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

Nikhil Krishnan[1], Joseph Knight [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 are appropriate for bacterial communities that display intricate and heterogeneous morphologies, such as wrinkly *B. subtilis* 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* biofilm expansions. 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 *B. subtilis* biofilm wrinkles in enabling 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 colonies growing on hard substrates, as a key mechanism for population rescue in biofilms that display complex morphologies.

Introduction

Analogously to other populations, microbial communities expand their habitat by invading surrounding virgin territory in a process termed spatial range expansion[1,2]. Range expansions are typically driven by individuals growing preferentially at the front, where resources are readily available[3]. 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[4], genetic demixing[5], rapid loss of genetic diversity[6], and expansion load[7]. In particular, mutants that emerge behind the front will remain trapped in the bulk of the colony, even if they carry a significant growth advantage[8,9].

These phenomena have been extensively studied in microbial range expansions characterised by smooth morphologies, where cells are densely and homogeneously packed so that cells' positional re-arrangement is very limited [1,3,10,11]. However, microbial communities in nature often adopt a biofilm lifestyle in which cells can adhere to each other and to their surrounding[12,13] through the production of an extracellular matrix, resulting in local mechanical stress build-up and emergent complex three-dimensional architecture [14,15]. A particularly striking example of such architectures is found in the morphology of wrinkly *Bacillus subtilis* biofilms[14], which exhibit large aerial projections, here termed wrinkles, that support liquid channels underneath[16]. These channels have been shown to play a role in nutrient and waste transport[17], indicating that they can provide a connecting route between the inside and the outside of the biofilm.

In this study, we show that these channels support the passage of bacteria across *B. subtilis* biofilms, providing an escape route for trapped mutants that is unavailable in smooth colonies. Interestingly, we also find that motility is required for bacteria to be able to escape. Overall, our results point to rich and uncharted evolutionary dynamics that can take place in complex biofilms characterized by heterogeneity in cell density, spatial structure and phenotype.

Results

To investigate the fate of trapped clones during the growth of *B. subtilis* biofilms, we designed an assay to detect the escape of cells initially trapped well behind the front (Fig 1A). We inoculated a smaller droplet of a 'test' clone (inner droplet) within a larger droplet of a 'resident' clone (outer droplet). The inner droplet is placed immediately after the outer droplet has dried to ensure that the test clone is completely surrounded by the resident one. The inoculation is carried out on a thin agar slab containing Msgg media, which promotes biofilm growth in *B. subtilis* (See Methods)[18], placed atop a thicker layer of agar containing the same media. After two days during which the biofilm develops and wrinkles emerge, the thinner slab is transferred to a new base layer of agar containing antibiotic that favours the growth of the initially trapped 'test' clone, 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

To understand whether wrinkle formation alters the ability of trapped cells to escape the biofilm core, we carry 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). After approximately two days of growth, long radially extending wrinkles emerge (Fig 1C). As previously shown[16,17], these wrinkles support a network of inter-connected channels beneath them with depth and width spanning over many cell lengths (Supporting Information, Fig. S3). The biofilm is then transferred to a base agar layer supplemented with a high concentration of phleomycin, which provides a selective advantage for the inner droplet. Approximately 24 hours after the transfer, domains of fluorescent cells emerge from the outer end of the wrinkles at the edge of the biofilm (Fig 1C). We find that the result is highly reproducible, often showing multiple escape events at the end of different wrinkles within the same biofilm, with almost perfect correlation between the location of the escape event and the presence of a nearby wrinkle (Table 1, Supporting Information Fig. S1).

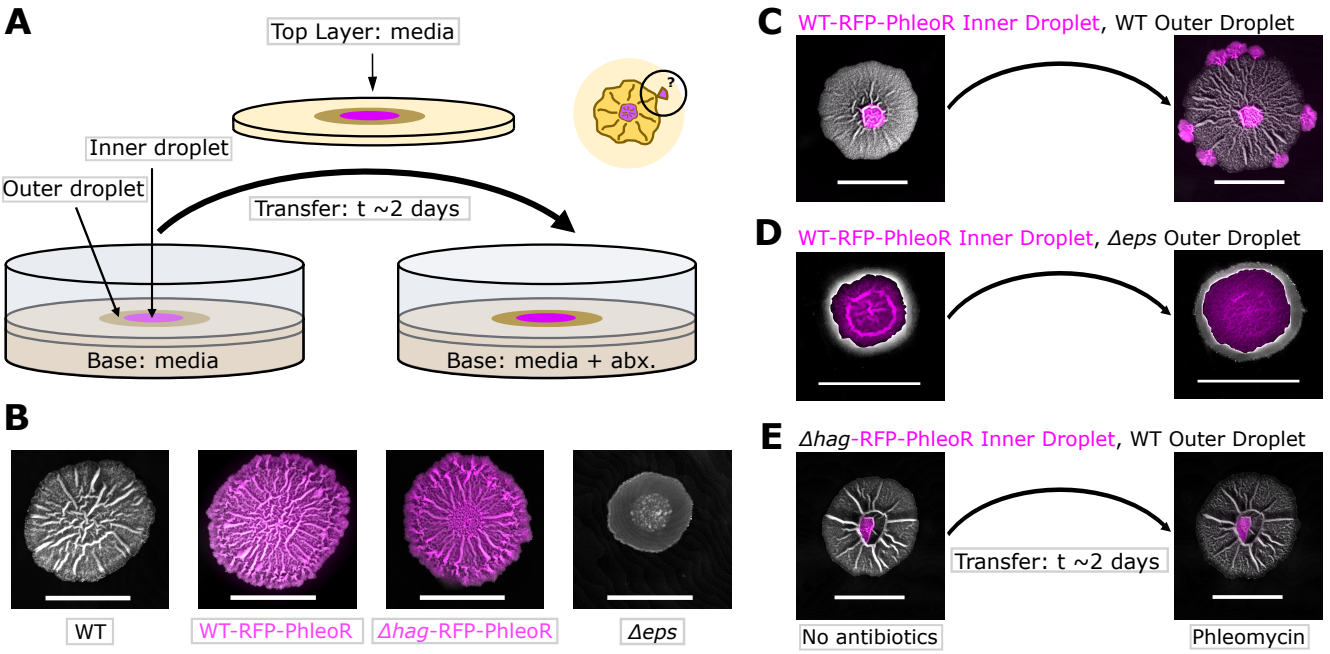

Figure 1. Initially trapped cells emerge at the edge of the expanding biofilm in the presence of wrinkles. (A) *B. subtilis* biofilms are inoculated using two strains. One strain is inoculated first as a larger outer droplet. As soon as the outer droplet has dried, a smaller inner droplet is placed on top. The biofilm is inoculated 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 and transfer to phleomycin for two further days of growth. (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, which appears to be co-located with the ends of radial wrinkles. (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.

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. To test whether wrinkles are indeed necessary to observe escape, we repeated our experiment using a strain of *B. subtilis* with *eps* knocked-out as the outer droplet. *Eps* encodes a major component of the *B. subtilis* biofilm matrix and its absence has been shown to result in wrinkle-less biofilms[18,19]. In contrast to the biofilms which exhibit wrinkles, here we consistently find that the inner clone remains caged by the surrounding biofilm, despite the large selective advantage induced by the presence of the antibiotic (Fig 1D, Supplemental Information, Fig. S2). We conclude that the presence of wrinkles is necessary for escape.

	On Wrinkle	Total	Prop.
Rep. 1	7	9	0.78
Rep. 2	3	3	1.00
Rep. 3	7	7	1.00
Rep. 4	5	5	1.00
Rep. 5	3	4	0.75
Rep. 6	7	10	0.70
Sum	32	38	0.84

Table 1. Escape of initially trapped cells in the presence of wrinkles is recorded. The total number of domains of escaped cells at the edge of the biofilm for each replicate is indicated in the column labelled “Total". The number of these domains that emerge at the mouth of the wrinkles is indicated in the column “On Wrinkle". The proportion of domains that emerge at the mouth of the wrinkles for each replicate is indicated under “Prop." and visually indicated by the blue shading.

Motility is required for trapped cells to escape through wrinkles

We then wondered whether cells escape the centre by simply growing and filling the channel underneath the wrinkle or require active motion. To address this, we repeated the experiment with the wild-type strain as the outer droplet (WT), and a flagellum knock-out, unable to swim, as the inner droplet (Δhag-RFP-PhleoR). Upon conducting this experiment for over 20 replicates, no escape of the inner droplet cells was observed (Fig 1E), despite the significant fitness advantage (See Supplemental Information, Fig. S2). We conclude that cells need to be flagellated in order to escape the biofilm core.

Discussion and Conclusion

The emergence of wrinkles in many biofilm-forming species has intrigued scientists over the last few decades. While these wrinkles are understood to support liquid channels and support nutrient and waste flow in *B. subtilis*, their function, if any, remains poorly understood[16,17]. In this study, we propose and investigate a novel role, which impacts clonal population dynamics during biofilm expansion. We find that the channels emerging underneath the wrinkles provide a connecting route between the inside and the outside of the biofilm that can be used by cells to move across the densely packed cellular community. From an evolutionary point of view, this result points to a stark difference between the fate of trapped mutant clones in wrinkly biofilms compared to smooth colonies. While in the latter trapped cells are unable to escape, even if carrying a significant fitness advantage, in the former advantageous mutants are able to bypass the surrounding cells, travel across the biofilm, reach the front of the expansion and potentially rescue the population upon an environmental shift. It has been shown that in microbial spatial expansions, the vast majority of spontaneous genetic diversity lies trapped in the bulk of the colony[8,9]. Our result suggests that complex three-dimensional architectures, typical of natural biofilms, enable the population to tap into this mutational reservoir

and significantly increase the potential adaptability of the bacterial community.

Interestingly, we find that active motility is a requirement for escape. It has previously been shown that in some conditions a net fluid flow is present from the outside towards the inside of *B. subtilis* biofilms[16]. A possibility is that active motility allows the cells to swim against this flow to reach the edge of the expansion. Additionally, the channels underneath the wrinkles have been shown to remain open only for a limited amount of time[16], so it is also possible that motility is needed to reach the outer end before closure. Additional experiments are needed to disentangle the exact escape mechanisms cells use, but overall, our results point to motility as a key and currently understudied phenotype in mature biofilms growing on hard substrates.

Finally, from a fundamental perspective, we emphasize that the key difference that distinguishes complex three-dimensional biofilms from conventional smooth colonies 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, requires cells to produce an extracellular matrix. Yet, we find that only motile cells can escape, which is a mutually exclusive regulational pathway to matrix production in *B. subtilis*. Therefore, phenotypic heterogeneity is a second fundamental requirement for our observations. Taken together, this points to an urgent need for novel mesoscopic models that can accommodate cell-to-cell variability to investigate the rich and unexplored population dynamics of complex biofilms.

Methods

Bacterial strains, media, and culture conditions

The strains used are biofilm-forming *B. subtilis* strains derived from NCIB 3610. In escape experiments, for the outer droplet, we used either the wild-type NCIB 3610, or a strain of the same background in which *epsA-O*, the operon encoding genes responsible for the exopolysaccharide component of the matrix, was replaced with *tet*, which confers tetracycline resistance. For the inner droplet, we used two reporter strains expressing RFP. The background of these strains is a more readily transformable strain of NCIB 3610, 3A38[20] with phleomycin resistance. One of these two strains had no further modifications while the other had the *hag* gene, which encodes the principle flagellar filament and is required for swimming and swarming motility, replaced with *kanR*, conferring resistance to kanamycin. The *hag* knockout was confirmed to be unable to spread via swimming motility on 0.5% LB agar plates.Wild-type NCIB 3610 and reporter strains were a gift from the lab of James Locke. The *eps* knockout was a gift from Agnese Seminara. Strain details are enumerated in Supplemental Information, Table S1.

Strains were grown in LB (Miller's lysogeny broth base, Invitrogen) or a defined Msgg medium which promotes biofilm growth (5 mM phosphate buffer pH 7.0; 100 mM MOPS buffer pH 7.0, adjusted with NaOH; 0.5% (v/v) glycerol; and 0.5% (w/v) monosodium glutamate; 2 mM $MgCl_2$; 0.7 mM $CaCl_2$; 50 μM $MnCl_2$; 50 μM $FeCl_3$; 1 μM $ZnCl_2$; 2 μM thiamine HCl).

Msgg agar plates were prepared a day before an experiment by autoclaving 1.5% (w/v) agar (VWR Chemicals), with MOPS buffer, Phosphate buffer, and glycerol from stocks. After cooling, the rest of the ingredients were added from filtered stocks. Monosodium glutamate stocks were made fresh from stocks on the day of preparation. 30 mL molten Msgg agar was poured

into 128 by 86 mm rectangular plates (Nunc OmniTray Single-Well Plate, Thermo Scientific), and 10 mL was poured into 100 mM Petri dishes. Once the agar had set, plates were allowed to dry overnight at room temperature. The following day, Msgg agar plates were further dried for 1 hour in a laminar flow hood. After drying, the thinner Msgg agar layers in the 100 mM Petri dish were sterilely transferred to each of the thicker Msgg agar plates. The thinner circular Msgg agar layers were trimmed to allow proper lid closure.

Overnight *B. subtilis* cultures were grown from single colonies in LB at 37 °C with appropriate selection antibiotics: 0.25 μg/mL phleomycin (Invitrogen), 10 μg/mL kanamycin (Sigma-Aldrich), and 20 μg/mL tetracycline (Sigma-Aldrich). Overnight cultures were adjusted to $OD_{600} \sim 1$ before 2 μL of the initial outer droplet were placed on the Top layer of the Msgg agar once it was placed on a base layer in a laminar flow hood and allowed to dry for $\sim$ 3 minutes. Once the outer droplet was dried a 0.5 μL inner droplet was placed so that it is completely encompassed by the outer droplet and as close to concentric as possible, and allowed to dry for $\sim$ 1 minute. Msgg agar plates were sealed with parafilm and incubated at 30° C. After two days of incubation, the top layer of Msgg agar was transferred to another base layer of Msgg agar containing 10 μg/mL phleomycin (Sigma-Aldrich), which was prepared a day earlier, dried overnight on the bench and in the laminar hood as previously described. At the time of transfer, it was confirmed via fluorescence microscopy that the inner droplet remained encompassed by the outer droplet. Progress of the biofilm was monitored via time-lapse or daily still images with a fluorescence microscope.

Microscopy and image processing

Biofilms were imaged using Zeiss Axio Zoom.V16 stereo microscope equipped with a Zeiss PlanApo Z 0.5 ×/0.125 FWD 114-mm objective. During time-lapses, images were taken every 40 minutes while the samples were incubated at 30° C in a custom-made incubation chamber enclosing the entire microscope setup. Images were taken of the biofilm from above through a highly transparent glass heated to 32° C to prevent condensation.

The brightness and contrast of images were adjusted using the FIJI distribution of ImageJ[21].Fluorescence images had the background removed using the rolling ball method as implemented in FIJI.

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Author contributions statement

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