Chapter

Extending an Eco-Evolutionary Understanding of Biofilm-Formation at the Air-Liquid Interface to Community Biofilms

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Abstract

Growing bacterial populations diversify to produce a number of competing lineages. In the *Pseudomonas fluorescens* SBW25 model system, Wrinkly Spreader mutant lineages, capable of colonising the air-liquid interface of static microcosms by biofilm-formation, rapidly appear in diversifying populations with a fitness advantage over the ancestral wild-type strain. Similarly, a biofilm is rapidly produced by a community containing many biofilm-competent members, and selection by serial transfer of biofilm samples across microcosms results in a gradually changing community structure. Both the adaptive radiation producing Wrinkly Spreaders and the succession of biofilm communities in these static microcosms can be understood through evolutionary ecology in which ecological interactions and evolutionary processes are combined. Such eco-evolutionary dynamics are especially important for bacteria, as rapid growth, high population densities and strong selection in the context of infections can lead to fast changes in disease progression and resistance phenotypes, while similar changes in community function may also affect many microbially mediated biotechnological and industrial processes. Evolutionary ecology provides an understanding of why bacterial biofilms are so prevalent and why they are such a successful colonisation strategy, and it can be directly linked to molecular analyses to understand the importance of pathways and responses involved in biofilm-formation.

Keywords: adaptive radiation, air-liquid (A-L) interface biofilms, evolutionary ecology, experimental evolution, fitness, microcosms, oxygen gradients, *Pseudomonas*, Wrinkly Spreaders

1. Introduction

Our research interests have focussed on air-liquid (A-L) interface biofilm-formation by the model pseudomonad *Pseudomonas fluorescens* SBW25 and the adaptive Wrinkly Spreader in experimental microcosms (see our recent reviews [1, 2]), and we have recently begun to extend our investigations into biofilm-formation by communities dominated by similar biofilm-competent pseudomonads. Our

research has also developed from a molecular biology perspective [1] towards a more evolutionary and ecological understanding [2] of why biofilms are such a successful colonisation strategy used by bacteria in a wide variety of environments.

In contrast to our changing perspectives, we realise that although biofilm research is interdisciplinary, it appears dominated by molecular biologists working with medically relevant model species with a focus on a mechanistic understanding of biofilm-formation which has remained unchanged from that of the early biofilm pioneers [3, 4]. However, contemporary biofilm research includes a wide range of other disciplines, including evolutionary ecology which provides a framework for understanding how the cooperation needed between bacterial cells to produce biofilms is established and maintained, how bacteria diversify and adapt within these structures, and how biofilm communities respond to changing environmental conditions.

We note that although biofilm reviews addressing evolutionary ecology are published regularly, evolutionary ecology content is negatively correlated with molecular biology and medical content in those reviews with a wider focus.[1] This should be of concern, as any mechanistic understanding of biofilms lacking an evolutionary ecological element will not be able to evaluate the importance of these structures nor make long-term predictions about persistence or function in a wide range of medical, biotechnological and industrial contexts. Furthermore, these negative correlations suggest that the medical molecular microbiology community is ignoring or is unaware of the contributions evolutionary ecology could make towards understanding and mitigating the impact of biofilm-associated disease.

2. Importance of an eco-evolutionary perspective in biofilm research

Evolutionary ecology seeks to understand how ecological interactions can affect selection and adaptation and the consequences of evolutionary change [5–7]. These interactions occur within and between populations, as well as with the environment, and ecological processes involving these interactions explain community dynamics and succession. In contrast, evolutionary processes are usually considered as driving lineages through time, and when subject to selection can result in adaptive changes and ultimately speciation (we use the term lineage here to include mutations, alleles and genotypes, individuals and mutants, and species, all of which can be followed through time and across generations to investigate ecological interactions or evolution). However, ecological and evolutionary changes are directly linked and can occur on the same time-scale [8, 9]. Such eco-evolutionary dynamics are especially important in bacterial populations and communities, where growth rates and numbers are high and selective pressures can be extreme, leading to the rapid fixation of adaptive mutations and striking changes in phenotype or community function.

Evolution research should not therefore be limited to examining fossils or contemporary ecosystems but can be undertaken over relatively short time-scales

[1] We have assessed changing interests in biofilm research by undertaking a simple content analysis of open access reviews published between 2000 and 2004 and 2014–2019 listed by Google Scholar and PubMed on 10 October 2019 (n = 40), scoring each for medical (M), molecular biology (MB), and evolutionary or ecological (EE) content. No significant differences were seen in each content type between dates (Wilcoxon, $P < 0.05$) or between contents for each date (Kruskal-Wallis, $P < 0.05$). In early publications we found a significant correlation between M & MB (Spearman $\rho = -0.83$, $P < 0.0001$), but not between M & EE ($P = 0.12$) or MB & EE ($P = 0.96$). In recent publications there were significant correlations between M & EE ($\rho = -0.74$, $P = 0.0002$) and MB & EE ($\rho = -0.43$, $P = 0.06$), but not between M & MB ($P = 0.49$).

in experimental evolution studies using microbial populations and microcosms [10–20]. In particular, the ease with which bacterial populations can be cultured, short generation times and large population sizes which allow mutations to accumulate (diversification) and be identified, the ability to freeze isolates indefinitely, and undertake genetic analyses, make bacteria an ideal model to explore aspects of evolutionary ecology.

Two significant eco-evolutionary processes are particularly relevant to biofilm research. The first of these are ecological interactions which help assemble, stabilise or change community structure [21–23] (community change is often referred to as succession). The main two-way interactions between members of a community are mutualism, commensalism, competition and predation. Cooperation, one example of mutualism where both partners benefit, is usually considered an intraspecific or within-lineage interaction, though it can also occur between closely related lineages or lineages with very similar phenotypes as in the case of community biofilms. External forcing such as physical disturbance can alter ecological interactions (**Figure 1a**) and the impact of this can be measured in terms of system stability and productivity, and possibly even by a change in function. Evolutionary processes, including selection, speciation, drift and dispersal also effect community composition and diversity [21, 23, 24].

The second significant eco-evolutionary process relevant to biofilm research is adaptive radiation [5], the evolution of diversity through random mutation and selection (**Figure 1b**), which in the context of bacteria, can happen very rapidly within a few generations. Developing populations accumulate mutations or diversify, and those mutants with a fitness advantage over their competitors can be considered successful or adaptive. Although evolution is normally thought of as the slow accumulation of mutations with small additive effects on fitness, bacterial microcosms are usually dominated by the first adaptive lineage to appear or by adaptive lineages which appear early on in the process of diversification [14].

Adaptive lineages often make use of new ecological opportunities with key innovations that allow them to interact with the environment in a fundamentally different way [5, 25, 26]. Ecological interactions also occur between lineages and will result in the fixation or loss of particular mutations. These interactions clearly link community change and adaptive radiation, as they help determine the importance of novel ability, such as biofilm-formation, brought in by immigration or key innovation resulting from mutation. In terms of the cooperation required for

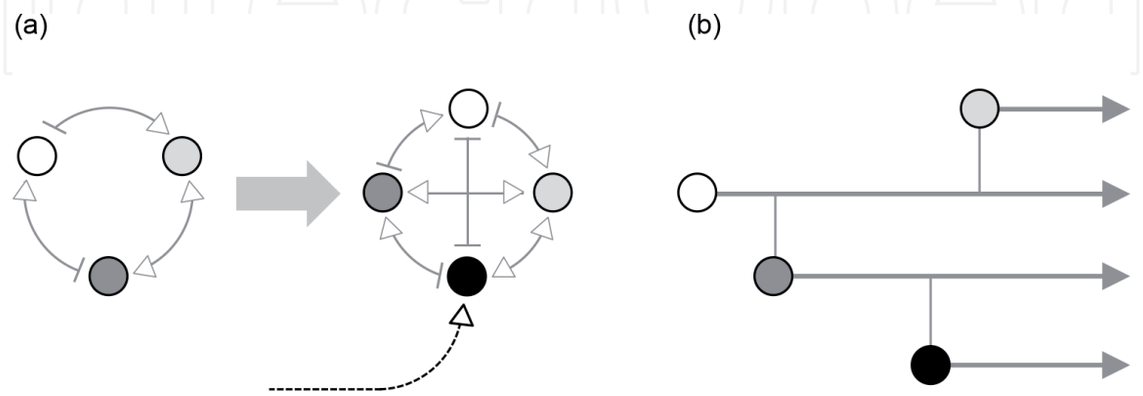

Figure 1.
Eco-evolutionary processes involve ecological interactions and adaptive radiation. Basic ecological interactions determine community dynamics which can change over time, for example, by the immigration (dashed line) of a new member with a novel ability (arrows and bars linking nodes represent positive and negative interactions between community members, respectively) (a). Adaptive mutations occurring in diversifying populations established by a common ancestor can lead to new lineages with key innovations which then compete with other lineages (vertical lines represent mutations giving rise to new lineages) (b).

biofilm-formation, kin selection ensures that cost of construction, often considered in terms of public goods or common pool resources such as the extracellular polymeric substances (EPS) which provide the main structural element of biofilms, is spread across all members who then all share in the benefits [15, 27].

Cooperation is further stabilized in biofilms by spatial separation of producers and cheaters who do not contribute to the cost of construction and a reduction of distance over which the benefits of cooperation act [19]. It is important to note that where external forcing or selection occurs, or where there is an ecological opportunity, community structures will change and lineages continue to adapt, until the theoretical end-point of evolution in a community known as an evolutionary stable community is achieved [28].

3. The SBW25 model system

Pseudomonas fluorescens SBW25 was originally isolated from the sugar beet (*Beta vulgaris* subsp. *vulgaris*) phyllosphere and has been used in experimental evolution studies where the appearance of mutant lineages with altered colony morphologies (these are sometimes referred to as morphotypes or morphs) in diversifying populations have allowed the dynamics of diversification and the fitness of adaptive mutations to be readily investigated [1, 2, 13, 14, 19, 29]. In this system, competitive trade-offs between lineages result in negative frequency-dependent selection and indicate that the major driver of adaptive radiation is competition for limited resources [29].

Fitness, a measure of an individual's reproductive success, is determined at the population level in such microcosms. For bacteria, fitness is readily assessed by comparing the maximum growth rate (v_{Max}) of one population with that of a second, reference population. Simple growth rate comparisons are typically used to infer the success of mutations for which enzymatic or regulatory changes are being investigated, but a more meaningful ecological comparison can be made by growing the two populations together, allowing them to interact with one another and to compete for limiting resources. Competitive fitness [14, 30, 31][2] can be readily determined using co-cultures if the two populations produce different colony morphologies allowing viable number counts to be made on agar plates, or if they can be labelled using fluorescent markers, to allow more rapid enumeration with automated cell counters.

The evolutionary consequences of ecological processes are readily studied using microcosms. They provide defined environments for bacterial growth, and because they are reproducible, treatments can be replicated, experiments are repeatable, and selective pressures can be changed by altering resources or inocula. Nonetheless, the use of microcosms in evolution studies faces some criticisms, including the fact that they are unnatural and very simple environments, and that these studies are essentially contrived [15]. However, although populations may be founded in these synthetic environments, evolutionary and ecological dynamics are interpreted in terms of recent evolutionary history which may span 10–60,000

[2] The competitive fitness (W) of one population (A) compared to a reference population (B) is determined as the ratio of Malthusian parameters (m_A/m_B) where $m = ln$ [final numbers/initial numbers] for each population over the period of the assay [30] (m is scaled here for generation time using ln as a correcting factor [31]). When $W_{A,B}$ is greater than one, A has a competitive advantage over B (and B is at a disadvantage), when $W_{A,B}$ is equal to one, the two populations are neutral, and when $W_{A,B}$ is less than one, A is at a disadvantage (and B has a competitive advantage). As W might be dependent on the initial ratios of the two competing populations, it can show a frequency-dependent response. The selection coefficient (s) is also often used as a measure of survival and success ($s = 1 - W$).

generations in 1 day–25 years (for example, in our system which is described in the following sections, and Lenski's long-term evolution experiment (LTEE) [32]) during which the populations adapt to these environments. Microcosms are not used to replicate the complexity of natural environments but are rather models in which key factors involved in the process of adaptive radiation can be tested [15]. These studies are of course contrived, in the sense that they are designed and in some cases the outcomes are inevitable, but the value of such an approach is that they can be initiated at any point along the evolutionary process and are not limited by the initial diversity or time (for example, the fitness of an adaptive lineage or mutant compared to the ancestral strain can be immediately explored by using genetic manipulation to produce the mutation without having to wait until it appears naturally) [15].

In liquid cultures, wild-type SBW25 populations diversify as random mutations occur, dividing the initially homogeneous or isogenic population into a number of related but diversified lineages. One re-occurring lineage frequently found in static microcosms was the Wrinkly Spreader (WS) mutant class, named after the wrinkled and flat colonies produced on agar plates which are readily distinguished from the smooth and rounded colonies produced by wild-type SBW25 (**Figure 2a**) [29] (quantitative aspects of the WS phenotype are referred to as wrinkleality [1, 35]). WS mutants are further distinguished by an altered niche preference in static microcosms, where they form a robust and well-attached physically cohesive-class biofilm [36] at the air-liquid (A-L) interface, rather than growing throughout the liquid column like wild-type SBW25 (**Figures 2b** and **3b**) [29] (A-L interface biofilms are sometimes referred to as a pellicle [37]).

Wrinkly Spreaders are considered to be adaptive (evolved) lineages because they have a competitive fitness advantage over their ancestor, wild-type SBW25, which does not normally form biofilms in static microcosms [29, 38]. However, in shaking microcosms WS mutants are disadvantaged because they cannot form biofilms [38] and on agar plates the WS phenotype is genetically unstable [39]. Biofilm-formation by Wrinkly Spreaders and SBW25 [40] is neither unusual nor peculiar, as many other soil, plant and water-associated pseudomonads form A-L interface biofilms in static microcosms under the same conditions [36].

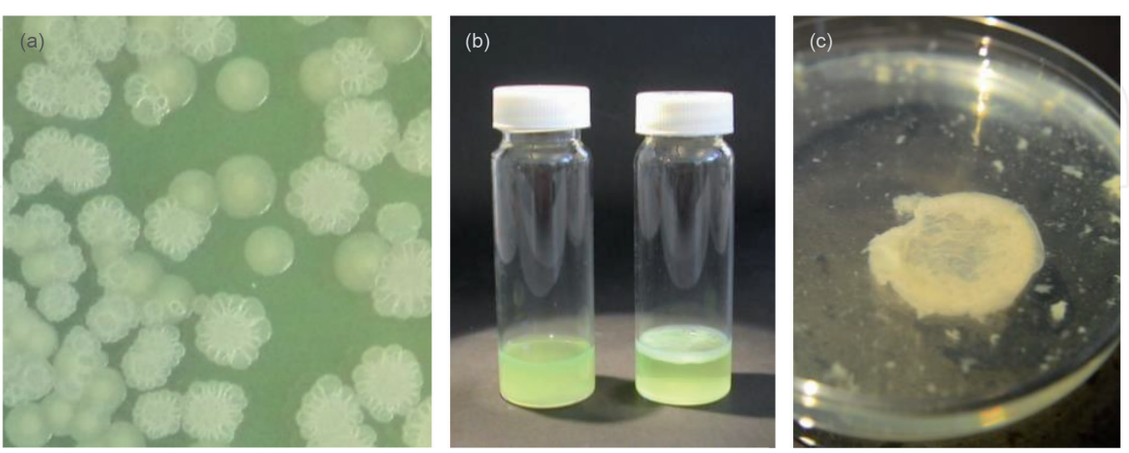

Figure 2.
Ancestral SBW25 and adaptive Wrinkly Spreaders. Wild-type SBW25 and Wrinkly Spreader colonies are readily identified on agar plates (a). In static microcosms (b), wild-type SBW25 grows throughout the liquid column (left microcosm) and the Wrinkly Spreader forms a robust biofilm at the A-L interface (right microcosm). These microcosms are 28–30 ml glass vials containing 6 ml growth medium; they are incubated with shaking which provides a homogeneous and unstructured environment with good aeration, or statically which leads to a heterogeneous and structured environment dominated by an O_2 gradient [29, 33]. When tipped out, the WS biofilm retains shape (c) demonstrating just how robust these structures are (see **Figure 4** *for more biofilm images). Photographs: A. Spiers.*

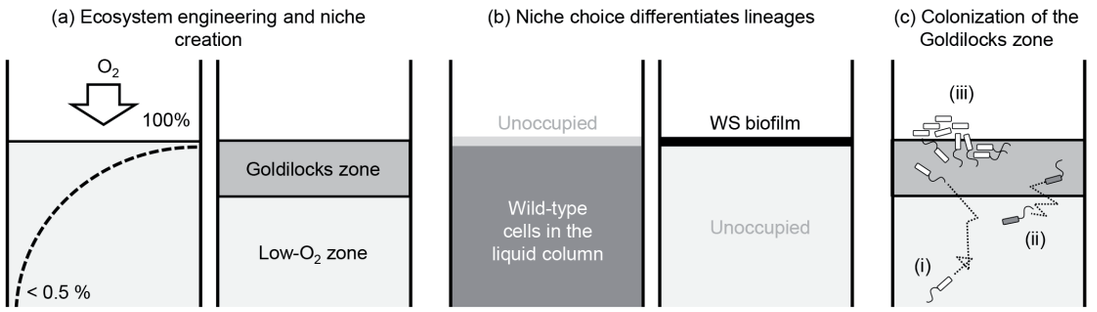

Figure 3.
The success of the Wrinkly Spreader in static microcosms can be understood from an evolutionary ecological perspective. The ecosystem engineering of the initial wild-type SBW25 colonists produces an O_2 gradient (dotted curve) which creates an O_2-rich upper zone (the Goldilocks zone) and a lower depleted zone (a). Wild-type SBW25 and Wrinkly Spreaders show different niche preferences with the WS colonising the top of the Goldilocks zone at the A-L interface (b). The WS biofilm-forming strategy is a more efficient use of resources than constant aerotaxis (swimming) to counter Brownian motion, microcurrents and vibrations which would move cells away from the optimal growth zone (c) (cell tracks indicate (i) aerotaxis towards the goldilocks zone and (ii) displacement from this region; WS biofilms (iii) are formed at the A-L interface).

The distinctive WS colony morphology allowed an investigation of the genes required for biofilm-formation, as mini-transposon mutants of the archetypal WS with wild-type-like colony morphologies were also defective in biofilm-formation [38]. This approach identified the cellulose biosynthesis (*wss*) operon required for the production of partially acetylated cellulose which was the primary biofilm matrix or EPS [38, 41]. However, the WS colony morphology and biofilm also involves an additional EPS, poly-β-1,6-N-acetyl-D-glucosamine (PNAG), as well as lipopolysaccharide (LPS), and interactions between cellulose, PNAG, LPS, and cells are required to maintain biofilm strength and integrity [42, 43]. Mini-transposon analysis also identified a chemotaxis-like (*wsp*) operon with a diguanylate cyclase (DGC) response regulator [38, 44–46]. Subsequent sequence analysis of this operon from the archetypal WS determined the presence of a single nucleotide mutation changing one amino acid residue in the methylesterase subunit [45] which acts as a negative regulatory component of the system. This results in the over-activation of the DCG, leading to increased c-*di*-GMP levels and the activation of the cellulose synthase complex. Mutations in other Wsp subunits, regulators and DGCs activated the WS phenotype in a series of independently isolated mutants [35, 43, 45, 47–49].

This understanding of the underlying molecular biology of the WS phenotype allowed a mechanistic link to be made between adaptive mutation and fitness [45] and demonstrated how easily perturbations c-*di*-GMP homeostasis could result in a key innovation through the activation of a system normally repressed in wild-type SBW25 [1, 2]. The relative ease of recovering WS lineages from diversifying populations of wild-type SBW25, demonstrating a change in niche preference and determining the competitive fitness advantage compared to the ancestral strain, also makes the SBW25 system a model for demonstrating evolution in laboratory classes [50, 51].

The microcosm system has therefore since been used to examine how wild-type colonists modify their environment [33], cells access the A-L interface [52], different environmental conditions drive WS evolution, phenotype and fitness [35, 53], and whether quorum regulation might be involved in biofilm-formation [54]. In the following subsections, we describe how the ecosystem engineering of the colonists provides the ecological opportunity and creates the niche for adaptive WS lineages and explain why biofilm-formation is the better strategy for colonizing this new niche.

4. Ecosystem engineering, ecological opportunity and niche creation

Sterile static microcosms have a uniform O_2 distribution throughout the liquid column. However, after inoculation the metabolic activity of wild-type SBW25 cells rapidly produces a steep O_2 gradient, with less than 0.1% normal levels of dissolved O_2 below 1 mm after 5 h [33]. The ecosystem engineering by these early colonists is driven by O_2 uptake levels which exceed the O_2 flux from the air above into the liquid column, and as a result the initially spatially homogeneous and unstructured environment is divided into an upper high-O_2 zone and a lower O_2-depleted zone (**Figure 3a**). The transition between the two zones is arbitrary but reflects a significant change in growth by wild-type SBW25. Further growth makes the O_2 gradient even more extreme, with less than 1% O_2 found below the top 200 μm layer of the liquid column after 5 days [33].

This depletion of O_2 is an example on a bacterial scale of the social dilemma known as the tragedy of the commons. In this, O_2 is a shared and limiting resource known as the commons, and if used selfishly and without restraint by members of the community it will be depleted and eventually destroyed [55]. Despite the growing difference between high and low-O_2 zones, wild-type SBW25 cells remain distributed throughout the liquid column though there is an appreciable accumulation of cells at the top [52]. Growth rates will be higher in this region which we have described as the Goldilocks zone[3] of optimal growth [2, 53], rather than lower down, as growth is limited by O_2 availability rather than by nutrient levels in this microcosm system [33, 53].

The ecosystem engineering of the initial colonists is also an example of niche creation (niche construction or biogenic habitat formation) [19, 56], as the high-O_2 zone now represents an ecological opportunity [5, 25, 26] for any adaptive lineage capable of colonizing this region more successfully than the initial colonists. Adaptive radiation and niche creation are inter-linked [5, 19, 25, 26, 57], and in this system the high-O_2 zone is colonized primarily by the Wrinkly Spreaders by biofilm-formation at the A-L interface (**Figure 3b** and **c**). Single-cell confocal Raman spectroscopy has demonstrated that WS cells recovered from within the biofilm have the same spectral profile as those grown under high-O_2 conditions, while cells recovered from the liquid column below the biofilm are more similar to those grown under low-O_2 conditions [58].

WS cells under high-O_2 conditions also grow faster than those under low O_2-conditions [33]. However, although WS cells do not grow faster than wild-type SBW25 cells under high O_2-conditions [33], their rapid domination of the A-L interface and subsequent population growth displaces the wild-type colonists from this region in a process known as niche exclusion. WS growth at the A-L interface further reduces O_2 flux into the lower parts of the liquid column in a density dependent manner, effectively limiting the growth of any non-biofilm-forming competitor and WS biofilms have more impact on niche divergence as populations lacking WS produce shallower O_2 gradients [59].

As the WS biofilm population increases, the division between the high and low-O_2 zones also moves up into the biofilm [33], allowing further niche differentiation within the biofilm structure itself. Substantial fitness variation has been observed

[3] 'Goldilocks and the Three Bears', written by Robert Southey, is a tale about a girl called Goldilocks who enters the home of a family of bears while they are away. She tests their chairs, beds and breakfast porridge, always choosing the one most favourable for her, before eventually being chased away when the bears return. The 'Goldilocks zone' is also used to refer to the habitable zone around a star where the temperature is just right for liquid water to exist on an orbiting planet. Here we use the term, stricto sensu, to mean the A-L interface plus the high-O_2 zone immediately below it.

between independently isolated WS [38, 43, 47, 49], suggesting multiple lineages may develop in these populations and compete with one another as Red Queens [60][4] and further competition occurs with resident cheater lineages which no longer produce cellulose [61] and do not contribute to biofilm-formation [62–64].

Fluorescent microscopy suggests WS cells are most active near the top surface of the biofilm [33] and electron micrographs show that it is a very porous structure [65] (**Figure 4**). It is possible that continuous growth near the top progressively limits the growth of cells lower down in a manner known as the Ancestors' inhibition effect [61], though this can also be interpreted as altruistic behaviour by cells which push their descendants up into better O_2 conditions and help suffocate neighbouring competitors [19, 61]. Spatial separation caused by the clumping of WS cells

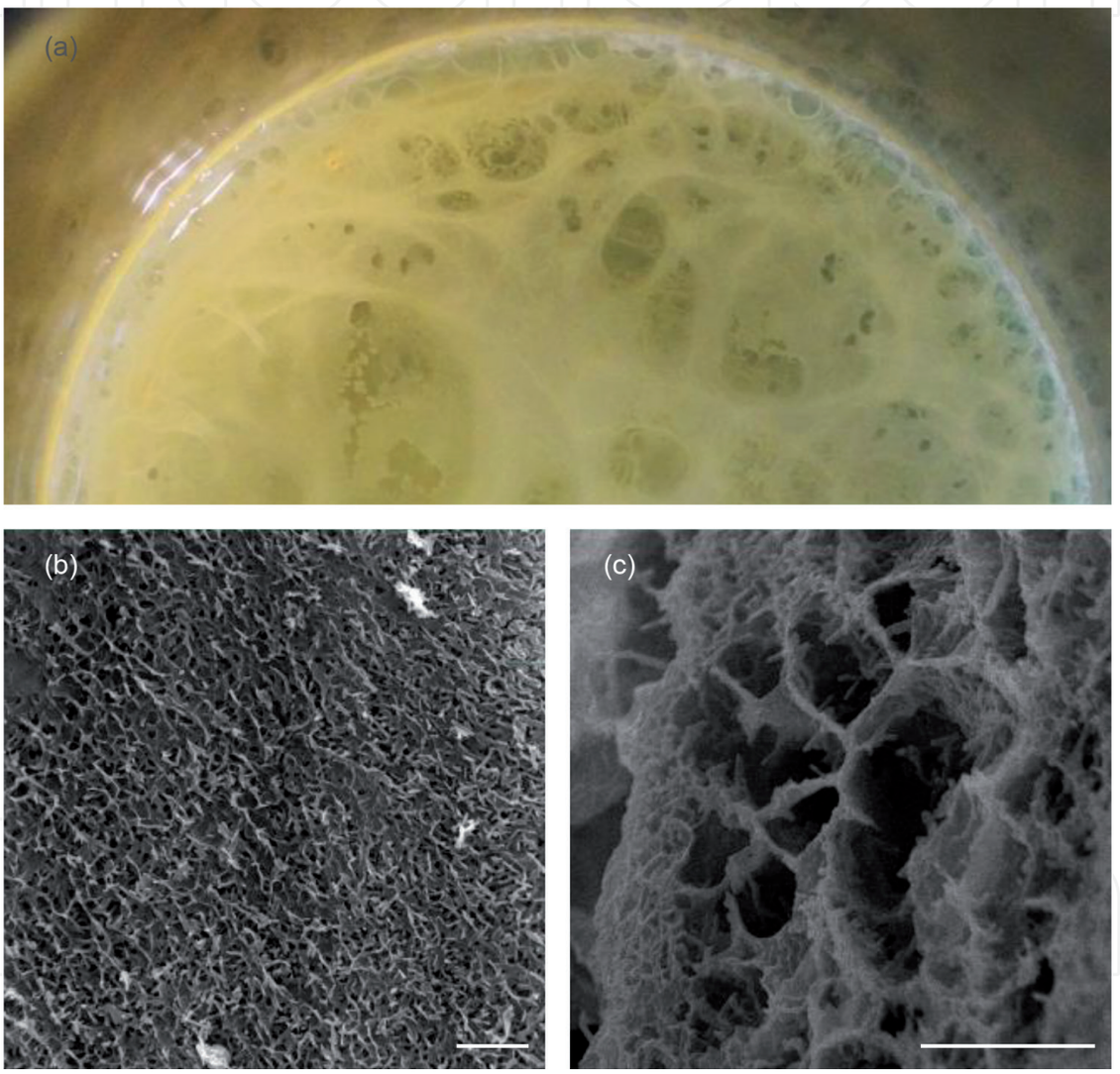

Figure 4.
The Wrinkly Spreader biofilm is a complex structure with voids and fibres apparent at different levels of magnification. Shown are views of biofilms in situ from above (a) and by electron microscopy (b and c) (scale bars represent 10 μm; the mean wild-type SBW25 bacterial body length is 3 μm [34] and individual cells are just visible in (c)). Photographs: (a) A. Spiers, (b and c) O. Moshynets.

[4] The Red Queen is a character in 'Through the looking-glass, and what Alice found there', written by Lewis Carrol. In the Red Queen's race, she and Alice were constantly running yet remained in the same spot. The Red Queen has been adopted as an evolutionary hypothesis which states that lineages must constantly adapt and evolve in order to compete successfully against others which are adapting and to a constantly changing environment. (The Red Queen should not to be confused with the Queen of Hearts who appears in an earlier story by Lewis Carrol.)

by the production of cellulose and the exclusion of cheaters, plus the continued development of the O_2 gradient within developing biofilms [33] which limits the distance over which the benefits of cooperation act, will help stabilize cooperation in biofilms [19] and allow kin selection to provide a competitive advantage to WS lineages. Biofilm development, including increasing depth and total biomass, as well as lineage and total population levels, ultimately ends with system failure when it rips and sinks to the bottom of the microcosm vial [2].

5. Biofilm-formation is the best strategy for colonising the high-O_2 zone

Aerobic motile bacteria such as SBW25 could gain access to the high-O_2 zone by aerotaxis [66], using flagella-mediated swimming motility and following the O_2 gradient up towards the A-L interface. Aerotaxis could also be used to maintain position against the physical displacement of cells caused by random diffusion, micro-currents and random knocks and vibrations occurring in microcosms during incubation. Although SBW25 is known to be capable of swimming, swarming and twitching motilities, we only recently demonstrated that wild-type and WS cells are aerotaxic [52] and that the average swimming velocity [34] is sufficient to overcome the negative effects of random diffusion on cell localization [52].

However, random diffusion still has a significant effect on maintaining position in the high-O_2 zone, and we were able to demonstrate this using modified

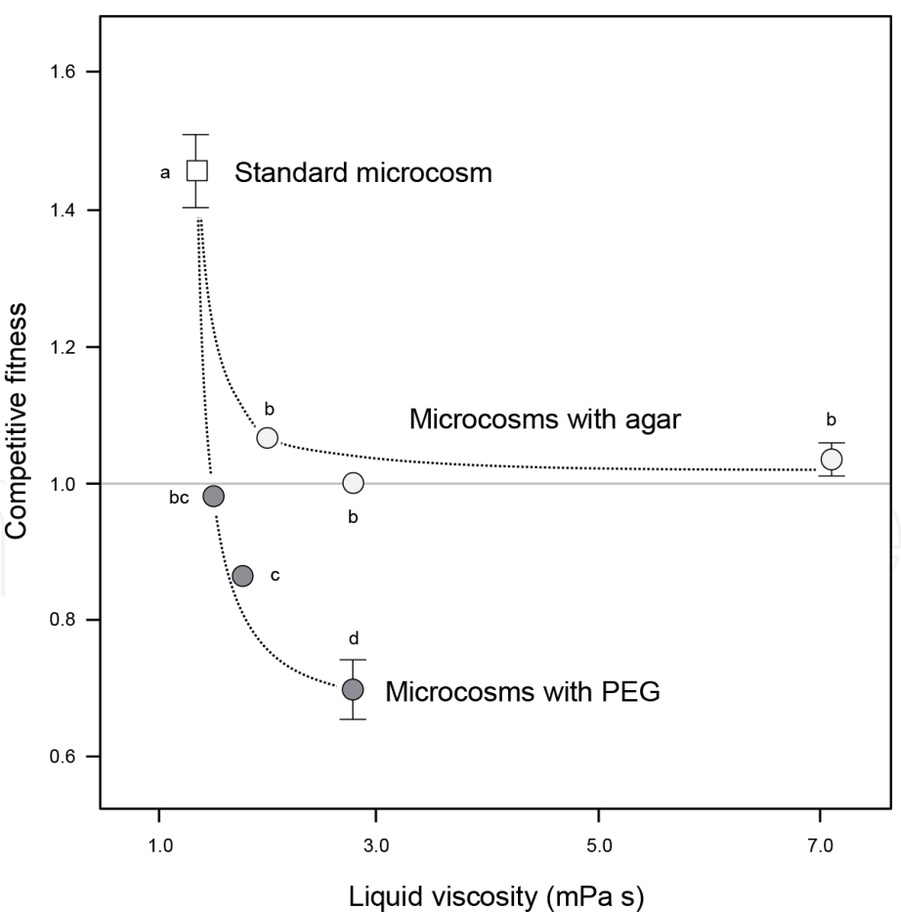

Figure 5.
WS fitness decreases with increasing liquid viscosity. Agar (light grey circles) and polyethylene glycol (dark grey circles) were used to increase the viscosity of standard microcosms (white square) and the competitive fitness of the archetypal WS determined in comparison with wild-type SBW25 under Fe-limited conditions where it cannot form a biofilm. Means are shown with standard errors. Dotted lines suggest trends and differences between means were investigated by Tukey-Kramer HSD; means sharing the same letters are not significantly different ($\alpha = 0.05$). Data are replotted from [52] (Supplementary Information).

microcosms in which we had added low concentrations of agar or polyethylene glycol to increase viscosity, as diffusion is inversely dependent on liquid viscosity [52]. Both wild-type and WS cell localization improved with increasing viscosity and, furthermore, WS competitive fitness was found to decrease with increasing viscosity (**Figure 5**) [52]. This indicates that WS biofilm-formation is a better strategy allowing the colonization of the high-O_2 zone and more specifically, of the A-L interface, than constant aerotaxis.

We argue that the need to remain in place at the top of the liquid column efficiently in order to make use of greater O_2 availability is the fundamental explanation for the success of A-L interface biofilm-formation in static microcosms by motile aerobic such as the pseudomonads [36] where growth is limited by O_2-availability rather than by nutrients [53]. The success is determined by a cost-benefit trade-off, in which resource costs required for biofilm-formation by the community or constant aerotaxis by individual cells are balanced against population gains.

6. Biofilms are not equivalent structures or of equal value

Although biofilm-formation has been extensively investigated for a wide range of model bacteria, SBW25 is the only strain for which multiple A-L interface biofilms with qualitatively different phenotypes have been reported. Wild-type SBW25 produces a cellulose-based but fragile and poorly attached 'viscous mass' (VM) [36] biofilm when induced by exogenous Fe [40], and a genetically modified strain over-expressing the *wss* operon produces a similarly fragile biofilm [38]. In addition to the Wrinkly Spreaders, Fuzzy Spreaders have also been recovered from diversifying populations of SBW25 in static microcosms [29]. Though these were initially thought to be adaptive mutants which grew at the bottom of static microcosms and were adapted to anoxic conditions, they have subsequently been shown to produce fragile and short-lived A-L interface biofilms in which cells aggregate because of altered LPS expression [67]. A range of other biofilm-forming mutants have also evolved from genetically manipulated strains of SBW25, including the CBFS and PWS mutants which utilise PNAG as the primary biofilm matrix [43, 68].

WS and WS-like phenotypes are often caused by loss-of-function mutations affecting negative regulators, less frequently by promoter activation or gene-fusion mutations, and finally by rare mutations resulting in intragenic gain-of-function [47]. In general, these biofilm-forming lineages have a fitness advantage compared to non-biofilm-forming competitors [38, 43, 47, 49]. However, possible negative pleiotropy and epistasis effects [11, 14] might contribute to a lower-than-expected fitness advantage in some cases, and the accumulation of additional mutations not associated with the WS phenotype may also have a negative effect on fitness in a process known as Muller's ratchet [14].

In order to better understand the links between WS mutation, phenotype, and fitness, it has been necessary to develop quantitative assays to describe WS biofilms and an experimental approach to test the effect of physical disturbance on biofilm-formation and fitness. Variations in WS phenotype or wrinkleality [1, 35], including microcosm growth, biofilm strength and attachment levels, can be determined using a combined biofilm assay [69] that can quantitatively differentiate WS isolates recovered from different environments, whilst careful use of orbital shakers can provide intermediate levels of disturbance between static and shaking conditions.

Using this approach, we can differentiate CBFS, VM and WS biofilms on the basis of competitive fitness compared to a non-biofilm-forming strain. Under static conditions CBFS fitness is greater that either VM or WS biofilms, suggesting that the CBFS biofilm is the most cost-effective solution to colonising the A-L interface.

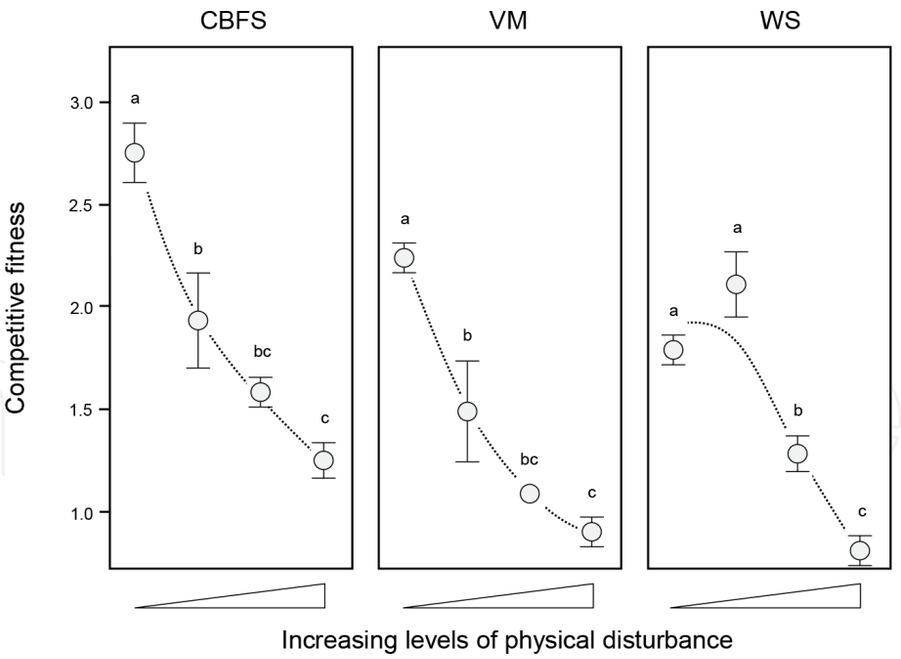

Figure 6.
The adaptive advantage of biofilms is dependent on levels of physical disturbance. Competitive fitness assays were used to assess the adaptive advantage of CBFS, VM and WS biofilms compared to a non-biofilm-forming competitor across a range of levels of physical disturbance from static to shaken conditions. Means are shown with standard errors. Dotted lines suggest trends and differences between means were investigated by Tukey-Kramer HSD; means sharing the same letters are not significantly different (α = 0.05). Data and analyses will be reported in full elsewhere (A. Koza and A. Spiers).

However, as the level of physical disturbance increases, CBFS and VM biofilms fail and sink before the more resilient WS biofilms. As a result, their fitness decreases before WS fitness (**Figure 6**). At maximum levels of disturbance where no biofilm can form, VM and WS fitness is lower than CBFS fitness. This suggests that the VM and WS phenotypes which continue to produce cellulose but cannot form biofilms are more costly than the CBFS phenotype which does not utilise this particular EPS. We noted that in these microcosms CBFS aggregates accumulated on the vial walls above the liquid line. Stranded cells may have better access to O_2 than those remaining in the liquid column and this may further increase competitive fitness.

We are also able to differentiate CBFS, VM and WS biofilms on the basis of structure and rheology, which, when combined with our fitness analyses, suggests that the CBFS biofilm is the most cost-effective structure allowing the colonisation of the A-L interface. It falls between the more costly and over-engineered WS and barely adequate VM biofilms and provides a greater fitness advantage because the levels of physical disturbance static microcosms are subject to will neither increase, which might favour the WS biofilm, nor fall, which may favour the VM biofilm.[5]

7. Community biofilm-formation in static microcosms

As the evolutionary dynamics of diversifying SBW25 populations and the fitness advantages of biofilm-formation in static microcosms are increasingly well understood, we have begun to consider the drivers of biofilm-formation in community-based multi-species biofilms [70–72]. Communities artificially established in microcosms from mixed inocula are particularly interesting as strong

[5] This 'neither too much nor too little' evaluation suits Red Queens who choose to compete for the occupation of the Goldilocks zone in our microcosms.

selection would be expected to play a role in community assembly with a rapid loss of redundant members who do not contribute to the new system. There are simple organising principles in microbial communities especially where competitive interactions are dominant [73, 74]. However, with the exception of WS-like biofilms initiated through mutation, cell-to-cell communication is thought to co-ordinate biofilm-formation and ensure that all members contribute to the cost of production without cheating [15, 27]. As a result, biofilm-formation is seen largely as a cooperative undertaking by closely related lineages, yet this appears to conflict with the view that competitive interactions generally dominate microbial communities.

In order to investigate the relative importance of cooperation and competition in community biofilms, we have developed a model system using soil-wash inocula which include biofilm-competent pseudomonads [36] and our static microcosms in which O_2 is the growth-limiting factor. This typically resulted in very fragile and poorly attached VM-like biofilms within 2–3 days with substantial growth also occurring throughout the liquid column. Preliminary trails suggested that growth levels were sensitive to different media and aeration conditions, and treatment with antibiotics, copper and perchlorate had differential effects on growth, biofilm strength and attachment levels, demonstrating that different selective pressures could alter community productivity and biofilm-formation.

We then undertook a serial transfer experiment selecting for biofilm-formation by transferring biofilm samples using a wire-loop across a series of 10 microcosms for a total of 60 days of incubation. Under such selection, we expected to see replicate communities dominated by robust WS-like biofilms and a decrease in strain diversity as non-biofilm-formers and uncompetitive strains were lost. We also expect to see a significant reduction in the number of bacteria growing below the biofilm in the liquid column, as competition for access to O_2 should drive ecological change and result in more 'effective' biofilm-formation.

However, replicate communities continued to produce weak biofilms despite their physically cohesive appearance [36], suggesting that the selective pressure for biofilm-formation was not particularly strong. Nonetheless, a significant loss of diversity was observed, and an analysis of random isolates suggested that the proportion of biofilm-formers increased, and a phenotypic shift occurred between the initial and final selected communities (**Figure 7**), confirming that these communities were subject to selective pressure. Although we expected to see the selected communities dominated by one or a few 'super' biofilm-formers, they appeared to be dominated by a mix of lineages with very similar phenotypes. This is perhaps not surprising, as our preparation of the soil-wash inocula would have selected for fast-growing aerobic and biofilm-competent bacteria such as *Pseudomonas* spp. from the original soil community (environmental filtering within the soil would also have selected for related lineages and lineages with similar phenotypes). Such mixes may be stable, as the coexistence of related lineages and the coexistence of unrelated lineages with similar phenotypes, is possible because they may not exhibit significant levels of negative interactions and might even facilitate one another [75].

We also found significant levels of growth in the liquid column below the biofilms, suggesting that lineages were colonising the A-L interface and low-O_2 region from the biofilm transfer samples and that migration was occurring between these two zones. It is possible that biofilm-competent lineages might avoid competition at the A-L interface by choosing a less competitive niche lower down the liquid column in a biochemical trade-off [76] in which lower growth rates resulting from O_2-limitation are balanced by the cost of biofilm-formation which would have been required at the A-L interface.

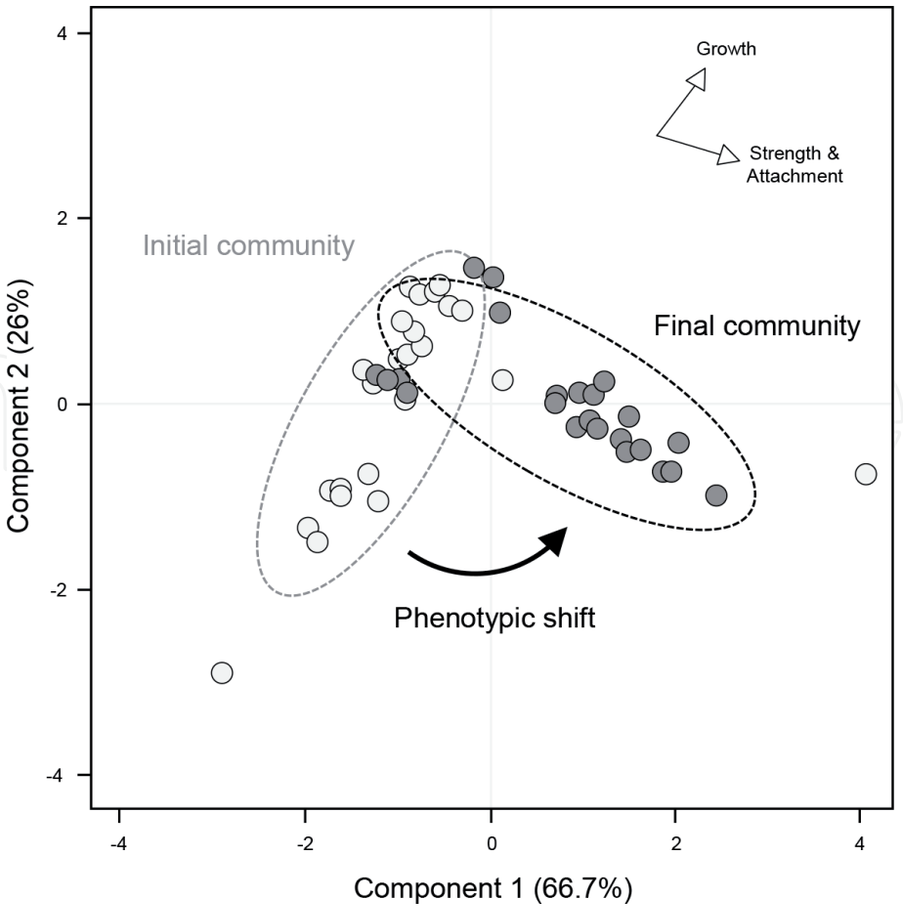

Figure 7.
Serial transfer of biofilm samples results in changes in biofilm characteristics of individual community members. Isolates were sampled from the initial (light grey circles) and final communities (dark grey circles) after serial transfer of biofilm material by wire loop across 10 microcosms over 60 days. Principal component analysis (PCA) of isolate biofilm characteristics, including total microcosm growth, biofilm strength and attachment levels, shows a phenotypic shift occurring between initial and final communities. Data and analyses will be reported in full elsewhere (R. Jerdan and A. Spiers).

Although this research is still on-going and will be published in full elsewhere, our current focus is to better understand the levels of competition occurring within the community biofilm and the role of the low-O_2 region in maintaining diversity in selected communities. A future goal is to investigate the dynamics of diversification of wild-type SBW25 in these communities in order to see how competition within the community biofilm effects WS evolution and fitness.

8. Conclusions

Biofilm research is interdisciplinary but is increasingly fragmented and polarised, with interest still dominated by molecular biologists working with medically relevant model species and a mechanistic focus on biofilm-formation. This perspective limits our understanding of more complex community-based biofilms, as ecological interactions and evolutionary processes play important roles in the development and success of these structures, with immigration and adaptive radiation introducing novel abilities or key innovations which may have a significant impact on community function. Biofilm research is now at the stage where an eco-evolutionary perspective should be included to produce a more comprehensive and holistic understanding of biofilms in a wide range of contexts, from model systems to biofilm-associated disease, biotechnology and industry.

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Conflict of interest

The authors declare that there are no conflicts of interests.

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