Adaptation of *Bacillus thuringiensis* to plant colonisation affects differentiation and toxicity

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Abstract

Although certain isolates from the *Bacillus cereus* group (*Bacillus cereus sensu lato*) are used as probiotics, safety concerns remain due to pathogenic traits. For example, toxin production might shift as an adaptive survival strategy in natural niches (the soil and plant rhizosphere). Therefore, it is crucial to explore bacterial evolutionary adaptation to the environment. Herein, we investigated *Bacillus thuringiensis* (Cry-) adaptation to the colonisation of *Arabidopsis thaliana* roots, and monitored changes in cellular differentiation in experimentally evolved isolates. Isolates from two populations displayed improved iterative ecesis on roots and increased toxicity against insect larvae. Molecular dissection and recreation of a causative mutation revealed the importance of a non-sense mutation in the *rho* transcription terminator gene. Transcriptome analysis revealed how Rho impacts various *B. thuringiensis* genes involved in carbohydrate metabolism and virulence. Our work suggests that evolved multicellular aggregates have a fitness advantage over single cells when colonising plants, creating a trade-off between swimming and multicellularity in evolved lineages, in addition to unrelated alterations in pathogenicity.

Introduction

Microbes can be both beneficial and harmful to human life. Probiotics are living microorganisms that have health benefits if administered in adequate amounts[1]. They have many applications including preventing gastrointestinal tract infections, optimising the growth of livestock, and enhancing the growth properties of crops. However, humans and other animals suffer from numerous diseases caused by pathogenic microbes. *Bacillus thuringiensis* and *Bacillus cereus* (*sensu stricto*), members of the *Bacillus cereus* group, are spore-forming, soil-dwelling organisms with plant growth-promoting potential[2,3]. *B. thuringiensis* has been successfully applied as a commercialised bio-

pesticide for decades due to its entomopathogenic properties, while *B. cereus* is a causative agent of foodborne diseases. Cases of human disease have also been reported from *B. thuringiensis* infections[4,5]. *B. cereus* group species have a complex ecological lifestyle, and can be isolated as resistant spores or metabolically active vegetative cells from soil, rhizosphere, plant tissues, and living or dead insects[6]. Under certain conditions cells develop biofilms, in which microbes thrive by forming tightly packed, multicellular aggregates, surviving harsh conditions[7]. *Bacillus* spp. are recruited by small molecules secreted by plants, and can colonise plant roots by producing an exopolysaccharide matrix[8]. Root-colonising bacteria (rhizobacteria) can benefit plants by promoting growth and preventing infections by plant pathogens[9]. Varieties of biocontrol products contain *Bacillus* spp., including *B. cereus* group organisms[10]. However, our current understanding of their ecology is limited, especially the association between plant-promoting effects and infection risk. Toxic potential might prevent the application of *B. cereus* in plant biologicals, or lead to disease outbreaks[3]. Our work aimed to uncover the physiology of *B. thuringiensis* experimentally evolved on plant roots.

Besides traditional molecular genetics, experimental evolution (EE) is an effective approach for exploring the evolutionary dynamics of microbes under different environmental niches[11]. From simple growth conditions such as shake flasks[12] and chemostat cultures[13], to complex environmental niches including eukaryote hosts[14–16], EE has been successfully applied to microbes, revealing unique insights connecting microbial phenotypes and genotypes. Despite the prevalence of biofilms in nature, most EE has been performed using planktonic bacteria, hence we lack a detailed understanding of adaptation within biofilm populations[17]. A fascinating 'bead transfer model' was developed to unravel the genetic diversification of the opportunistic pathogen *Burkholderia cenocepacia*[18,19]. After long-term EE, *B. cenocepacia* diversified into different morphotypes with enhanced community

productivity generated by niche complementarity effects[20]. Similarly, diverse colony morphotypes arose during EE of highly wrinkled floating biofilms of *Bacillus subtilis*, called pellicles[8,21,22 23].

Compared with well-studied evolved biofilms of *B. subtilis*, knowledge on the *B. cereus* group remains limited. EE of *B. cereus* group strains has been performed within diverse hosts from nematodes to vertebrates to dissect host-microbe interactions and the evolution of virulence pathways[15,24]. Such approach demonstrated reciprocal coevolution between *B. thuringiensis* and its nematode host *Caenorhabditis elegans*[15], in contrast to one-sided adaptation that favoured mutational landscape changes in certain toxin genes. Laboratory evolution of *B. cereus* group biofilms remains limited, despite the relevance of biofilms in various environmental niches, where the evolutionary ecology of virulence can also be followed[25]. Herein, we devised an EE setup associating *B. thuringiensis* (Cry-) with the model plant *Arabidopsis thaliana* to scrutinise the parallel evolution of this bacterium. After 40 cycles of laboratory evolution in the plant rhizosphere, bacterial lineages displayed enhanced root colonisation ability compared with the ancestral strain. Intriguingly, single isolates from two of the evolved lineages tended to re-colonise a new root more efficiently compared with the other lineages, in addition to exhibiting altered bacterial differentiation and pathogenicity. Investigation of a key mutation in the gene encoding the Rho transcription termination factor in these linages demonstrated how transcriptional rewiring alters cell fate decisions in *B. thuringiensis*.

Results and Discussion

Experimental evolution on plants selects for improved root colonisers

EE of *B. thuringiensis* 407 Cry$^-$ (Bt407) was initiated using six parallel linages colonising the roots of 7-day-old *A. thaliana* seedlings under hypotonic conditions generally applied for *Bacilli*[26]. Initially, inoculated planktonic bacterial cells developed biofilms on plant roots. Subsequently, these biofilms were re-established on new roots after transplanting seedlings to a new culture after 2 days (Fig. 1a).

Plant-associated biofilms formed on roots were quantitatively monitored by assessing the productivity (bacterial colony forming units [CFU]/root length) after every 5th transfer. Notably, one of the six lineages (linage B) yielded CFU values below the detection limit after five transfers. We speculate that the strong population bottleneck (i.e. only few cells re-establishing a new biofilm) led to the disappearance of this lineage, since only a few hundred bacterial cells were attached to roots during the initial stages (Fig. 1b). Although these ecosystems were independent, biofilm productivity increased gradually in the other five populations throughout the whole EE period. The increased biomass provides evidence for successful adaptation during EE, as reported in previous studies[18,23].

Unsurprisingly, evolved strains formed more extensive root biofilms than the Bt407 ancestor, several times thicker in places (Fig. S1). To test the adapted traits, three colonies were isolated from each linage after the 40th transfer, and examined for biofilm establishment on roots. The majority of the evolved isolates exhibited significant root colonisation enhancement compared to the ancestor strain (Fig. 1c).

Next, colonised plants were transferred to fresh medium hosting new seedlings, and CFU values were quantified. This capacity, termed as root re-colonisation, was strikingly increased in two of the evolved linages (E and F; Fig. 1d). When a new seedling was provided, the biofilm cells of lineages E and F tended to detach from the older seedlings and colonise the new ones more efficiently.

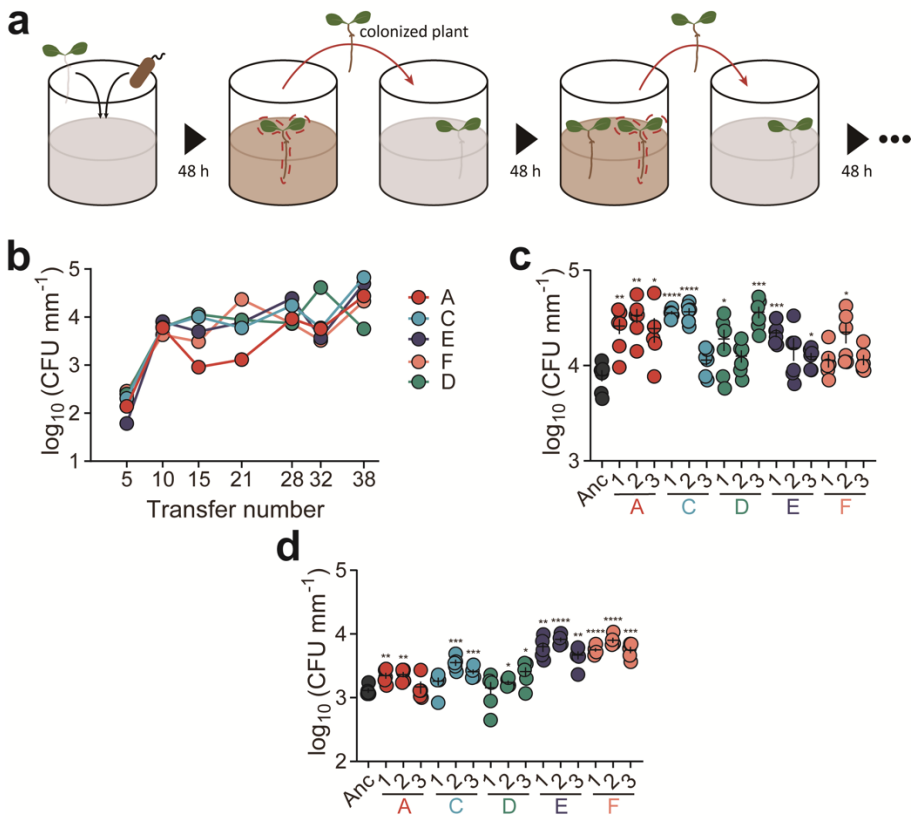

Fig. 1 Experimental evolution setup and productivity assessment. **a,** Experimental evolution model of root-associated biofilms. Parallel linages of the Bt407 (Cry$^-$) strain were initiated on a 1-week-old *A. thaliana* seedling to form biofilm in MSNg medium in 48-well microliter plates agitated at 50 rpm. Colonised plants were subsequently transferred to a well containing a sterile seedling, and steps were repeated for 40 transfers. **b,** Productivity (CFU mm^{-1}) of plant-colonised Bt407 (Cry$^-$) lineages are shown at roughly every 5th transfer. **c,** Plant-colonised biofilm productivity (CFU mm^{-1}) of evolved isolates (n = 6 biologically independent plantlet samples of similar length). The central values (horizontal lines) represent the mean and the error bars represent standard error of the mean. Asterisks indicate significant differences between each group and the ancestor (*p <0.05; **p <0.01; ***p <0.001; ****p <0.0001; two tailed t-test with Welch's corrections). **d,** Re-colonised biofilm productivity (CFU mm^{-1}) of the evolved isolates (n = 6 biologically independent plantlet samples of similar length). Significance assessment was conducted as described for panel **c**.

Shifts in multicellular behaviour accompany adaptation to root colonisation

Colonisation of the rhizosphere by *Bacilli* depends on various multicellular traits[26–28]. We specifically examined the evolved isolates from lineages E and F, and compared the differentiation properties to the ancestor and the isolates from other evolved lineages (A, C and D). Primarily, colonisation of a new niche depends on bacterial motility[29]. *B. thuringiensis* displays two types of flagella-driven motility: single cells swim, while rafts of cells swarm aided by surfactants. Surprisingly, evolved isolates from the E and F lineages were greatly reduced in swimming motility, while other lineages exhibited motility comparable with the ancestor (Fig. 2a, Supplementary Fig. 2). Swimming motility is required for air-liquid interface biofilm development of *B. cereus* in 24 h cultures[30], whereas under flow conditions, non-motile cells form dense and thick biofilms. Notably, EE was performed using mildly shaken cultures, possibly facilitating random contact between bacterial debris and seedlings. Thus, swimming might not provide a benefit for bacteria to recolonise plants in this setup, and there might be a fundamental trade-off between the free-swimming state and living in communities. By contrast, when surface spreading was tested using increased agar concentration (i.e. 0.7% agar) to determine swarming, the isolates from linages E and F displayed enhanced swarming compared to the ancestor (Fig. 2b, Supplementary Fig. 2). Studies have shown that defects in swarming ability can cause poor root colonisation in *Bacilli*[31,32]. Swarming cells suppress cell division and cell elongation, which is either a requirement for, or an indicator of, swarming motility. In line with this, cell elongation was observed in the evolved E and F lineages (Fig. 2c).

Next, we tested sporulation in the evolved linages. After 48 h, the Bt407 ancestor strain had sporulated efficiently in a defined sporulation medium (HCT), while cells from lineages E and F were packed in aggregates with reduced levels of complete sporulation (Fig. 2d). When exposed to harsh

conditions or competitors, sporulation serves as a secondary defensive strategy in addition to being embedded in biofilm communities. Herein, we found that evolved isolates exhibited delayed or less frequent sporulation in minimal medium, which may contribute to the efficacy of root colonisation. At the cost of reducing sporulation efficiency, evolved biofilm aggregates might have a fitness advantage over swimming planktonic cells when competing for limited nutrients and/or colonising plants.

Additionally, cryo-Scanning Electron Microscopy (cryo-SEM) was used to examine bacterial ultrastructure and cell length (Fig. 2e), which further confirmed longer chains of unseparated cells of linage E in addition to slightly increased single cell length, compared to the ancestor strain.

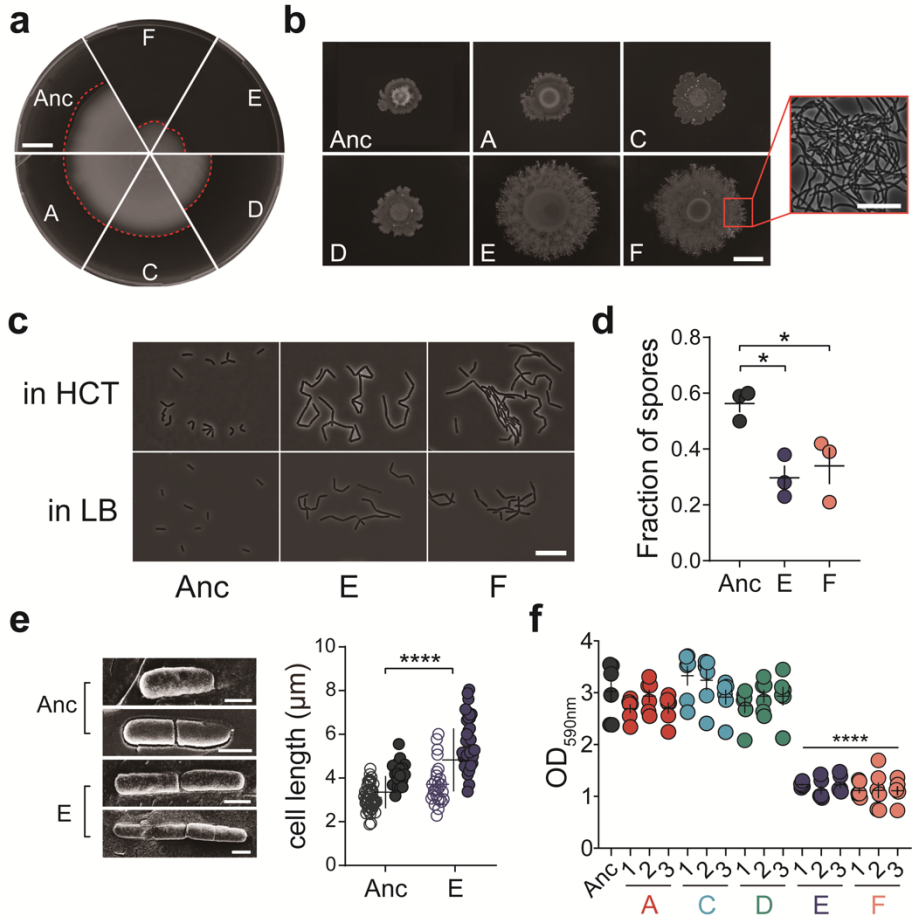

Fig. 2 Certain evolved lineages demonstrate elongated and sessile bacterial traits. a, Swimming motility of Bt407 ancestor and representative evolved isolates. Scale bar indicates 1 cm. Images are

representative of three independent biological replicates. **b,** Swarming radius of the Bt407 ancestor and evolved isolates. The framed area in F shows filamentous growth at the colony edge. Scale bars indicate 1 cm and 10 μm, respectively. Images are representative of three independent biological replicates. **c,** Cell morphology of planktonic cultures at OD_{600} of 1 in HCT and LB media. Scale bar indicates 10 μm. Images are representative of three biologically independent bacterial cultures in HCT and LB media, respectively. **d,** Sporulation of the ancestor strain and evolved lineages E and F (n = 3 independent biological samples per group). Vegetative cells and heat-resistant spores, respectively, were counted after a 48 h incubation in HCT medium at 30°C. Error bars indicate the standard error of the mean. Statistically significant differences were examined using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons (*p <0.05). **e,** Cryo-SEM imaging of the Bt407 ancestor and evolved lineage E (left). Calculated length of single bacterial cells (open symbols) and chains (closed symbols) are shown on the right. The central values (horizontal lines) indicate the means (n = 67 for Bt407 ancestor and n = 64 for evolved lineage E) and the error bars represent standard deviation. Asterisks indicate significant differences (****p <0.0001; two tailed t-test with Welch's corrections). Scale bar indicates 1 μm. **f,** Surface-attached (submerged) biofilm formation, quantitated by Crystal Violet staining and subsequent solubilisation by 70% ethanol. The central values (horizontal lines) represent the mean (n = 6 biologically independent samples) and the error bars represent the standard error of the mean. Asterisks indicate significant differences between each group and the ancestor (****p <0.0001; two tailed t-test with Welch's corrections).

To further elucidate the sessile, non-motile properties of certain evolved isolates, *in vitro* biofilm formation was assayed under different scenarios. Pellicle biofilm formed at the air-liquid interface generally requires motility as a pivotal factor during formation[33]. As expected, pellicle formation in LBGM[34,35] (LB with glycerol and $MnSO_4$) medium was greatly reduced in the evolved

lineages E and F, providing additional evidence for pellicle formation being heavily dependent on swimming motility (Supplementary Fig. 3a). Pellicles in evolved lineage A showed partial impairment, potentially indicating that pellicle formation could also be linked to other altered traits.

Next, the isolates were tested for submerged biofilm development at the plastic surface under slight shaking conditions. Both imaging and quantitative data demonstrated a significantly lower degree of submerged biofilm formation in linages E and F, compared to the ancestor and other EE linages (Fig. 2f and Supplementary Fig. 3b). Meanwhile, the total growth of the ancestor and evolved strains was similar in the tested medium (Supplementary Fig. 3c). Although pellicle formation and surface-attached biofilm formation are common techniques for screening biofilm formation in the laboratory, the list of genes required for these two biofilm models differs. The reduced biofilm formation of evolved isolates from linages E and F in these two microenvironments implied spatial specialisation of certain evolved strains, meaning that microbes may have tended to form aggregates in the medium instead of swimming to the air-liquid interface or the bottom of the microliter plates.

Competitive advantage of evolved linages during interaction with plants

Following fundamental *in vitro* physiological tests, we wondered why evolved isolates showed enhanced root colonisation, and whether they responded differently to plant materials compared with the ancestor. As proposed by Beauregard and colleagues[26], MSN medium supplemented with 0.5% cellobiose (MSNc) can be used to assess the plant-induced biofilm formation properties of *B. subtilis*. Cellobiose, a plant disaccharide resulting from hydrolysed cellulose, is similar to that found on plants. However, MSNc medium alone did not induce biofilm formation as reported previously. Consistent with the literature on other *Bacilli*, we found that the ancestor strain could not form floating biofilms in this medium after 48 h, but produced homogenous planktonic cultures displaying poor growth (Fig. 3a). By contrast, the evolved isolates formed various

levels of floating aggregate-resembling biofilms. Dense structural aggregates (round shape and up to 10 mm diameter) with high compactness were observed for linage E and F (Fig. 3a). Similarly, cellobiose also elevated the formation of aggregates in other evolved lineages, though varying in shape (stick-like) and size (up to 10 mm long) from those of lineages E and F. Cellobiose is a common component of the plant cell wall, which is present in soil and decaying plants, and can be utilised by some *B. cereus* strains as a carbon source[36]. The elevated aggregation phenotype of evolved isolates suggests that an altered plant material-associated metabolic capacity and/or biofilm induction was favoured under the EE regime.

To further explore the impact of plant polysaccharides on biofilm formation of the evolved isolates, pellicle formation was tested in LB medium supplemented with various plant polysaccharides. Among the polysaccharides previously investigated[26], xylan could induce dense and robust pellicle formation in the evolved isolates, whereas the pellicles of the ancestor remained thin and fragile (Fig. 3b). This result is somewhat surprising as no previous study has reported that LB medium, a rich medium, could induce pellicle formation with plant polysaccharide. Even in a nutrient-rich medium, evolved isolates could utilise plant polysaccharide as a signal and/or utilise it during biofilm assembly. Moreover, morphological differences were apparent among the evolved isolates; although the pellicles of lineages E and F were dense and compact, the absence of wrinkled structures suggests a distinct fundamental population behaviour compared with the other evolved isolates from linages A, C and D. Therefore, plant polysaccharide and disaccharide could be a major factor, and possibly the only factor, causing the adaptation of evolved strains, and thus contribute to enhanced root colonisation.

As the evolved linages displayed altered differentiation properties and distinct biofilm formation in response to plant-derived polysaccharides and disaccharide, competitive colonisation between the ancestor and evolved isolates was directly quantified on *A. thaliana* seedlings.

Specifically, seedlings were seeded with combinations of ancestor and selected evolved isolates at a one-to-one ratio, carrying mKATE2 and GFP reporter, respectively. Within each evolved lineage, isolates displaying the highest root colonisation ability were selected for this assay. Concurrently, a control mixture of the ancestor labelled with two distinct fluorescent reporters was also assayed. Colonised biofilms were visualised by confocal laser scanning microscopy (CLSM) after incubation for 48 h with the seedlings. Unsurprisingly, evolved isolates had a competitive advantage over the ancestor in terms of root colonisation (Fig. 3c and d). Importantly, the ancestor strain was most significantly outcompeted by evolved lineages E and F, compared with the more subtle competitive advantage of the other three lineages. Strikingly, cells in lineages E and F showed elongated and aggregated morphology on plant roots (Supplementary Fig. 3d). Using a previously described method, the relative frequencies of each strain were quantified in root-colonised biofilms, based on total pixel volume[37]. The frequency of evolved lineages E and F presented at a ratio above 85%, while other evolved lineages displayed frequencies of 60 to 80% (Fig. 3d). By contrast, the ratio of control ancestor mixtures remained approximately equal (Supplementary Fig. 4a). Taken together, the competition assay results demonstrated that evolved strains were better at forming root colonisation biofilms than the ancestor. In lineages E and F, elongated cell bundles attained the highest fitness, almost excluding colonisation of the ancestor.

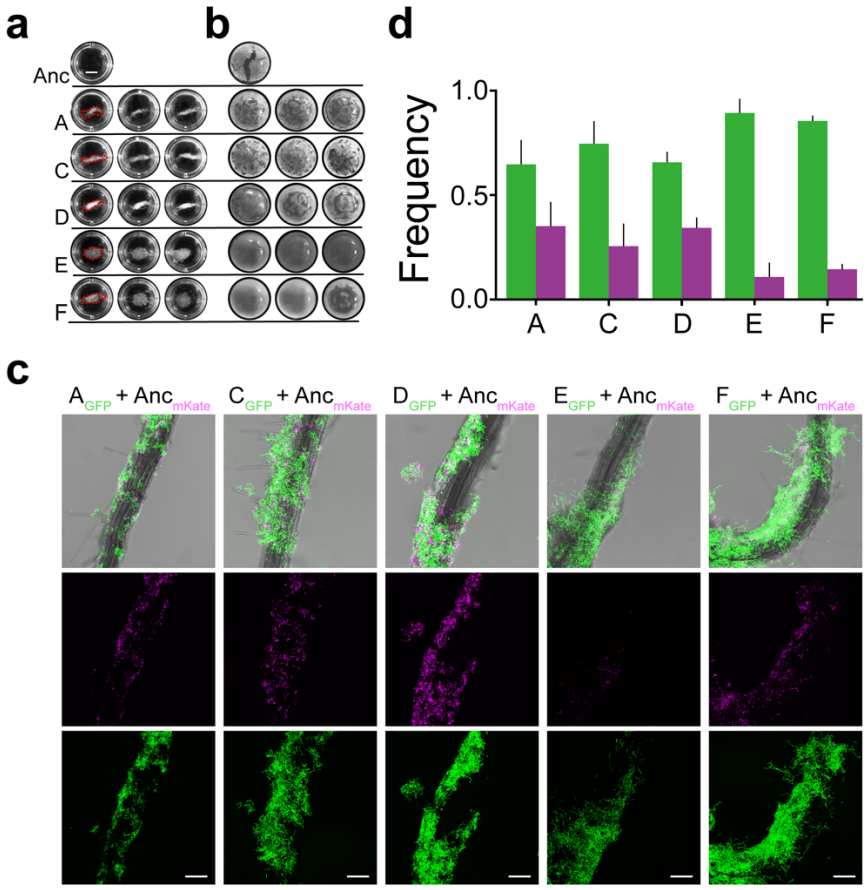

Fig. 3 Biofilm formation induced by plant polysaccharides is promoted in evolved lineages. a, Images of biofilm-aggregates in response to MSN medium supplemented with the plant polysaccharide cellobiose (0.5%) (MSNc). Biofilm analyses are representative of three biological replicates. Scale bar indicates 3 mm. **b,** Pellicle formation of the ancestor and evolved isolates induced in LB medium supplemented with the plant polysaccharide xylan (0.5%). Scale bar indicates 3 mm. **c,** *A. thaliana* roots colonised by a 1:1 mixture of Bt407 ancestor (magenta) and evolved isolates (green) imaged by CLSM. The top row shows an overlay of fluorescence channels and the bright field image. Images are representative from three independent seedlings. Scale bar indicates 50 µm. **d,** Frequencies of each strain in the root-colonised biofilm. Bars represent the mean (n = 3) and the error bars represent standard deviation. Colour codes are as in panel c.

Intriguingly, when competition assays lasted for two cycles (4 days), very few vegetative ancestral bacteria were captured by CLSM (Supplementary Fig. 4b). Conversely, the aggregates formed by evolved lineages E and F maintained the ability to colonise plants, showing delayed sporulation compared with the ancestor. This phenomenon was only observed for evolved lineages E and F. Ancestor-only cultures displayed a high degree of sporulation under these conditions. In view of the delayed sporulation observed for linage E and F, these results may suggest that increased competitive re-colonisation of roots could be due to both increased aggregation/biofilm establishment and delayed sporulation on the root surface. We assumed that increased aggregate formation and delayed sporulation are associated traits in response to elevated root colonisation. To test this hypothesis, we measured the sporulation kinetics of all strains in MSNc medium. In line with the results from CLSM imaging, cells of evolved lineages E and F exhibited the lowest sporulation efficacy at 96 h (Fig. 4a and b). Other evolved lineages also sporulated more slowly than the ancestor in MSNc medium, accompanied by an aggregating phenotype. Together, microscopic observations and sporulation frequency analyses suggested three distinct phenotypes for the ancestor and the evolved isolates (Fig. 4c). The aggregation phenotype typically provides bacteria or yeast with ecological benefits such as better nutrient uptake and protection from harsh environments[38,39], and based on our results, possibly increased biofilm formation on the plant root surface.

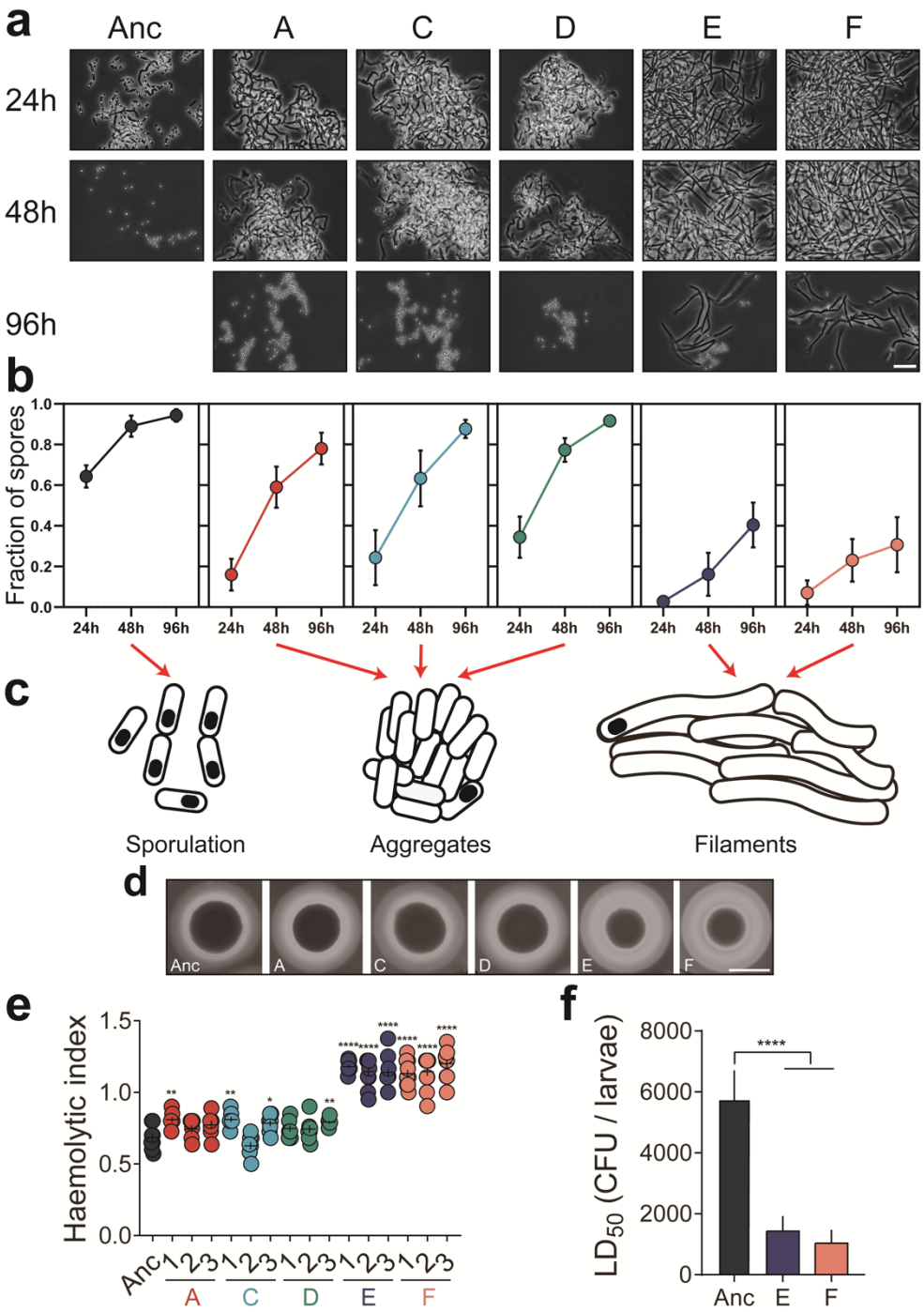

Fig. 4 Sporulation kinetics, aggregation response, and pathogenesis of the ancestor and evolved isolates. a, Microscopic observations of strains in MSNc medium harvested at different timepoints. Images are representative of three independent cultures. Scale bar indicates 10 μm. **b,** Sporulation percentage of strains in MSNc medium at three timepoints. Symbols represent the mean of three replicative assays, and error bars represent the standard deviation. **c,** Graphic representation of the

three distinct cell morphologies observed. **d,** Representative images of haemolytic activity from ancestor and evolved isolate colonies. Scale bar indicates 10 mm. **e,** Haemolytic indices (as described in Methods). The central values (horizontal lines) represent the mean (n = 8), while the error bars represent the standard error of the mean. Asterisks indicate significant differences between each group and the ancestor (*p <0.05; **p <0.01; ***p <0.001; ****p <0.0001; two-tailed t-test with Welch's corrections). **f,** LD_{50} values of ancestor and evolved isolates based on mortality toward insect larvae (*Galleria mellonella*). Bars represent mean LD_{50} values (n = 3), and error bars represent standard deviation. Asterisks indicate significant differences between each group and the ancestor (****p <0.0001; two-tailed t-test with Welch's corrections).

Plant-adapted E and F lineages display enhanced pathogenesis

Although *B. thuringiensis* is well known for its entomopathogenic traits, the strain used in our study is an acrystalliferous derivative of *B. thuringiensis* strain 407, and therefore less virulent toward insects than its corresponding wild-type strain. Like *B. cereus*, Bt407 Cry^- could be considered a potential opportunistic pathogen that may cause food poisoning through the synthesis of pore-forming cytotoxins haemolysin BL (Hbl), non-haemolytic enterotoxin (Nhe), and cytotoxin K (CytK)[40], for which genes are present in this strain. Expression of these toxin genes is regulated by various transcriptional regulatory systems such as PlcR, ResDE, Fnr and CcpA. Generally, these regulatory systems are synchronised with other bacterial behaviours including motility, biofilm formation and metabolism. In addition, differentiation to a swarmer cell, an attribute that was enhanced in the evolved linages, has been associated with increased virulence properties in *B. cereus*[41]. Thus, we wondered if the adapted Bt407 linages might also exhibit re-shaped virulence properties. Firstly, haemolytic activity of the evolved isolates was assayed on brain heart infusion (BHI) agar medium containing sheep blood. Evolved lineages E and F displayed an increased hemolytic zone, a sign of

more pronounced haemolysis, compared with the ancestor and other evolved lineages (Fig. 4d and e). Moreover, *in vivo* pathogenesis was tested against *Galleria mellonella*, a popular lepidopteran model, via injection into the haemolymph of insect larvae. Various concentrations of vegetative cells were employed to determine dose-response curves and LD_{50} values. Evolved isolates from linages E and F exhibited a significant several-fold increase in LD_{50} compared to the ancestor strain (Fig. 4f), consistent with the haemolytic activity assay results. Taken together, both *in vitro* and *in vivo* assays revealed enhanced virulence properties of evolved lineages E and F. Alteration in phenotypes such as motility and biofilm formation might be closely linked to the pathogenicity of evolved strains. However, exactly how alternative non-host niches (the plant rhizosphere in this case) may affect the investment in cooperative virulence remains relatively unexplored. In *Pseudomonas aeruginosa*, it has been shown that the formation of aggregates confers enhanced virulence by selective upregulation of quorum sensing systems[42]. Accordingly, autoaggregation of evolved lineages E and F may result in increased synthesis or secretion of effectors that promote virulence.

Genomic analysis reveals key mutations related to evolutionary adaptation

To identify whether evolved isolates harboured mutations responsible for the observed evolved phenotypic properties, whole-genome sequencing (WGS) analysis was carried out on three isolates from each evolved linage and the ancestor. The analysis revealed a total of 58 mutations on the chromosome, most of which are non-synonymous and assigned to specific genes. Most mutations that were identified in all three isolates of a certain linage were related to metabolic processes such as peptidoglycan and amino acid metabolism (Supplementary Dataset 1). Specifically, mutations in evolved lineages A, C and D were present in genes related to peptidoglycan/cell wall metabolism. By contrast, lineages E and F harboured mutations in genes related to transcription and translation, which

led to the hypothesis that certain genes (e.g., *rho*) might be responsible for different cell fate decisions in these evolved lineages.

Unlike *Escherichia coli*, the Rho-dependent transcription termination gene in *B. subtilis* is non-essential[43]. Inactivation of *rho* alters the global transcriptome, but allows robust growth in rich medium[44], and the dispensability of Rho in transcription provides plasticity in *B. subtilis* fitness. Based on these previous observations, we speculated that Rho-dependent termination might play a similar role in Bt407, and the nonsense mutation within the *rho* gene in linages E and F might strongly influence diverse physiological processes. Notably, the observed mutation ($rho^{Glu54stop}$) in linages E and F presumably abrogates the full function of Rho since the stop codon is located before the RNA binding domain (Fig. 5a).

Reintroduction of a nonsense mutation in *rho* imitates the key phenotypes of evolved linages

To dissect important genotype-phenotype relationships, the nonsense mutation in *rho* was targeted, given its pivotal role in differentiation processes in *B. subtilis*[45]. The $rho^{Glu54stop}$ mutation (Fig. 5a) observed in linage E and F was reintroduced into the Bt407 ancestor strain using homologous recombination. Similar to a previous study on *B. subtilis*[45], the nonsense mutation in *rho* impaired the swimming motility of Bt407, which partially imitated the severely reduced swimming radius of isolates from evolved lineage E (Fig. 5b). In line with the above results, the $rho^{Glu54stop}$ strain was unable to form robust pellicles, displaying a flat and less wrinkled biofilm structure (Fig. 5b).

In minimal medium[46], cell chaining and bundle formation of the constructed $rho^{Glu54stop}$ strain was comparable to isolates from evolved linage E (Supplementary Fig. 5a). Similarly, aggregate formation could also be observed in the $rho^{Glu54stop}$ strain in response to cellobiose, although not as robust as the evolved E and F linages (Fig. 5c). Additionally, the higher root colonisation ability of the $rho^{Glu54stop}$ strain was confirmed, demonstrated by elevated CFU values compared with the

ancestor (Fig. 5d). In agitated LB cultures, where ancestor cells were mostly dispersed, the *rho* mutant and the linage E isolate both displayed an elongated cell morphology and reduced cell separation, determined by cryo-SEM imaging (Supplementary Fig. 5b and c). Furthermore, inactivation of *rho* led to higher levels of haemolytic activity and insect larvae toxicity compared with the ancestor, in line with the results from evolved linages E and F, and suggesting that Rho directly or indirectly impacts the transcription profile of certain genes responsible for pathogenesis (Fig. 5e and f). Combining these results, we hypothesised that the altered differentiation properties of evolved E and F linages are primarily caused by the nonsense mutation in *rho*.

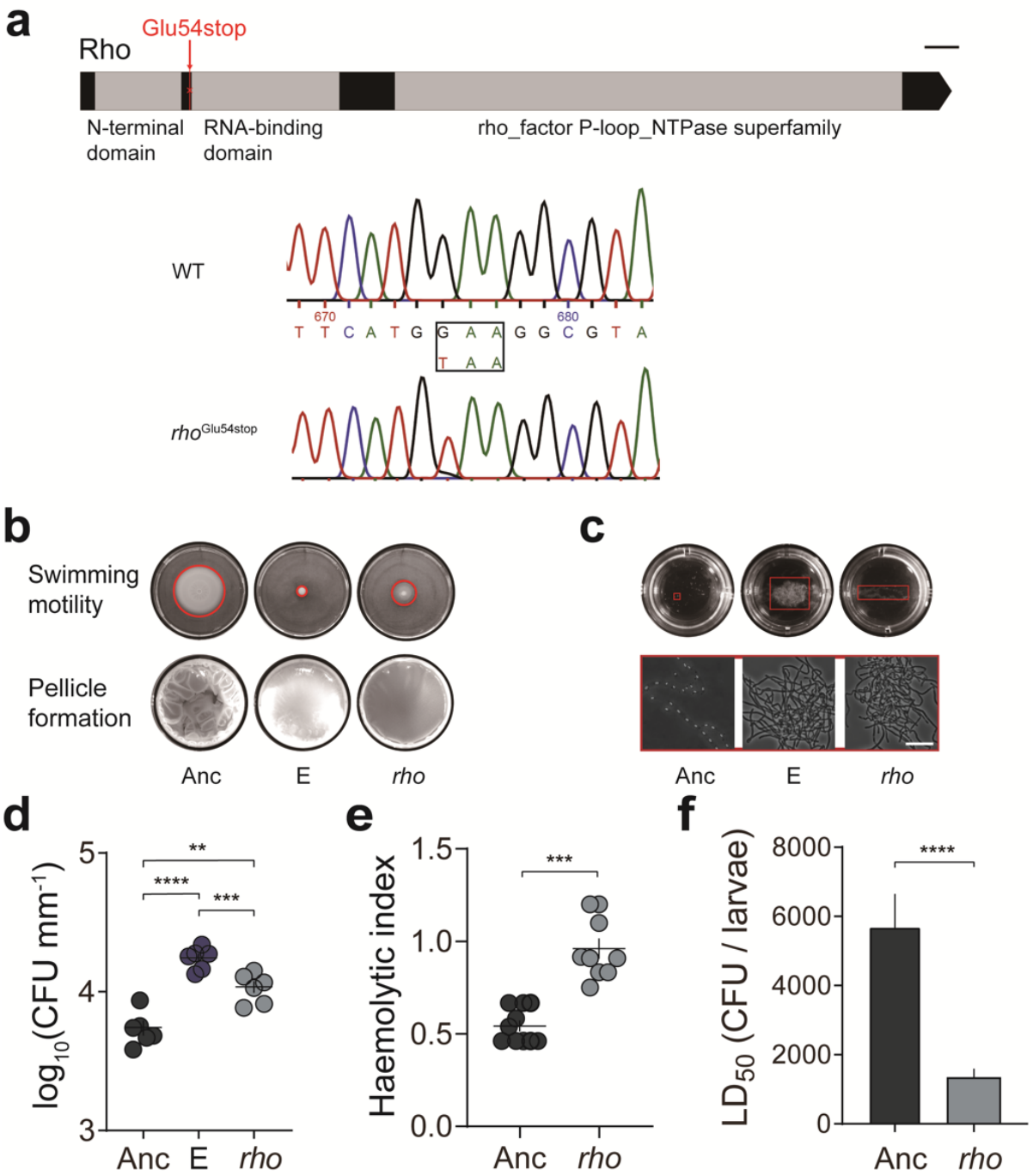

Fig. 5 The Rho transcriptional terminator plays a crucial role in cell fate decisions. a. Schematic illustration of the location of the *rho*^{Glu54stop} mutation. The conserved motifs were identified through the NCBI conserved domain database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Scale bar indicates 50 nt. **b,** Motility and biofilm formation in the *rho*^{Glu54stop} strain. Images are representatives of at least three independent replicates. **c,** The plant disaccharide cellobiose promotes

aggregate formation. Images are representative of at least three independent replicates. Scale bar indicates 10 µm**. d,** Increased plant colonisation of the E lineage isolate and the *rho*^{Glu54stop} strain. The central values (horizontal lines) represent the mean (n = 6 biologically independent seedlings for all groups) and the error bars represent the standard error of the mean. For multiple comparisons, ordinary one-way ANOVA and Tukey comparison tests were employed (**p <0.01; ***p <0.001; ****p <0.0001). **e,** Haemolytic activity is increased in the *rho*^{Glu54stop} strain. The central values (horizontal lines) represent the mean (n = 10 biologically independent sampled cultures) and the error bars represent SEM (***p <0.001; two-tailed t-test with Welch's corrections). **f,** *In vivo* toxicity of the *rho*^{Glu54stop} strain. The central values (horizontal lines) represent the mean (n = 3) and the error bars represent standard deviation (****p <0.0001; two-tailed t-test with Welch's corrections).

The nonsense mutation in *rho* reshapes the transcriptional landscape of *B. thuringiensis*

As a global modulator of transcript length, Rho is essential in certain species such as the enterobacterium *E. coli*, and mutations in *rho* alter cellular fitness in the presence of various nutrients and antibiotics[47,48]. Unsurprisingly, mutations in *rho* have been found in numerous laboratory evolution studies[49–52]. In *B. subtilis*, Rho-mediated transcriptome changes affect different cell differentiation programs such as cell motility, biofilm formation, sporulation, and antibiotic resistance[45,53].

In order to reveal a potential molecular mechanism for how Rho affects the global gene expression profile of Bt407, we compared the transcriptomes of the ancestor strain, one isolate from evolved lineage E, and the *rho*^{Glu54stop} strain. Analysis of the transcriptomic data revealed that 377 and 270 genes were significantly (5% false discovery rate threshold) up- or downregulated, respectively, in the evolved isolate from linage E compared with the ancestor (Supplementary Dataset S2). In the case of the *rho*^{Glu54stop} strain, 523 and 378 genes were up- or downregulated, respectively.

Most importantly, the evolved strain and the *rho*^{Glu54stop} strain shared a similar pattern of differentially expressed genes (DEGs) and Gene Ontology (GO) terms categories (Fig. 6a–c).

As expected based on the phenotypic assays, haemolytic activity, and *in vivo* toxicity assays, genes with GO terms related to motility and chemotaxis were downregulated, while genes associated with pathogenesis were significantly upregulated, in both the evolved isolate and the *rho*^{Glu54stop} strain, compared with the ancestor (Fig. 6c, d, and Supplementary Dataset 3). In parallel with linages E and F harbouring mutations in genes related to the basic cellular processes of transcription and translation, downregulation of genes involved in translation, and to a lesser extent transcription, was observed in the transcriptome analysis (Fig. 6c). Adaptation of metabolic pathways related to plant polysaccharides was implied by the upregulation of genes responsible for the metabolism of various carbohydrates such as cellobiose, pyruvate and galactose (Fig. 6d, Supplementary Dataset 3). For instance, UDP-galactose, which is generated by the enzyme GalE, may serve as a substrate for the production of the extracellular polymeric substance (EPS) matrix, thereby helping microbes to form biofilms on plants. In *B. subtilis*, a *galE* mutant exhibited a decreased level of root colonisation[26]. Herein, upregulation of *galE* in the evolved isolate and the *rho*^{Glu54stop} strain may imply that the higher production rate of galactose contributes to enhanced root colonisation in the evolved strains. Furthermore, RNA-seq data verified the adaptive cellobiose-related metabolic process in the evolved isolate and the *rho*^{Glu54stop} strain (Fig. 6d), concurrent with our hypothesis that adaptive metabolism of cellobiose successfully led to enhanced root colonisation ability of the evolved isolates. Notably, the cellobiose-specific phosphotransferase system in *Klebsiella pneumoniae* contributes to biofilm formation[54]. We reasoned that altered carbohydrate utilisation may also influence sporulation of the *rho*^{Glu54stop} strain, for which only ~40% of cells were sporulating after 48h in MSNc medium (Supplementary Fig. 5d and e). Our transcriptome data revealed that genes related to carbohydrate metabolism were upregulated in evolved isolates exhibiting more extensive root colonisation.

Exploring the genomic features of bacterial adaptation to plants revealed that genomes of plant-associated bacteria encode significantly more carbohydrate metabolism functions than non-plant-associated bacterial genomes[55]. In summary, we hypothesise that elevated carbohydrate metabolism and altered cellular physiology led to more pronounced aggregate formation by the *rho* mutant, providing higher fitness for root colonisation.

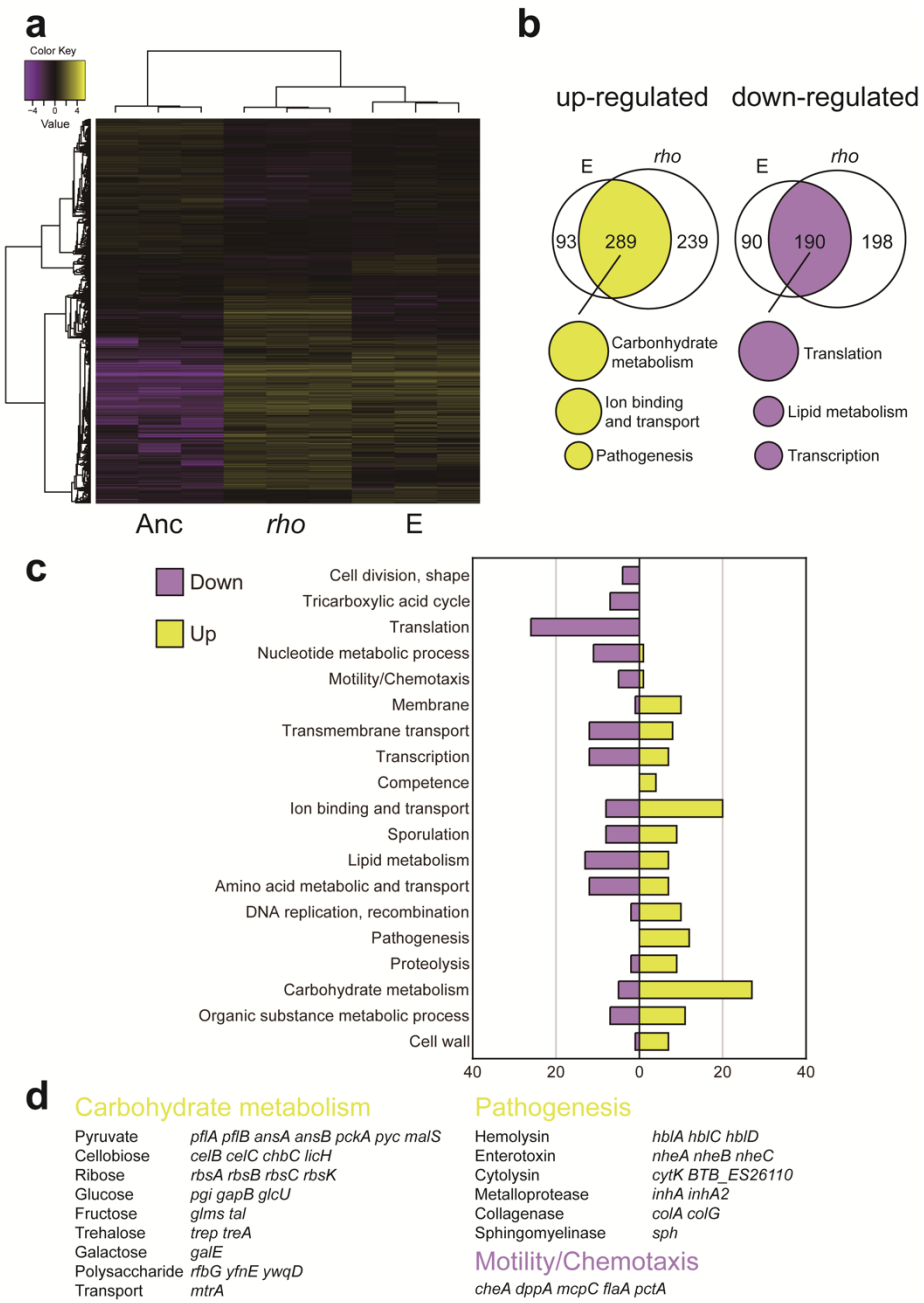

Fig. 6 An evolved isolate from linage E and the *rho*^{Glu54stop} strain share similar gene expression patterns with the ancestor. a, Heatmap showing the relative expression levels of differentially expressed genes (DEGs) among the strains (n = 3 biologically replicates for each strain). TMM-normalised FPKM gene expression values were hierarchically clustered according to samples and genes. In the map, FPKM values are log2-transformed and median-centred by gene. The colour key gives the log2 value scale (negative and positive values represent gene expression below and above the median, respectively). **b,** Venn diagrams of genes up- or downregulated in the evolved isolate from lineage E and the reconstructed *rho*^{Glu54stop} strain, compared with the ancestor. Top functions of grouped DEGs are listed underneath. **c,** Gene Ontology (GO) terms of the corresponding DEGs in the evolved strain and the *rho*^{Glu54stop} strain. **d,** Representative functions and related DEGs. Yellow and magenta indicate up- and downregulated expression, respectively, in the evolved strain and the *rho*^{Glu54stop} strain, relative to the ancestor.

Conclusion

An EE approach was designed to investigate the genotypic and phenotypic evolution of *B. thuringiensis* as biofilms associated with *A. thaliana*. Upon interaction with plant seedlings, evolved isolates accumulated mutations related to specific metabolic pathways, providing them with higher fitness during root colonisation. Certain evolved linages acquired a loss-of-function mutation, leading to disrupted Rho-dependent transcription termination, which eventually facilitated adaptive responses to a constantly changing environment, and efficient recolonisation of plant seedlings. Fortuitously, loss of Rho function begat enhanced pathogenesis of these plant-adapted linages, highlighting how pathogenesis may shift during environmental adaptation in the *Bacillus cereus* group. In nature, bacteria tend to form multicellular aggregates in order to survive environmental stressors[56,57]. When cultivated in both soil and liquid soil extract, *B. cereus* employs a multicellular behaviour to grow

and translocate[58]. Therefore, evolved isolates form dense aggregates in response to plants or plant polysaccharides, implying that plant-derived carbon may provide a driving force during laboratory evolution.

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Methods

Bacterial strains, media and cultivation conditions

Supplementary Table 1 includes all bacterial strains, plasmids and primers used in this study. Bacterial strains were routinely cultured on Lysogeny broth (LB-Lennox, Carl Roth; 10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl) plates (1.5% agar) and stored at −80 °C with 28% glycerol added. The following antibiotic concentrations were applied for cloning and plasmid maintenance: kanamycin (50 μg/mL), ampicillin (100 μg/mL), erythromycin (5 μg/mL), and tetracycline (10 μg/mL). X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at 40 μg/mL.

A. thaliana used in this study is belonging to ecotype Col-0. Plant seeds (around 30) were routinely surface-sterilized in 2 mL centrifuge tubes using ethanol (70%, vol/vol) for 10 min. Subsequently, seeds were further sterilized in 1 mL sodium hypochlorite (1 % , vol/vol) and vigorously mixed using an orbital mixer for 15 minutes, thereafter seeds were washed in sterile distilled water by repeated centrifugation and removal of supernatant for 5 times. After sterilization, seeds were suspended in 100-200μl of water, subsequently planted on Murashige and Skoog basal medium (Sigma) 0.5% agar plates with 0.05% glucose. Plates were sealed with parafilm and incubated at 4°C for 3 days, followed by placing in a plant chamber with an angle of 65° (21°C, 16h light and 20°C, 10h dark). After 6 days, comparable seedlings ranging from 0.8-1.2cm in length were selected for subsequent experiments.

Construction of *rho* mutant

Mutant with specific point nucleotide changes was created by homologous recombination using a marker-less replacement method described by Janes and colleagues[59]. First, the homologous fragments with desired mutations were PCR amplified and cloned into the modified pMAD shuttle vector[60], which contains additional I-SceI restriction site[61] (kindly provided by Dr. Toril Lindbäck). A successful clone carrying the intended mutation in *rho* gene was verified by Sanger sequencing (Eurofins Genomics) and electroporated into Bt407 to obtain blue colonies on erythromycin/X-gal plates. Integration of the vector into the chromosome was stimulated as described by Janes and colleagues[59]. Subsequently, pBKJ223 encoding I-SceI restriction enzyme was introduced resulting in double-stranded break at the chromosome and promoting a second recombination event[62]. Finally, genomic DNA was extracted from white colonies those that have lost erythromycin resistance, and mutation was verified by Sanger sequencing of PCR fragment spanning the region of interest.

EE of root colonization

EE involving six parallel replicates was performed with an acrystalliferous derivative of *B. thuringiensis* 407 (Bt407cry-, referred as Bt407). The setup devised here was adapted from the concept of long-term biofilm EE on polystyrene beads[6], but instead of inert substrate, plant root was used for subsequent colonization by Bt407. The root colonization was carried out as described generally[26]. Week-old *A. thaliana* seedlings (around 10 millimeter) were transferred into 300 uL of MSNg medium (5 mM potassium phosphate buffer, 100 mM MOPS, 2 mM MgCl2, 0.7 mM CaCl2, 0.05 mM MnCl2, 1 µM ZnCl2,2 µM thiamine, 0.2% NH4Cl plus 0.05% glycerol as carbon source) in 48-well plates subsequently inoculated with Bt407 culture (OD600 = 0.02). Then the plates were put on a laboratory shaker at 90 rpm in the climate chamber. After 48h, the seedlings with mature biofilm were washed gently to remove unattached cells in fresh MSNg medium, before they were

transferred to a new plate hosting fresh medium and seedlings. When there were two seedlings floating in the medium, adhered cells immigrate from the old seedling to the new one. Every 48h, the procedure was repeated to facilitate repetitive rounds of root colonization. Colony forming units (CFU) per millimeter defined here as biofilm productivity were assessed regularly for the old seedlings. For this, the seedlings were washed and mixed with 100 μL glass sand in 1 mL 0.9% NaCl buffer, vortexed for 5min vigorously. The bacterial solution was diluted and plated for recording CFU. After 40 transfers, biofilm cells on the newly colonized plants were plated, and three single colonies were randomly selected and preserved for later analysis.

Biofilm assays

For pellicle formation assay, Bt407 cells were cultured overnight in LB medium at 37°C, subsequently diluted 1:100 in 3 mL of LB, allowed to grow up to $OD_{600} < 0.5$, after which bacterial cultures were adjusted to $OD_{600} = 0.3$. Pellicle formation was assayed in 1 ml LBGM or xylan supplemented LB media in a 24-well microtiter plate. LBGM was formulated as an efficient biofilm-inducing media for *B. cereus* group bacteria, which include glycerol (1%) and $MnSO_4$ (100 μM) besides LB broth. For xylan supplemented LB medium, xylan was used at a 0.5% (w/v) as plant-derived polysaccharide to induce pellicle formation. For each well of microtiter plates, 10 microliters of adjusted cultures were added into the medium and incubated at 30°C for 48 hours. Images were taken using a Panasonic DC-TZ90 camera.

Submerged biofilm formation was evaluated using a low nutrient medium (EPS, described previously[63]). Overnight cultures of different strains were adjusted at OD600 of 0.2, 100 μL of which was added into 24-well plates containing 2 mL of EPS medium, and incubated at 30°C at 50 rpm for 20h. For quantitative analysis, total growth was measured at OD_{620}. After that, all planktonic cultures were removed and plates were washed with 0.9% NaCl buffer. After air-drying, adhered biofilm were

stained with crystal violet solution (0.3%, 2mL). After the solution was discarded from the wells, the unbound stain was removed with washing, the biofilm bound crystal violet was solubilized with 70% ethanol, and its absorbance was detected at OD_{590}.

Motility assays

Soft agar plates (0.3% agar) were prepared for swimming assays. Overnight cultures of different strains were adjusted to OD_{600} of one, five μL were spotted on plates, and subsequently, the plates were incubated at 30°C. The swimming radius was measured after 20h. Swarming assay was conducted on TrA medium (1% tryptone and 0.5% NaCl) containing 0.7% agar. Similar to swimming assay, overnight cultures of strains were adjusted to OD_{600} of 1, then 5 μL were spotted on TrA agar medium, followed by incubation at 30°C. Images were obtained by an Axio Zoom V16 stereomicroscope (Carl Zeiss) equipped with a 0.5 × Plan Apo objective and LED cold-light sources.

Sporulation assay

Sporulation efficiency was evaluated in a defined sporulation medium HCT[64] and MSNc[26]. Briefly, overnight cultures of strains were diluted in 10 mL sporulation medium to obtain the exponentially growing cultures. Flasks were incubated at 30 °C and 200 rpm and at certain time points samples were harvested and sonicated to disrupt cell aggregates (2 × 12 pulses of 1 s with 30% amplitude; Ultrasonic Processor VCX-130, Vibra-Cell, Sonics, Newtown). Half of the sample was heated at 70 °C for 20 min or left untreated, and both were serially diluted with 0.9% NaCl buffer and plated at LB agar plates. The sporulation efficiency was represented by the fraction of spores, calculated as the ratio of CFU in heat-treated compared to untreated samples.

Haemolytic assays and insect larvae experiments

Haemolytic index was measured using 1.5% BHI agar plates supplemented with 5% defibrinated sheep blood (Thermo Scientific). After overnight incubation at 30°C, the hemolysis area and colony sizes were determined using ImageJ software and the hemolytic index was calculated as previously described[65]. For virulence experiment, wax moth larvae (*Galleria mellonella* larvae, obtained from Creep4you A/S) were used. Four dilutions (approximately 10^3 to 10^6 of cells) of overnight cultures were prepared, corresponding CFU was determined, and 10 µL of each dilution were injected via the hindmost left prolegs of larvae using 10-µL Hamilton syringes, while the control larvae were treated with 0.9% NaCl buffer. The experiment was repeated 3 times with a minimum of 20 larvae in each group. After incubation at 37°C for 24h, the mortality of each group was recorded. The LD_{50} of each strain was evaluated based on mortality data and calculated by the probit regression analysis employing IBM SPSS Statistics 20.

Microscopy

For bright-field images of pellicles and colonies, Axio Zoom V16 stereomicroscope (Carl Zeiss) was used, equipped with a Zeiss CL 9000 LED light source, a PlanApo Z 0.5 × objective, and AxioCam MRm monochrome camera (Carl Zeiss).

Confocal laser scanning microscopy imaging was performed as described previously[37]. For root attached biofilms, colonized plants were washed with sterilized ddH_2O twice and placed onto glass slides. Images were obtained using a 63 × /1.4 OIL objective. Fluorescent reporter excitation was performed with the argon laser at 488 nm and the emitted fluorescence was recorded at 484–536 nm and 567–654 nm for GFP and mKate, respectively. Z stack series were requited with 1 µm steps and stacks were merged using ImageJ software.

For cryo-electron microscopy imaging, bacteria were grown overnight at 30°C, OD_{600} was adjusted to 1 using NaCl buffer (0.9%),1 ml of the adjusted cultures were centrifuged, washed three times using NaCl buffer (0.9%), and the resulting cell pellets were subjected to cryo-SEM analysis. Cryo-fixation was performed via high pressure freezing (HPF) before observed by cryo-SEM. The HPF was performed by the HPF instrument HPM100 (Leica Microsystems) in standard conditions. After freezing, the sample was mounted into a cryo-sample holder in liquid nitrogen attached to a Leica VCT100 cryo transfer arm (Leica Microsystems) and transferred into a Leica MED020 freeze-fracture and coating system. After the freeze-fracturing in the MED20 cryo-preparation chamber (Leica Microsystems), the biofilm freezing samples were sublimated at -90°C for about 90 sec and coated with 6 nm of a C/Pt alloy. The biofilm samples were then transferred by the VCT 100 shuttle (Leica Microsystems) into a Quanta 3D FEG cryo-SEM (Thermo Fisher Scientific) and observed at 2kV at -160°C. The cryo-SEM imaging was performed at the Core Facility for Integrated Microscopy, Copenhagen University, Denmark. The image analysis and measurement of the bacteria length were performed by using ImageJ software.

Genome resequencing

Genomic DNA were extracted from overnight cultures using 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. thuringiensis* Bt407 genome (GenBank accession no. CP003889.1). Identified mutations in each strain are listed in Supplementary Dataset 1. Raw sequencing data has been deposited to the NCBI Sequence Read Archive (SRA) database under BioProject accession number: PRJNA673616.

RNA extraction and transcriptome analysis

Overnight grown strains were diluted to an OD_{600} of 1.0 in LB medium, grown to late-log phase, cultures were collected by centrifuging, and flash frozen in liquid nitrogen. Total RNA was extracted by combining phenol-chloroform–isopropanol treatment using the High Pure RNA Isolation Kit (Roche, Germany)[66]. Concentration and quality of extracted RNA was examined by Nanodrop and Agilent RNA 6000 Nano Chips. Ribo-off rRNA Depletion Kit (Bacteria) (Vazyme Biotech) was used to deplete ribosomal RNA. *In vitro* fragment libraries were prepared using the Illumina Truseq RNA Library Prep Kit v2, library qualities were controlled by Agilent Tapestation 2200. Paired-end fragment reads were generated on an Illumina NextSeq sequencer using TG NextSeq® 500/550 High Output Kit v2 (300 cycles). Reads were quality-trimmed using an error probability of 0.05 (Q13) as threshold.

Transcriptome reads were first pre-processed with cutadapt 1.1 (http://code.google.com/p/cutadapt/) to remove remains of Illumina adapters (error rate 10% and overlap ≥10 nt; "-e 0.10 -O 10"), then quality-trimmed using prinseq-lite[67] to remove the first 10 bases, trim from both ends with a quality threshold ≥ 20, and retain reads with a mean quality ≥ 28 and a length ≥ 30 nt ("-trim_left 10 -trim_qual_right 20 -trim_qual_left 20 -min_qual_mean 28 -min_len 30"). Read pairs for which both

mates passed the QC checks were recovered using cmpfastq.pl (http://compbio.brc.iop.kcl.ac.uk/software/cmpfastq.php), were generated, followed by differential gene expression analyses using Trinity 2.10.0 with RSEM 1.3.3 to estimate gene/transcript abundances and DESeq2 v1.22.2 run in R 3.5.3 (http://www.R-project.org/) for statistical testing[68]. In RSEM, the bowtie2[69] v2.4.1 aligner was employed to map (with a max. insert size of 1000; "--max_ins_size 1000") the read pairs onto the annotated protein-coding (CDS) transcript sequences of Bt407 (GenBank: GCF_000306745.1, file "cds_from_genomic.fna"). Genes that have p-value adjusted for multiple testing (p_{adj}; corresponding to the false discovery rate [FDR]) ≤ 5% were considered as DEGs and included for further analysis (Supplementary Dataset S2). A heatmap with hierarchical tree clustering of genes and replicate samples according to gene expression profiles was computed using the "analyze_diff_expr.pl" utility script in Trinity[65]. For that, gene/transcript abundances were converted (with the "run_TMM_normalization_write_FPKM_matrix.pl" utility) to TMM-normalized (trimmed mean of M values) FPKM (fragment per kilobase per million mapped reads) expression values. The Pearson correlation coefficient was chosen as distance metric and average linkage was chosen as clustering method (options "–sample_dist sample_cor –sample_cor pearson –sample_clust average"). A log2 cut-off of 0 and a p-value cut-off of 0.05 were set ("-C 0 P 0.05") to select DEGs (other options included "--min_rowSums 10 --min_colSums 0"). Functional analysis of DEGs was carried out using the Genome2D webserver[72] and GSEA-Pro v3.0 webserver (http://gseapro.molgenrug.nl/), combining FACoP (http://facop.molgenrug.nl/) and database (UniProt and SubtiWiki) information (Supplementary Dataset S3).

Raw sequencing data have been deposited to the NCBI Sequence Read Archive (SRA) database under BioProject accession number: PRJNA673616.

Statistical analysis

Unless indicated otherwise, all experiments were performed with at least three biological replicates. Statistical analysis of bacterial traits comparison between evolved isolates and the ancestor (e.g., root colonization assays) was analyzed and illustrated using Graphpad Prism 8. Specifically, the statistical differences were calculated using Student's *t* test with Welch's correction (assuming unequal s.d.). For comparison across multiple groups (e.g. comparison among the ancestor, evolved strain E and constructed *rho* mutant), ordinary one-way ANOVA analysis and Tukey tests were employed.

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Competing Interests

The authors declare that there are no competing financial interests in relation to the work described.

Authors contributions

Y.L. and Á.T.K. conceived the project; Y.L. performed experiments; M.A. and J.K.Y.P created mutant strain; M.E.R. performed the cryo-SEM analysis; G.M., N.J.T. and O.A.Ø. contributed to data analysis; Y.L. and Á.T.K. wrote the manuscript, with all authors correcting the final version.

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