Diversification of *B. subtilis* during experimental evolution on *A. thaliana* leads to synergism in root colonization of evolved subpopulations

Christopher Blake[1], Mathilde Nordgaard Christensen[1], Gergely Maróti[2], Ákos T. Kovács[1,*]

[1] Bacterial Interactions and Evolution Group, DTU Bioengineering, Technical University of Denmark, 2800 Kongens Lyngby, Denmark

[2] Institute of Plant Biology, Biological Research Centre of the Hungarian Academy of Sciences, 6726 Szeged, Hungary

*For correspondence. E-mail: atkovacs@dtu.dk; Søltofts Plads Building 221, 2800 Kgs Lyngby, Denmark, Tel. +45 4525 2527; Fax +45 4588 4922

Keywords: *Bacillus subtilis*; experimental evolution; root colonization; biofilm; motility; morphotype

Summary

The soil bacterium *Bacillus subtilis* is known to suppress pathogens as well as promote plant growth. However, in order to fully exploit the potential as natural fertilizer, we need a better understanding of the interactions between *B. subtilis* and plants. Here, *B. subtilis* was examined for root colonization through experimental evolution on *Arabidopsis thaliana*. The populations evolved rapidly, improved in root colonization and diversified into three distinct morphotypes. In order to better understand the adaptation that had taken place, single evolved isolates from the final transfer were randomly selected for further characterization, revealing changes in growth and pellicle formation in medium supplemented with plant polysaccharides. Intriguingly, certain evolved isolates showed improved root colonization only on the plant species they evolved on, but not on another plant species, namely tomato, suggesting *A. thaliana* specific adaption paths. Finally, synergism in plant root colonization was observed for a mix of all three morphotypes, as the mix performed better than the sum of its constituents in monoculture. Our results suggest, that genetic diversification occurs in an ecological relevant setting on plant roots and proves to be a stable strategy for root colonization.

Significance Statement

Understanding how plant-growth-promoting rhizobacteria colonize plant roots is crucial to fully utilize their potential for agricultural applications. Here, we utilized experimental evolution of the PGPR *Bacillus subtilis* on *Arabidopsis thaliana* to study root colonization. We revealed that evolving populations rapidly improve in root colonization and diversify into distinct morphotypes. Notably, improved root colonization by evolved isolates was observed on *A. thaliana*, not on tomato. Moreover, isolates of distinct morphotypes interacted during root colonization and the mixture of morphotypes showed higher productivity then predicted. These findings suggest that genetic diversification might be a stable strategy to maximize root colonization.

Introduction

The interplay between root-associated microbial communities and the plant itself is essential for the productivity and health of the plant (Vandenkoornhuyse *et al.,* 2015). In general, these microbial communities are highly diverse and comprise various complex interactions, both beneficial as well as deleterious (Trivedi *et al.,* 2020). The plant host species tends to be one of the main drivers of variation in the composition of the plant root microbiome, indicating an active selection of microbes by the plant (Micallef *et al.,* 2009; Fitzpatrick *et al.,* 2018). Especially beneficial microbes, e.g. plant growth promoting rhizobacteria (PGPR), are suggested to be actively recruited from the surrounding bulk soil (Yi *et al.,* 2011; Pieterse *et al.,* 2014).

One widely studied PGPR is the Gram-positive, non-pathogenic, spore-forming bacterium *Bacillus subtilis* (Cazorla *et al.,* 2007; Todorova & Kozhuharova, 2010; Luo *et al.,* 2015). *B. subtilis* is commonly found in soil and has been isolated from various plant species (Cazorla *et al.,* 2007). It is widely recognized to aid plants through multiple direct as well as indirect manners (Blake *et al.,* 2021). *B. subtilis* is known to promote plant growth either through production of phytohormones or by improving nutrient availability (Arkhipova *et al.,* 2005; Saravanakumar *et al.,* 2011; Sivasakthi *et al.,* 2014). In addition, *B. subtilis* protects the plant against pathogens, either directly by competing for space and nutrients and by producing antimicrobials, or indirectly by triggering induced systemic resistance (Lugtenberg & Kamilova, 2009; Pieterse *et al.,* 2014). One of the main mechanisms of plants to attract and select for *B. subtilis,* is the secretion of root exudates, which act as chemoattractant and nutrient source for microbes (Zhang *et al.,* 2014). Subsequently, *B. subtilis* needs to evade the innate immune response of the plant and tightly attach to the root, often in the form of biofilms, providing further opportunities for co-evolution with their host plant (Chen *et al.,* 2013; Deng *et al.,* 2019). Even though some *B. subtilis* strains are already applied as biocontrol agents within agriculture, its success under field conditions varies immensely (Peng *et al.,* 2011; Wei *et al.,* 2016). The main cause of this variation, has been suggested to be the inability of *B. subtilis* to colonize and persist in the rhizosphere for longer periods of time (Shoda, 2000).

Up until now, most studies analyzing the importance of bacterial properties for successful root colonization by *B. subtilis* used either constructed mutant strains or distinct natural isolates (Beauregard *et al.,* 2013; Chen *et al.,* 2013; Allard-Massicotte *et al.,* 2016; Dragoš *et al.,* 2018a). This revealed, amongst other things, that the capability of *B. subtilis* to colonize plant roots is tightly linked to its chemotactic response towards root exudates, as well as its ability to form biofilms (Beauregard *et al.,* 2013; Chen *et al.,* 2013; Allard-Massicotte *et al.,* 2016). Indeed, *B. subtilis* strains exhibiting strong pellicle formation - a type of biofilm formed at the liquid-air interface – have been observed to show enhanced root colonization, while weak pellicle forming strains showed reduced root colonization (Chen *et al.,* 2013). Biofilms consist of cells, which are packed tightly together, embedded in a self-produced extracellular matrix (ECM), which for *B. subtilis* consists mainly of an exopolysaccharide (EPS) and the protein TasA (Branda *et al.,* 2006; Arnaouteli *et al.,* 2021). Dragoš *et al.* (2018a) have shown that both EPS and TasA are required for successful root colonization, as both mutants, Δ*eps* and Δ*tasA*, showed a reduced number of cells on plants, compared to the WT. On the contrary, inoculation of plant roots with a mixture of Δ*eps* and Δ*tasA* led to a significantly higher root colonization compared to the WT, as matrix components are shared and division of labor occurs between complementing Δ*eps* and Δ*tasA* mutants (Dragoš *et al.,* 2018a). Matrix components such as TasA and EPS are regarded as public goods, i.e. compounds that are costly to produce but provide a collective benefit for the overall population (Branda *et al.,* 2006; Zhang *et al.,* 2015; Dragoš *et al.,* 2018a). Public goods like matrix components, however, do not only provide the basis for division of labor, where resources are used and shared efficiently, but might also allow exploitation, where cheaters are able to utilize the public good without contributing to its production (van Gestel *et al.,* 2014; Dragoš *et al.,* 2018a, Dragoš *et al.,* 2018b).

Recently, a new study emerged using experimental evolution to analyze bacterial traits important for plant microbial interactions (Batstone *et al.,* 2020). In general, experimental evolution might be less constrained by preconceptions and is a powerful tool to analyze how microorganisms adapt to a wide range of environments as well as to study evolutionary trade-offs (Kawecki *et al.,* 2012). By allowing

cells to grow until the population reaches a high density, and subsequently transferring only a part of the population to new, fresh medium, continued growth is granted and therefore natural selection will drive the population over generations to adapt to the given conditions (McDonald, 2019). Batstone *et al.* (2020) used experimental evolution to evolve *Ensifer meliloti* on distinct *Medicago truncatula* genotypes in a greenhouse system in soil for five plant generations. *E. meliloti* rapidly adapted to its local host genotype, meaning that derived bacteria were both more beneficial and achieved higher fitness on the plant host genotype they shared an evolutionary history with (Batstone *et al.,* 2020).

In this study, we established a laboratory model system to inspect the evolution of *B. subtilis* on plant roots. 14-16d old *A. thaliana* seedlings were inoculated with *B. subtilis* in a minimal medium, after 2 days, the colonized root was subsequently transferred to fresh medium containing a new sterile seedling (14-16d old). This allowed us to follow evolving populations for 20 transfers and select for a regular root recolonization, including chemotaxis, biofilm formation and dispersal. We observed that evolving populations rapidly improved in root colonization and quickly diversified into distinct morphotypes. When these morphotypes were mixed together, they interacted synergistically on plant roots. Intriguingly, even though variants with reduced biofilm formation ability displayed lessened root colonization in mono-culture, these could evolve and persist in most populations benefitting from the matrix overproduced by another morphotype. Finally, the evolved isolates exclusively increased colonization of *A. thaliana*. The lack of such increase on tomato (*Solanum lycopersicum*) roots suggested an *A. thaliana*-specific adaptation.

Results

B. subtilis shows rapid improvement and diversification during evolution on plant roots

To examine how the PGPR *B. subtilis* evolves on plant roots and adapts in root colonization, five parallel populations (from A to E), started from a single clonal lineage of *B. subtilis* strain DK1042 (natural competent derivative of the undomesticated NCBI 3610 (Konkol *et al.,* 2013)), were passaged

on *A. thaliana* seedlings for 20 transfers, spanning over a total of 40 days. To follow possible improvements in root colonization during the experimental evolution, colony forming unit (CFU) per gram of roots was assessed after transfer 1, 5, 10, 17 and 20. As expected, root colonization improved quickly in all evolving populations. The number of cells colonizing the roots increased on average about 10-fold, considering all five populations, from the first to the 5th transfer and stayed at similar level afterwards (Fig. 1A). Tukey´s test confirmed that T5, T10 and T20 were significantly different from T1. Furthermore, in all five populations, three very distinct morphological variants were observed in the CFU assays. These include a Snow (SN) variant with hyper-wrinkled, whitish colony morphology, a Smooth (SM) variant with round, smooth and shiny colony morphology and a Wrinkled (WR) variant with a colony morphology comparable to the ancestor (Fig. 1B). Genetic stability of the three distinct morphotypes was confirmed by repeated passaging on solid medium and in liquid culture for a chosen representative example of each morphotype isolated from three populations (population A, D, and E). SN was observed in all five populations at T5 reaching in average a final frequency of around 20% (Fig. 1CD). SM was observed in all five populations at T10, except for population E, where it was observed after T15. The final frequencies of the morphotypes were highly similar across all five populations, except population B, where SM was below detection level at T20 (Fig 1D).

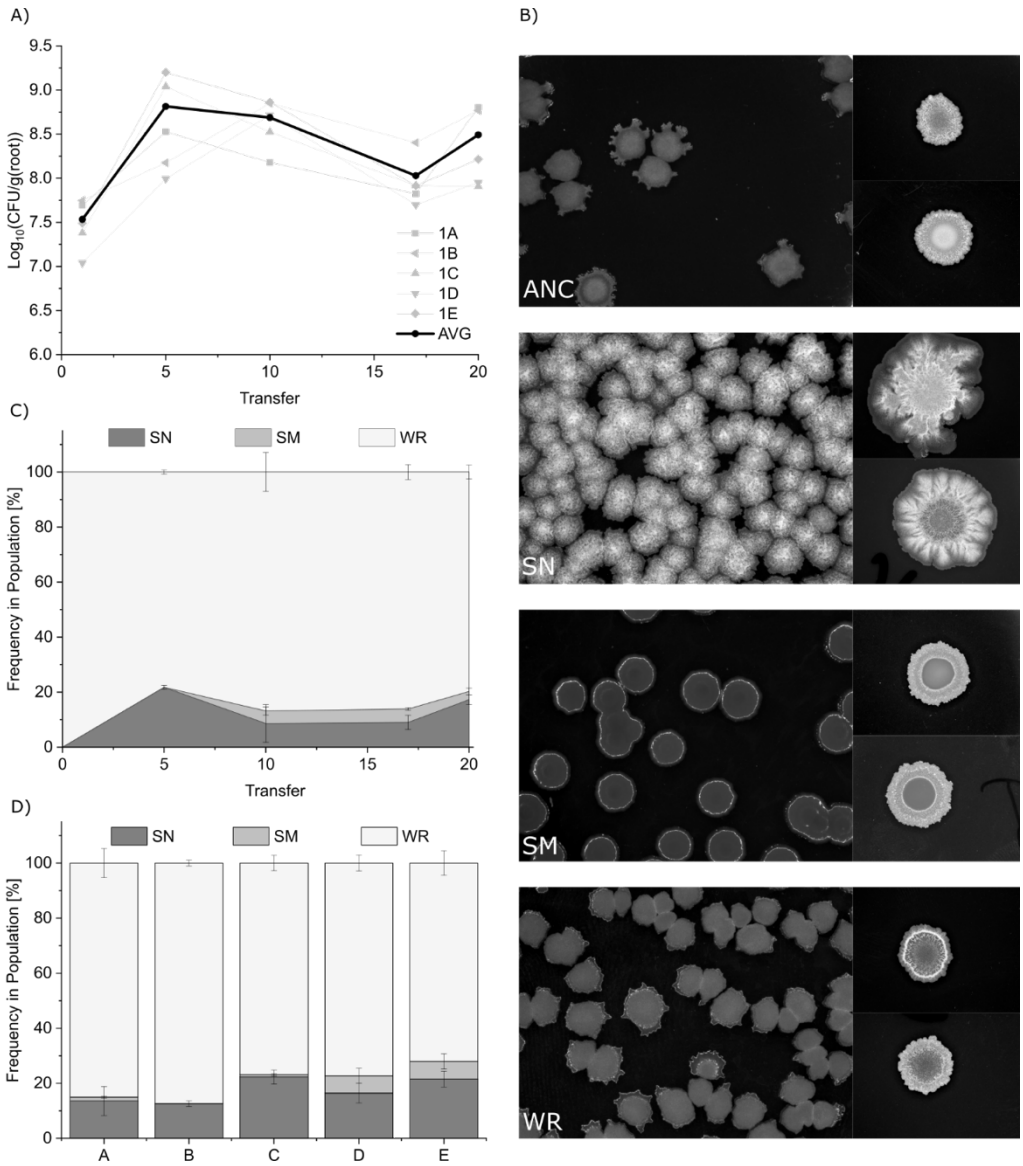

Fig. 1. Adaptation in root colonization and diversification of *B. subtilis* on *A. thaliana*. A. Changes in root colonization during experimental evolution was monitored as CFU per gram root for T1, T5, T10, T17 and T20 in populations A to E. B. Distinct colony morphologies were identified and described based on colony architecture when plated (left) or spotted (right) on 1.5% LB-Agar after 48h incubation at 30°C. C. Relative frequencies of different morphotypes in subsequent evolutionary time points in average of all five populations (n=5). D. Final (T20) relative frequencies of different morphotypes in all five parallel evolved populations (n=3). For panel C and D, data represent mean and error bars represent standard error.

Pellicle formation and growth on plant polysaccharides correlate with distinct morphotypes

To further explore the phenotypic traits of the three distinct morphotypes, morphologically distinct isolates from the final transfer were randomly selected, and tested for growth and biofilm pellicle formation in response to plant polysaccharides. As *B. subtilis* was evolved in a minimal salt medium

(see Experimental procedures), bacterial cells were dependent on carbon sources provided by the plant for their growth. We hypothesized, therefore, that evolved isolates could adapt to the environment by improving the utilization of plant polysaccharides. Indeed, most evolved isolates showed altered planktonic growth in MSNc + 0.5% xylan. Growth patterns correlated well with the specific morphotypes, i.e. prolonged growth of SN, and higher growth rate as well as higher maximum optical density (OD) of SM compared to the ancestor (Fig. 2A).

Pellicle formation has been proven to be not only a great indicator for biofilm productivity in general, but also to correlate with root colonization by *B. subtilis* strains (Chen *et al.,* 2013). Here, we tested pellicle formation in LB medium in the presence of the plant polysaccharide xylan in 24-well plates, since Beauregard *et al.* (2013) observed that xylan induces biofilm formation in *B. subtilis*. After incubating for 48h at 30°C, pellicle complexity and architecture of evolved isolates were analyzed in comparison to the ancestral strain. As expected, pellicle formation by the evolved isolates mostly correlated with morphological appearance of colonies on solidified LB medium (Fig. 2B). Similar to their colony morphology, SN variants produced robust whitish pellicles, indicating overproduction of matrix components (Fig. 2B). On the contrary, SM variants were unable to form a stable pellicle, only a thin film was observed on the liquid-air interface (Fig. 2B). Notably, no major changes between pellicles formed by WR compared to the ancestral strain were observed (Fig. 2B).

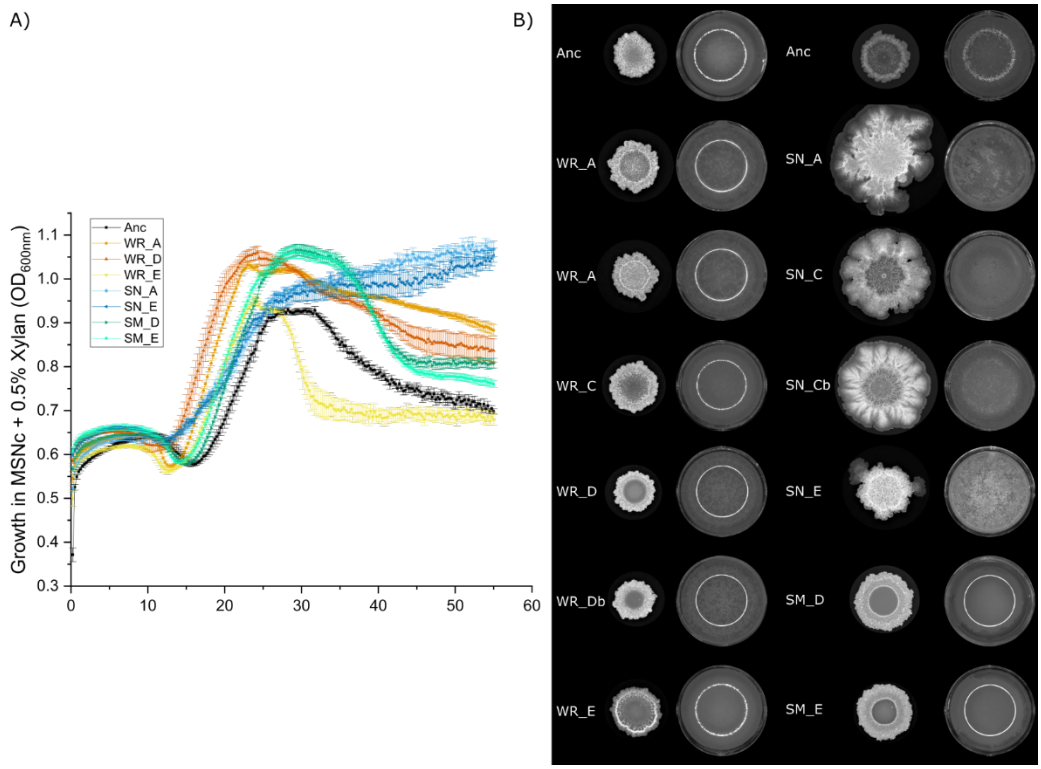

Fig. 2. Growth and pellicle formation of evolved isolates from final transfer (T20) in response to plant polysaccharides compared to the ancestor. A. Growth of evolved isolates and ancestor in MSNc + 0.5% xylan was followed at 24°C for 56h (n=6, 2 independent overnight cultures with each 3 replicates). Data represent mean and error bars represent standard error. B. Colony and pellicle morphology developed by evolved isolates and ancestor. Colony morphology was tested by spotting 2µl of ON culture on 1.5% LB-Agar and incubating for 48h at 30°C. Pellicle morphology was studied by inoculating overnight cultures into 1 mL LB medium supplemented with 0.5% xylan at a starting OD_{600} of 0.075. Images of colonies and pellicle were obtained after 48h at 30°C.

Improvements in plant root colonization tend to be A. thaliana specific

Biofilm formation on the root by *B. subtilis* is initiated by the recognition of plant signals, and the composition of those signaling components is suggested to be plant species-specific (Warembourg *et al.,* 2003; Beauregard *et al.,* 2013). Therefore, we wondered whether improvements in root colonization might be specific and limited to the plant species they evolved on. To test this, we inoculated both 14-16d old *A. thaliana* seedlings as well as 6-8d old tomato seedlings with either our evolved isolates from the final transfer or with the ancestral strain and quantified plant colonization after 48h.

Intriguingly, we observed that 7 out of 12 isolates showed improved root colonization compared to the ancestral strain on *A. thaliana*, while none improved on tomato (Fig. 3). This indicates that, *B. subtilis* adapted specifically to root colonization of *A. thaliana*. Interestingly, improvements in root colonization tended to be only partially in accordance with the morphotypes of the tested isolates. All four tested SN isolates, tended to improve in root colonization on *A. thaliana*. This is consistent with previous studies, which showed that enhanced biofilm formation leads to increased root colonization (Chen *et al.,* 2013). On the contrary, WR and SM isolates showed varying results. Remarkably, only one of the SM isolates showed reduced root colonization on both plant species in accordance with highly reduced pellicle formation, while the other SM isolate performed similar to the ancestor. Notably, the two SM isolates evolved in distinct populations and might therefore harbor distinct mutations, explaining this variation.

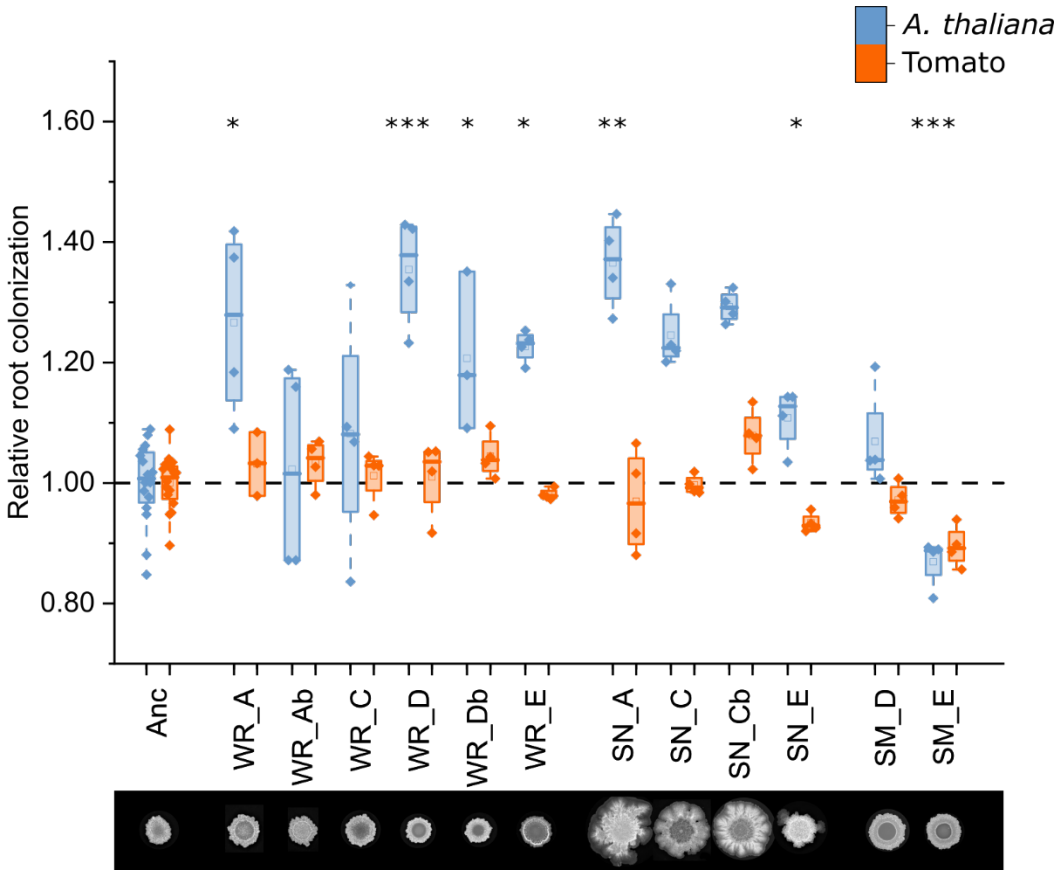

Fig. 3: Root colonization by evolved isolates from the final transfer (T20) compared to the ancestor. Both 14-16 days old *A. thaliana* (blue) and 10-12 days old tomato seedlings (orange) were inoculated with evolved isolates originating from different populations (populations A, C, D, and E) as well as the ancestor and incubated for 48h in a plant chamber. The biofilm on the roots was disrupted through

vortexing with small glass beads and the suspension was plated on LB-agar medium to allow CFU counting. For each isolate, relative root colonization was calculated by dividing the log10-transformed CFU value of each replicate with the mean of the log10-transformed CFU of the ancestor from the same experimental setup. Here, the pooled CFU of the ancestor from all experimental set-ups is shown. The dashed line represents the mean of the ancestor. ANOVA followed by Post-hoc Dunnett´s test were performed to test for significance between each isolate and the ancestor (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

Diversification involves mutations related to biofilm development, motility and metabolic pathways

To understand the genetic basis of the observed diversification genomes of selected SN, SM and WR isolates were re-sequenced. The genome of the ancestor was previously re-sequenced (Dragoš *et al.,* 2018b), that revealed differences in the genome sequence of our laboratory DK1042 stock compared to the database. In total, 43 non-overlapping mutations (SNPs) were detected in the seven re-sequenced genomes, of which 38 were single-nucleotide variants, 3 were deletions, and 2 were insertions (Table 1). Both SN isolates harbored a single mutation, located in the gene coding for the biofilm master regulator, SinR. The two SM isolates shared a mutation in the *flhB* gene, important for flagellar assembly, as well as numerous mutations in the *gtaB* and *yvzE* genes, coding for UTP-glucose-1-phosphate uridylyltransferases. Each of them harbored additional SNPs, a non-synonymous mutation in the glycerol facilitator coding gene, *glpF*. Further, a synonymous mutation in *ydaD* gene was detected in SM_D, while SM_E additionally carried non-synonymous mutations in the sporulation specific genes, *spoVR* and *dacB*. Finally, the distinct WR isolates from different evolved populations shared no overlapping mutations, but commonly harbored SNPs in genes related to motility (*fliI*, *fliR*, and *motB* in WR isolates from population A, D, and E, respectively). Two of the WR isolates contained mutations in *pdeH* encoding a c-di-GMP degrading phosphodiesterase next to few additional mutations in these isolates.

Snow is exploited by other co-colonizing morphotypes in mixed root colonization

Finally, we wondered how the SM isolates, which showed reduced pellicle biofilm development in general and significantly reduced root colonization for one isolate, maintained low frequencies

throughout the evolution experiment and was not outcompeted over time during the evolution. We hypothesized that its root colonization in the evolving populations might be facilitated by other co-occurring morphotypes and SM might exploit the matrix produced by the other morphotypes. Therefore, we tested the impact of diversity on individual and group performance in root colonization by inoculating *A. thaliana* seedlings with SN, SM and WR in monoculture as well as with a mix of all three morphotypes with equal starting frequencies (1:1:1) for three different populations each (populations A, D, and E).

As expected, in monoculture, all three tested SN isolates showed improved root colonization compared to the ancestor, while SM was reduced and WR showed no major changes compared to the ancestor (Fig. 4). Even though the mix of all three morphotypes performed better in all three populations than the ancestor, its CFU was the overall highest only in population D, while in population E, it reached similar cell abundance as SN in monoculture, and the total root-colonizing cell number was lower in population A than SN in monoculture. In population D, all three morphotypes seemed to benefit from each other, as the CFU of each of the SN, SM and WR were higher in the co-culture than predicted, suggesting a mutualistic interaction. On the contrary, in population A and E only SM and WR benefited from the co-culture, while SN showed highly reduced CFU in the mix compared to its prediction. Therefore, SN might be exploited by its co-occurring morphotypes WR and SM in these populations.

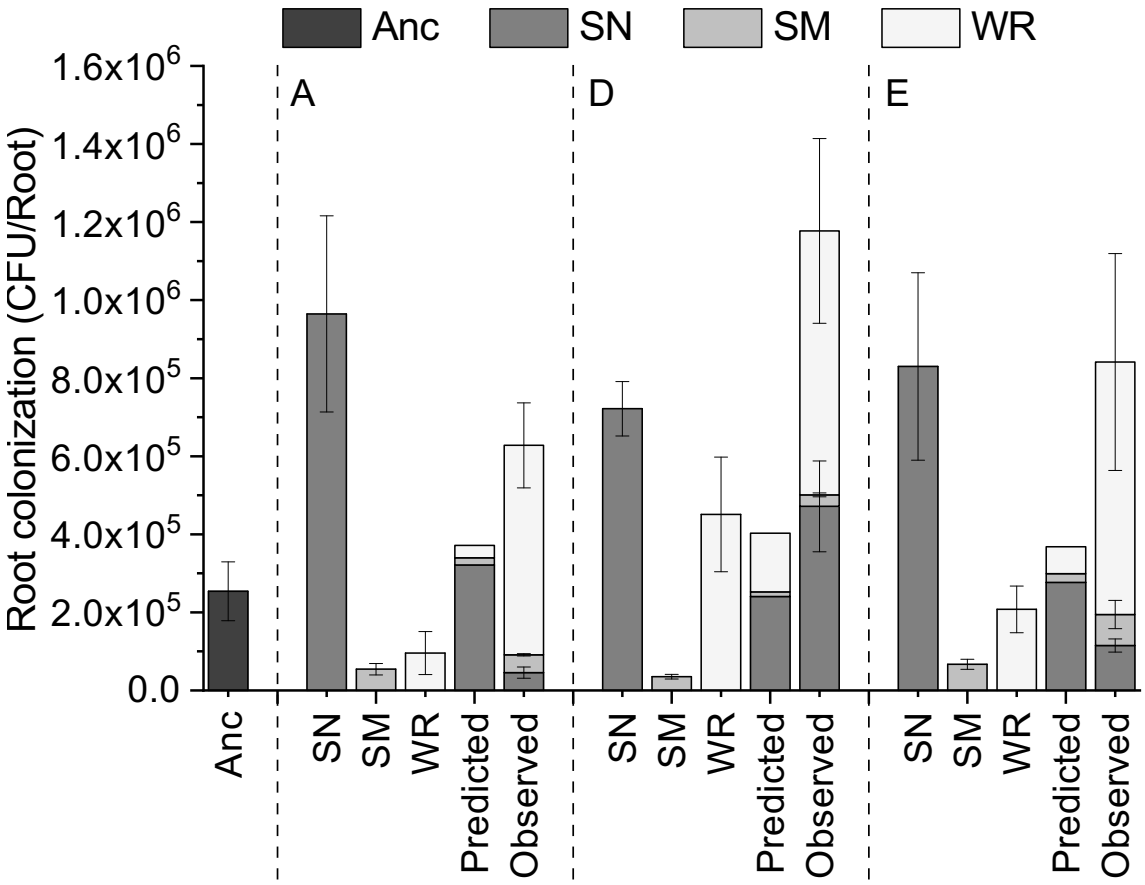

Fig. 4. Root colonization of morphotypes in monoculture as well as in a 1:1:1 mix or the ancestor. 14-16d old *A. thaliana* seedlings were inoculated with either the mix or the strains in monoculture and incubated for 48h in a plant chamber. The biofilm on the roots was disrupted by vortexing with small glass beads and the suspension was plated on solidified LB medium to allow CFU counting. Predicted colonization was calculated as the product of the proportion of each morphotype in the inoculum (1/3) and its performance (CFU per root) in monoculture. Observed colonization is the total colonization of the mixed community in the experimental setup. Bars represent the mean and error bars represent standard error for four replicates (9 replicates for ancestor pooled from different days).

Discussion

The PGPR *B. subtilis* is a promising sustainable alternative to traditionally used agrochemicals, as it harbors many important plant growth-promoting and biocontrol properties while having reduced negative effects on biodiversity and the environment (Blake *et al.,* 2021; Arnaouteli *et al.,* 2021). Even though its potential seems immense under laboratory conditions and in greenhouse trials, the success varies greatly when applied in fields (Peng *et al.,* 2011; Bardin *et al.,* 2015; Moreira & de Mio, 2015; Wei *et al.,* 2016). To improve the application of *B. subtilis* within agriculture, we need a deeper understanding of the underlying mechanisms of how it interacts with and colonizes plants. Previous studies using either mutant strains or distinct natural isolates of *B. subtilis* identified both chemotaxis as well as biofilm formation as necessary bacterial properties for successful root colonization (Chen *et al.,* 2013; Allard-Massicotte *et al.,* 2016; Dragoš *et al.*, 2018a). In this work, we used experimental evolution to analyze how *B. subtilis* evolves on *A. thaliana* roots, by continuously transferring a previously colonized root to a blue cap bottle containing fresh medium and a sterile root, thereby allowing only root-colonizing cells to be carried on throughout the experiment. In contrast to the experimental evolution study by Batstone *et al.* (2020) that similarly examined plant-microbe interaction, we focused solely on initial and re-colonization of plant roots by *B. subtilis* in liquid minimal media with small plant seedlings. As expected, *B. subtilis* populations rapidly increased in root colonization during the experimental evolution, indicating fast adaptation to plant roots. In order to test whether genetic adaptation had taken place, single evolved isolates from the final transfer (T20) were randomly selected for further characterization. Intriguingly, when testing evolved isolates from the final transfer for improved root colonization of *A. thaliana* and tomato seedlings, we only observed improvements on *A. thaliana* indicating *A. thaliana*-specific adaptation paths. Furthermore, we observed that evolving populations quickly diversified into three distinct morphotypes, including a hyper-wrinkled SN variant, a round and smooth SM variant, and a variant highly similar in colony morphology to the ancestor, termed WR. Finally, distinct morphotypes interacted synergistically during root colonization (Fig. 4). Depending on the evolved population they originated from, SN, SM

and WR interacted either in a mutualistic way, as all three morphotypes benefited, or SM and WR exploited SN, as they most likely capitalized on overproduced matrix components.

Previous work proved that rapid diversification is commonly observed in microbial evolution and also occurs in *B. subtilis* biofilms (Dragoš *et al.,* 2018b). Here, we demonstrated that diversification of *B. subtilis* also plays an important role in evolution during ecologically- and biotechnologically-relevant plant root colonization. Diversification of *B. subtilis* during evolution on *A. thaliana* roots seems highly reproducible, as the same evolutionary pattern and highly similar morphotype frequencies could be observed in the final transfer of all five parallel lines. In addition to the hyper-wrinkled SN variant, from which all tested isolates showed hyper-robust biofilm formation in the form of complex colonies and thick pellicles, a SM morphotype also emerged that seemingly had lost its ability to form a stable pellicle when inoculated solitarily. In accordance with previous work by Chen *et al.* (2013), who tested correlation of pellicle formation with root colonization of distinct natural isolates of *B. subtilis*, the evolved isolates displaying a more robust pellicle formation, namely SN, also improved in root colonization. On the contrary, one of the tested two SM isolates showed reduced root colonization, while the other SM isolate performed similar to the ancestor on plant roots. Notably, the isolates with similar morphological properties, obtained either from distinct or same population(s), displayed variable plant root colonization properties, suggesting that dissimilar traits of the analogous morphotypes are either due to additional mutations or alternatively, distinct mutations led to similar morphological, but slightly different plant colonization consequences (Poltak & Cooper, 2011).

To get a better understanding of the genetic basis behind the adaptive mechanisms, genomes of seven isolates were re-sequenced. In both of the tested SN isolates, a mutation in the gene encoding the biofilm regulator SinR was found. An identical mutation in the *sinR* gene was previously observed resulting in hyper-wrinkled colony morphology and increased expression of biofilm-related genes (Richter *et al.,* 2018; Kampf *et al.,* 2018). Further, *ΔsinR* mutant *B. subtilis* with hyper-robust biofilm displayed increased number of cells attached to plant roots (Chen *et al.,* 2013). These previous observations explain the enhanced biofilm formation and root colonization of the SN isolates. The

sequenced SM isolates harbored multiple mutations in *gtaB* and *yvzE* genes, coding for UTP-glucose-1-phosphate uridylyltransferases. Intriguingly, acetylation of GtaB was demonstrated to be important for proper biofilm formation in *B. subtilis* and a *gtaB* deletion mutant showed reduced pellicle development (Reverdy *et al.,* 2018), suggesting the relevance of *gtaB* mutations in the observed SM morphotypes. Two of the three WR variants carried distinct mutations in the *pdeH* gene that encodes a c-di-GMP degrading phosphodiesterase, and additional, again distinct, mutations in the intergenic region upstream of *ydaJ* that encodes a lipoprotein that may modify the extracellular polysaccharide synthesized by YdaLMN proteins (Bedrunka & Graumann 2017). Notably, perturbation of these genes slightly alters colony morphologies (Bedrunka & Graumann 2017). Finally, all SM and WR morphotypes carried mutations in genes related to motility. While motility is not essential for biofilm formation, motile strains are benefited in biofilm development compared to non-motile derivates (Hölscher *et al.,* 2015), therefore these mutations might additionally impact the efficiency of plant colonization. Nevertheless, overexpression of the *eps* operon in the SN morphotypes due to *sinR* mutation, could also possibly impact motility due to the EpsE clutch (Blair *et al.,* 2008). However, the role and impact of motility on the evolution of *B. subtilis* during plant root colonization will be examined in future studies.

Remarkably, evolved isolates showed improvements in root colonization only on *A. thaliana*, but not on tomato. In nature, the plant host species tend to be important drivers for diversity of the plant microbiome, indicating active selection by the plant (Compant *et al.,* 2019). Furthermore, PGPR have been shown to respond species-specifically in chemotaxis towards their original host plant where these were isolated from (Zhang *et al.,* 2014). Distinct plant species produce and secrete distinct arrays of root exudates, which act not only as a major carbon source for PGPR, but also as chemical signals for bacterial chemotaxis and are thus important initiators of root colonization (Warembourg *et al.,* 2003; Beauregard, 2015). Similar to root exudates, the composition of plant polysaccharides, which are known inducers of biofilm formation, differs between plant species (Beauregard *et al.,* 2013). Lastly, the sheer differences in the physiological architecture of plant roots, including size, thickness,

abundance of lateral roots and root hairs, might provide even more variation in environmental conditions between distinct plant species, thereby allowing plant species-specific adaptations (Saleem *et al.,* 2018). Although the Arabidopsis-specific aspects of the adaptation mechanism still need to be revealed, the evolved isolates performed differently on the two plant species.

A general observation from several experimental evolution studies with biofilm models is that the productivity of evolved populations not necessarily equals to the sum of productivities of its members in monocultures, as interactions between distinct evolved variants might occur (Brockhurst *et al.,* 2006; Poltak & Cooper, 2011; Ellis *et al.*, 2015). These can be both positive or negative, either mutualistic interactions, e.g. cross-feeding and niche construction, or antagonistic interactions, e.g. cheats and exploitation, might evolve (Day & Young, 2004; Poltak & Cooper, 2011, Dragoš *et al.,* 2018b). Here we extend these observations with a biotechnological relevant system on plant roots, describing the evolution of both exploitative as well as mutualistic interactions between the evolved morphotypes in distinct evolutionary lineages. The number of cells colonizing plant roots with an initially equal mix of SN, SM and WR is higher compared to its prediction in all three tested evolutionary lineages. In two of the three tested evolved populations, even though the mix performed better than the ancestor, the three members population did not outperform the monoculture of SN. Furthermore, SN showed strongly reduced CFU in these two mixes compared to its predicted value, suggesting that WR and SM benefited, most likely due to exploiting the enhanced matrix production by SN, without having the cost of the production. Similar to our study, Dragoš *et al.* (2018b) observed the evolution of a non-producing and matrix-exploiting smooth morphotype in *B. subtilis* pellicles. The described smooth variant integrated and resided in the biofilm produced by co-occurring morphotypes, therefore utilizing the matrix without having the cost (Dragoš *et al.,* 2018b). However, the absolute CFU of the tested isolates from population D on the plant root was higher in the mix compared to all three constituents in monocultures. Furthermore, all three morphotypes seemed to benefit from being in this mix, as they showed higher CFU than predicted, indicating that synergism evolved in this population between SN, SM and WR. The observation that interactions between the

three morphotypes differed in distinct evolved populations indicates that biodiversity both evolved and is maintained differently in the parallel populations (Poltak & Cooper, 2011). Nevertheless, further experiments are needed to reveal whether similar ecological interactions exist among the isolates from distinct lineages.

In conclusion, our results suggest that *B. subtilis* adapts rapidly to root colonization on *A. thaliana.* Our findings are relevant for the application of *B. subtilis* as a PGPR in agriculture, as they imply that genetic diversification might be a stable strategy for root colonization, and a mix of distinct phenotypic variants might be most beneficial. In addition to a potential increase in net productivity on plant roots, a diverse mix might be more resistant to invasions by non-cooperating cheaters (Brockhurst *et al.,* 2006), therefore, enhancing also the maintenance of the formed biofilm on the root.

Experimental procedures

Bacterial strains and culture conditions

Throughout this study, the ancestral strain *B. subtilis* DK1042 (natural competent variant of the undomesticated NCIB 3610 (Konkol *et al.,* 2013)) and evolved derivatives were grown in Lysogeny Broth (LB) (Lennox, 10 g·l^{-1} Tryptone, 5 g·l^{-1} Yeast Extract and 5 g·l^{-1} NaCl) over night (ON) at 37°C. For formation of complex colonies, 2µl of exponential phase culture were spotted on LB medium solidified with 1.5% agar and incubated at 30°C for 48h. To assay pellicle formation, the OD_{600} of a bacterial culture in exponential phase was adjusted to 5, and 15µl were inoculated into 1ml of LB + 0.5% xylan (from beechwood, Carl Roth) in 24-well microtiter plates and incubated at 30°C for 48h. In order to analyze growth in response to plant polysaccharides, the OD_{600} of a bacterial culture in exponential phase was adjusted to 0.1 in a minimal salt medium (MSN) (5mM potassium phosphate buffer pH 7.0, 0.1M 3-(N-morpholino)propanesulfonic acid pH 7.0, 4 mmol·l^{-1} $MgCl_2$, 0.05 mmol·l^{-1} $MnCl_2$, 1 µmol·l^{-1} $ZnCl_2$, 2 µmol·l^{-1} thiamine, 700 µmol·l^{-1} $CaCl_2$, 0.2 % NH_4Cl) supplemented with 0.5% cellobiose (Carl Roth) (MSNc) and 0.5% xylan, and 100µl was transferred to a 96-well plate. The well plate was incubated in a plate reader (BioTek Synergy HTX Multi-Mode Microplate Reader) for 58h at 24°C, orbital shaking, and the OD_{600} was measured every 15 minutes in a 9-point measurement at different points of each well, to exclude errors of clumping and inhomogeneous suspensions. For root colonization experiments MSN supplemented with 0.05% glycerol (MSNg) was used.

Seedling preparation

In this study, both *A. thaliana* ecotype Col-0 and tomato (*Solanum lycopersicum L.*, Maja Buschtomato, Buzzy Seeds, NL) seedlings were used. Seeds were surface sterilized in Eppendorf tubes by shaking vigorously with an orbital mixer for 12 minutes in 1mL 2% sodium hypochlorite. Afterwards, seeds were washed five times in 1 ml sterile MiliQ water through alternating centrifugation and removal of liquid solution. After sterilization, 8-10 seeds were plated on pre-dried 1% MS-agar (Murashige and Skoog basal salts mixture; Sigma) (2.2 g l^{-1}) ensuring that the seedlings have sufficient space for proper

root development and plates were sealed with parafilm. *A. thaliana* seeds were stratified in the dark at 4°C for 2-3d and subsequently incubated in a plant chamber with a day/night cycle of 16h light at 24°C and 8h dark at 20°C for 12-14d at an angle of 65°. Tomato seeds were moved to the plant chamber without stratification and incubated for 6-8d.

Experimental evolution

We established an experimental evolution approach inspired by the bead transfer model of Poltak & Cooper (2011) to study *B. subtilis* evolution on plant roots, similar to the setup exploited for *Bacillus thuringiensis* (Lin *et al.,* 2020). Therefore, every other day, a previously colonized plant seedling was transferred to a new sterile seedling, allowing only bacterial cells that attach to the new root to continue in the experiment, enabling selection for a continuous cycle of dispersal, chemotaxis towards the plant root, and biofilm formation on the root. To start the experimental evolution, five 14-16d old *A. thaliana* Col-0 seedlings (population A to E) were placed in 100mL blue cap bottles containing 27ml of MSNg, and inoculated with 3ml of bacterial culture of *B. subtilis* DK1042 adjusted to OD_{600} of 0.2, reaching a starting OD_{600} of 0.02. To ensure that only the roots but not sprout or leaves provided surface for colonization, the seedlings were pulled through a sterile nylon mesh floating on top of the liquid medium, leaving only the roots submerged in the medium (Harris *et al.,* 2019). Seedlings were incubated for 48 hours under static conditions in a plant chamber with a day/night cycle of 16h light at 24°C and 8h dark at 20°C. Before the transfer, the mesh of the previously colonized root was removed and the seedling was carefully washed in sterile MilliQ water, in order to remove non-colonizing cells. Subsequently, the colonized seedling was placed in fresh medium containing a new sterile seedling, allowing re-colonization. Evolving populations were continuously passaged every 48h for a total of 40 days (20 subsequently colonized seedlings per population). To follow the development of the populations, the bacterial cells colonizing the old roots were plated on LB agar medium after initial colonization and transfer 5, 10, 17 and 20. In order to disperse the biofilm from the seedling for the quantification, roots were detached from the sprout, carefully washed in sterile MilliQ, and

vortexed vigorously for 3 minutes in 1ml of 0.9% NaCl with small glass beads (Glass beads Ø0.75-1.00 mm, Carl Roth). The resulting cell suspensions were plated for CFU quantification and saved as frozen stocks for later comparison.

Morphological diversification

Distinct morphotypes of evolved populations were determined according to characteristics in colony morphology (shape, size and color) on LB agar medium. To ensure different morphotypes were genetically stable, random colonies of each variant were picked and passaged for three transfers in liquid LB and on LB agar medium. In order to analyze the development of the ratio of each morphotype during the evolution, a bacterial culture prepared from the frozen stock of each population for transfer 5, 10, 17, and 20, as well as of the ancestor as starting point 0, was grown to log-phase, diluted, plated on LB-agar and incubated for 16h at 30°C. Subsequently, morphotypes were identified, counted and ratios were calculated.

Root colonization productivity

In order to analyze whether evolved isolates were improved in root colonization compared to the ancestor, and whether those improvements were specific for the plant species the isolates evolved on, root colonization was tested on both *A. thaliana* Col-0 and tomato seedlings. Therefore, three single isolates were picked randomly from the final populations (i.e. T20) of linages A, C, D, and E. 14-16d old *A. thaliana* and 6-8d old tomato seedlings were inoculated with each of the evolved isolates in four replicates as described previously for initiation of the experimental evolution. After confirmation of equal variance with Levene´s test and normality of data with Shapiro–Wilk test, changes in root colonization were analyzed for significance with Dunnett´s post hoc test. Furthermore, to test the impact of diversity on the individual and mixed performance in root colonization, 14-16d old *A. thaliana* seedlings were inoculated with a 1:1:1 mix of pre-grown cultures of the three distinct morphotypes (SN, SM and WR), originating from the same evolved population (population 1, 4 and 5).

Seedlings were incubated together with the bacterial inoculant for 48h in the plant chamber to allow root colonization. Afterwards, seedlings were carefully washed in sterile MilliQ water, the biofilm formed on plant roots was disrupted and the bacterial suspensions were plated on LB agar medium to allow CFU quantification. For mixed colonization, the predicted root colonization was calculated by multiplying the root colonization of each constituent in mono culture with the ratio in the inoculum (1/3) (Loreau & Hector, 2001; Poltak & Cooper, 2011).

Microscopy

Bright field images of whole pellicles and colonies were obtained with an Axio Zoom V16 stereomicroscope (Carl Zeiss, Germany) equipped with a Zeiss CL 9000 LED light source and an AxioCam MRm monochrome camera (Carl Zeiss, Germany).

Genome resequencing and genome analysis

Resequencing of evolved isolates was performed as previously (Dragoš *et al.,* 2018b; Dragoš *et al.,* 2018c; Martin *et al.,* 2020; Thérien *et al.,* 2020; Gallegos-Monterrosa *et al.,* 2021). Genomic DNA of selected evolved isolates from the final transfer was extracted from 2ml ON cultures using the EURx Bacterial and Yeast Genomic DNA Kit. Paired-end libraries were prepared using the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina. Paired-end fragment reads were generated on an Illumina NextSeq sequencer using TG NextSeq® 500/550 High Output Kit v2 (300 cycles). Primary data analysis (base-calling) was carried out with "bcl2fastq" software (v2.17.1.14, Illumina). All further analysis steps were done in CLC Genomics Workbench Tool 9.5.1. Reads were quality-trimmed using an error probability of 0.05 (Q13) as the threshold. In addition, the first ten bases of each read were removed. Reads that displayed ≥80% similarity to the reference over ≥80% of their read lengths were used in the mapping. Non-specific reads were randomly placed to one of their possible genomic locations. Quality-based SNP and small In/Del variant calling was carried out requiring ≥8 × read coverage with ≥25% variant frequency. Only variants supported by good quality bases (Q ≥ 20) were considered and only when

they were supported by evidence from both DNA strands in comparison to the *B. subtilis* NCIB 3610 genome and pBS plasmid (GenBank accession no. NZ_CP020102 and NZ_CP020103, respectively). Identified mutations in each strain are listed in Table 1. Raw sequencing data has been deposited to the NCBI Sequence Read Archive (SRA) database under BioProject accession number: PRJNA705297

References

Allard-Massicotte, R., Tessier, L., Lécuyer, F., Lakshmanan, V., Lucier, J. F., Garneau, D., *et al.* (2016) *Bacillus subtilis* early colonization of *Arabidopsis thaliana* roots involves multiple chemotaxis receptors. *mBio* **7:** e01664-16.

Arkhipova, T.N., Veselov, S U., Melentiev, A.I., Martynenko, E.V., and Kudoyarova, G.R. (2005) Ability of bacterium *Bacillus subtilis* to produce cytokinins and to influence the growth and endogenous hormone content of lettuce plants. *Plant Soil* **272:** 201-209.

Arnaouteli, S., Bamford, N., Stanley-Wall, N., and Kovács, Á.T. (2021) *Bacillus subtilis* biofilm formation and social interactions. *Nat Rev Microbiol* (in press)

Bardin, M., Ajouz, S., Comby, M., Lopez-Ferber, M., Graillot, B., Siegwart, M., and Nicot, P.C. (2015) Is the efficacy of biological control against plant diseases likely to be more durable than that of chemical pesticides? *Front Plant Sci* **6:** 566.

Batstone, R T., O'Brien, A.M., Harrison, T.L., and Frederickson, M.E. (2020) Experimental evolution makes microbes more cooperative with their local host genotype. *Science* **370:** 476-478.

Beauregard, P. B. Chai, Y. Vlamakis, H. Losick, R. and Kolter, R. (2013) *Bacillus subtilis* biofilm induction by plant polysaccharides. *Proc Natl Acad Sci U S A* **110:** E1621-E1630.

Beauregard, P. B. (2015) Not just sweet talkers: How roots stimulate their colonization by beneficial bacteria. *Adv Bot Res* **75:** 1-20.

Bedrunka, P., and Graumann, P.L. (2017) Subcellular clustering of a putative c-di-GMP-dependent exopolysaccharide machinery affecting macro colony architecture in *Bacillus subtilis*. *Environ Microbiol Rep* **9:** 211-222.

Blair, K.M., Turner, L., Winkelman, J.T., Berg, H.C., and Kearns, D.B. (2008) A molecular clutch disables flagella in the *Bacillus subtilis* biofilm. *Science* **320:** 1636-1638.

Blake, C., Christensen, M.N., and Kovács, Á.T. (2021) Molecular aspects of plant growth promotion and protection by *Bacillus subtilis*. *Mol Plant Microbe Interact* **34:** 15-25.

Branda, S.S., Chu, F., Kearns, D B., Losick, R., and Kolter, R. (2006) A major protein component of the *Bacillus subtilis* biofilm matrix. *Mol Microbiol* **59:** 1229-1238.

Brockhurst, M.A., Hochberg, M.E., Bell, T., and Buckling, A. (2006) Character displacement promotes cooperation in bacterial biofilms. *Curr Biol* **16:** 2030-2034.

Cazorla, F.M., Romero, D., Pérez-García, A., Lugtenberg, B.J.J., Vicente, A.D., and Bloemberg, G. (2007) Isolation and characterization of antagonistic *Bacillus subtilis* strains from the avocado rhizoplane displaying biocontrol activity. *J Appl Microbiol* **103:** 1950-1959.

Chen, Y., Yan, F., Chai, Y., Liu, H., Kolter, R., Losick, R., and Guo, J.H. (2013) Biocontrol of tomato wilt disease by *Bacillus subtilis* isolates from natural environments depends on conserved genes mediating biofilm formation. *Environ Microbiol* **15:** 848-864.

Compant, S., Samad, A., Faist, H., and Sessitsch, A. (2019) A review on the plant microbiome: ecology, functions, and emerging trends in microbial application. *J Adv Res* **19:** 29-37.

Day, T., and Young, K.A. (2004) Competitive and facilitative evolutionary diversification. *BioScience* **54:** 101-109.

Deng, Y., Chen, H., Li, C., Xu, J., Qi, Q., Xu, Y., et al. (2019) Endophyte *Bacillus subtilis* evade plant defense by producing lantibiotic subtilomycin to mask self-produced flagellin. *Commun Biol* **2:** 368.

Dragoš, A., Kiesewalter, H., Martin, M., Hsu, C.Y., Hartmann, R., Wechsler, T., *et al.* (2018a) Division of labor during biofilm matrix production. *Curr Biol* **28:** 1903-1913.

Dragoš, A., Lakshmanan, N., Martin, M., Horváth, B., Maróti, G., Falcón García, C., Lieleg, O., and Kovács, Á.T. (2018b) Evolution of exploitative interactions during diversification in *Bacillus subtilis* biofilms. *FEMS Microbiol Ecol* **94:** fix155

Dragoš, A., Martin, M., Falcón García, C., Kricks, L., Pausch, P., Heimerl, T., *et al.* (2018) Collapse of genetic division of labor and evolution of autonomy in pellicle biofilms. *Nat Microbiol* **3:** 1451-1460

Ellis, C.N., Traverse, C.C., Mayo-Smith, L., Buskirk, S.W., and Cooper, V.S. (2015) Character displacement and the evolution of niche complementarity in a model biofilm community. *Evol* **69:** 283-293.

Fitzpatrick, C.R., Copeland, J., Wang, P. W., Guttman, D.S., Kotanen, P.M., and Johnson, M.T. (2018) Assembly and ecological function of the root microbiome across angiosperm plant species. *Proc Natl Acad Sci U S A* **115:** E1157-E1165.

Gallegos-Monterrosa, R., Nordgaard Christensen, M., Barchewitz, T., Köppenhöfer, S., Priyadarshini, B., Bálint, B., *et al.* (2020) Impact of Rap-Phr system abundance on adaptation of *Bacillus subtilis*. *bioRxiv* https://doi.org/10.1101/2020.09.01.278184

Harris, S.L., Pelaez, C.A., and Shank, E.A. (2019) Monitoring bacterial colonization and maintenance on *Arabidopsis thaliana* roots in a floating hydroponic system. *J Vis Exp* **147:** e59517.

Hölscher, T., Bartels, B., Lin, Y. C., Gallegos-Monterrosa, R., Price-Whelan, A., Kolter, R., *et al.* (2015) Motility, chemotaxis and aerotaxis contribute to competitiveness during bacterial pellicle biofilm development. *J Mol Biol* **427:** 3695-3708.

Kampf, J., Gerwig, J., Kruse, K., Cleverley, R., Dormeyer, M., Grünberger, A., *et al.* (2018) Selective pressure for biofilm formation in *Bacillus subtilis*: differential effect of mutations in the master regulator SinR on bistability. *mBio* **9:** e01464-18.

Kawecki, T.J., Lenski, R.E., Ebert, D., Hollis, B., Olivieri, I., and Whitlock, M.C. (2012) Experimental evolution. *Trends Ecol Evol* **27:** 547-560.

Konkol, M.A., Blair, K.M., and Kearns, D.B. (2013) Plasmid-encoded ComI inhibits competence in the ancestral 3610 strain of *Bacillus subtilis*. *J Bacteriol* **195:** 4085-4093.

Lin, Y., Alstrup, M., Pang, J.K.Y., Maróti, G., Er-Rafik, M., Tourasse, N., *et al.* (2020) Adaptation of *Bacillus thuringiensis* to plant colonisation affects differentiation and toxicity. *bioRxiv* https://doi.org/10.1101/2020.12.03.410076

Loreau, M., and Hector, A. (2001) Partitioning selection and complementarity in biodiversity experiments. *Nature* **412:** 72-76.

Lugtenberg, B., and Kamilova, F. (2009) Plant-growth-promoting rhizobacteria. *Annu Rev Microbiol* **63:** 541-556.

Luo, C., Zhou, H., Zou, J., Wang, X., Zhang, R., Xiang, Y., and Chen, Z. (2015) Bacillomycin L and surfactin contribute synergistically to the phenotypic features of *Bacillus subtilis* 916 and the biocontrol of rice sheath blight induced by *Rhizoctonia solani*. *Appl Microbiol Biotechnol* **99:** 1897-1910.

Martin, M., Dragoš, A., Otto, S.B., Schäfer, D., Brix, S., Maróti, G., and Kovács, Á.T. (2020) Cheater-mediated evolution shifts phenotypic heterogeneity in *Bacillus subtilis* biofilms. *ISME J* **14:** 2302-2312.

Moreira, R. R., and De Mio, L. L. M. (2015) Potential biological agents isolated from apple fail to control Glomerella leaf spot in the field. *Biol Control* **87:** 56-63.

McDonald, M.J. (2019) Microbial Experimental Evolution–a proving ground for evolutionary theory and a tool for discovery. *EMBO Rep* **20:** e46992.

Micallef, S.A., Shiaris, M.P., and Colón-Carmona, A. (2009) Influence of *Arabidopsis thaliana* accessions on rhizobacterial communities and natural variation in root exudates. *J Exp Bot* **60:** 1729-1742.

Peng, G., McGregor, L., Lahlali, R., Gossen, B.D., Hwang, S.F., Adhikari, K.K., Strelkov, S.E, and McDonald, M.R. (2011) Potential biological control of clubroot on canola and crucifer vegetable crops. *Plant Pathol* **60:** 566-574.

Pieterse, C.M., Zamioudis, C., Berendsen, R.L., Weller, D.M., Van Wees, S.C., and Bakker, P.A. (2014) Induced systemic resistance by beneficial microbes. *Annu Rev Phytopath* **52:** 347-375.

Poltak, S.R., and Cooper, V.S. (2011) Ecological succession in long-term experimentally evolved biofilms produces synergistic communities. *ISME J* **5:** 369-378.

Reverdy, A., Chen, Y., Hunter, E., Gozzi, K., and Chai, Y. (2018) Protein lysine acetylation plays a regulatory role in *Bacillus subtilis* multicellularity. *PLoS One* **13:** e0204687.

Richter, A., Hölscher, T., Pausch, P., Sehrt, T., Brockhaus, F., Bange, G., and Kovács, Á.T. (2018) Hampered motility promotes the evolution of wrinkly phenotype in *Bacillus subtilis*. *BMC Evol Biol* **18:** 155.

Saleem, M., Law, A.D., Sahib, M.R., Pervaiz, Z.H., and Zhang, Q. (2018) Impact of root system architecture on rhizosphere and root microbiome. *Rhizosphere* **6:** 47-51.

Saravanakumar, D., Kavino, M., Raguchander, T., Subbian, P., and Samiyappan, R. (2011) Plant growth promoting bacteria enhance water stress resistance in green gram plants. *Acta Physiol Plant* **33:** 203-209.

Shoda, M. (2000) Bacterial control of plant diseases. *J Biosci Bioeng* **89:** 515-521.

Sivasakthi, S., Usharani, G., and Saranraj, P. (2014) Biocontrol potentiality of plant growth promoting bacteria (PGPR)-*Pseudomonas fluorescens* and *Bacillus subtilis*: A review. *African J Agricult Res* **9:** 1265-1277.

Thérien, M., Kiesewalter, H.T., Auria, E., Charron-Lamoureux, V., Wibowo, M., Maróti, G., *et al.* (2020) Surfactin production is not essential for pellicle and root-associated biofilm development of *Bacillus subtilis*. *Biofilm* **2:** 100021

Todorova, S., and Kozhuharova, L. (2010) Characteristics and antimicrobial activity of *Bacillus subtilis* strains isolated from soil. *World J Microbiol Biotechnol* **26:** 1207-1216.

Trivedi, P., Leach, J.E., Tringe, S.G., Sa, T., and Singh, B.K. (2020) Plant–microbiome interactions: From community assembly to plant health. *Nat Rev Microbiol* **18:** 607-621.

van Gestel, J., Weissing, F.J., Kuipers, O.P., and Kovács, Á.T. (2014) Density of founder cells affects spatial pattern formation and cooperation in *Bacillus subtilis* biofilms. *ISME J* **8:** 2069-2079.

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015) The importance of the microbiome of the plant holobiont. *New Phytol* **206:** 1196-1206.

Warembourg, F.R., Roumet, C., and Lafont, F. (2003) Differences in rhizosphere carbon-partitioning among plant species of different families. *Plant Soil* **256:** 347-357.

Wei, F., Hu, X., and Xu, X. (2016) Dispersal of *Bacillus subtilis* and its effect on strawberry phyllosphere microbiota under open field and protection conditions. *Sci Rep* **6:** 22611.

Yi, H.S., Yang, J.W., Ghim, S.Y., and Ryu, C.M. (2011) A cry for help from leaf to root: Above ground insect feeding leads to the recruitment of rhizosphere microbes for plant self-protection against subsequent diverse attacks. *Plant Signal Behav* **6:** 1192-1194.

Zhang, N., Wang, D., Liu, Y., Li, S., Shen, Q., and Zhang, R. (2014) Effects of different plant root exudates and their organic acid components on chemotaxis, biofilm formation and colonization by beneficial rhizosphere-associated bacterial strains. *Plant Soil* **374:** 689-700.

Zhang, F., Kwan, A., Xu, A., and Süel, G.M. (2015) A synthetic quorum sensing system reveals a potential private benefit for public good production in a biofilm. *PLoS One* **10:** e0132948.

Zhu, B., and Stülke, J. (2017) SubtiWiki in 2018: from genes and proteins to functional network annotation of the model organism Bacillus subtilis. *Nucl Acids Res* **46:** D743-748

Table 1. List of mutations detected in the genomes of re-sequenced morphotypes. Functions of the gene products were retrieved from SubtiWiki Database (Zhu & Stülke, 2017)

SN		SM		WR			Position	Gene	Mutation	Function of gene product
SN_A	SN_E	SM_D	SM_E	WR_A	WR_D	WR_E				
		✓					472036	*ydaD*	288G>T	general stress protein
				✓			478790	*intergenic*	C>G	upstream region of *ydaJ* that codes for a lipoprotein that may modify the extracellular polysaccharide synthesized by YdaLMN proteins
					✓		478876	*intergenic*	T>del	
		✓					1002770	*glpF*	Pro78Ala	glycerol facilitator
			✓				1016370	*spoVR*	Glu229Gly	spore cortex synthesis
				✓			1391100	*thiU*	Pro192Gln	thiamine uptake
						✓	1433817	*motB*	Gly226Asp	flagellar motor rotation
				✓			1696818	*fliI*	Thr304Lys	flagellar-specific ATPase
					✓		1706108	*fliR*	Ala89_Ala93del	part of the flagellar Type III secretion system
		✓	✓				1707666	*flhB*	Glu347fs	part of the flagellar Type III secretion system
			✓				2424537	*dacB*	Ala7Thr	sporulation-specific penicillin-binding protein
✓	✓						2552926	*sinR*	Ala85Thr	control of biofilm formation
				✓			2578188	*pstBA*	Ser216Pro	phosphate ABC transporter ATP-binding protein
					✓		3258864	*pdeH*	Asp137fs	degradation of c-di-GMP
				✓			3258936		Asp116Tyr	
		✓	✓				3666149	*gtaB*	483G>A	UTP-glucose-1-phosphate uridylyltransferase
		✓	✓				3666155		489G>A	
		✓	✓				3666161		Glu165Asp	
		✓	✓				3666171		Arg169Cys	
		✓	✓				3666173		507C>T	
		✓	✓				3666176		510C>T	
			✓				3666182		516T>C	
		✓	✓				3673066	*yvzE*	Phe27Val	putative UTP-glucose-1-phosphate uridylyltransferase
		✓	✓				3673068		Phe27Leu	
		✓	✓				3673077		90A>T	
		✓	✓				3673098		Asp37Glu	
		✓	✓				3673101		114A>T	