to changes in
252 other environmental conditions such as nutrient concentration, temperature, and oxygen concentration,
253 it is necessary to understand a wider gene regulatory network. Nevertheless, the conditions for biofilm
254 life cycle generation can be better organized as per the presence or absence of hysteresis. Of note, this
255 classification will probably be applicable to biofilm life cycle studies focusing on other microbial
256 species.

257 A morphological diagram was created for understanding the colony morphology of *B. subtilis* in the
258 1990s[34, 61], in which one of the five colonies was formed depending on the two parameters of nutrient
259 concentration and agar concentration in the medium. These five colonies are called Eden cluster-like[40-
260 42], diffusion-limited aggregation (DLA)-like[43-46, 62, 63], concentric ring-like, disk-like[34, 35] and dense
261 branching morphology (DBM)-like[36-39], respectively. The concept of cell type was not established at
262 the time, but it is now well understood and the colony pattern can be explained as follows. The
263 dominant cell type is a matrix producer in Eden cluster-like and DLA-like colonies; disk-like and
264 DBM-like colonies have motile cells as the predominant cell type. Finally, in the concentric ring-like
265 colony, the dominant cell type switches between matrix producers and motile cells. Thus, among the

266 five patterns, concentric colonies had multiple dominant cell types, which created unsolved problems.
267 The mechanism of pattern formation was unknown, and it was not understood or reproduced by a
268 mathematical model[64]. Our proposed model for cell-type regulation has solved this problem.
269 Furthermore, the concentric colonies presented uncertainties even with respect to the formation
270 conditions, such as an extremely narrow agar concentration range[21] and the dependence on culture
271 temperature[19]. Apparently, parameters other than agar and nutrient concentrations were likely involved
272 in the formation of concentric colonies. In other words, introducing some kind of a stress parameter is
273 effective for concentric pattern formation. We obtained the conditions to stably form concentric
274 colonies by introducing environmental pH. By drawing the morphology diagram in the three-
275 parameter space, it is possible to understand the entire cell population morphology correctly and in
276 detail. By fusing our cell-type regulation model with a multilevel morphogenesis model, we expect to
277 obtain a simulation model that accurately predicts the growth of multicellular systems with non-
278 uniform cell state distribution.
279 Robust biofilms and other complex cell populations of microorganisms are social structures involved
280 in the organization of diverse cell types. Therefore, understanding the mode of state regulation of each
281 cell should be the first key to understanding and controlling the behavior of the cell population as a
282 whole. Therefore, higher-order structures of cell populations are self-organized in a trans-hierarchical
283 manner: from gene-level and cell-level control to microscopic structures, macroscopic morphology
284 and function of cell populations, and interaction with the surrounding environment. Thus, the
285 construction of multilevel models for controlling the state of cell populations will strongly advance
286 the research of various multicellular societies.

287 # Methods

288 ## Experimental methods

289 *B. subtilis* wild-type strain OG-01 (JCM32485) was used[44, 65]. A very small quantity of bacteria

290 (endospores) was incubated overnight (10-16 h) in 5 mL of Luria–Bertani liquid growth medium (LB
291 Broth Base (Lennox L Broth Base), Gibco BRL Life Technologies, cat. no. 12780-052) at 35°C with
292 shaking at 140 rpm. For inoculation on solid agar media, this final pellet was diluted with the buffer
293 to an OD_{600} of 0.5.

294 A solution of 86 mM NaCl and 29 mM K_2HPO_4 was prepared. As a nutrient, 20 g l^{-1} peptone (Bacto
295 Peptone, BD Biosciences, cat. no. 211677) was added to the solution, and the pH was then adjusted to
296 a designated value by adding 6 M HCl. 0.7% agar (Bacto Agar, BD Biosciences, cat. no. 214010) was
297 added to the above liquid nutrient medium. After autoclaving at 121°C for 15 min, 20 mL of the
298 solution was poured into each plastic petri dish. The dishes were maintained at room temperature
299 overnight and dried at 50°C for 90 min.

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

305 Mathematical model of dynamic cell-type regulation.

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

308
$$\begin{aligned}
\frac{dS}{dt} &= \frac{c_S H}{a_S + b_S C} - d_S S \\
\frac{dH}{dt} &= \frac{c_H}{a_A + b_A A} - d_H H \\
\frac{dA}{dt} &= \frac{c_A}{a_A + b_A S} - d_A A \\
\frac{dC}{dt} &= c_C X A - d_C C
\end{aligned} \tag{1}$$

where a_*, b_*, c_*, d_* ($* = S, H, A, C$) are positive constants. There are two cases where cell-type control is not hysteretic (Fig. 5b) and hysteretic (Fig. 5c), 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. 5a) 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. 5d. Then, the following can be proved[60]: There exists $I_{SH}^* > 0$ such that

1. If $I_{SH} \le I_{SH}^*$, then the steady states are anhysteretic (Fig. 5b).
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. 5b).

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

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

Case 2 above (the external activation of Spo0A~P and SigH is larger than a threshold $I_{SH} > I_{SH}^*$) shows the situation in Fig. 5d, and it is also in agreement with the experimental results, thus suggesting that $I_A = I_{\mathrm{AbrB}}$ it plays the role of a parameter corresponding to the environmental pH reduction.

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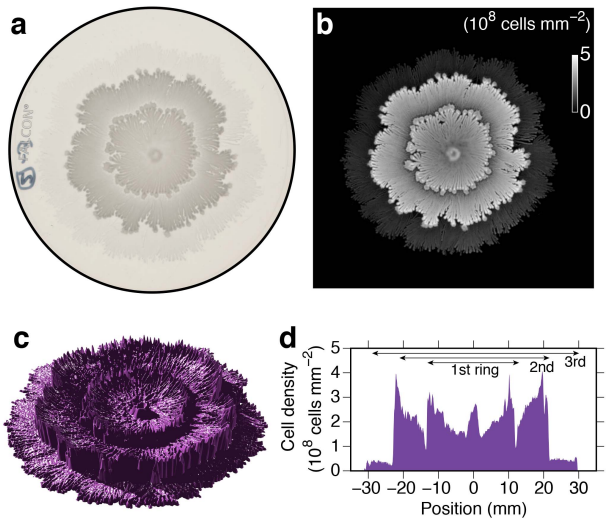

Fig. 1 Concentric rings-like colony embodies the life cycle. a Raw image of transmitted light obtained by a flat scanner. The center is the inoculation point. The diameter of the dish is 88 mm. **b** Two-dimensional cell density. **c** Three-dimensional reconstruction of the colony. **d** Cross-sectional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

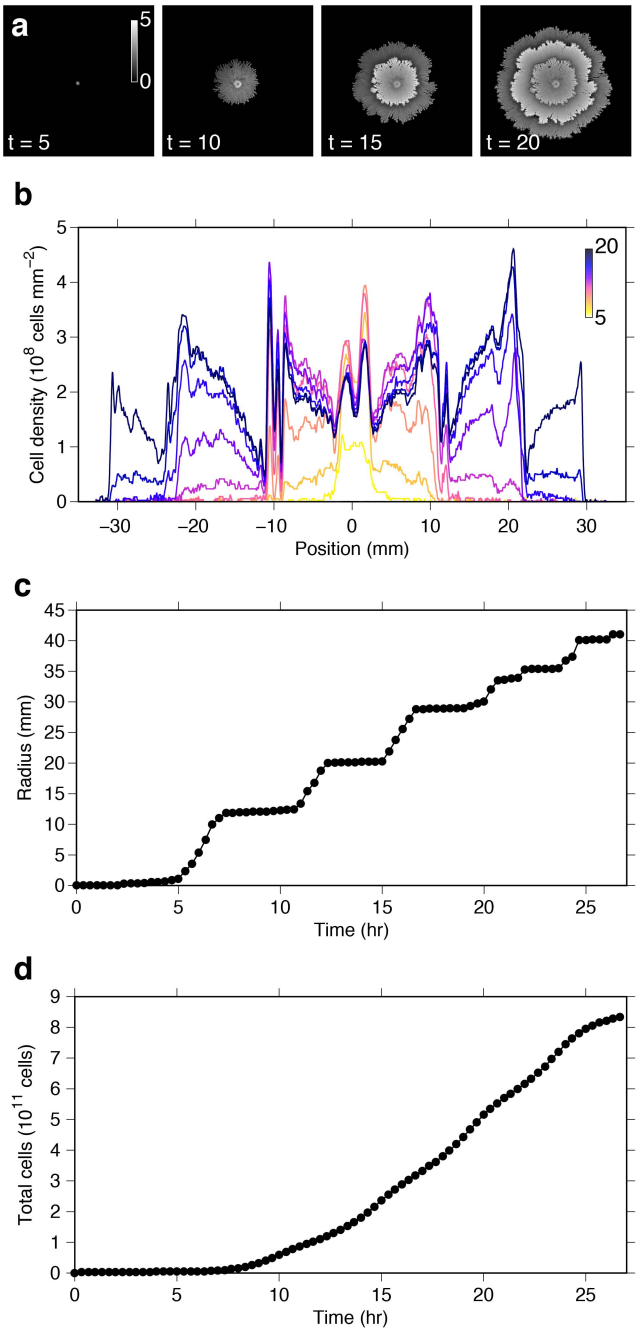

Fig. 2 Growth dynamics of concentric rings-like colony. a Two-dimensional cell density. Color bar, cell density (10^8 cells mm^{-2}). Incubation time, 5, 10, 15, and 20 h after inoculation. **b** Cross-section cell density. The center is the inoculation point. **c** Increasing colony radius. **d** Increasing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

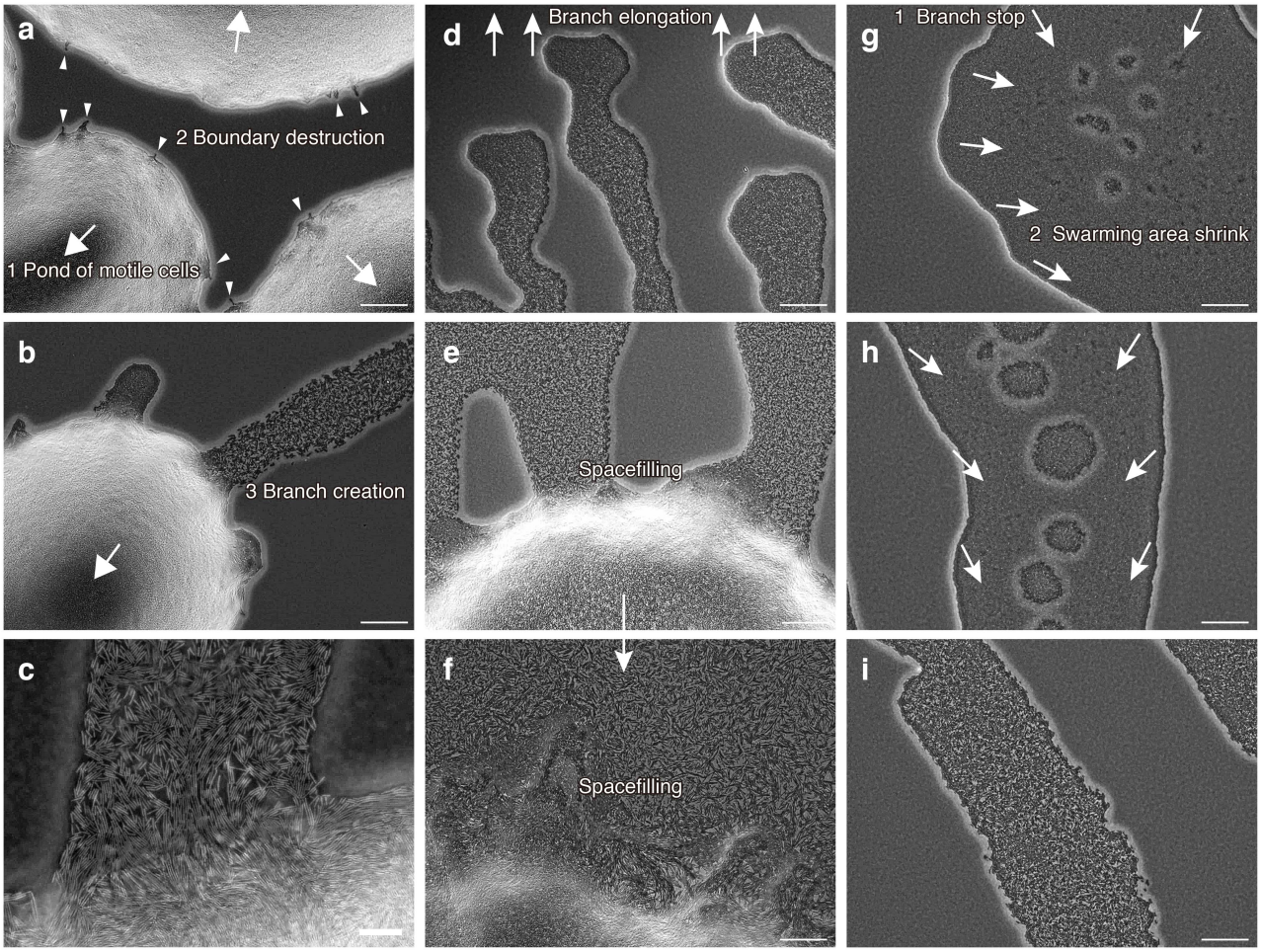

Fig. 3 Microscopic images of phase transition during colony expansion. a-c Transition from the growth phase to the migration phase. Motile cells appear slightly inside colony boundaries (large arrows in **a** and **b**). The number of motile cells increased to form bacterial ponds where cells organize 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**).

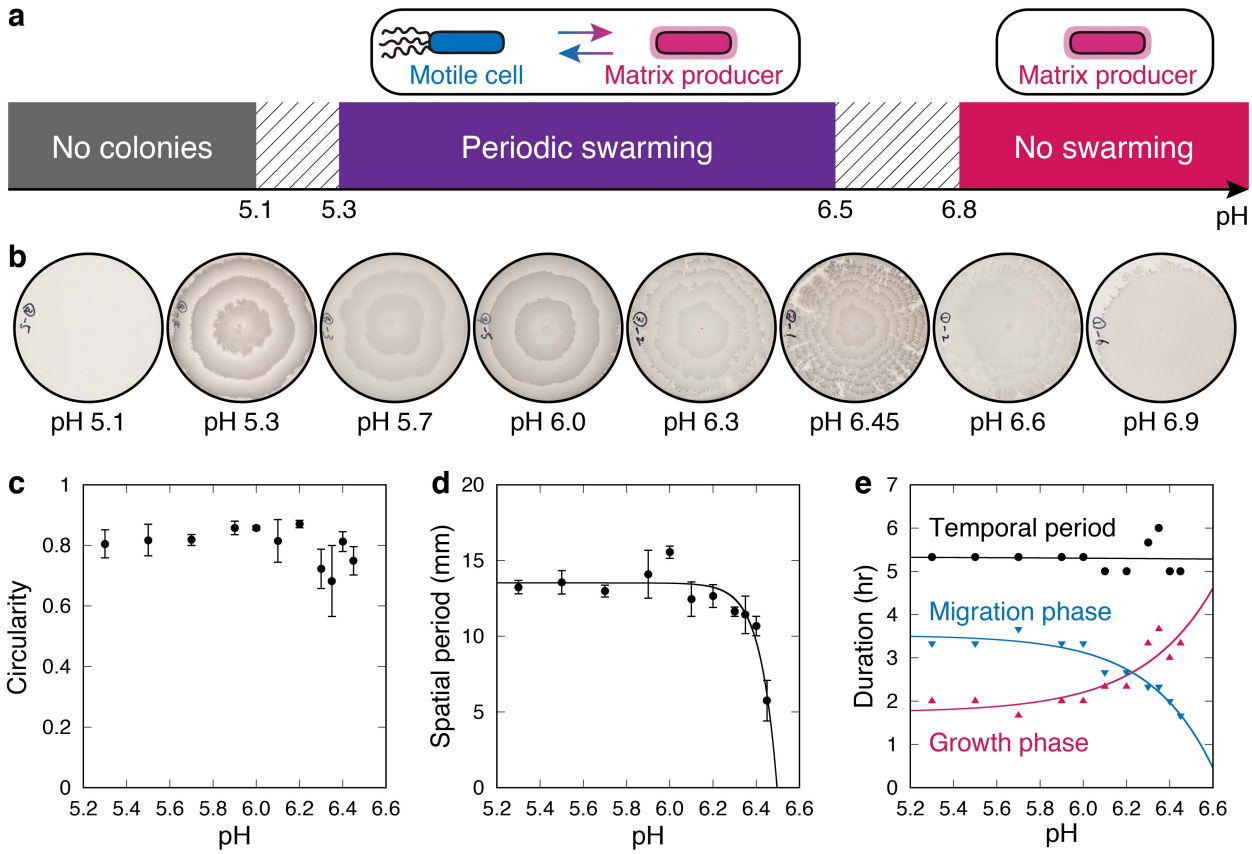

Fig. 4 Effect of environmental pH on periodically expanding colonies. a Environmental pH conditions for forming periodically expanding colonies. **b** Colonies under different environmental pH conditions. **c** Circularity of colony ring (defined by circularity of second ring). **d** Spatial period (width 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**).

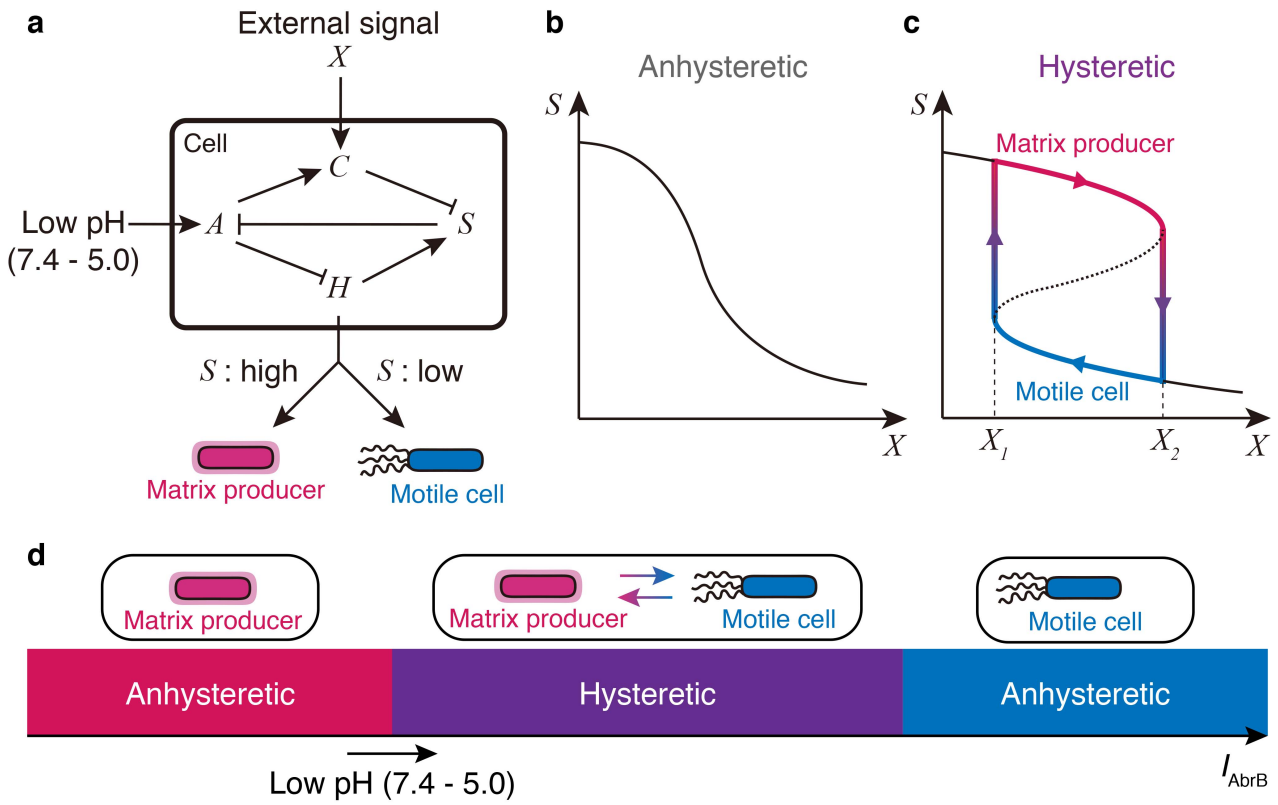

Fig. 5 A model for the response of cells and cell populations to environmental pH. a A model for cell-type selection for environmental pH and cell density. **b, c** Two types of cell-type controls. Anhysteretic (**b**) and hysteretic (**c**). **d** A model for cell population-type selection. The horizontal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