Numerical simulation of wrinkle morphology formation and the evolution of different *Bacillus subtilis* biofilms

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**ABSTRACT**

Wrinkle morphology is a distinctive phenomenon observed in mature biofilms that are produced by a great number of bacteria. The wrinkle pattern depends on the mechanical properties of the agar substrate and the biofilm itself, governed by the extracellular matrix (ECM). Here we study the macroscopic structures and the evolution of *Bacillus subtilis* biofilm wrinkles using the commercial finite element software ABAQUUS. A mechanical model and simulation are set up to analyze and evaluate bacteria biofilm’s wrinkle characteristics. We uncover the wrinkle formation mechanism and enumerate the quantitative relationship between wrinkle structure and mechanical properties of biofilm and its substrate. Our work can be used to modify the wrinkle pattern and control the biofilm size.

**Key words** | ABAQUS, bacteria biofilms, biofilm mechanical property, wrinkle morphology

**INTRODUCTION**

Bacterial biofilms refer to communities that self-assemble into a cohesive extracellular matrix on solid surfaces or pellicles floating on top of liquids (Isaeva 2012). This leads to numerous problems and applications, in a variety of industrial and medical settings (Costerton et al. 1999). Biofilms can cause contaminant problems but also be useful for waste water treatment, bioremediation (Singh et al. 2006), microbial leaching (Eisele & Gabby 2014), and microbial fuel cells (Gardel et al. 2012). In both problematic and the beneficial aspects, it is of much interest to be able to control the growth of biofilms, especially the biofilm wrinkle structure.

*Bacillus subtilis* is one of the best-studied Gram-positive bacteria and a good model organism to study biofilm formation (Curtis et al. 2007). In our experiments, we observe that the differences in the wrinkle structures of *B. subtilis* biofilms are driven by the mechanical properties of the agar substrate, as shown in Figure 1(a)–1(c). A recent study showed that *B. subtilis* biofilm wavelength tends to decrease with increasing agar concentration, resembling the agar-dependence of inter-spoke arc-lengths, and is moderately affected by the glucose concentration during yeast colony expansion (Chen et al. 2014). Asally et al. (2012) also investigated wrinkled biofilms of *B. subtilis*, and discovered that the wrinkle formation is induced by mechanical forces that promote vertical mechanical buckling, which is in turn caused by localized cell death, as shown in Figure 1(d)–1(h). They found that the deletion of genes implicated in biofilm development alters the mechanical stiffness of mutant biofilms.

These experimental observations clearly show wrinkle formation, but to better understand biofilm growth, theoretical modeling and quantitative analysis would be needed of wrinkle formation mechanisms, the dependence of the wrinkle pattern on mechanical properties of the *B. subtilis* biofilm and its substrate, and the wrinkle’s dynamic evolution. Guided by experimental observations, we study the wrinkle’s macroscopic structures and the evolution of *B. subtilis* biofilms using ABAQUS. Our simulation results show a positive correlation between the stiffness of *B. subtilis* biofilm and substrate and the width of the wrinkle pattern. In addition, we find a negative correlation between stiffness and the height of the wrinkle pattern. These two simulation results are consistent with the experimental results. Furthermore, we set up a mechanical model to study the wrinkle formation mechanism.

**MATERIALS AND METHODS**

We grew colonies on 1.5 and 3wt% agar gel with a minimal media, MSgg, designed to induce biofilm formation and sporulation: 5 mM potassium phosphate (pH 7)/100 mM 3-(N-morpholino)propanesulfonic acid sodium salt (MOPS) (pH 7)/2 mM MgCl₂/700 μM CaCl₂/50 μM MnCl₂/50 μM...
FeCl₂/1 μM ZnCl₂/2 μM thiamine/0.5% glycerol/0.5% glutamate/50 g/mL tryptophan/50 g/mL phenylalanine. The agar solution is cooled to 55 °C before adding the remaining ingredients. We typically used 100-mm diameter petri-dishes containing 12 mL of media to obtain a 30 mm diameter biofilm that exhibits the changes of the wrinkle characteristics in 2 days. The plates were covered with lids and cooled overnight at room temperature, then spotted within 24 h.

The bacteria were transferred to the surface of the agar by spotting with 0.1 μL of bacterial culture at OD₆₀₀ = 1. Before inoculating the plates, the lids were removed to allow the surface to dry for 5–10 minutes. The drop was allowed to dry for 5–10 additional minutes with the lid off until the meniscus of the initial drop was no longer visible and the bacteria were left in a ‘coffee ring’ around the perimeter. The microscope was maintained at 32 °C using heating elements and fans. For 2-day time-lapse movies, biofilm colonies were grown in a Tupperware container stuffed with wet paper towels and sealed around the microscope using Glad Press 'n Seal plastic wrap to prevent evaporation.

As shown in Figure 2, the B. subtilis biofilm increased in height to hundreds of micrometers, spreading to reach a diameter of several centimeters, and forming different macroscopic wrinkles. Also, the wrinkle size of B. subtilis biofilm grown on 1.5 wt% agar gel was larger than that of B. subtilis biofilm grown on 3 wt% agar gel.

**MODEL AND NUMERICAL SIMULATION**

To investigate the possible role of mechanical forces in wrinkle formation, we turn to material sciences studies of wrinkling in the bonded, thin-layered films that are commonly used in optical coatings and electronic devices, such as microprocessors. We use these studies because, as the name implies, biofilms constitute thin biological films composed of bacterial cells that are embedded within an ECM.

**Numerical simulation of wrinkle morphology**

Here are the assumptions of our model:
1. The biofilm is homogeneous.
2. The biofilm is at the final growing stage. We do not consider nutrient diffusion, and ignore the effect of other
external conditions on the bacterial biofilm; for example, pH, illumination and temperature.

3. Cross-sections of *B. subtilis* biofilms revealed that cell death is localized at the bottom of biofilms, and more specifically at the center of the folded wrinkle structures. The regions of cell death provide a localized outlet for mechanical forces. Therefore, wrinkle formation appears to be initiated by localized cell death, which spatially focuses lateral forces, and thereby promotes vertical buckling of the biofilm. So we apply equivalent displacement loads in the left and right sides on the axis of *B. subtilis* biofilms.

Based on these assumptions, we established a two-dimensional *B. subtilis* biofilm wrinkle structure model, as shown in Figure 3.

In the two-dimensional plane model, the agar substrate is 2 mm long and 2 mm thick; the *B. subtilis* biofilm is also 2 mm long, while its thickness is only 0.02 mm. *B. subtilis* biofilms generated from ΔabrB, ΔsrfA and WT have varying stiffnesses due to differences in ECM production, as shown in Table 1. Besides these three mutant biofilm stiffnesses, we also considered other different mutant *B. subtilis* biofilm stiffnesses which need further experimental work to verify.

**Finite element simulation results**

We simulated the wild type *B. subtilis* biofilm (E = 25 kPa) wrinkle structure by using a two-dimensional planar model of the bacterial biofilm that forms nine waves with the total length of approximately 2 mm; therefore we know that the wrinkle wavelength is about 220 μm, as shown in Figure 4.

We simulated the formation of wrinkle structure of different mutant *B. subtilis* biofilms to study the change of the wrinkle wavelength and wave height, as shown in Figure 5(b). The corresponding mechanical properties are shown in Table 1. For example, for a biofilm grown by deleting *abrB* gene of wild type *B. subtilis* biofilm (Young’s modulus

50 kPa), we obtained a wrinkle number of six, and correspondingly, a wrinkle wavelength of 330 μm, as shown in Figure 5(a). The detailed stress distribution in one wrinkle wave is shown in Figure 5(c). Across the biofilm thickness, there is a central layer with zero stress, tensile stresses above the central layer and compressive stresses below.

A comparison of Figures 4 and 5(a) shows that when the elastic modulus of B. subtilis biofilm increases, the width of the wrinkle structure increases. We varied the Young’s modulus to represent different mutant B. subtilis biofilms, as shown in the first and third columns of Table 2. Then we obtained the wrinkle number, wave height and wavelength of these biofilms, as shown in the last three columns of Table 2.

A quantitative relationship between B. subtilis biofilm’s mechanical stiffness and the wavelength and wave height can be seen. A positive correlation between stiffness and the width of the wrinkle can be found, which is also consistent with experimental measurements (Asally et al. 2012). Furthermore, a negative correlation between stiffness and the height of the wrinkle was obtained, as shown in Figure 6. These results show that the mechanical stiffness of B. subtilis biofilm, which is at least in part determined by the ECM, is a key property determining the wrinkle pattern during biofilm development.

When the effects of the mechanical properties of the agar substrate using a similar finite element simulation were investigated, a quantitative relationship between agar percentage of substrate and wrinkle wavelength and wave height was obtained, which is consistent with the experimental results (Chen et al. 2014). A negative correlation between the agar percentage and the wrinkle wavelength was found and a positive correlation between the agar percentage and the wrinkle wave height, as shown in Figure 7(b). The simulation results for the relationship
between the agar percentage and the stiffness of the substrate are shown in Figure 7(a) (Chen et al. 2014).

Wrinkle formation mechanism

A mechanical model was introduced to study B. subtilis biofilm buckling induced by dead cells, which pushed up the biofilm from underneath with a vertical force $F$. For a cycle of wrinkle structure as the object, the mechanical principle is schematically shown in Figure 8.

Here, $L$ is the wavelength of the wrinkle structure, $h$ is the thickness of bacterial biofilm, $b$ is the breadth of bacterial biofilm, $\omega$ is the flexural equation, $x$ is the distance between the point and the origin. The flexural equation for biofilm after buckling is:

$$\omega = f(x)$$ (1)

We can obtain the approximate differential equation of deflection line from basic mechanics:

$$\frac{d^2 \omega}{dx^2} = \frac{M}{EI}, I = \frac{bh^3}{12}$$ (2)

Here, $M$ is the bending moment, $E$ is the mechanical stiffness, and $I$ is the inertia moment of bacterial biofilm.

The flexural equation can be obtained through the integro-differential equation:

$$\omega = \int \left( \frac{M}{EI} \right) dx + Cx + D$$ (3)

Here, $C$ and $D$ are constants. The boundary condition is:

$$\begin{cases} x = 0, \omega = 0 \\ x = \frac{L}{2}, \omega' = 0 \\ x = L, \omega = 0 \end{cases}$$ (4)

After the boundary conditions are applied, the flexural equation becomes:

$$\omega = \frac{Fx}{48EI} (3L^2 - 4x^2), 0 \leq x \leq \frac{L}{2}$$ (5)

From Equations (1)–(5) we find that the deflection of B. subtilis biofilm has a linear relationship with force, and

<p>| Wrinkle characteristics under different Young’s modulus |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate length (mm)</th>
<th>Young’s modulus (kPa)</th>
<th>Wrinkle’s number</th>
<th>Wrinkle wavelength (μm)</th>
<th>Wrinkle wave height (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECM production ↓↓</td>
<td>2</td>
<td>5</td>
<td>14</td>
<td>142</td>
<td>250</td>
</tr>
<tr>
<td>ΔsrfA</td>
<td>2</td>
<td>8</td>
<td>12</td>
<td>166</td>
<td>240</td>
</tr>
<tr>
<td>ECM production ↓</td>
<td>2</td>
<td>15</td>
<td>11</td>
<td>182</td>
<td>220</td>
</tr>
<tr>
<td>WT</td>
<td>2</td>
<td>25</td>
<td>9</td>
<td>220</td>
<td>190</td>
</tr>
<tr>
<td>ECM production ↑</td>
<td>2</td>
<td>35</td>
<td>8</td>
<td>250</td>
<td>150</td>
</tr>
<tr>
<td>ΔabrB</td>
<td>2</td>
<td>50</td>
<td>6</td>
<td>330</td>
<td>120</td>
</tr>
<tr>
<td>ECM production ↑↑</td>
<td>2</td>
<td>65</td>
<td>4</td>
<td>500</td>
<td>90</td>
</tr>
</tbody>
</table>

Figure 6 | Wrinkle characteristics change with Young’s modulus of B. subtilis biofilms. The line marked by triangles indicates the relationship between bacterial biofilm mechanical stiffness and wrinkle wavelength. The line marked by squares shows the relationship between bacterial biofilm mechanical stiffness and wrinkle wave height. The line marked by stars indicate the wrinkle width for WT, ΔsrfA, and ΔabrB observed in the experiment.
the deflection changes with the elastic modulus of biofilm, as shown in Figure 9. Here, the force depends on the number of dead cells, which increases with time. The relation between force and time is linear, based on experimental observation (Wilking et al., 2015).

We next consider the wrinkle formation mechanism in B. subtilis biofilms. First, we obtained the vertical force produced by the biofilm deflection induced by dead cells, which is shown by the dashed and dotted line in Figure 10. Secondly, we obtained the lateral force from active cells induced biofilm deflection, which is shown by the dotted line in Figure 10. The combination of both vertical and lateral forces is shown by the dashed line in Figure 10. The biofilm deflection evolution from experimental observations is shown by the solid line in Figure 10.

From simulation results we found that the vertical force produced by dead cells provided the dominant role for wrinkle formation at the beginning of biofilm growth. After about 2 days, however, the biofilm deflection depends more on the lateral force. Eventually, the deflection depends on both vertical and lateral forces, finally, and reaches a stable value.

CONCLUSION AND DISCUSSION

In our work, we studied B. subtilis biofilm wrinkle formation mechanism, and wrinkle structure depending on the mechanical stiffness of agar substrate and biofilm itself.

In our model, B. subtilis biofilms with different Young’s modulus correspond to different ECM productions by
mutant biofilms. The ECM is composed primarily of complex, extracellular polysaccharides, which have high molecular weights and high concentration. The complex and extracellular polysaccharides together serve as cross-links thus making the polymer chains entangled. Protein can also act as cross-linkers. The polysaccharides and proteins together bestow the biofilm’s structural integrity (Wilking et al. 2011). Differences in the composition and production of ECM contribute to the broad range in biofilm mechanical properties. In our simulation we assume the biofilm to be homogeneous, while our experimental observations of B. subtilis biofilms exhibit a fascinating life cycle with material properties that are dynamic and heterogeneous. We still need a fuller understanding of biofilm material properties to establish more reasonable models to describe biofilm wrinkle morphology.

From our work, we found that the mechanical properties of the agar substrate can change the wrinkle pattern. The properties of the agar substrate which influence adsorption, adhesion, and diffusion and thus regulate the physiology of bacteria and their growth into biofilms include stiffness, mechanical stability, elasticity, and topography (Renner & Weibel 2011).

For the biofilm wrinkle formation mechanism, we studied two extreme cases. In the first, we considered the lateral force from active cells induced biofilm buckling, as shown in Figure 6. In the second case, we considered the vertical force produced by dead cells induced biofilm buckling, as shown in Figure 9. In fact, both lateral force and vertical force play important roles in wrinkle formation. That is why differences are observed between the simulation results and the experimental observations, as shown in Figure 10.

In Figure 10, the dash dot line indicates the simulated wrinkle wave height induced by vertical force. In region 1, at the beginning of the B. subtilis biofilm growth, the experimental measurement shown as the solid line is slightly larger than the theoretical prediction from vertical force. That is because there is also a contribution from a small lateral force produced by active cells. After wrinkles form in region I, the effect of vertical force produced by dead cells on the biofilm buckling is not apparent. Due to the existence of water channels beneath biofilm wrinkles, as shown in Figure 11 (Wilking et al. 2013), the dehydration of dead cells, gravity, and lateral force contribute increasingly to wrinkle size, as shown in region II of Figure 10. Biofilm grows thicker with time, the vertical force gradually increases with biofilm thickness, and after about 4 days, both the vertical force and the lateral force contribute to the wrinkle pattern, as shown in region III of Figure 10. When the biofilm becomes old enough, nutrients are completely depleted and nearly all cells turn to spores, and the wrinkle size reaches a stable value, as shown in region IV of Figure 10.

Overall, the architecture of mature wild-type B. subtilis biofilms grown on agar exhibits a characteristic, wrinkled structure. Wrinkles are filled with water extracted from agar, forming a network of channels that enhance nutrient and waste transportation, which are necessary for biofilm

![Figure 10](https://iwaponline.com/wst/article-pdf/73/3/527/463823/wst073030527.pdf)

**Figure 10** | Simulation results and experimental observations of wrinkle wave height. The solid line indicates the wrinkle wave height observed in the experiment. The dashed and dotted line indicates the simulated wrinkle wave height induced by vertical force produced by dead cells. The dotted line indicates the simulated wrinkle wave height produced by vertical and lateral forces combined.

![Figure 11](https://iwaponline.com/wst/article-pdf/73/3/527/463823/wst073030527.pdf)

**Figure 11** | Characterization of channels in B. subtilis biofilms. Series of microscopy images of a region near the center of the biofilm. Injection of an aqueous dye reveals a network of channels beneath the wrinkles. The image is from Wilking et al. (2013).
growth and physiology. Our understanding of biofilm wrinkle formation mechanism can help to facilitate or inhibit biofilm growth by mechanically adjusting the wrinkle size.

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