Liquid channels within *B. subtilis* biofilms allow the escape of trapped clones and population rescue

Nikhil Krishnan[1], Joseph Knight [1], Abhirup Mookherjee[1], Luis Ruiz Pestana[2], and Diana Fusco[1,*]

[1]Cavendish Laboratory, Department of Physics, University of Cambridge, Cambridge, UK

[2]Department of Civil and Architectural Engineering, University of Miami, Coral Gables, USA

[*]df390@cam.ac.uk

ABSTRACT

Bacterial communities undergoing spatial expansions, such as colonies and biofilms, typically exhibit a severe loss of genetic diversity due to gene surfing, a phenomenon by which only cells close to the expanding edge have a chance of contributing to the next generation. As a result, mutants that emerge behind the front will remain trapped in the bulk of the population, even if they carry a significant growth advantage. While these findings are applicable to smooth, uniformly dense colonies, where cell spatial re-arrangement is limited, it is unclear whether they hold in bacterial communities that display complex three-dimensional morphologies, such as wrinkly biofilms.

We employ an experimental procedure that enables a rapid switch of the chemical environment with minimal physical manipulation of the cell community to investigate the fate of trapped clones carrying a selective advantage in wrinkly *B. subtilis* biofilms. We find that these clones are able to use the channels underneath the wrinkles to traverse long distances and take over the expansion front. Moreover, we find that active motility is required to accomplish this task. Collectively, our findings reveal an intriguing novel role of wrinkles, as they enable potentially advantageous mutants to propel themselves towards the leading edge of the expansion and rescue the population upon environmental changes. Our results also point at motility, which has traditionally received little attention in bacterial communities growing on hard substrates, as a key mechanism for population rescue in biofilms that display complex morphologies.

Introduction

Spatial range expansions, which describe the growth of a population into surrounding virgin territory, are typically driven by individuals replicating preferentially at the front, where resources are readily available[1]. As a result, only individuals sufficiently close to the front have a chance of contributing to the next generation, leading to well-appreciated evolutionary consequences, such as gene surfing[2], genetic demixing[3], rapid loss of genetic diversity[4], and expansion load[5]. In microbial colonies, these effects are so extreme that fast growing mutants emerging behind the front will remain trapped in the bulk of the

colony without contributing to the expansion[6,7].

These phenomena have been extensively studied in colonies characterised by smooth morphologies, where cells are densely and homogeneously packed so that positional re-arrangement is very limited [1,8–10]. However, microbial communities in nature often adopt a biofilm lifestyle in which cells adhere to each other and to their surrounding[11,12], resulting in mechanical stress build-up and emergent three-dimensional architectures [13,14]. Such an example is observed in wrinkly *Bacillus subtilis* biofilms[13], which exhibit large aerial projections - wrinkles - that support liquid channels underneath[15]. These channels have been shown to play a role in nutrient and waste transport[16], thus providing a connecting route between the inside and the outside of the biofilm.

Here, we show that these channels support the passage of motile bacteria, providing an escape route for trapped mutants that is unavailable in smooth colonies. Our results point to rich, uncharted evolutionary dynamics that can occur in complex biofilms characterized by heterogeneity in cell density, spatial structure and phenotype.

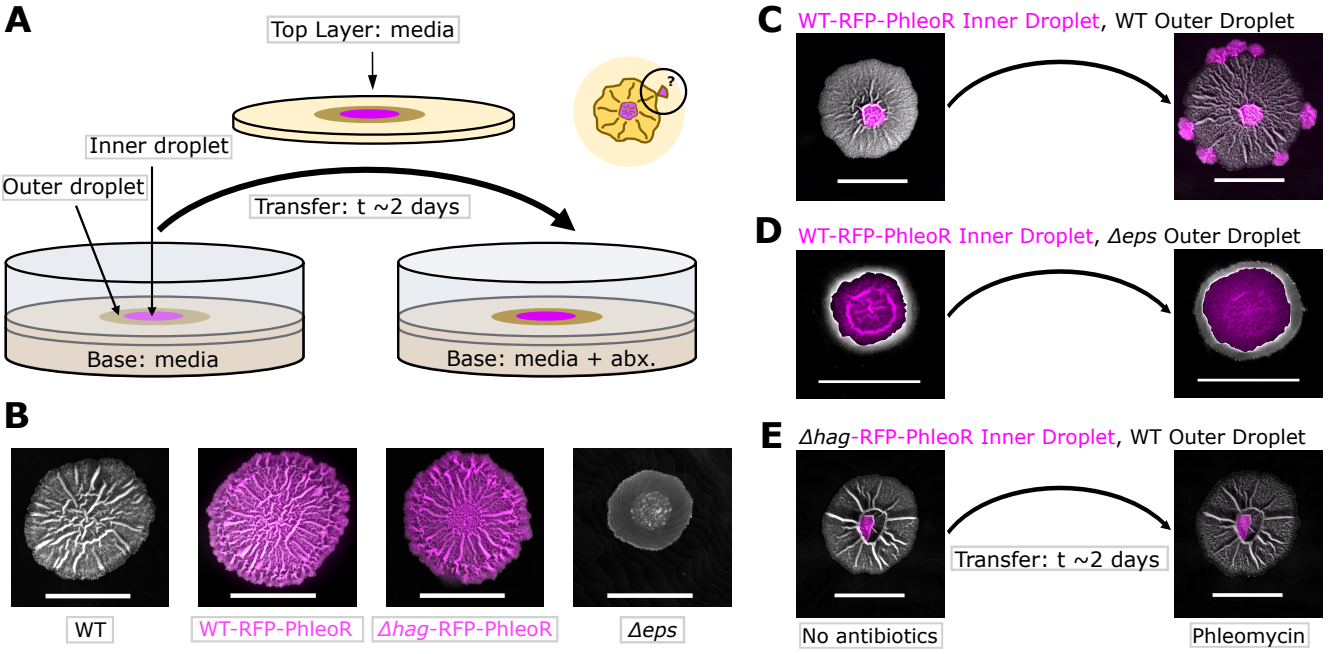

Figure 1. Initially trapped cells emerge at the edge of the expanding biofilm in the presence of wrinkles. (A) *B. subtilis* biofilms are inoculated using two strains. One strain is inoculated first as a larger outer droplet. Once dry, a smaller inner droplet is placed on top. The inoculation is carried out on an Msgg agar plate that consists of a thick base layer on top of which sits a thinner layer. After the biofilm matures over the course of two days, the thin layer is transferred to a new base agar layer containing Msgg media and antibiotics (abx.) selecting for the inner droplet strain. Fluorescent images are taken after the transfer to examine whether cells from the inner droplet are found at the edge of the biofilm expansion with further incubation. (B) Morphological features of the four strains used in this work after two days of growth with no antibiotic followed by two further days of growth on phleomycin. (C) When the outer droplet is the wild-type biofilm-forming strain (no fluorescence), and the inner droplet is an analogous strain expressing RFP and resistant to phleomycin, domains of fluorescent cells emerge at the edge of the biofilm and are found to be co-located with the ends of radial wrinkles (See Table S1, Supplemental Movie 1). (D) If the outer droplet is an *eps*-deficient strain, unable to form wrinkles and the inner droplet is the same as in (C), escape of the inner droplet cells to the front of the expanding colony is not observed. This was confirmed across 2 replicates. (E) If the outer droplet is the same as in (C) and the inner droplet is a flagellum-null strain constitutively expressing RFP and resistant to phleomycin, escape of the inner droplet cells to the front of the expanding colony is not observed. This negative result was confirmed across 20 replicates. Scale bars in (B)-(E) are 10 mm. Images are a composite of brightfield and RFP images.

Results

To investigate the fate of advantageous trapped clones in *B. subtilis* biofilms, we designed an assay to detect the escape of fitter cells, initially caged in the bulk of the population, upon environmental change (Fig 1A). We inoculated a small droplet of a 'test' resistant clone (inner droplet) within a larger droplet of a 'resident' susceptible clone (outer droplet) on a thin agar slab containing Msgg media (See Methods) placed atop a thicker layer of agar containing the same media. After two days during which wrinkles emerge, the thinner slab is transferred to a new base layer containing antibiotic, inducing a sudden environmental change with no alteration to the spatial structure of the biofilm.

Trapped clones can invade the expansion front in wrinkly biofilms We initially carried out our transfer experiment using NCIB3610, wild-type *B. subtilis* (WT) as the outer droplet, and a corresponding strain carrying a constitutive fluorescent reporter and phleomycin resistance as the inner droplet (WT-RFP-PhleoR). 24 hours after the transfer, domains of fluorescent cells reproducibly appear from the outer end of the wrinkles at the edge of the biofilm (Fig 1C, Table S1, Fig. S1).

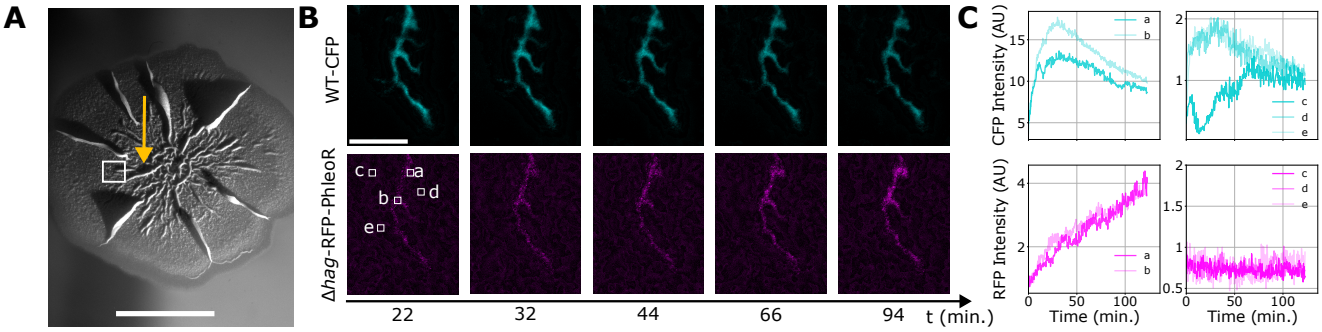

Figure 2. High-magnifcation fluorescent microscopy reveals that flagellated cells infiltrate liquid channels faster and deeper than non-flagellated cells. (A) We place a droplet of a concentrated WT-CFP and Δ*hag*-RFP-PhleoR cell mixture on a small opening made at the base of a large wrinkle (yellow arrow). The biofilm subsequently imbibes the droplet. The scale bar indicates 10mm. The white frame indicates the approximate region of the images in (B). (B) Wild-type cells expressing CFP (WT-CFP), falsely coloured in cyan, quickly occupy liquid channels and are able to spread further to branching channels with a smaller diameter (See Supplemental Movie 2). Δ*hag* -RFP-PhleoR cells, falsely coloured in magenta, are slower to fill the largest channel shown and are unable to spread into smaller branching channels (See Supplemental Movie 3). Scale bar indicates 0.5 mm. (C) Five regions roughly 55 x 55 μm in size are chosen, as shown in (B). Regions a and b are chosen to represent easily accessible, large channels. Here, the wild-type shows a rapid increase in signal, followed by a decay due to passage of the cells to other regions. In contrast, non-flagellated cells show a much slower dynamics. Regions c-e are chosen to represent smaller, branching channels. These regions are successfully explored by the flagellated cells, but no detectable signal is observed for the non-flagellated ones. Average intensity is calculated from the contrast-adjusted images shown in (B) of the CFP and RFP signal, in each region (see Methods).

Wrinkles are necessary for escape to occur Given the spatial correlation between wrinkles and escape location, we hypothesized that the channels underneath the wrinkles are used as an escape route[15,16] (Fig. S3), making wrinkles a necessary feature for escape to occur. We repeated our experiment using an *eps*-null strain as outer droplet, which lacks a major component of the extracellular matrix, thus generating wrinkle-less biofilms[17,18]. In contrast to the previous case, we consistently find that the test clone remains caged by the surrounding biofilm, despite its large selective advantage (Fig 1D, Fig. S2). We conclude that the presence of wrinkles is necessary for escape.

Motility is required for trapped cells to escape We then wondered whether cells escape the centre by simply growing and filling the channel underneath the wrinkle or by actively moving along the channel. To address this, we repeated the experiment with the wild-type strain as the outer droplet (WT), and a resistant flagellum-null strain, unable to swim, as the inner droplet (Δhag-RFP-PhleoR). No escape of the inner droplet cells was observed over 20 replicates (Fig 1E).

Upon gently introducing in the biofilm centre a concentrated mixture of flagellated (WT-CFP) and non-flagellated (Δhag-RFP-PhleoR) cells (Fig. 2A,B), we observe that flagellated cells can navigate more swiftly and thoroughly the interconnected network of channels within the biofilm (Fig. 2C), explaining why only motile cells can escape.

Discussion

We investigated how biofilm wrinkles impact the clonal population dynamics during expansion. We find that the channels underneath the wrinkles provide a connecting route across the biofilm that can be used by cells to move across the densely packed cellular community. From an evolutionary standpoint, this result points to a stark difference between the fate of advantageous mutants emerging behind the front in smooth colonies, where they would remain trapped in the bulk, and wrinkly biofilms, where the same mutants would be able to bypass the surrounding cells and potentially rescue the population upon an environmental shift. Because in range expansions the vast majority of spontaneous genetic diversity lies trapped in the bulk of the colony[6,7], the emergence of complex three-dimensional architectures may enable the population to tap into this mutational reservoir and significantly increase the resilience of the community. Intriguingly, we find that active motility is a requirement for escape, as it enables cells to more efficiently find their way out through the intricate channels network. The result highlights the potential importance of this currently understudied phenotype in mature biofilms growing on hard substrates.

From a fundamental perspective, we emphasize that the key factor that leads to a distinct evolutionary fate of trapped clones in our study compared to previous work is *heterogeneity*. The presence of channels within the packed cellular environment points to large variability in cell density as a first fundamental requirement for our observations. These channels need wrinkles as a support, which, in turn, require cells to produce an extracellular matrix. Yet, we find that only motile cells can escape. Because motility and matrix production are mutually exclusive regulatory pathways in *B. subtilis*[19], phenotypic heterogeneity is a second fundamental requirement. Altogether, our results urge for cross-scale models that can accommodate cell-to-cell variability while capturing macroscopic morphological features to investigate the rich, unexplored population dynamics of complex biofilms.

Data availablity

Data and code are available at `https://github.com/FuscoLab/2023_Krishnan_bsubtilis_escape`

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Author contributions statement

NK performed the experiments and analysis, conceptualized the experiment, and wrote the manuscript. JK and AM performed the experiments. DF and LRP conceptualized the experiment and wrote the manuscript.