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

Sohei Tasaki[1,2*], Madoka Nakayama[3], Izumi Takagi[4,5], Wataru Shoji[6]

[1]Institute for the Advanced Study of Human Biology (WPI-ASHBi), Kyoto University Institute for Advanced Study (KUIAS), Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

[2]RIKEN Center for Biosystems Dynamics Research (RIKEN BDR), 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan

[3]Department of General Engineering, Sendai National College of Technology, 48 Nodayama, Medeshima-Shiote, Natori, Miyagi 981-1239, Japan

[4]Mathematical Institute, Tohoku University, 6-3 Aramaki-aza-Aoba, Aoba-ku, Sendai, Miyagi 980-8579, Japan

[5]Institute for Mathematical Sciences, Renmin University of China, No 59 Zhongguancun Street, Haidian District, Beijing 100872, China

[6]Frontier Research Institute for Interdisciplinary Sciences (FRIS), Tohoku University, Sendai, Japan

[*]Corresponding Author: tasaki.sohei@gmail.com

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 of a biofilm is produced by a group. 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.

The simplest model of bacterial biofilm life cycle generation is concentric circular colonies formed by a single species of bacteria (Fig. 1). *Proteus mirabilis*[15-18] and *Bacillus subtilis*[18-23] are known to form concentric colonies. During their formation, the dominant cell type for colony growth switches periodically between migrating and non-migrating states. In case of *B. subtilis*, the migrating state is motile cells, and they 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]. Most of the cells in the non-migrating state are called matrix producers, which do not move and proliferate 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, 30]. Therefore, the cell group looks like a thread that continues to grow, and its growth mode is called chaining or bundling. 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[31, 32] are known to coexist to assist the slow two-dimensional expansion in this case, improving sliding mobility on the medium surface[33], 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, is exactly the essence of the life cycle of biofilms.

Although the outline of concentric colony formation is known in this way, the details of its mechanism remain 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 known[24]. Among them, the control between motile cells and matrix producers is particularly important for the life cycle of the cell population. The genes that characterize each of these two types are designed to be mutually exclusive because of self-activation and mutual repression. When this mutual exclusivity becomes stronger, hysteresis occurs in cell-type regulation and a life cycle is generated 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 in cell-type control of the cell population, and it depends on environmental conditions. Investigating the conditions for forming concentric colonies is essential for understanding the life cycle generation mechanism of cell populations.

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

Except for concentric colonies, the dominant cell type is often defined as either a motile cell or matrix producer. First, colonies formed by swarming motile cells are formed in an agar concentration of 0.4–0.7%. It is classified into two types according to the nutrient concentration: disk-like (high nutrition)[34, 35] and dense branching morphology (DBM)-like (low nutrition)[36-39]. On the other hand, the slowly expanding colonies dominated by matrix producers are formed on a solid medium with an agar concentration of 0.7% or more. These colonies are also classified into two types, namely, Eden cluster-like (high nutrition)[40-42] and diffusion-limited-aggregation (DLA)-like (low nutrition)[43-46], depending on the nutrient concentration[44, 45]. In general, the classification is based on these five patterns. A detailed examination revealed that a small number of motile cells were mixed even on the surface of a hard medium with an agar concentration of 1%, which affected macroscopic colony morphology and expansion rate[47]. Particularly, a crater-like pattern in which cells gather at the tip of the colony is formed by only slightly lowering the environmental pH from the intracellular pH of pH 7.4. This is due to chemotaxis toward nutrients, which is a phenomenon that does not occur during swarming[27]. Chemotaxis is also observed in concentric colonies. Specifically, in the inner region after the end of the migration phase, slow swimming migration of the cell population occurs because of chemotaxis from the inside to the outside, and as a result, the ring tip becomes longer. The mechanism of this phenomenon is the same as that for crater-like pattern formation. On the other hand, when the agar concentration is low (0.5%), swift colony expansion via swarming is observed because the energy required for migration is low due to the availability of abundant water on the surface of the medium. However, only under certain conditions, the optimum pH of 7.4, the dominant cell type becomes matrix producers rather than motile cells, and lacework-patterned colonies may be formed[23]. Thus, other than concentric colonies, different stress parameters such as environmental pH alter the choice between motile cells and matrix producers.

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

Results

Concentric colonies embody the biofilm life cycle

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

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

Microscopic observation of phase switching in concentric colonies

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

Among those destroyed points, the breakage starts at points on the outer side of the colony boundary, and motile cells flow out from there to form branches (Fig. 3b, c). Here is the migration phase where motile cells are dominant (Fig. 3d-f). In this phase, the formed branches quickly expand outward because of swarming (Fig. 3d). Then, the number of break points near the root increases, and the gap between branches is filled with outflow cells from the inside (Fig. 3e, f). The end of the migration phase begins when the branches stop growing (Fig. 3g). Immediately thereafter, the swarming area shrinks from the tip of the branch toward the inner root (Fig. 3g-i). When there is no swarming region, the growth phase begins. In the growth phase, the cells do not move and proliferate, and the cell density increases. The cell density is considered to be measured by the cells themselves by the concentration of the secreted quorum-sensing factor ComX[50-52]. When the concentration exceeds a certain threshold, motile cells reappear and transition to the migration phase occurs (Fig. 3a). This is one round of the life cycle of *the B. subtilis* cell population.

Effect of environmental pH on periodically expanding colonies

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

Next, we investigated the properties of concentric colonies in this pH range. First, the circularity of the ring was constant, although there was some variation in the pattern transition region near the neutral pH (Fig. 4c). The spatial period (width of the swarm ring) seemed to increase monotonically as pH decreased (Fig. 4d). 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. 4e). We found that while the total time period was almost constant, the growth phase duration decreased as the pH decreased, and 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 between 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 information. The regulation between two cell types, motile cells, and matrix producers is well studied, particularly the switch between planktonic and biofilm states[24, 53-56]. The two cell types are designed as mutually exclusive gene expression states. We created a simple mathematical model to investigate cellular state dynamics (Fig. 5a). 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. 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[50-52]. In the following equation, its concentration is denoted as X, which is an input from C to the cell-type regulation system[50, 51, 57]. 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[58, 59].

Here we considered the most minimal and smooth dynamic model of these four variables ("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. 5b, c)[60]. 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. 5b) or hysteretic (Fig. 5c), considering the increase/decrease in the cell density signal X. The latter hysteretic control is necessary for biofilm life cycle generation or 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 of the cell population decreases as it disperses in the motility state, and switches to the matrix-producing state when it falls below a certain threshold X_1; Growth phase: As the cell population grows and matures in the matrix-producing state, X increases, and when it exceeds a certain threshold $X_2 \left(> X_1\right)$, it 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[58, 59]. These two are potential candidates for inputting environmental pH information. It is not easy to determine which of these two elements that 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. 5a, d; “Methods”)[60]. Therefore, we proposed a model of cell-type regulation using AbrB as the input point for environmental pH information (Fig. 5a). On the basis of this cell-type regulation model, the generation mechanism of the life cycle of the cell population in the parameter range that performs hysteretic control could be explained (Fig. 5c), and the dependence on environmental conditions could be understood.

Discussion

We have shown the mechanism of formation of *B. subtilis* concentric colonies that expand periodically and embody the life cycle of the cell population. By performing culturing experiments and their quantitative analysis, the dynamics of colony growth and its environmental pH dependence were clarified from a multilevel perspective from the cell level to the macroscopic morphological level. The mechanism of cyclic stagnation and expansion is that the predominant cell type switches between motile cells and matrix producers. Based on the accumulation of molecular biology research to date, the outline of the gene regulatory network responsible for the regulation between these two cell types of *B. subtilis* has been established. We proposed a model of cell-type regulation for environmental conditions and cell density by combining concentric colony culture experiments under different environmental pH conditions. The mathematical properties of the minimal smooth model that describes the dynamics also suggested the input location of environmental pH in the cell-type regulation system. The obtained model should be a general control mechanism that selects an appropriate cell population structure depending on the situation. Particularly, the S-shaped curve of the set of equilibrium points obtained by this mathematical model analysis makes it possible to understand the hysteretic control of the cell population that forms concentric colonies, and subsequently, the biofilm life cycle generation mechanism.

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

A morphological diagram was created for understanding the colony morphology of *B. subtilis* in the 1990s[34, 61], in which one of the five colonies was formed depending on the two parameters of nutrient concentration and agar concentration in the medium. These five colonies are called Eden cluster-like[40-42], diffusion-limited aggregation (DLA)-like[43-46, 62, 63], concentric ring-like, disk-like[34, 35] and dense branching morphology (DBM)-like[36-39], respectively. The concept of cell type was not established at the time, but it is now well understood and the colony pattern can be explained as follows. The dominant cell type is a matrix producer in Eden cluster-like and DLA-like colonies; disk-like and DBM-like colonies have motile cells as the predominant cell type. Finally, in the concentric ring-like colony, the dominant cell type switches between matrix producers and motile cells. Thus, among the five patterns, concentric colonies had multiple dominant cell types, which created unsolved problems. The mechanism of pattern formation was unknown, and it was not understood or reproduced by a mathematical model[64]. Our proposed model for cell-type regulation has solved this problem.

Furthermore, the concentric colonies presented uncertainties even with respect to the formation conditions, such as an extremely narrow agar concentration range[21] and the dependence on culture temperature[19]. Apparently, parameters other than agar and nutrient concentrations were likely involved in the formation of concentric colonies. In other words, introducing some kind of a stress parameter is effective for concentric pattern formation. We obtained the conditions to stably form concentric colonies by introducing environmental pH. By drawing the morphology diagram in the three-parameter space, it is possible to understand the entire cell population morphology correctly and in detail. By fusing our cell-type regulation model with a multilevel morphogenesis model, we expect to obtain a simulation model that accurately predicts the growth of multicellular systems with non-uniform cell state distribution.

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

Methods

Experimental methods

B. subtilis wild-type strain OG-01 (JCM32485) was used[44, 65]. A very small quantity of bacteria (endospores) was incubated overnight (10-16 h) in 5 mL of Luria–Bertani liquid growth medium (LB Broth Base (Lennox L Broth Base), Gibco BRL Life Technologies, cat. no. 12780-052) at 35°C with shaking at 140 rpm. For inoculation on solid agar media, this final pellet was diluted with the buffer to an OD_{600} of 0.5.

A solution of 86 mM NaCl and 29 mM K_2HPO_4 was prepared. As a nutrient, 20 g l^{-1} peptone (Bacto Peptone, BD Biosciences, cat. no. 211677) was added to the solution, and the pH was then adjusted to a designated value by adding 6 M HCl. 0.7% agar (Bacto Agar, BD Biosciences, cat. no. 214010) was added to the above liquid nutrient medium. After autoclaving at 121°C for 15 min, 20 mL of the 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. 5a), 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 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.

Acknowledgments

This work was supported by JSPS KAKENHI, Grant Number 19K03645 (S.T.), and MEXT KAKENHI, Grant Number 17H06327 (S.T.).

References

1. Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as Complex Differentiated Communities. *Annu Rev Microbiol* **56**, 187-209 (2002).

2. Donlan RM. Biofilms: microbial life on surfaces. *Emerg Infect Dis* **8**, 881-890 (2002).

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

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

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

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

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

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

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

10. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **9**, 34-39 (2001).

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

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

13. Algburi A, Comito N, Kashtanov D, Dicks LMT, Chikindas ML. Control of Biofilm Formation: Antibiotics and Beyond. *Appl Environ Microbiol* **83**, e02508-02516 (2017).

14. Galié S, García-Gutiérrez C, Miguélez EM, Villar CJ, Lombó F. Biofilms in the Food Industry: Health Aspects and Control Methods. *Front Microbiol* **9**, (2018).

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

16. Rauprich O, Matsushita M, Weijer CJ, Siegert F, Esipov SE, Shapiro JA. Periodic phenomena in *Proteus mirabilis* swarm colony development. *J Bacteriol* **178**, 6525-6538 (1996).

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

18. Yamazaki Y*, et al.* Periodic growth of bacterial colonies. *Physica D: Nonlinear Phenomena* **205**, 136-153 (2005).

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

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

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

22. Shimada H*, et al.* Dependence of local cell density on concentric ring colony formation by bacterial species *Bacillus subtilis*. *J Phys Soc Japan* **73**, 1082-1089 (2004).

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

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

25. Kearns DB, Losick R. Swarming motility in undomesticated *Bacillus subtilis*. *Mol Microbiol* **49**, 581-590 (2003).

26. Kearns DB, Chu F, Rudner R, Losick R. Genes governing swarming in *Bacillus subtilis* and evidence for a phase variation mechanism controlling surface motility. *Mol Microbiol* **52**, 357-369 (2004).

27. Kearns DB. A field guide to bacterial swarming motility. *Nat Rev Microbiol* **8**, 634-644 (2010).

28. Marvasi M, Visscher PT, Casillas Martinez L. Exopolymeric substances (EPS) from *Bacillus subtilis*: polymers and genes encoding their synthesis. *FEMS Microbiol Lett* **313**, 1-9 (2010).

29. Smith TJ, Blackman SA, Foster SJ. Autolysins of *Bacillus subtilis*: multiple enzymes with multiple functions. *Microbiology* **146**, 249-262 (2000).

30. Chai Y, Norman T, Kolter R, Losick R. An epigenetic switch governing daughter cell separation in *Bacillus subtilis*. *Genes Dev* **24**, 754-765 (2010).

31. Nakano MM, Magnuson R, Myers A, Curry J, Grossman AD, Zuber P. *srfA* is an operon required for surfactin production, competence development, and efficient sporulation in *Bacillus subtilis*. *J Bacteriol* **173**, 1770-1778 (1991).

32. Nakano MM, Xia LA, Zuber P. Transcription initiation region of the *srfA* operon, which is

controlled by the *comP-comA* signal transduction system in *Bacillus subtilis*. *J Bacteriol* **173**, 5487-5493 (1991).

33. van Gestel J, Vlamakis H, Kolter R. From cell differentiation to cell collectives: *Bacillus subtilis* uses division of labor to migrate. *PLoS Biol* **13**, e1002141 (2015).

34. Wakita J-i, Komatsu K, Nakahara A, Matsuyama T, Matsushita M. Experimental investigation on the validity of population dynamics approach to bacterial colony formation. *J Phys Soc Japan* **63**, 1205-1211 (1994).

35. Wakita J-i, Kuninaka H, Matsuyama T, Matsushita M. Size distribution of bacterial cells in homogeneously spreading disk-like colonies by *Bacillus subtilis*. *J Phys Soc Japan* **79**, 094002 (2010).

36. Grier D, Ben-Jacob E, Clarke R, Sander LM. Morphology and microstructure in electrochemical deposition of zinc. *Phys Rev Lett* **56**, 1264-1267 (1986).

37. Sawada Y, Dougherty A, Gollub JP. Dendritic and fractal patterns in electrolytic metal deposits. *Phys Rev Lett* **56**, 1260-1263 (1986).

38. Ben-Jacob E, Garik P. The formation of patterns in non-equilibrium growth. *Nature* **343**, 523-530 (1990).

39. Wakita J-i, Ràfols I, Itoh H, Matsuyama T, Matsushita M. Experimental investigation on the formation of dense-branching-morphology-like colonies in bacteria. *J Phys Soc Japan* **67**, 3630-3636 (1998).

40. Eden M. 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 (1961).

41. Vicsek T, Cserző M, Horváth VK. Self-affine growth of bacterial colonies. *Physica A: Statistical Mechanics and its Applications* **167**, 315-321 (1990).

42. Wakita J-i, Itoh H, Matsuyama T, Matsushita M. Self-affinity for the growing interface of

bacterial colonies. *J Phys Soc Japan* **66**, 67-72 (1997).

43. Witten TA, Sander LM. Diffusion-limited aggregation, a kinetic critical phenomenon. *Phys Rev Lett* **47**, 1400-1403 (1981).

44. Fujikawa H, Matsushita M. Fractal growth of *Bacillus subtilis* on agar plates. *J Phys Soc Japan* **58**, 3875-3878 (1989).

45. Matsushita M, Fujikawa H. Diffusion-limited growth in bacterial colony formation. *Physica A: Statistical Mechanics and its Applications* **168**, 498-506 (1990).

46. Fujikawa H, Matushita M. Bacterial fractal growth in the concentration field of nutrient. *J Phys Soc Japan* **60**, 88-94 (1991).

47. Tasaki S, Nakayama M, Shoji W. Self-organization of bacterial communities against environmental pH variation: Controlled chemotactic motility arranges cell population structures in biofilms. *PLoS One* **12**, e0173195 (2017).

48. Blackman SA, Smith TJ, Foster SJ. The role of autolysins during vegetative growth of *Bacillus subtilis* 168. *Microbiology* **144**, 73-82 (1998).

49. Vollmer W, Joris B, Charlier P, Foster S. Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiol Rev* **32**, 259-286 (2008).

50. Magnuson R, Solomon J, Grossman AD. Biochemical and genetic characterization of a competence pheromone from *B. subtilis*. *Cell* **77**, 207-216 (1994).

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

52. Okada M*, et al.* Structure of the *Bacillus subtilis* quorum-sensing peptide pheromone ComX. *Nat Chem Biol* **1**, 23-24 (2005).

53. Kearns DB, Losick R. Cell population heterogeneity during growth of *Bacillus subtilis*. *Genes*

Dev **19**, 3083-3094 (2005).

54. Kobayashi K. Gradual activation of the response regulator DegU controls serial expression of genes for flagellum formation and biofilm formation in *Bacillus subtilis*. *Mol Microbiol* **66**, 395-409 (2007).

55. Chai Y, Chu F, Kolter R, Losick R. Bistability and biofilm formation in *Bacillus subtilis*. *Mol Microbiol* **67**, 254-263 (2008).

56. Cairns LS, Hobley L, Stanley-Wall NR. Biofilm formation by *Bacillus subtilis*: new insights into regulatory strategies and assembly mechanisms. *Mol Microbiol* **93**, 587-598 (2014).

57. Grossman AD, Losick R. Extracellular control of spore formation in *Bacillus subtilis*. *Proc Natl Acad Sci USA* **85**, 4369-4373 (1988).

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

59. Wilks JC*, et al.* Acid and base stress and transcriptomic responses in *Bacillus subtilis*. *Appl Environ Microbiol* **75**, 981-990 (2009).

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

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

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

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

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

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

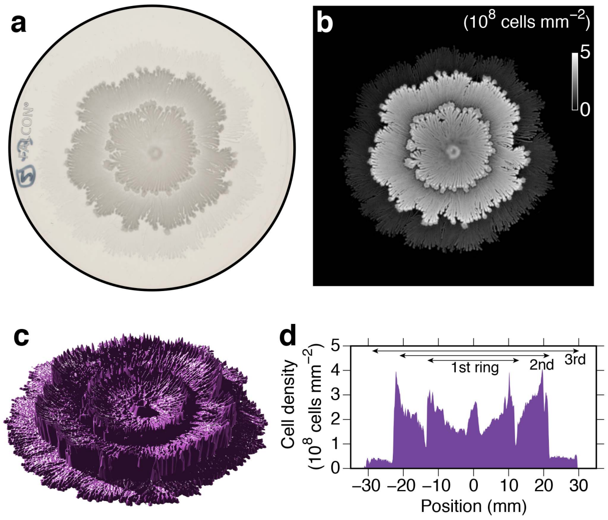

Fig. 1 Concentric rings-like colony embodies the life cycle. a Raw image of transmitted light obtained by a flat scanner. The center is the inoculation point. The diameter of the dish is 88 mm. **b** Two-dimensional cell density. **c** Three-dimensional reconstruction of the colony. **d** Cross-sectional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

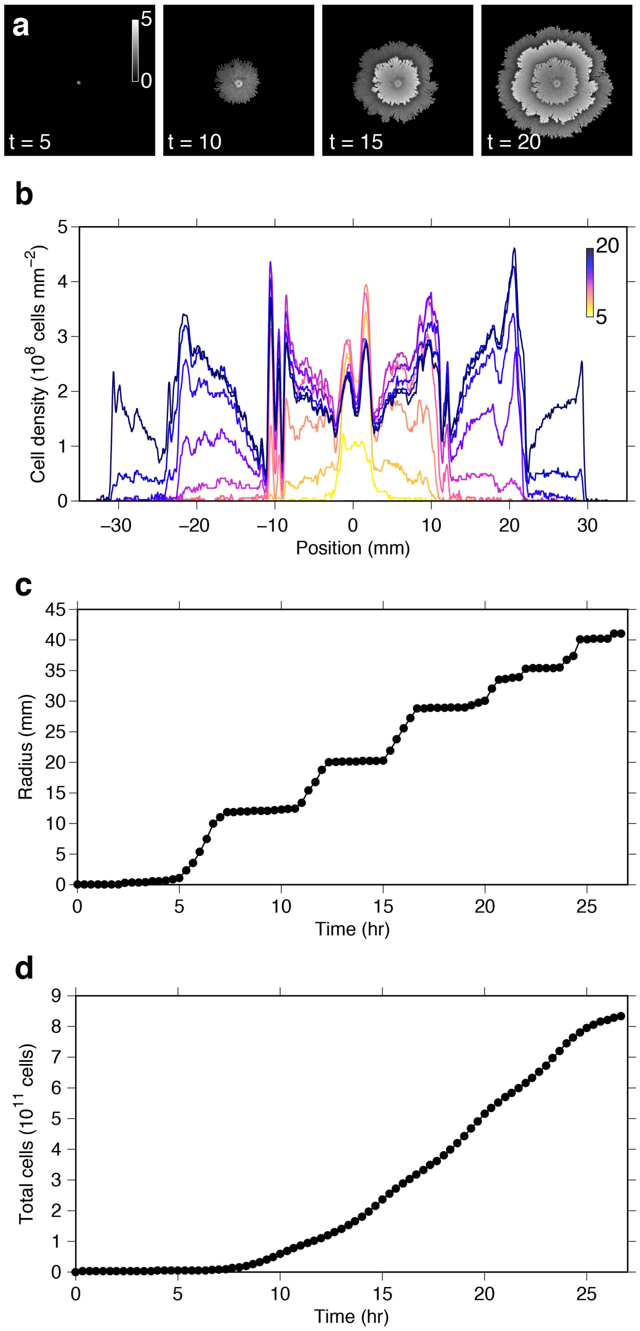

Fig. 2 Growth dynamics of concentric rings-like colony. **a** Two-dimensional cell density. Color bar, cell density (10^8 cells mm^{-2}). Incubation time, 5, 10, 15, and 20 h after inoculation. **b** Cross-section cell density. The center is the inoculation point. **c** Increasing colony radius. **d**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

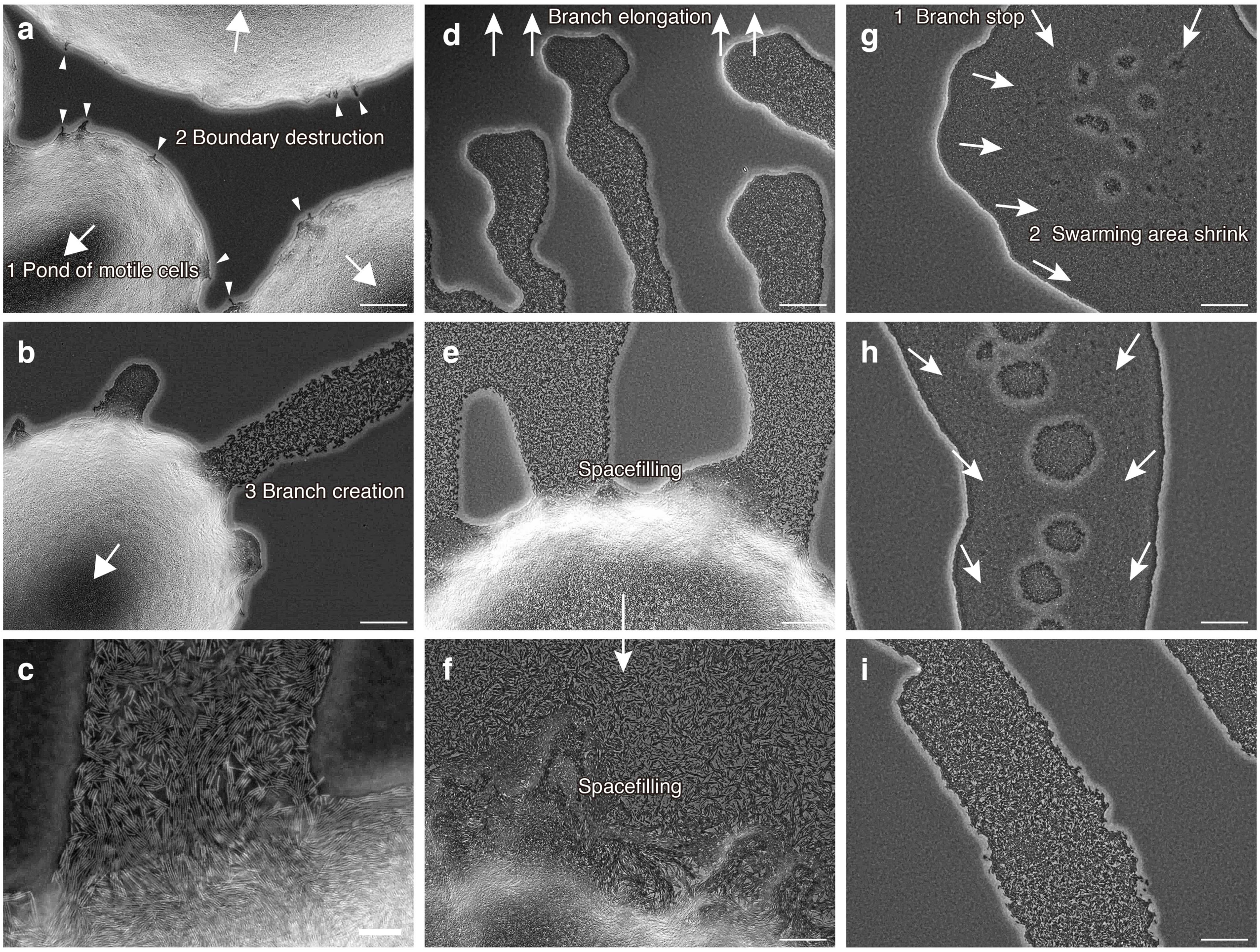
a
2 Boundary destruction
1 Pond of motile cells
d
Branch elongation
g
1 Branch stop
2 Swarming area shrink
b
3 Branch creation
e
Spacefilling
h
c
f
Spacefilling
i

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**).

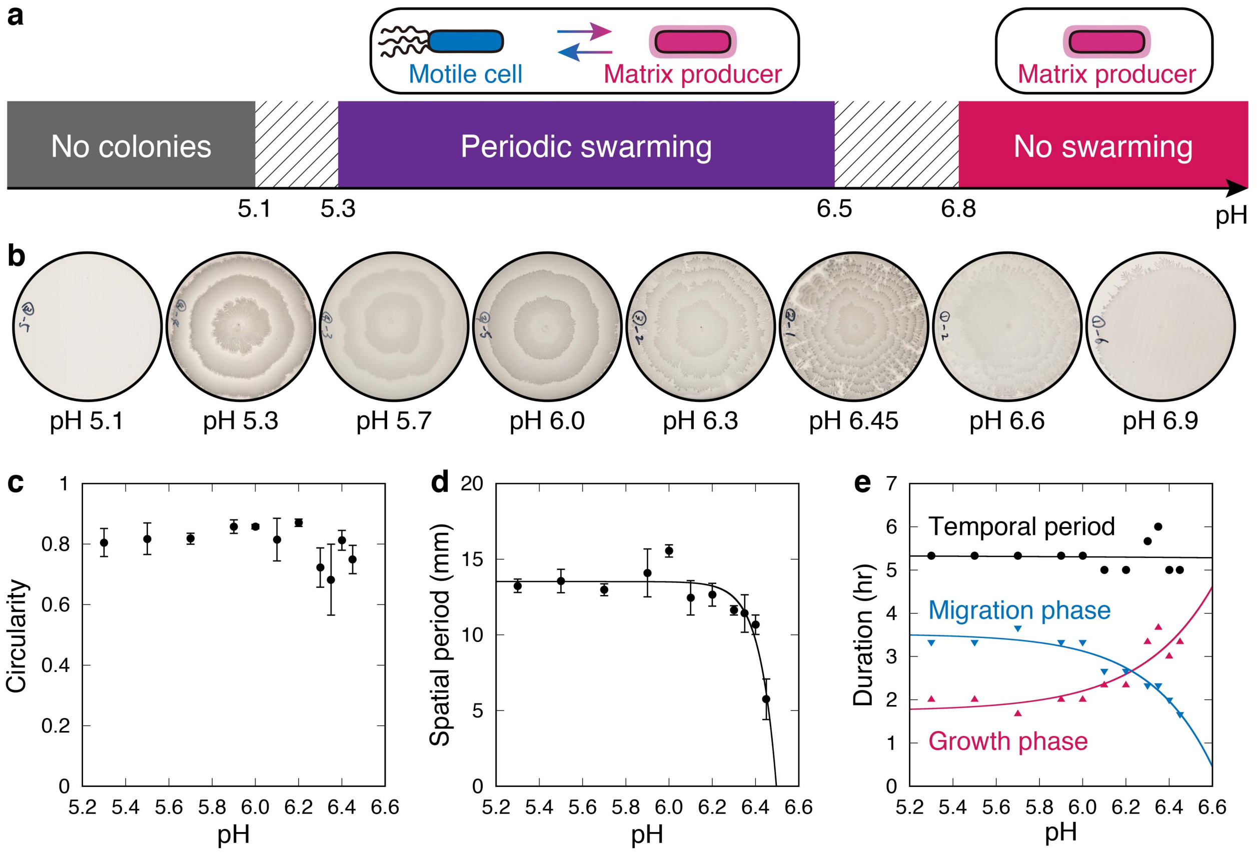

Fig. 4 Effect of environmental pH on periodically expanding colonies. a Environmental pH conditions for forming periodically expanding colonies. **b** Colonies under different environmental pH conditions. **c** Circularity of colony ring (defined by circularity of second ring). **d** Spatial period (width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

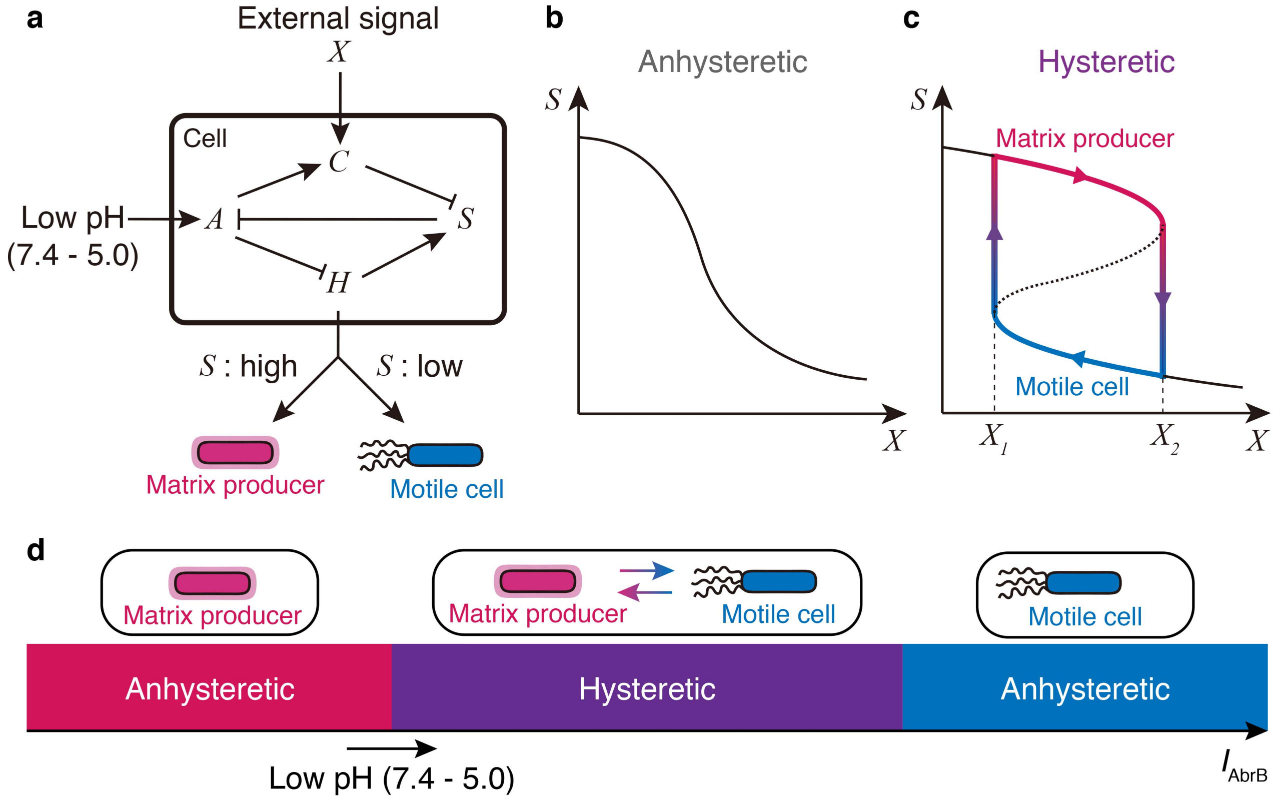
a
External signal
X
Cell
C
Low pH
(7.4 - 5.0)
A
S
H
S : high
S : low
Matrix producer
Motile cell
b
Anhysteretic
S
X
c
Hysteretic
S
Matrix producer
Motile cell
X1
X2
X
d
Matrix producer
Matrix producer
Motile cell
Motile cell
Anhysteretic
Hysteretic
Anhysteretic
Low pH (7.4 - 5.0)
IAbrB

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").