



Evolved Biofilm: Review on the Experimental Evolution Studies of *Bacillus subtilis* Pellicles

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Abstract

For several decades, laboratory evolution has served as a powerful method to manipulate microorganisms and to explore long-term dynamics in microbial populations. Next to canonical *Escherichia coli* planktonic cultures, experimental evolution has expanded into alternative cultivation methods and species, opening the doors to new research questions. *Bacillus subtilis*, the spore-forming and root-colonizing bacterium, can easily develop in the laboratory as a liquid–air interface colonizing pellicle biofilm. Here, we summarize recent findings derived from this tractable experimental model. Clonal pellicle biofilms of *B. subtilis* can rapidly undergo morphological and genetic diversification creating new ecological interactions, for example, exploitation by biofilm non-producers. Moreover, long-term exposure to such matrix non-producers can modulate cooperation in biofilms, leading to different phenotypic heterogeneity pattern of matrix production with larger subpopulation of “ON” cells. Alternatively, complementary variants of biofilm non-producers, each lacking a distinct matrix component, can engage in a genetic division of labor, resulting in superior biofilm productivity compared to the “generalist” wild type. Nevertheless, inter-genetic cooperation appears to be evanescent and rapidly vanquished by individual biofilm formation strategies altering the amount or the properties of the remaining matrix component. Finally, fast-evolving mobile genetic elements can unpredictably shift intra-species interactions in *B. subtilis* biofilms. Understanding evolution in clonal biofilm populations will facilitate future studies in complex multispecies biofilms that are more representative of nature.

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Introduction

Humans have been “evolving” plants and animals long before Darwin described the laws of natural selection. In parallel with the domestication of higher organisms, we unintentionally “evolved” microbes, selecting for their metabolic capabilities that improved digestibility, longevity or taste of available foods [1]. Nowadays, exploiting Darwin's legacy, and decades of population biology and genetics, controlled laboratory evolution of microbes serves as a powerful tool in biotechnology, providing microbial strains with highly desirable properties [2–4]. Nevertheless, despite its undeniable applicative potential, laboratory evolution has flourished as a tool of basic science. The first studies launched soon after the release of “On The Origin of Species” provided direct experimental validation of Darwin and Wallace's theory: William

Dallinger cultivated unicellular eukaryotes for 7 years, gradually increasing the temperature and eventually reporting lineages that tolerated 70 °C, but were no longer able to grow efficiently at their ancestral temperature [5]. For 30 years, we have witnessed the laboratory evolution of *Escherichia coli* batch cultures that have now passed 70,000 generations in 2018 [6,7]. These studies have allowed the quantification of adaptation dynamics by natural selection [8–10], the measurement of mutation rate changes [11,12], and also revealed how microbes adapt to exploit new carbon sources [13] or diversify into coexisting ecotypes [14,15].

In addition to the prominent explorations of Richard Lenski, the number of systematic evolution studies on alternative experimental models is rising [3,16–18]. Those models involve different microbial species and alternative cultivation methods that are feasible for

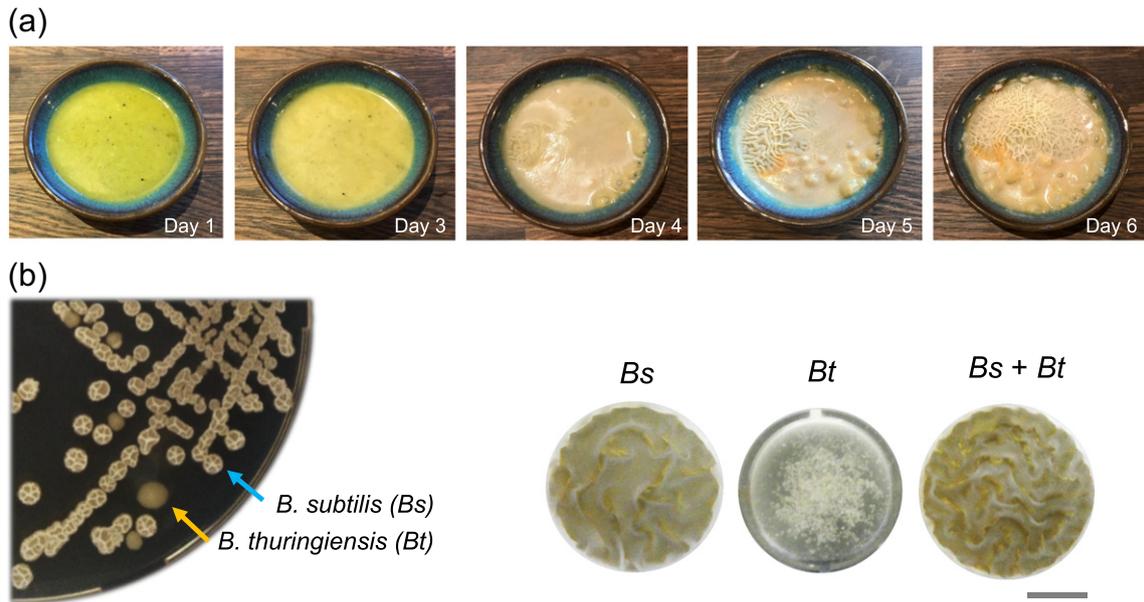


Fig. 1. Spontaneous pellicle biofilm formation by *B. subtilis* in a leek soup made outside of the laboratory. (a) Potatoes, leek, and commercial broth were cooked, smashed using a kitchen blender and left at room temperature for 6 days. After 4 days, pellicle formation could be observed. (b) The pellicle was collected, disrupted mildly, and inoculated onto LB agar to examine colony morphology. Two distinct colony types, complex and unstructured, were later identified as *B. subtilis* and *B. thuringiensis*, respectively, using 16S DNA sequencing. Biofilm formation was re-examined using filter-sterilized soup medium (right) revealing that only the *B. subtilis* isolate was capable of forming a pellicle. Scale bar indicates 0.5 cm. The “soup experiment” was reproduced several times at different households within Europe. Strain isolation and identification was performed twice, each time confirming the presence of *B. subtilis* and *B. thuringiensis*.

long-term studies. The emergence of new experimental systems has opened possibilities to address new research question on, for instance, niche expansion, adaptation to biofilm or predatory lifestyles, and evolution of inter- and intra-species interactions and multicellularity. Here, we emphasize pellicle biofilm of *Bacillus subtilis* as a fascinating and feasible model for experimental evolution. We also provide an overview of recent findings on biofilm evolution that derive from this model.

Pellicle Biofilms as a Feasible Model for Experimental Evolution

Biofilm is the most common life form of bacteria, where they thrive in packed, multicellular aggregations, embedded in a self-produced matrix. Depending on the type of occupied surface, biofilms can be categorized into submerged (solid–liquid interface), colony (solid–air interface), or pellicles (liquid–air interface) types. Submerged and pellicle biofilms differ in nutrients and oxygen availability, and the preferred niche to establish biofilm seems to depend on bacterial species. For instance, marine bacteria like *Vibrio cholerae* form surface-associated biofilms [19,20], certain species like *Pseudomonas aeruginosa* can exhibit both submerged [21] or pellicle lifestyle [22,23], while *B. subtilis* is predominantly known for

architecturally complex colonies and wrinkled pellicle formation [24–26]. Pellicle lifestyle is stimulated by an oxygen gradient that emerges when bacteria reach high cell density in static liquid medium. Active, aerotaxis-dependent motility drives *B. subtilis* cells toward the oxygen-rich liquid–air interface [25], where bacteria switch from motile to sessile lifestyle and engage in matrix production [27]. Biofilm matrix production depends on de-repression of *epsA-O* and *tapA-sipW-tasA* operons encoding for enzymes required for exopolysaccharide (EPS) and amyloid fiber protein TasA, respectively [26,28,29]. De-repression takes place when the master regulator Spo0A reaches a threshold phosphorylation level, at which state it represses the production of AbrB that acts as a repressor of *epsA-O* and *tapA-sipW-tasA* operons, and Spo0A triggers the synthesis of SinI, which in turn can inhibit the matrix genes repressor, SinR [30,31]. The expression of the *B. subtilis* matrix genes is under complex regulatory control and is activated upon various environmental signals [24,32].

Interestingly, clonal populations of *B. subtilis* exhibit phenotypic heterogeneity of matrix genes expression, as only a subpopulation of cells appears to be in the so called “ON state” and produce the extracellular matrix [33,34]. Despite such phenotypic diversity, *B. subtilis* pellicles are extremely robust, containing cell aggregates that cannot be disintegrated using standard laboratory methods (e.g., rigorous mixing using a vortex), as they

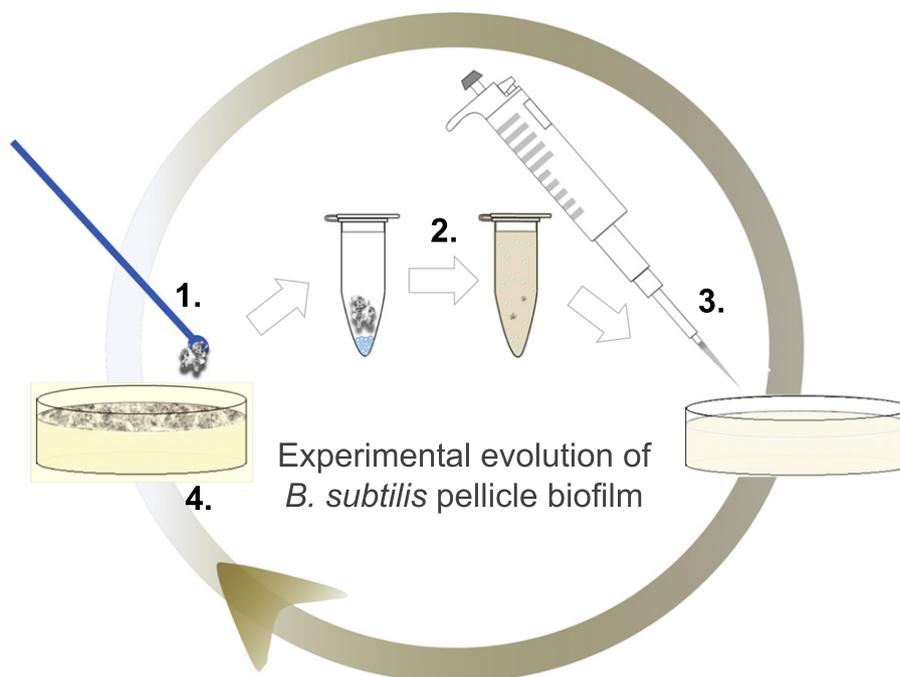


Fig. 2. Experimental evolution of the *B. subtilis* pellicle biofilm. (1) Cells are incubated in a static biofilm-promoting medium that allows pellicle formation. Due to its robustness, the pellicle can be collected easily from the air–liquid interface using a plastic inoculation loop. (2) The pellicle is transferred into a sterile Eppendorf tube filled with few milligrams of glass sand and sterile physiological solution. The glass sand will allow partial disruption of the pellicle using a vortex. After the disruption step, samples can be obtained for analysis (e.g., productivity assay or population structure) or storage. (3) A small fraction of cells is inoculated into fresh biofilm-promoting medium. (4) Cells are allowed to establish a pellicle, and the transfer cycle can be repeated.

require multiple sonication rounds to disperse. The *B. subtilis* biofilm surface complexity has even been compared to fruiting bodies of slime mold, where cells pile up into stalk-like structures with dormant spores on top [26].

Although the *B. subtilis* pellicle is one of the best understood biofilm systems at a molecular level, we still do not understand where it occurs in nature and why it evolved into such a sophisticated form. The most popular biofilm proficient laboratory strains (*B. subtilis* NCBI 3610 and PS216) were isolated from soil, probably in a spore form, and therefore, their viability during collection remains unknown. Although *B. subtilis* is commonly described as a soil bacterium [24,35], processed food appears to be an additional source of *B. subtilis* isolates reported in the NCBI genome database (A. Dragoš, unpublished observation), possibly as food contamination.

Interestingly, complex pellicle development can be repeatedly observed in soups based on potatoes and commercial bouillon (Fig. 1A), and *B. subtilis* can be isolated as the main bacterial resident of these pellicles as confirmed by 16S sequencing. Perhaps, *B. subtilis* is one of few species that can survive cooking and establish large populations, due to its ability to colonize liquid–air interfaces. Soup pellicles also comprised *Bacillus thuringiensis*,

which is incapable of pellicle formation in monoculture. Therefore, *B. subtilis* pellicles can in fact form spontaneously under non-natural conditions in food and can even facilitate the growth of the co-incorporating species (Fig. 1B).

Indeed, pellicle biofilms may represent a distinct arena for interactions between different species or bacterial strains. As compared to the planktonic state and biofilms grown on solid surfaces, pellicles likely allow intermediate mixing between cell lineages. Such intermediate mixing not only allows for intra-species exploitation [36] but can also facilitate the evolution of cooperative interactions (see Glossary for selected terms) like a division of labor [34,37,38].

Finally, pellicle biofilms serve as a feasible experimental model system for several reasons. First, pellicles are easy to cultivate and collect as they float on the liquid surface and stick to the plastic loop that allows collection of the entire biofilm material for unbiased quantification (Fig. 2). Second, the remarkable robustness of pellicles allows sampling and imaging without disrupting their native structure [34,36,39,40]. Third, as pellicles float at the liquid surface, they can be exposed to various bioactive compounds at different time points of biofilm development, as could easily be injected

into the medium with a syringe, without major effects on biofilm formation and structure.

B. subtilis is one of the most biotechnologically potent bacteria, serving as a probiotic [41], plant growth promoting rhizobacterium (PGPR) [42], and hydrophobic additive to construction materials [43]. All these beneficial properties of *B. subtilis* are directly associated with its ability to form biofilms. Extensive knowledge of the molecular genetics of biofilm formation largely facilitates linking the observed phenotypic changes during laboratory evolution with mutations in specific genes making the *B. subtilis* pellicle a perfect model to study molecular evolution.

Rapid Diversification in Pellicle Biofilms of *B. subtilis*

One of the most intriguing features of biofilms is the presence of spatial structures that facilitates the appearance of distinct interactions [16,18]. Thus, in contrast to the well-mixed environments of planktonic cultures, the spatially heterogeneous micro-niches promote diversification. Early experimental evolution studies have revealed that biofilms are a hotspot of diversification manifested by genetic differentiation [44,45]. Genetic diversification of *Burkholderia cenocepacia* and *P. aeruginosa* biofilms can easily be traced through the appearance of distinctive morphotypes with distinct biofilm formation properties [45,46]. Complex interactions develop among the morphotypes including exploitation of the secreted biofilm matrix component and metabolic cross-feeding [45,47]. Analogous to Gram-negative model systems, the genetic diversification of *B. subtilis* pellicles can be observed during experimental evolution [36]. Within a few hundred bacterial generations, four morphotypes can be isolated from the *B. subtilis* pellicles of parallel populations: wrinkly, rough, spreader, and smooth, each of which has distinct measurable differences in surface complexity and hydrophobicity. The overall hydrophobicity of colonies, the so-called non-wetting behavior, depends on both the complexity of the biofilm surface [48,49] and the matrix components produced [50–53]. These morphotypes coexist in the evolving pellicles and accompany increased productivity of the biofilm, where productivity is defined as the total cell number residing in the biofilm. Genetic dissection of the detected mutations revealed that the wrinkly colonies likely evolved from the rough morphotypes, while the spreader and smooth variants share common evolutionary history. The morphotypes exhibit disparate matrix gene expression profiles in correlation with their capacity to form surface floating biofilms in mono-cultures, highest in the wrinkly isolates, followed by the rough and spreader colonies, while the smooth morphotypes have the lowest matrix gene expression that is inferior to the ancestor. In line with the lowest matrix gene expression, the smooth

morphotype is unable to create a floating biofilm, but this ability can be rescued by the secreted matrix components. Thus, the smooth phenotype is able to exploit the cooperative products of the other morphotypes. Importantly, the smooth variant localizes on the bottom layer of the pellicle in the co-cultures consisting of the four morphotypes, supporting previous findings on the role of the extracellular matrix in positioning of cells, and there are certain limits to its exploitability [54,55].

While these experiments highlight the diversification and establishment of both cooperative and exploitative scenarios in biofilm-proficient variants, reduced spatial structures or mitigated ability to form biofilms can also select for increased matrix production. *B. subtilis* strains lacking single cell motility display increased spatial segregation followed by the rapid emergence of matrix overexpressing mutants, so called wrinkly spreader (WS) phenotypes [56]. The appearance of WS is driven by strong selection pressure acting toward colonization of the air-medium interface and is not due to an altered mutation frequency in the mutants. The WS isolates carry mutations in *SinR*, resulting in enhanced matrix gene expression and a speeding up of the establishment of floating films compared to non-motile ancestors. By contrast, motile strains do not benefit from the WS phenotype, and therefore, the frequency of WS appearance is negligible [56]. Comparable suppressor mutants with enhanced matrix gene expression can be observed in *B. subtilis* strains lacking the phosphodiesterase, *YmdB* [57], suggesting a strong selection pressure on reformation of biofilm matrix production in deficient strains. Correspondingly, the evolution of enhanced biofilm formation via mutations in biofilm regulatory pathways was also observed in other bacterial species [47,55,58–60]. Importantly, mutations in the *B. subtilis* regulatory pathway are accompanied with alterations in gene expression heterogeneity of matrix production [57].

The Impact of Intra-species Interaction on the Evolution of Phenotypic Heterogeneity

The emergence of variants with lower or diminished matrix production in the *B. subtilis* biofilm raises the question of how such non-producers affect matrix production in the cooperative members of the population. Mutants deficient in production of EPS are not able to form robust pellicles alone, but can take advantage of the EPS-producing wild type, incorporate into the biofilm, and simultaneously save metabolic costs and exploiting a public good [34,39,61,62]. Experimental evolution has been performed on pellicles established by cooperative wild-type cells harboring a reporter for EPS production and non-producers that lack EPS production. These experiments revealed that in response to *eps*

mutant incorporation into the *B. subtilis* pellicles, the wild type undergoes evolutionary adaptation by shifting the phenotypic heterogeneity of *eps* gene expression and becomes a matrix overproducer (hyper-ON), so that most of the cells express the *eps* genes [61]. At the same time, the fraction of *eps* mutants increases in the population that may result in the collapse of these populations where no pellicle is produced upon re-inoculation of the bacterial culture. By contrast, the wild type generally tempers *eps* gene expression in the absence of *esp* mutants and most cells will be in the hyper-OFF state under the conditions used.

The hyper-ON phenotype is caused by variable non-synonymous mutations in the *rsiX* gene that codes for the anti- σ^X factor. σ^X is an extracytoplasmic function sigma factor involved in responding to cationic antimicrobial agents [63]. Importantly, σ^X positively regulates the expression of the *abh* gene, whose gene product negatively affects the biofilm repressor, AbrB [64]. Therefore, modification of the σ^X -Abh-AbrB pathway likely shifts the phenotypic heterogeneity of matrix gene expression in *B. subtilis*. Adjusting biofilm matrix production by an ECF sigma factor is not restricted to *B. subtilis*, mutation in AlgT of *P. aeruginosa* similarly boosts alginate production [65].

Although the long-term impact of cheating on cooperative populations was examined previously [66–68], the study on *B. subtilis* pellicle biofilm exposed for the first time that adaptation of the matrix producing strains in the presence of *eps* mutants is feasible by the modification of gene expression heterogeneity.

Experimental Evolution of Pellicles Is Influenced by Phage-Mediated Arms Race

Generally, experimental evolution in the laboratory reveals how bacterial populations adapt to a certain condition. Concurrently, experimental evolution might also expose altered interactions within the population that is driven by genetic rearrangements of prophage regions. While *eps* mutants can incorporate into the pellicles established by wild-type *B. subtilis*, strains lacking both EPS and TasA components are unable to exploit the produced matrix efficiently and cannot integrate into biofilms, although these strains have strong fitness advantages in planktonic cultures where matrix production is a metabolic burden [39]. However, experimental evolution containing a mixture of wild-type and $\Delta eps \Delta tasA$ double mutants revealed that the incorporation of matrix deficient cells can increase if the population is repeatedly exposed to a sporulation bottleneck. Importantly, the incorporation of the evolved matrix deficient cells into the pellicle reduced biofilm productivity. Interestingly, the altered intra-species interaction between the two *B. subtilis* strains (i.e., the enhanced incorporation frequency of

matrix deficient cells) was driven by hybrid phages spontaneously released by the evolved populations. These hybrid phages could transfer the improved biofilm-incorporation properties to the ancestral double mutants, suggesting the *de novo* evolved interference competition defined the evolutionary trajectory of mixed strain populations. In addition, this experimental approach also highlights the influence of bacteriophages in the evolution of biofilms as observed for other microbes [69].

Experimental Evolution Aids Testing Stability of Cooperation in Pellicle Biofilms

Experimental evolution studies on *B. subtilis* pellicles were used to inspect the stability of mutual cooperation or division of labor in biofilms. While single cultured *eps* and *tasA* mutants cannot form robust pellicles, in a co-culture they are able to establish a wild type like biofilm [29,34,39]. The combination of *eps* and *tasA* mutant strains at an optimal ratio (i.e., 30% *eps* and 70% *tasA*) results in superior productivity compared to the wild type that produces both components [34]. As noted above, the two major operons involved in matrix production are both heterogeneously expressed, however, the differences in the regulators influencing the *epsA-O* and *tapA-sipW-tasA* operons result in distinct subpopulations producing either or both components of the matrix in addition to cells in an OFF state [34]. Therefore, the wild-type strain might already follow a phenotypic division of labor as suggested before [70]. Individual-based simulations identified that genetic division of labor during biofilm formation is prominently influenced by the reciprocal, symmetrical exchange of public goods under relatively low-diffusion conditions and certain metabolic constraints [34]. Interestingly, the benefits of the *in vitro* observed genetic division of labor can be also detected during plant root colonization of *B. subtilis* that depends on the biofilm matrix components.

In spite of the apparently beneficial effect of a genetic division of labor, experimental evolution of the co-culture revealed that the division of labor collapses in the longer time scale [40]. Within a few transfers, pellicle productivity declines and is accompanied by a shift in population composition toward the EPS-producing mutant majority. Although EPS is more easily exploited in the mixed cultures compared to the more privatized protein fiber component, disparity in the adaptive potential of cooperating strains might lead the population toward collapse. Indeed, preadaptation of *eps* or *tasA* strains for solitary biofilm formation (see below) facilitated their long-term coexistence [40]. These experiments highlight that cooperation collapse does not necessarily lead to evolutionary dead-end and population extinction, but might be the birthplace of innovation.

Evolution of New Biofilm Strategies Is Revealed by Experimental Evolution of Mutant Strains

While robust pellicle biofilm production of freshly isolated *B. subtilis* strains depends on the presence of both EPS and TasA components [71], engineered strains producing only one of these matrix components can evolve novel strategies to increase their ability to colonize the air-medium interface [40]. Both *eps* and *tasA* mutant strains evolve to colonize the surface using a distinct strategy, but both exploit the remaining matrix component.

The *tasA* mutant strain that preserves EPS production evolves to increase the expression of the *eps* operon by multi-faceted mechanisms. As the transcriptional regulation of the *B. subtilis* matrix genes involves numerous intertwined pathways, delicate modification of diverse components can frequently lead to enhanced transcription. Indeed, the evolution of the EPS-overproducer can be observed in all parallel populations that started as *tasA* mono- or as *tasA* + *eps* co-cultures. The nucleotide changes in the genomes of the evolved isolates are diverse, emphasizing the plethora of adaptation routes to enhancing matrix gene expression. The evolved traits of the *tasA* mutant can be mimicked synthetically by overexpression of the *eps* operon. Enhanced EPS production in the overexpression strain, as well as in the evolved isolates, creates a slimy pellicle phenotype with increased viscous properties [40]. Therefore, harnessing a “MAKE MORE” strategy, increased EPS synthesis augments the surface colonization properties in the absence of the matrix protein component.

Similarly, the *eps* mutant evolves to utilize the remaining matrix component, the amyloid fiber; however, the adaptation strategy is different. As overexpression of *tasA* does not improve the air-medium colonization in the absence of EPS, the evolved isolates follow an “ALTER” strategy [40]. Substitution of selected amino acids to cysteine has drastic impacts on TasA amyloidogenesis, resulting in thicker fibers with increased oligomerization, as determined by electron microscopy and native gel electrophoresis, respectively. Reintroduction of the evolved *tasA* genes to the ancestral *eps* mutant restores the improved traits. Intriguingly, none of the TasA proteins and its homologs coded in the sequenced *Bacillus* genomes contain cysteine [72], suggesting that the altered TasA protein is only advantageous in the absence of EPS. Indeed, introduction of evolved *tasA* gene into an EPS proficient strain weakens the characteristic hydrophobicity of pellicles, which might explain why TasA proteins generally lack cysteine in biofilm forming *Bacilli*. The modifications of hydrophobicity could also be caused by altered interactions with the hydrophobin protein of *B. subtilis*, BslA, dimerization of which is mediated by disulfide bond formation [73]. Deletion of the *bslA* gene

in one of the evolved isolates harboring a TasA variant with cysteine substitution abolishes the improved biofilm formation on the air-liquid interface [40]. Importantly, while the evolved traits of EPS-overexpressing isolates are exploited by ancestral like variants, the modified TasA component is more privatized and therefore the evolved population is more homogeneous producing almost exclusively the cysteine-containing TasA protein.

Discovery of hidden evolutionary pathways in mutant backgrounds goes beyond the biofilms of *B. subtilis*, and is readily observed in other species, including the parallel evolution of WS morphotypes in *Pseudomonas fluorescens* when commonly observed adaptation routes are removed [74]. Recently, evolutionary solutions to suboptimal cell shape were studied using a spherical mutant of *P. fluorescens*, again demonstrating that even seemingly lethal mutations are vincible by compensatory mutations [75].

Experimental Evolution of *B. subtilis*: out of the Biofilms

We note that besides the above-mentioned examples, *B. subtilis* has also been scrutinized to understand adaptation independently of the biofilm setup [2]. For example, trait loss can be observed in prototrophic *B. subtilis* cultivated in rich medium. After 6000 generations, less than 0.001% of the population that was propagated in sporulation medium is able to grow in minimal medium [76]. Similarly, reductive evolution (e.g., loss of sporulation, secondary metabolite plipastatine synthesis, acetate production, and motility) is observed in *B. subtilis* when cultivated under relaxed selection for sporulation [77,78]. By contrast, when undomesticated *B. subtilis* is cultured for 2 months in batch cultures, colony morphology diversification is witnessed both in static and shaken conditions [79]. Colony morphology robustness correlates with matrix gene expression and WS variants contains non-synonymous mutations in SinR similar to those observed during biofilm selections [56,57]. Finally, numerous adaptation studies have been performed previously using *B. subtilis* to reveal the impact of ultraviolet radiation [80], low pressure [81], and high temperature [2]. All these studies add to our understanding of how *B. subtilis* evolves in the laboratory under diverse conditions.

Future Perspectives

Although microbes predominantly live in multispecies communities, to date, the evolution of biofilms has mostly been explored in single species setups. While we need to understand better the evolution of complex systems, we should also appreciate that pathogenic or host-associated biofilms often consist

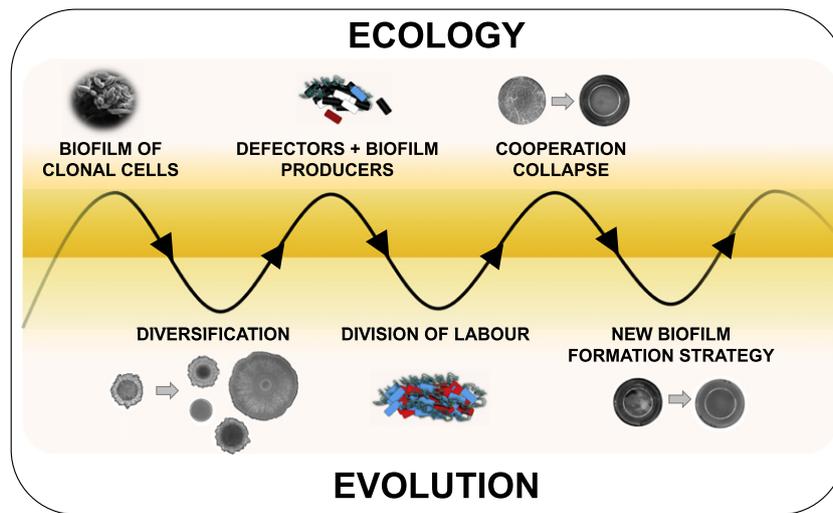


Fig. 3. Summary of evolution studies performed using *B. subtilis* pellicle biofilm. Evolutionary events (presented at the bottom) can lead to new ecological interactions (presented on top) and vice versa, creating an eco-evolutionary feedback loop.

of solitary species [82–84]. Such natural single-species biofilms can exhibit tremendous metabolic and morphological diversity with complex interactions [85]. The experimental evolution of *B. subtilis* pellicles allows the development of similar colony complexity to be followed. The experimental model has enabled evolution to be studied from multiple starting points (clonal cells, matrix producer *versus* matrix non-producer, two types of matrix producers), each of which could potentially emerge from a clonal biofilm population (Fig. 3). The outcomes from several compatible studies have been assembled into an eco-evolutionary feedback model, where the emergence of new ecological interactions shapes the evolutionary fate of the population (Fig. 3).

Understanding the complexity that may arise in single species biofilms will facilitate future studies in multispecies setups. As *B. subtilis* can colonize plant roots [34,86,87] and belongs to PGPR, the experimental co-evolution of bacteria with plant hosts provides a large potential to optimize biofertilizers. It was already shown that a pre-engineered bacterium can rapidly evolve into a plant endosymbiont [88], but the directed evolution of root colonizers in planta has never been performed in the laboratory. Comparing the evolutionary fate of a *B. subtilis* root-associated biofilm with previously observed adaptation in pellicles [36] could reveal the role of plant hosts in shaping evolution in biofilms. Alternatively, co-evolution could be used to optimize PGPR microbial consortia [89], considering the possible pitfalls of a genetically diverse, cooperative community (e.g., collapse of a division of labor) observed in single species system [40].

It was reported recently that *B. subtilis* can colonize fungal hyphae [90], providing an interesting platform to study bacteria-fungi co-adaptation. Previous work

revealed that weak-Crabtree yeast *Lachancea kluyveri* adapts to the presence of bacteria by elevated ethanol production, likely as a self-defense strategy [91]. How does *B. subtilis* adapt to fungi? Next to our basic understanding of bacteria–fungi coevolution, directed evolution could be applied to select for pathogenic fungi antagonists, or alternatively, strains supporting beneficial fungi could in the future be used as biofertilizers.

Recently, the coevolution of *B. thuringiensis* and *Caenorhabditis elegans* has been studied, revealing possible trade-offs between pathogenic lifestyle and biofilm formation [92]. Although the molecular mechanism of biofilm formation in *B. thuringiensis* varies from that of *B. subtilis* [93], a similar mechanism (involving residual secreted matrix components) could lead to biofilm revival when environmental selection changes.

Finally, prophage dynamics during evolution are not well understood and recent findings suggest that phage-mediated interference competition can modulate population structure in biofilms [39,94]. Moreover, there is an increasing number of reports on lytic phage activity as a proxy for biofilm formation [95,96]. By contrast, the *E. coli* biofilm matrix protects bacteria from phage attack [97]. In the light of the promises of phage therapy as an alternative antimicrobial strategy, understanding how phage affects biofilm evolution is of major importance.

“We would like to watch an entire microbial community of thousands of species evolve over time,” Rich Lenski recently said [7]. Scaling up species diversity will bring challenges, calling for the development of new tools, experimental models and protocols. Overcoming those challenges can “upgrade” experimental evolution into a powerful research tool in ecology.

Glossary of Evolutionary Terms Used Specifically in this Review for Microbes

See also Ref. [16] for evolutionary terms used for the microbiology field

- *cooperative interaction/scenario*: interaction between two microbes where both benefit
- *cooperative members of the population*: cells that provide public goods which benefit other members of the population
- *division of labor*: type of cooperation where cooperating individuals specialize in different tasks
- *morphotype*: a strain that exhibits a distinct colony morphology
- *non-producer*: a mutant strain lacking synthesis of certain (secreted) compound
- *privatized (good)*: a compound that only benefits the producer organism
- *public good*: a secreted compound that is freely accessible for other members of the population, i.e., “public”

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EPS, exopolysaccharide; PGPR, plant growth promoting rhizobacterium; WS, wrinkly spreader.

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