Analysis of biofilm expansion rate on agar substrates with different stiffness under limited nutrition

Jin Wu (✉ wujindeyouxiang@126.com)
University of Science and Technology Beijing https://orcid.org/0000-0001-8508-9064
Xianyong Li
Rui Kong
Jiankun Wang
Xiaoling Wang

Research Article

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Abstract

In this study, we explore the growth of *Bacillus subtilis* biofilm on substrates with different agar concentrations, and obtain the area, morphology information and fluorescence intensity of biofilms by using optical imaging technologies. We find that expansion rate variations of biofilms growing on substrates with high and low agar concentration (1.5wt%, 2.0wt%, 2.5wt%) are not in the same phase. In the first three days’ growth, the interaction stress between biofilm and each agar substrate increases, which makes the biofilm expansion rate decreases before wrinkle pattern IV (branches) comes up. After three days, in the later growth stage after wrinkle pattern IV appears, the biofilm has larger expansion rate growing on 2.0wt% agar concentration, which has the larger wrinkle distance in wrinkle pattern IV reducing energy consumption. The above findings are further verified through images of the fluorescence intensity of matrix producing cells of each biofilm. Our study shows that the stiff substrate does not always inhibit the biofilm expansion, although it does in the earlier stage, after that, mature biofilms acquire larger expansion rate by adjusting the growth mode through the wrinkle evolution even in nutrient extremely depletion.

01 Introduction

Biofilms are tightly packed communities of bacteria embedded in the extracellular matrix of polysaccharides secreted by the bacteria themselves [1, 2]. It can survive as long as there is water, nutrients and adhesive surface [3]. The substrate hardness of adhesive surfaces has a large span, such as oceans and lakes, wound surfaces, respiratory mucosa, metals on the surface of industrial pipelines and ship hulls [4], which can be seen everywhere in nature and daily life. Biofilm sense the changes of the external environment by secreting chemicals and transmitting ionic electrical signals, to adjust their own growth state in time to adapt to the environment [5, 6].

The biofilm, an active soft material, also exhibits rich morphological patterns. We find that the morphology of *Bacillus subtilis* biofilm begins to evolve regularly after experiencing from the central to labyrinthine networks area, then wrinkle pattern changes into radial ridges, branches in turn [7, 8]. The wrinkle evolution is the growth mechanism by which bacteria regulate their physiological state in response to the environmental change. For example, low oxygen level can engender more wrinkles on the surface of *Bacillus subtilis* biofilms [9]. Changing the concentration of agar substrate can change the spatiotemporal wrinkling patterns of *Vibrio cholerae* biofilm [10]. Wrinkle morphology runs through the whole biofilm growth, the morphology variation reflects different biofilm growth stage.

The experiments have proved that the biofilm growth is affected by ambient factors, such as nutrient concentration, temperature, pH value, substrate stiffness and so on [11]. In the laboratory, the substrate stiffness is altered by changing its agar concentration, the existing research shows that the higher agar concentration the slower the biofilm growth rate [10, 12, 13]. It is generally believed that high agar concentration decreases the nutrient diffusion coefficient to hinder the biofilm growth [10]. Substrate stiffness not only change the biofilm expansion, but also affect rim irregularity, wrinkling morphology,
phenotype development and distribution, and so on. Under the same nutrient concentration, biofilms become thicker and have greater rim irregularity when growing on a soft substrate than when growing on a hard substrate [12]. For *Vibrio cholerae* biofilms growing on soft agar substrate, wrinkles first appear in the peripheral region and propagate inward. In contrast, for biofilms growing on hard agar substrate, wrinkles first appear in the central region and propagate outward [10]. The agar concentration in the range of 0.5–2.5% does not affect the velocities of swarming bacteria significantly but relates to the switching period between different cell behaviors. Specifically, in the growth phase, bacterial cells spend most of their time multiplying without moving actively. When the cell density reaches a certain level, they start swarming rapidly in random directions. the switching period between bacterial swarming and growth was shorter at 0.5% agar substrate compared with 1.5 or 2.5% [14].

In the existing experiment work about biofilm growth, the culture period is usually within 3 days, the corresponding findings are based on this. In our recent experiments, we increase the biofilm growth time for much longer period like two weeks and even more than one month, and we observe more interesting phenomena. The burgeon wrinkle pattern is first discovered [15] after 20 days growth of the biofilm. After 35 days, the biofilm edge grows into a complex branching structure, which allows the biofilm to expand outward at a faster speed to adapt the extremely nutrient-lacking environment [16].

In this paper, we focus on the effect of substrate stiffness on the biofilm expansion growth. In the experiment, expansion rate of *Bacillus subtilis* biofilms growing on three substrates with different agar concentrations (1.5wt%, 2.0wt% and 2.5wt%) increase rapidly within 3 days and then get slowly, in this period, the area expansion rate of biofilm growing on 2.0wt% agar concentration substrate is larger than those growing on 1.5wt% and 2.5wt% agar concentration substrates. To explanation, we divide the biofilm into different regions according to wrinkle morphology pattern and determine which patterns account for its growth.

## 02 Materials And Methods

### 2.1 Biofilm inoculation and culture

We construct a triple fluorescent *Bacillus subtilis* strain (NCIB3610 sacA: : *P* _hag_ – mkate2 *Kan<sup>R</sup>) amyE: _P*_tapA_ – _cfp_ Spc<sup>R</sup> ywrK: _P*_sspB_ – _citrus_ Cm<sup>R</sup>) to represent different cell differentiation. The blue fluorescent protein CFP reports on the tapA promoter, which corresponds to the amyloid protein in matrix producing cells. We use 1.5wt%, 2.0wt% and 2.5wt% agar gel containing minimal salts glutamate glycerol (MSgg) medium designed to induce biofilm formation and sporulation. MSgg is composed of 5 mm potassium phosphate (PH7), 100mm MOPS (PH7), 2mm MgCl<sub>2</sub>, 700μ mCaCl<sub>2</sub>, 50μm MnCl<sub>2</sub>, 50μm FeCl<sub>2</sub>, 1μm ZnCl<sub>2</sub>, 2m thiamine, 0.5% glycerol, 0.5% glutamate, 50μg / ml tryptophan, 50μg / ml phenylalanine and 50μg / ml threonine. The agar solution is cooled to 55°C before adding the remaining ingredients. We use 10-cm-diameter petri dishes containing a 2-mm-thick layer of agar substrate. We cover the dishes with lids, let them cool overnight at room temperature, and spot
within 24h. These conditions speed up the cell differentiation and make three main phenotypes coming up within two days.

We transfer the bacteria to the surface of the agar by spotting with 0.1 μl of bacterial culture at OD$_{600}$ = 1.0. Before inoculating the plates, we remove the lids and allowed the surface to dry for 5–10 min. We allow the drop to dry for 5–10 additional minutes with the lid off, until the meniscus of the initial drop is no longer visible and the bacteria are left in a coffee ring around the perimeter whose radius is about 1 mm. To prevent evaporation during recording of time-lapse movies, we place the spotted plate in a Tupperware container stuffed with wet paper towels and seal around the microscope using the Glad Press’n Seal plastic wrap. The temperature of the microscope is maintained at 37°C using the heating elements and fans.

In our experiment, only the agar gel concentration substrate is changed, the remaining conditions is consistent. Each experiment is repeated at least 3 times.

2.2 Measurement of biofilm thickness

We calculate the thickness of biofilms based on the Lambert–Beer law, which holds that when a beam of parallel monochromatic light penetrates an object, the thickness of the object is positively correlated with its absorbance.

We measure the optical density (OD) of images and calculate the biofilm thickness (H) through equations (1) and (2). Where $I$ is intensity of the transmitted light through the substrate and biofilm, and $I_0$ is that through the transparent substrate. $\lambda$ is attenuation length. In previous studies, we have conducted multiple cutting experiments on biofilms to measure the thickness information. We obtain a linear statistic between the thickness and the OD with the attenuation coefficient being 1.2 mm [17].

$$OD = \lg \left( \frac{I_0}{I} \right)$$

1

$$H = OD \cdot \lambda$$

2

03 Analysis Of Biofilm Growth Characteristics

3.1 Biofilm image analysis

The higher the agar concentration, the stiffer the solid substrate, which has a heterogeneous effect on the growth and morphology of biofilm. The biofilm area changes significantly on days 3 and 12, the biofilm growing on 2.0wt% and 2.5wt% agar concentration substrates have an obvious labyrinth wrinkles pattern II comparing with the biofilm growing on 1.5wt% agar concentration substrate, as shown in Fig. 1.
The biofilm can be divided into four regions from the biofilm center to the edge according to the wrinkle pattern characteristics, regions with various patterns are named as concentric rings (I), labyrinthine networks (II), radial ridges (III) and branches (IV), as shown in Fig. 2A. In the first three days, biofilms grow rapidly and the agar concentration is negatively correlated with the biofilm area change, as shown in Fig. 2B. With the consumption of nutrients and water, the overall biofilm growth trend tends to ease after three days, but the area difference between biofilms growing on substates of 1.5wt% and 2.0wt% agar concentrations is getting smaller and smaller, which decreases to zero on the day 9; and then the area expansion of biofilm growing on 2.0wt% agar concentration substate is larger than that the biofilm growing on 1.5wt% agar concentration substate; the biofilm growing on 2.5wt% agar concentration substate have the slowest expansion rate and the smallest area.

The expansion rate of biofilm area gradually slows down with the time. Within the first three days, the biofilm patterns evolve from I to II, and then to III, during this period, the area expansion rate of biofilm growing on 1.5wt% agar concentrations substate is larger than the biofilm growing on 2.0wt% and 2.5wt% agar concentrations substates, as shown in Fig. 2C, the area summation of I - III patterns does not change after three days. Meanwhile, the pattern IV begins to appear, as shown in Fig. 1(top), and the biofilm growth derives from the expansion pattern IV. The area expansion rates of biofilms growing on substates with 1.5wt% and 2.0wt% agar concentrations are similar on the 4th and 5th day, after that, the area expansion rate of biofilm growing on 2.0wt% agar concentration substate becomes largest. Until 12 days, we find that the biofilm area of patterns I - III is negatively correlated with the agar concentration, the biofilm area of pattern IV growing on 2.0wt% agar concentration is the largest, biofilm areas of pattern IV on 2.5wt% and 1.5wt% agar concentrations are similar, which are smaller than on 2.0wt% agar concentration, as shown in Fig. 2D.

3.2 Mechanical analysis of biofilm expansion in pattern III

Radial wrinkles on the biofilm form between day 2 and day 3, as shown in Fig. 3. According to the study about the evolution of wrinkles near spot like defects on film/substrate, for a given stiff film/compliant substrate, the initial wrinkle number is dependent on the size of the spot-like dust. As for the biofilm, the wrinkle pattern III is similar to the wrinkle in [18].

For precise characterization, the experimental pictures on day 3 are binarized, as shown in Fig. 4A. Area a is pattern I and II, which is mainly determined by the observation of experimental pictures. The wrinkles information of biofilm growing on 1.5wt% agar concentration is clearly visible, and the interface between patterns I, II and III is obvious. The biofilm wrinkles growing on 2.0wt% and 2.5wt% agar concentration in pattern II are not obviously different from pattern I and III, although wrinkles are serried but they are discontinuous and form branching structure in this pattern, as shown in Fig. 4A. For the number of radial wrinkles in biofilm III pattern, we choose radial wrinkles that run through biofilm pattern III and ignore the small wrinkles.
$a_1$ equals 4.5 mm and the number of radial wrinkles $N_1$ is 38 when biofilms growing on 1.5wt% agar substrate, $a_2$ equals 3.0 mm and $N_2$ is 25 when biofilms growing on 2.0wt% agar substrates, and $a_3$ equals 2.0 mm and $N_3$ is 18 when biofilms growing on 2.5wt% agar substrates. The larger the area of area $a$, the more radial wrinkles, proved that it is consistent with the evolution of wrinkles near spot-like defects on film, as shown in Fig. 4B. To compare the wrinkle distribution in pattern III, we obtain the mean distance between wrinkles along the blue circle by dividing the length of the blue circumference to the above wrinkle number, named $\lambda^{\text{III}}$. $\lambda^{\text{III}}_0$ equals to 1.0mm for 1.5wt% agar concentration, $\lambda^{\text{III}}_{02}$ equals to 1.29 mm for 2.0wt% agar concentration and $\lambda^{\text{III}}_{03}$ equals to 0.72 mm for 2.5wt% agar concentration.

From the mechanical point view, during the biofilm growth, various stresses such as the internal stress and biofilm/agar substrate adhering stress affect its expansion rate. According to the evolution of wrinkles on film / substrate [16], the radial (denoted as $\sigma_{rr}$) and hoop (denoted as $\sigma_{\theta\theta}$) stresses between biofilm and substrate are generated and can be expressed as [19],

$$\sigma_{rr} = -\sigma_0 \left[ 1 - e^{-(r-a)/l} \right]$$

$$\sigma_{\theta\theta} = -\sigma_0 \left[ 1 - v_b e^{-(r-a)/l} \right]$$

where $l$ is the transition length from uniaxial to equi-biaxial stress states, that is the radial distance of pattern $\text{III}$. $v_b$ is the biofilm Poisson's ratio, which is equal to 0.3 [20]. $\sigma_0$ is the internal stress of biofilm between cells and between cells and extracellular matrix, the interaction stress generated by biofilm growth/agar substrate and so on all affect the growth characteristics of biofilm. The internal stress is mainly distributed along the expansion direction of biofilm, especially at the growth edge. However, due to the heterogeneity of biofilm growth, there exists the internal stress along the cluster radial expansion direction locally. The internal stress direction is getting more uniform with the biofilm growth, as the cell density and the extracellular matrix increase [21].

According to previous studies [21] the correlation between the biofilm thickness and the internal stress magnitude can be expressed in the following equation:

$$\sigma_0 = 0.002746H^2 + 0.4943H + 73$$

where $H$ is the thickness of the biofilm, the unit is $\mu m$. $\sigma_0$ is the internal stress in the biofilm, the unit is Pa.
Through Eq. (5), the internal stress change in the 50-70h growth period is obtained, as shown in Fig. 4C. The value of $r$ ranges from the beginning of radial wrinkles to the bifurcation of wrinkles, $r_1 = 4.56.39\text{mm}$, $r_2 = 3.05.34\text{mm}$, $l_1 \approx 1.9\text{mm}$, $l_2 \approx 2.34\text{mm}$, and $l_3 \approx 1.99\text{mm}$. The mutual radi, I stress generated by biofilm and substrate is obtained through Eq. (3), which is only limited to pattern III, as shown in Fig. 4D.

The radial stress between biofilm and substrate is opposite to the direction of internal stress, and increases with time, which results in the decrease of the biofilm expansion rate. What’s more, the increase rate of the radial stress in biofilm growing on the 1.5wt% and 2.5wt% agar concentration substrate is larger than that of biofilm growing on the 2.0wt% agar concentration substrate, which explains why the expansion rate of biofilm growing on the 1.5wt% and 2.5wt% agar concentration substrate decreases faster in Fig. 2C.

Through Eqs. (3) and (4), $\sigma_{rr} \leq \sigma_{\theta \theta}$, which means there is the circumferential compressive stress at the biofilm edge, resulting in the formation of radial wrinkles. According to the evolution of wrinkles on film / substrate [18], when an equi-biaxial stress state $\sigma_{rr} = \sigma_{\theta \theta} = \sigma_0$ appears, the straight wrinkle evolves to the herringbone wrinkle. Due to the biofilm heterogeneity, these stresses distribute inhomogeneously, what’s more, with the biofilm expansion, these stresses change with time. When the relationship among these stresses meets the above conditions, the biofilm evolves from pattern III to pattern IV.

### 3.2 Energic Analysis Of Biofilm Growth In Pattern IV

By comparing images from the fluorescent channel of matrix producing cells, on day 12, the fluorescent intensity of matrix producing cells at the edge of the biofilm growing on 2.0wt% agar concentration substrate is the biggest, followed by the biofilm growing on 1.5wt% agar concentration substrate and then the biofilm growing on 2.5wt% agar concentration substrate. Matrix producing cells are able to transport a mixture of proteins and extracellular polysaccharides (EPS), which tightly encloses the cell populations in the biofilm. The research shows that EPS in the biofilm increases the difference in the concentration of macromolecules with that in agar matrix, and the resulting osmotic pressure promotes the transportation of nutrients in the biofilm, which is conducive to drives surface expansion [22, 23] and self-healing of the biofilm [24].

Figure 6 (A), (B) and (C) show part of biofilms with a growth period of 12 days. We observe that the wrinkle pattern of the biofilm growing on 2.0wt% agar concentration is much sparser with fewer wrinkles. The mean values of wrinkle distance $\lambda^{IV}$ of pattern IV growing on 1.5wt%, 2.0wt% and 2.5wt% agar concentration substrate are 0.11 mm, 0.15 mm and 0.08 mm respectively, which is correlated with the mean distance between wrinkles in pattern III, they are 1.0 mm for 1.5wt% agar concentration, 1.29 mm for 2.0wt% agar concentration and 0.72 mm for 2.5wt% agar concentration, respectively.
The biofilm is treated as an Föppl – von Karman (FvK) plate, in the wrinkled biofilm, elastic energy $U$ per unit area includes the bending and stretching energy. The total elastic energy per unit area in the wrinkled state has the form [25],

$$\frac{U}{U_0} = 1 - \left[ 1 - \frac{1}{\epsilon_{pre}} \left( \frac{n^2 H^2}{3 \lambda^2} + \frac{\lambda E_s}{4\pi H E_b} \right) \right]^2$$

$U_0$ is the biofilm energy without wrinkles, $U_0 = \frac{1}{2} h E_b \epsilon_{pre}^2$, $\bar{E}_b = E_b / \left( 1 - \nu_b^2 \right)$, where $E_b$ and $\nu_b$ are the Young’s modulus and the Poisson’s ratio of the biofilm. $\bar{E}_s = E_s / \left( 1 - \nu_s^2 \right)$, where $E_s$ and $\nu_s$ are the Young’s modulus and the Poisson’s ratio of the substrate, $\epsilon_{pre}$ is pre strain, $H$ is biofilm thickness, $\lambda$ is wavelength respectively.

The elastic energy of the biofilm decreases with the increase of the wrinkle distance, as shown in Fig. 6(E), the elastic energy increases with the increase of the modulus ratio $\bar{E}_s / \bar{E}_b$, as shown in Fig. 6(F). As the ratio $\bar{E}_s / \bar{E}_b$ continuously changes during the biofilm growth, resulting in the heterogeneous wrinkle distance distribution even in the same wrinkle pattern. From the energy aspect, the biofilm has larger wrinkle distance growing on the 2.0wt% agar concentration substrate, larger wrinkle distance and lower energy consumption induce faster biofilm expansion rate.

**04 Discussion And Conclusion**

1. The stiffness of substrate largely influences the biofilm growth rate, these influences are not monotonic, what is more, the change of biofilm expansion rate also affects wrinkle pattern evolution. The biofilm grows rapidly and the agar concentration is negatively correlated with the area change of biofilm within three days. After three days the area expansion rate of biofilm growing on 2.0wt% agar concentration substrate is larger than those growing on 1.5wt% and 2.5wt% agar concentration substrates.

2. Different wrinkle patterns are the evolution of biofilm adaptation to the environment. Wrinkles have many advantageous functions, such as optimize nutrient transport and consumption. The branch wrinkle of pattern IV is evolved from the radial ridge wrinkle of pattern III, which is similar to the branching of branches, the average wrinkle distances in pattern III and IV have an inherent relationship. In pattern IV, the bigger the average wrinkle distance the faster the biofilm expansion
rate, which is because that increasing the wrinkle distance can reduce energy consumption for wrinkle formation and leave more energy maintaining a certain plane expansion rate.

3. *Bacillus subtilis* mainly has three phenotypes, at different biofilm growth stages, the proportion and location of the three phenotypes change accordingly. Matrix producing cells secrete EPS to increase the osmotic pressure between agar substrate and improve the expansion ability of biofilm. We observed that in pattern IV, the fluorescence intensity of the cells that produce matrix at the growth front edge of the biofilm growing on the 2.0wt% agar substrate is the strongest, which corresponds to its growth rate.

**Declarations**

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**Authors and Affiliations**

School of Mechanical Engineering, University of Science and Technology Beijing, 100083, China

Jin Wu, Xianyong Li, Rui Kong, Jiankun Wang, Xiaoling Wang

**Corresponding authors**

Correspondence to Xiaoling Wang.

**Contributions**

Xiaoling Wang designed the research and performed field experiments. Jin Wu analyzed the data and wrote the manuscript. Xianyong Li, Rui Kong and Jiankun Wang had commented on the paper, and Xiaoling Wang revised the paper. All authors approved the final version of the manuscript before submission.

**Ethics Approval**

Not applicable.

**Consent to Participate**
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.

Conflict of interest

The authors declare no competing interests.

Consent to Publish

We confirm that this work is original and has not been published elsewhere, and not being considered for publication elsewhere.

Conflict of Interest

The authors declare no competing interests.

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Data Availability

Not applicable.

Code Availability

Not applicable.

References


**Figures**
Figure 1

Morphogenesis of *B. subtilis* biofilms grown at an air–solid interface. Transmission images of biofilms growing on 1.5wt%, 2.0wt% and 2.5wt% agar substrates at two time points. (Scale bars: 3 mm.)
Figure 2

(A) Experiment on biofilm morphogenesis. (Scale bars: 3 mm.) (B) The total area of biofilm and the area of I - III patterns versus time. (C) Comparison of area growth rates of biofilms growing on three agar substrates. (D) Comparison of the area of biofilms pattern (Ⅲ - Ⅳ) and (Ⅳ) on three agar substrates on day 12.
FIG.3. The picture of biofilm, day 2 and 3. (Scale bars: 3 mm.) \( \alpha \) is biofilm radius of I and II pattern regions; \( r \) is biofilm radius of I, II and III pattern regions; The red and blue circles are boundaries between II & III, and between III & IV, respectively. \( l \) is the transition length from uniaxial to equi-biaxial stress states, that is the radial distance of pattern III. \( \lambda \) is wrinkles wavelength. Subscript \( i = 1, 2, 3 \) represents that the biofilm growing on the substrate with agar concentration of 1.5wt%, 2.0wt% and 2.5wt% respectively.

Figure 3

See image above for figure legend
Figure 4

(A) Binary experiment picture on day 3. (Scale bars: 3 mm.) (B) The number of radial wrinkles in biofilm III pattern. (C) Biofilm internal stress with growth period of 50-70 hours. (D) Radial stress between biofilm and agar substrate. The direction of the radial stress between biofilm and agar substrate points to the center of the biofilm, indicating that it hinders the growth expansion.
Figure 5

Fluorescent images of matrix producing cells. (Scale bars: 300 μm.) day12
FIG. 6 (A–C) Amplification of biofilm pattern IV. (Scale bars: 300 μm.) (D) Binary picture of Fig.6B, λ is wrinkles distance. (E) The elastic energy of the biofilm versus wrinkles distance λ. (F) The elastic energy of the biofilm versus modulus ratios $E_s/E_b$. The elastic energy is normalized by $U_0$, which is the biofilm energy without wrinkles. $E_s/E_b$ represents the ratio of the biofilm modulus to substrate modulus.

Figure 6

See image above for figure legend