Cheater-mediated evolution shifts phenotypic heterogeneity in *Bacillus subtilis* biofilms

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Running title: Evolution of phenotypic heterogeneity in biofilms

Keywords: *Bacillus subtilis*, biofilm, phenotypic heterogeneity, experimental evolution, exopolysaccharide

ABSTRACT

Biofilms are closely packed cells held and shielded by extracellular matrix composed of structural proteins and exopolysaccharides (EPS). As matrix components are costly to produce and shared within the population, EPS-deficient cells can act as cheaters by gaining benefits from the cooperative nature of EPS producers. Remarkably, genetically programmed EPS producers can also exhibit phenotypic heterogeneity at single cell level. For instance, mature biofilms of *Bacillus subtilis* contain cells in an 'ON' state, expressing extracellular matrix genes, as well as cells in an 'OFF' state. Previous studies have shown that spatial structure of biofilms limits the spread of cheaters, but the long-term influence of cheating on biofilm evolution is not well understood. In addition, the influence of cheats on phenotypic heterogeneity pattern within matrix-producers, was never examined. Here, we examine the influence of EPS non-producers (cheaters) on phenotypic heterogeneity of matrix production within the populations of EPS producers (cooperators). We discovered that cheater-mediated evolution in pellicles leads to a transient shift in phenotypic heterogeneity pattern of cooperators, resulting in larger numbers of cells sustaining very high *eps* expression as depicted by hyper ON phenotype. Although, hyper ON strategy seems adaptive in presence of cheats, allowing favorable positioning of the WT in pellicles, it is soon substituted by hyper OFF phenotype and/or soon after by population collapse. This study provides additional insights on how biofilms adapt and respond to stress caused by exploitation in long-term scenario.

INTRODUCTION

Cooperative interactions are prevalent for all life forms [1], even for simple microbes that often exist in communities of matrix bound surface-attached cells called biofilms [2–6]. However, when costly products such as siderophores [7, 8], extracellular polymeric substances [9, 10], digestive enzymes [11], and signaling molecules [12, 13] are secreted and shared, cooperative behavior

becomes susceptible to cheating [2, 14–16], where mutants defective in cooperation can still benefit from cooperative community members [4, 5, 17]. It has been shown that spatially structured biofilms, where interactions with clonemates are common and diffusion of public goods is limited, may serve as natural defense against cheating [18–20]. However, long time scale studies have recently reported that biofilm defectors can spontaneously emerge and spread in biofilms by exploiting other matrix-proficient lineages [21–24]. In fact, a pioneering microbial evolution study on *Pseudomonas fluorescens* has already pointed towards dynamic evolutionary interplay between cooperation and exploitation in a biofilm mat [25], where emergence of cellulose overproducer (Wrinkly) allowed mat formation, but also created an opportunity for exploitation by non-producers (Smooth), eventually leading to so called 'tragedy of the commons' [4, 26, 27].

Taken together, biofilms are a suitable model to understand social interactions in an evolutionary time scale [23, 28–31]. Modelling and empirical data confirm that mutualism (beneficial to both actor and recipient) and altruism (beneficial to recipient but not to actor) play crucial roles in biofilm enhancement [32] but at the same time can lead to biofilm destabilization [25]. Can cooperators evolve tactics to evade exploitation and in turn, can cheats utilize evolution to enhance their selfish actions?

Recent studies showed that in well-mixed environment, cooperators adapt to cheats by reducing cooperation [14, 15, 33]. Such reduction could be achieved by various strategies, for instance decrease in motility [15], down regulation or minimal production in public goods [14, 15, 33], up-regulation of other alternative public goods [14], or bi-stable expression in virulence gene [2]. Interestingly, populations of cooperators often exhibit phenotypic heterogeneity at the single cell level [34, 35]. Therefore, an alternative and simple mechanism to modulate levels of cooperation in a population would be through changes in phenotypic heterogeneity pattern. However, the long-term effects of cheats on costly goods' expression at individual cell level have

never been examined. Understanding how heterogeneity of gene expression within the population is affected in the presence of cheats would provide better insight on microbial adaptation and stress response mechanisms.

Here, we address this question using pellicle biofilm model of *Bacillus subtilis* [36, 37]. Pellicle formation in *B. subtilis* involves, amongst others, aerotaxis driven motility and subsequent matrix production [38]. Aerotaxis is important for oxygen sensing to aid cells reach the surface, while matrix formation is significant to sustain cells to adhere to the surface and to each other. Exopolysaccharide (EPS) is a costly public good in *Bacillus subtilis* biofilms [10, 18, 39] and is heterogeneously expressed during biofilm formation with approximately 40% of cells exhibiting the ON state [39, 40]. We aimed to investigate the cheat-dependent alteration related to phenotypic heterogeneity in *eps* expression by the producer.

We reveal that cheating mitigation by the EPS producers involves a shift in phenotypic heterogeneity towards increased frequency of *eps*-expressing cells, which is achieved by a loss-of-function mutation in a single regulatory gene. We also demonstrate that although 'hyper ON' phenotype helps to reduce cheating, it is rapidly replaced by hyper OFF strategy. Our study uncovers an alternative anti-cheating mechanism based on changes in phenotypic heterogeneity and highlights meandering trajectories prior cooperation collapse.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Strain *B. subtilis* 168 $P_{hyperspank}$-mKATE P_{eps}-GFP (TB869) was obtained by transforming the laboratory strain, *B. subtilis* 168 $P_{hyperspank}$-mKATE (TB49) [10, 18], with genomic DNA from NRS2243 (*sacA*::P_{epsA}-*gfp*(Km)*hag*::cat) and selecting for Km resistance. The Δ*rsiX* strain with fluorescence reporters (TB959) was obtained by transforming TB869 with genomic DNA isolated from BKE23090 (168 *trpC*2 Δ*rsiX*::erm) [41]. Strains were maintained in LB medium (Lysogeny

Broth (Lennox), Carl Roth, Germany), while 2×SG medium was used for biofilm induction [10]. The Δ*eps* strains (TB608) was created previously [10].

Experimental evolution

Eight biological replicates of the co-cultures of 1:1 ratio of *B. subtilis* TB869 and TB608 were grown in 48-well plate containing 1ml 2×SG medium at 30°C for two days. Pellicles were harvested into Eppendorf tubes containing 500 µl sterile 2×SG medium and 100 µl of sterile glass sand, vortexed for 90 seconds, 10 µl fraction was transferred into 1ml 2×SG medium of a 48 well plate and incubated at 30°C static condition for two days. This cycle was continuously repeated 35 times. As control, four biological replicates of mono-cultures of *B. subtilis* TB869 were evolved using the same transfer method. Every 5th transfer cycle, harvested cultures were mixed with 15% glycerol and stored at –80°C.

Population ratio assay

At every 5th transfer, pellicle biofilm productivities and relative frequencies of mutants and WT were qualitatively assessed (colony forming units (CFU)/ml) using LB agar containing selective antibiotics. LB agar plates were incubated at 37°C for 16 h and colonies were counted. Three single clones of WT and of *Δeps* per population per timepoint were isolated from plates and stored at –80°C in the presence of 15% glycerol.

Quantitative assessment of hyper ON and hyper OFF populations

Ninety-five single isolates of ancestor or evolved TB869 strains obtained from the population per timepoint were allowed to form pellicles in 96-well PCR plate containing 100µl 2×SG medium. After incubation at 30°C for 24 h, the plate was vortexed for 90 seconds, bath sonicated (mini ultrasonic bath, Carl Roth, Germany) for 5 min and fluorescence was recorded using TECAN

Infinite F200 PRO microplate reader (excitation at 485/20 nm and emission at 535/10 nm for GFP; excitation at 590/20 nm and emission at 635/35 nm for RFP; using manual gain of 35 and 50 for GFP and RFP, respectively). Hyper ON and hyper OFF phenotypes were categorized based on cut off value of the WT ancestor mean ± 1SD. GFP intensity values above 134 arbitrary units (AU) were considered as hyper ON and values below 70 AU were categorized as hyper OFF.

Pellicle competition assay/Fitness assay

Competition assays were performed as previously described [10]. Specifically, strains of interest were premixed at 1:1 ratio based on their OD_{600} values and the mixture was inoculated into 2×SG medium at 1%. Cultures were grown for 48h under static conditions at 30°C and their relative frequencies were accessed using CFU counts (and selective antibiotics).

Stereomicroscopy to assess competition of WT and Δ*rsiX* against Δ*eps*

Fluorescent images of pellicles were obtained with an Axio Zoom V16 stereomicroscope (Carl Zeiss, Jena, Germany) equipped with a Zeiss CL 9000 LED light source and an AxioCam MRm monochrome camera (Carl Zeiss) and HE eGFP (excitation at 470/40 nm and emission at 525/50 nm), and HE mRFP (excitation at 572/25 nm and emission at 629/62 nm) filter sets. Images were taken at 3.5× and 55× magnifications. The exposure times for green and red fluorescence were set up to maximal possible values before reaching overexposure, using range indicator function. Zeiss software was used to obtain overlaid, artificially colored images of both fluorescence channels.

Qualitative assessment of Hyper ONs and Hyper OFFs via microscopy

Single clones of evolved WT (TB869) obtained from population ratio assay were allowed to form 1-day old pellicle. Harvested pellicles were subjected to microscopic analysis using an Axio Observer 780 Laser Scanning Confocal Microscope (Carl Zeiss) equipped with a Plan-Apochromat 63×/1.4 Oil DIC M27 objective, an argon laser for stimulation of fluorescence (excitation at 488

for green fluorescence and 561 nm for red fluorescence, with emission at 528/26 nm and 630/32 nm respectively). Zen 2012 Software (Carl Zeiss) and FIJI Image J Software [42] were used for image recording and processing, respectively.

Flow cytometry and data analysis

Frozen stocks of evolved populations were transferred onto LB-agar plates containing kanamycin (5µg/ml) to select solely for WT colonies. The plates were incubated overnight at 37°C, followed by inoculation of 10 randomly selected single colonies into 2×SG medium. After 24h-incubation at 30°C, the pellicles were harvested, sonicated and diluted accordingly. Flow cytometry was performed using BD FACSCanto II (BD Biosciences). To obtained average distribution of *eps* expression within populations, data obtained for all single isolates were subjected to binning using identical bin size. Next, a mean count for each bin by averaging individual counts within this bin obtained for single isolates, resulting in mean distribution of single cell level *eps* expression per population.

Genome re-sequencing and genome analysis

Genomic DNA of single clones from selected evolved populations were isolated using Bacterial and Yeast Genomic DNA kit (EURx) directly from –80°C stocks grown in LB medium for 5 h at 37°C with shaking at 220 rpm. For population sequencing analysis, approx. 100 colonies belonging to the evolved populations were harvested into 2ml LB broth and incubated at 37°C shaking at 220 rpm for 2-3 h. Re-sequencing was performed on an Illumina NextSeq instrument using V2 sequencing chemistry (2x150 nt). Base-calling was carried out with "bcl2fastq" software (v.2.17.1.14, Illumina). Paired-end reads were further analyzed in CLC Genomics Workbench Tool 9.5.1. Reads were quality-trimmed using an error probability of 0.05 (Q13) as the threshold. Reads that displayed ≥80% similarity to the reference over ≥80% of their read lengths were used in mapping. Quality-based SNP and small In/Del variant calling was carried out requiring ≥10× read coverage with ≥25% variant frequency. Only variants supported by good quality bases (Q ≥

30) on both strands were considered. Gene functions (product names) in *SI Appendix* Datasets were reported based on SubtiWiki [43].

RESULTS

'Hyper ON' matrix producers emerge during evolution with cheats

Exopolysaccharide EPS is one of the major components of *B. subtilis* biofilm matrix and mutants deficient in EPS production (Δ*eps*) are not able to form pellicle biofilms (Supplementary Fig. 1). In line with previous results [10, 18, 39], we confirmed that the Δ*eps* can take advantage of EPS-producing wild type (WT) and incorporate into the pellicle biofilm, resulting in lower productivity of the WT (Supplementary Fig. 1b) and reduced surface complexity of the pellicle (Supplementary Fig. 1a).

We were further interested if such social cheating could leave a phenotypic or genetic fingerprint in the population of the wild type *B. subtilis*. Previous studies have shown that cooperators can adapt to presence of cheats for example by decreasing the amount of released public goods and therefore minimizing cheating opportunities [2, 14, 15]. As *B. subtilis* exhibits phenotypic heterogeneity in *eps* matrix gene expression [39, 40] (Supplementary Fig. 2), we investigated how such heterogeneous expression is influenced by the presence of cheats in an evolutionary perspective.

To address this question, we co-cultured the EPS producers (wild type- WT) and cheating strain (Δ*eps*) for 10 biofilm growth cycles (~60 generations) (see methods). Based on previous studies [44, 45], we assumed that this evolutionary timeframe will be sufficient for evolution of adaptive mechanisms in the WT in response to social cheating, at the same time preventing the

diversification of the WT into a biofilm-deficient morphotype, which can be observed on longer evolutionary timescales [44].

During 10-cycle co-cultivation of WT and Δ*eps* strain, we observed a general trend of declining pellicle productivity accompanied by an increasing ratio of EPS non-producers in biofilms across all parallel populations (Fig. 1), indicating that the WT strain was constantly exposed to social cheating throughout the experiment.

Using confocal laser scanning microscopy, qualitative assessment of randomly selected isolates revealed that early populations of the EPS producers (5-10 transfer) which co-evolved with cheats exhibited a so called hyper ON phenotype, where the fraction of *eps* expressing cells was largely increased as compared to the ancestral strain (Fig. 1a, Supplementary Fig. 2). On the contrary, evolution in the absence of Δ*eps* mostly resulted in hyper OFF phenotype, with larger amounts of cells that do not express *eps* (Fig. 2a, Supplementary Fig. 2). To obtain a quantitative insight into the single-cell level expression of *eps* in the evolved WT populations, we performed flow cytometry measurements of P_{eps}-GFP harboring strain in pellicles formed by 10 randomly selected isolates per population (Fig. 2b, Supplementary Fig. 3, Supplementary Fig. 4). Although single isolates notably varied in their P_{eps}-GFP signal distributions (Supplementary Fig. 3), we spotted a general trend within the populations, that was in line with the microscopy results (Fig. 2b, Supplementary Fig. 4). Specifically, on average, the isolates from WT populations that co-evolved with Δ*eps,* were showing an increase in phenotypic heterogeneity, with a shift towards higher expression of *eps*, when compared with the ancestor (Fig. 2b, Supplementary Fig. 3). On the other hand, isolates that evolved without Δ*eps*, showed a substantial increase in OFF subpopulation (Fig. 2b, Supplementary Fig. 4).

As flow cytometry revealed large within-population variations of *eps* expression, we additionally screened 95 single isolates per population for average P_{eps}-GFP intensity (see methods) (Supplementary Fig. 5).

The results revealed major changes in distribution of P_{eps}-GFP intensity across the evolved and co-evolved WT populations as compared to the WT ancestor (Supplementary Fig. 5). Specifically, for all WTs evolved alone, the distributions narrowed and dramatically shifted to the left, so that the clear majority of isolates matched the hyper OFF category across the entire evolutionary time (Supplementary Fig. 5). On the contrary, in some co-evolved WT populations (population 3, 5 and 7) the distributions widened at the 10th transfer, resulting in increased frequency of hyper ON isolates (Supplementary Fig. 5).

Mutations in *rsiX* lead to Hyper ON phenotype

To unravel the genetic basis of the hyper ON phenotype that evolved in presence of cheats, several single isolates from the evolved populations were subjected to genome resequencing (for details see methods). The comparative analysis of sequencing data revealed that the WT isolates that co-evolved with cheats and exhibited the hyper ON phenotype, shared mutations in *rsiX* gene (Supplementary Dataset 1). The *rsiX* gene encodes for an anti-sigma factor that controls the activity of extracellular cytoplasmic function (ECF) sigma factor X which is involved in cationic antimicrobial peptide resistance important for cell envelope stress response [46]. Detected mutations resulted either in substitution of Valine 106 to Alanine or frameshift mutations in Serine 104 or Isoleucine 347 that could lead to change or loss of anti-SigX function. Indeed, we were able to recreate the evolved hyper ON phenotype in the pellicle solely by deleting the *rsiX* gene in the WT ancestor (Fig. 3a and b). Interestingly, a different type of frameshift mutation in Lysine 200 was found in one population of evolved WT alone but this population demonstrated a hyper OFF phenotype (Fig. 2a), suggesting that only certain types of

mutations in *rsiX* lead to the increase in number of *eps* expressing cells in the population or additional mutations have antagonistic effects in this isolate.

Mutation in *rsiX* contributes to competitive advantage of producer strains against cheats

As mutation in *rsiX* resulted in hyper ON phenotype that may be linked to elevated secretion of EPS, we hypothesized that Δ*rsiX* producers could support the spread of cheats. To better understand how ancestor WT and Δ*rsiX* interact with Δ*eps,* we cultivated the Δ*eps* in presence of EPS-containing supernatants obtained from the WT and Δ*rsiX* (Supplementary Fig. 6). Both supernatants could partially restore pellicle formation by Δ*eps* resulting in similar productivities of Δ*eps,* thereby not supporting our hypothesis on improved performance of the mutant in presence of hyper ON Δ*rsiX* strain.

In order to determine the effect of *rsiX* deletion on fitness of the WT in presence of cheats, we performed a series of competition assays. Apparently, the Δ*rsiX* showed two-fold increase in relative frequency (40%) (Fig. 4, Supplementary Fig. 7) when competed against the Δ*eps*, as compared to the WT ancestor (20%). Additionally, the significant fitness improvement of the WT strain evolved with cheats was already observed in 5th transfer and 10th transfer, mutually with an early occurrence of hyper ON phenotype in those populations. This was not the case for the WT evolved alone at 5th transfer (20%) (Fig. 4a, Supplementary Fig. 7). These results suggest that the early improvement in competitive strategies against cheats are caused by the *rsiX* mutation associated with hyper ON phenotype.

It is worth to mention that we could not detect any significant fitness costs or benefits linked to *rsiX* deletion in pairwise competition between Δ*rsiX* and WT in the liquid medium (Supplementary Fig. 8; relative fitness of Δ*rsiX* = 1.00 ±0.02 S.D.). Furthermore, we did not observe significant differences in productivities of WT and the hyper ON Δ*rsiX* mutant, when grown in monoculture pellicles (Supplementary Fig. 9), suggesting that positive effect of *rsiX*

mutation only manifests in presence of cheats. Similarly, different relative frequencies of Δ*eps* in pellicles formed by the ancestor or evolved matrix producers, did not result in different productivities of mixed pellicles (Supplementary Fig. 9). These results suggest that hyper ON phenotypes are vested on the increase in *eps*-expressing cells or limiting the spread of cheats but do not result in an increase in total yield.

It was previously demonstrated that increased matrix production can allow favorable positioning of a bacterial strain in the biofilm, thereby providing fitness advantage [47]. To test whether hyper ON phenotype can allow better positioning of the Δ*rsiX* in presence of Δ*eps,* we visualized 48 h grown pellicles formed by Δ*rsiX*:Δ*eps* and WT:Δ*eps* mixtures inoculated at 1:1 initial frequencies (Fig 5, Supplementary Fig. 10). While WT and Δ*eps* were 'well-mixed' with both strains present on the oxygen-rich surface of the pellicle, the Δ*rsiX* strain clearly dominated over the Δ*eps* occupying majority of the biofilm surface and marginalizing the Δ*eps* into small clusters (Fig 5, Supplementary Fig. 10). Therefore, deletion of *rsiX* that results in hyper-ON phenotype, provides fitness advantage over biofilm cheats, most likely by allowing favorable positioning of the matrix producers in the biofilm.

Hyper ON phenotype serves as a transient adaptive response to cheating

As the evolved WT isolates carrying point mutation in *rsiX* as well as the recreated Δ*rsiX* mutant (in ancestral genetic background) performed better in competition with Δ*eps* as compared to the WT ancestor, we reasoned that loss-of-function mutation in *rsiX* together with an associated hyper ON phenotype, might be an efficient evolutionary strategy against social cheating. Surprisingly however, prolonged evolution experiment eventually led to so called 'tragedy of the commons' as the Δ*eps* mutant took over in 6 out of 8 populations, completely abolishing the pellicle formation (Supplementary Fig. 11).

We next investigated changes in phenotypic heterogeneity pattern in late (transfer 15 onwards) co-evolved WT populations, similarly, as performed before for the early populations (see Supplementary Fig. 5). Specifically, we measured an average P_{eps}-GFP intensity in 95 randomly selected single isolates per population. Interestingly, in contrast to the early co-evolved WT carrying hyper ON phenotype, the results for late co-evolved populations revealed a strong shift towards low *eps* expression levels already in transfer 15 (Supplementary Fig. 12) and consistent for later evolutionary time points. The CLSM analysis on selected single isolates confirmed that the hyper ON phenotype observed until transfer 10 was now replaced by 'Hyper OFF' phenotype, which also developed in the control WT populations evolved in the absence of Δ*eps* (Supplementary Fig. 12).

To investigate the genetics behind this phenomenon all evolved WT populations from the last evolutionary time point (or the last time point prior the collapse) were re-sequenced. Curiously, both WT populations which resisted the invasion of Δ*eps* (population 5 and 8), but neither of the 'defeated' WT populations, carried mutations in *yvrG* gene (Supplementary Fig. 11, Supplementary Dataset 2) encoding for two-component histidine kinase involved in cell wall process. Finally, the *rsiX* mutation was not detected neither in the last populations before the collapse, with an exemption of population 7 with 1% hyper ON phenotype (Supplementary Fig. 11, Supplementary Dataset 1), nor at the last transfer point for the non-collapse, implying that this mutation was lost in parallel with the loss of hyper ON phenotype in the late populations.

DISCUSSION

Studies on evolution of cooperative behavior is important to understand how social behaviors are shaped in longer time scale. Moreover, exploring long term consequences of exposure to cheating allows to better understand how cooperation prevails in nature where environmental stress and exploitation exist inherently. Previous evolution studies on cheater-cooperator

interactions in spatially structured environment showed cheater mitigation via minimization of the cooperative trait [2, 14, 15]. On the contrary, here we show that cooperators initially respond to cheating by intensifying the cooperative behaviors through shift in phenotypic heterogeneity pattern towards hyper ON phenotype, where, in contrast to the ancestral strain, nearly all members of the population express the operon involved in EPS production. Further molecular analysis of the hyper ON isolates strongly suggests that this phenotype is triggered by loss-of-function mutation in *rsiX* gene. The product of *rsiX* represses the activity of ECF sigma factor, SigX that is involved in cell envelope stress response against cationic antimicrobial peptides [48]. Importantly, SigX has been previously shown to induce expression of *epsA-O* in *B. subtilis* via a complex regulatory pathway involving Abh and SlrR [49], explaining the observed enhanced in *eps* gene expression in *rsiX* mutant. Another example of matrix overproduction via ECF adaptation was also reported in Gram-negative bacterium *Pseudomonas aeruginosa* where mutations in another ECF called AlgT led to alginate overproduction and increased resistance to antimicrobials [50]. Therefore, adaptive boosts in matrix production through modulation of ECF is not exclusive for *B. subtilis* but seems to occur also in medically relevant Gram-negative pathogens like *P. aeruginosa*.

In contrast to previous studies that addressed long term cheating on diffusible siderophores, we explored evolutionary interplay between biofilm producers and non-producers in structured environment. Our results support previous observations on evolution of specific cheating-resisting mechanisms in co-operators, pointing towards ubiquity of this phenomenon. In addition, our work brings up two major findings 1) matrix producers can adapt to matrix non-producers by shifting phenotypic heterogeneity towards increased number of matrix-overexpressing cells 2) hyper ON phenotype is associated with favorable positioning of the matrix producers in the biofilm in presence of cheats, thereby limiting their numbers 3) hyper ON anti-cheating strategy is a short-term solution, which can either be replaced by another, yet unknown

strategy linked to hyper OFF phenotype, or followed by tragedy of the commons. As recently demonstrated, alternative EPS-independent biofilm formation strategies can emerge by single amino acid change is TasA [45]. It remains to be discovered whether shifts in phenotypic heterogeneity in response to long term cheating is general phenomenon that applies to different types of public goods.

Acknowledgements

This work was funded by the Deutsche Forschungsgemeinschaft (DFG) to Á.T.K. (KO4741/2.1) within the Priority Program SPP1617. M.M. was supported a FEMS Research and Training Grant (FEMS-RG-2017-0054). This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 713683 (H.C. Ørsted COFUND to A.D.). Work in the laboratory of Á.T.K. is partly supported by the Danish National Research Foundation (DNRF137) for the Center for Microbial Secondary Metabolites.

Competing Interests

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

Authors contributions

Á.T.K. conceived the project, M.M., A.D., S.B. and D.S. performed the experiments. G.M contributed with methods. M.M., A.D. and Á.T.K. wrote the manuscript, with all authors contributing to the final version.

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FIGURES

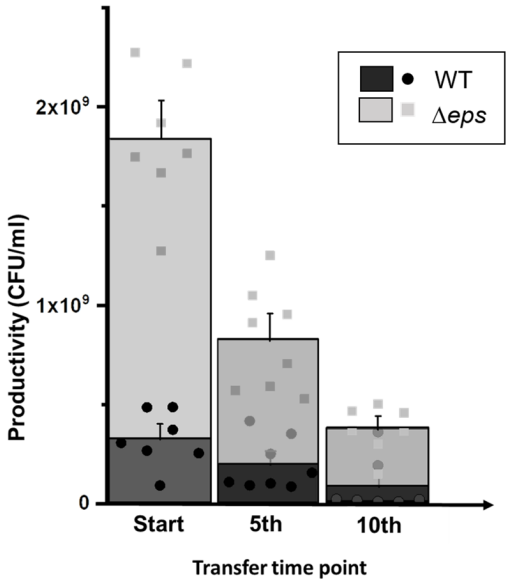

Fig. 1 Pellicle productivity. Total colony forming unit per ml of WT and Δ*eps* in 48 h old pellicles non-evolved (Start) (n=9), after experimental evolution at 5th (n=8 populations) and 10th transfer (n=7 populations). One population after 10th transfer was unable to form pellicle attributed to WT being totally outnumbered by Δ*eps*. Dots represent data obtained for all individual populations, while columns represent averages. Error bars correspond to standard error.

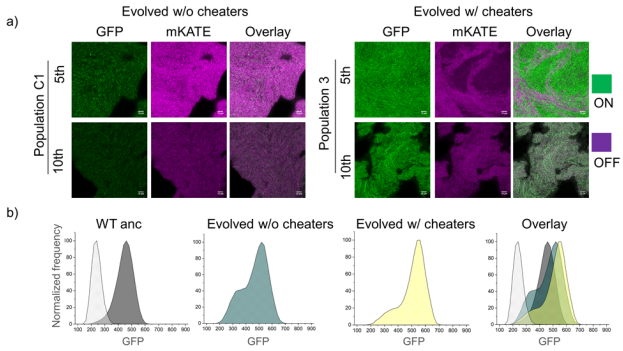

Fig. 2 Evolved WT biofilms in the presence of cheaters shows altered *eps* expression. **a** Qualitative assessment of *eps* gene expression based on confocal laser scanning microscopy of pellicles formed by single clones of WT evolved without cheaters belonging to population C1 (5th and 10th) and WT evolved with cheaters belonging to population 3 (5th and 10th transfer). Cells constitutively expressing mKATE (OFF cells) are represented in magenta and *eps* expressing cells (ON cells) are shown in green. Scale bar = 10 µm. **b** Flow cytometry analysis showing average distributions of fluorescence intensities of cells belonging to WT ancestor (dark grey), WT evolved without cheaters (blue), WT evolved with cheaters (yellow), and overlay of all 3 in comparison to WT non-labelled (light grey).

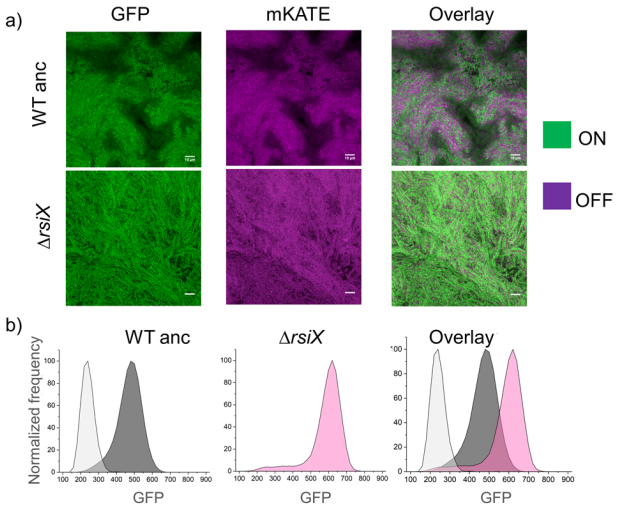

Fig. 3 Deletion of *rsiX* results in increased *eps* expression in pellicles. **a** Qualitative assessment of *eps* gene expression based on confocal laser scanning microscopy of pellicles formed by Δ*rsiX* (TB959) showing Hyper ON phenotype in contrast to WT ancestor (TB869). Cells constitutively expressing mKATE (OFF) are shown in magenta and *eps*- expressing cells (ON) are represented in green. Scale bar 10µm. **b** Flow cytometry results showing average distribution of fluorescence intensities of WT cells (dark grey), Δ*rsiX* cells (TB959) (pink) and overlay of the 2 in comparison to WT non-labelled (light grey).

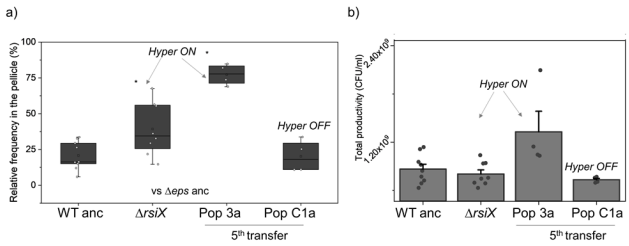

Fig. 4 a Pellicle competition assay of single clones belonging to producer populations (WT ancestor (n=9), Δ*rsiX* (n=8), evolved with (n=4) and without cheaters (n=4)) against Δ*eps* ancestor. **b** Productivity assay based on total CFU/ml of pellicles of co-cultures of Δ*eps* ancestor and single clones belonging to producer populations (WT ancestor (n=9), Δ*rsiX* (n=8), evolved with (n=4) and without cheaters (n=4)). Mean is represented in square within the box plots; median is denoted by horizontal line inside the boxes; whiskers represent the min and max; Error bars in bar graph are based on Standard error; single dots represent the individual data points (n); asterisk (*) represents the significant difference from the WT ancestor ANOVA (P value 0.05).

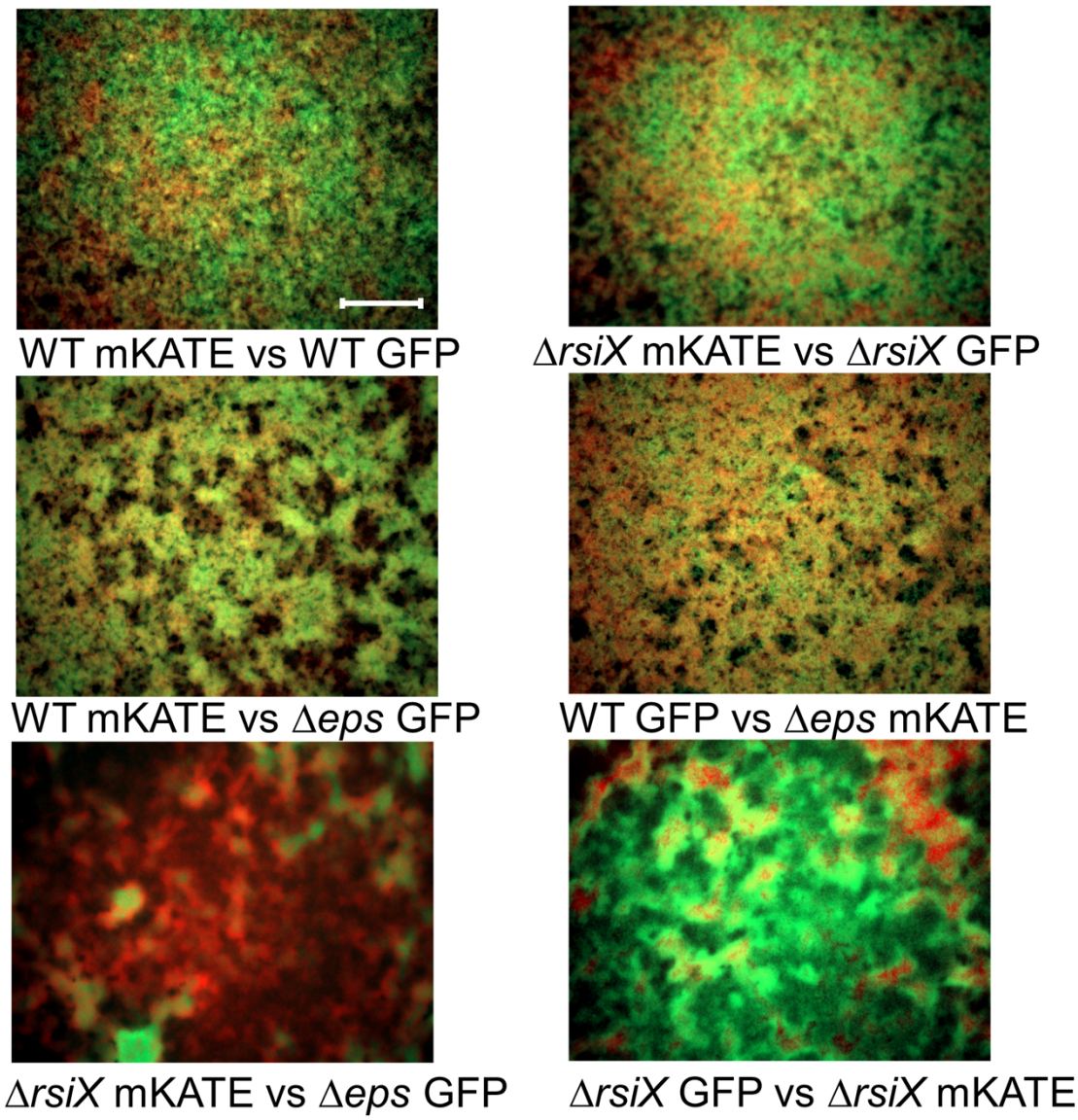

Fig. 5 Competition assay between fluorescently WT vs Δ*eps* and Δ*rsiX* vs Δ*eps*. Strains labelled with constitutively expressed GFP and mKate proteins, were inoculated in 1:1 initial frequency, pellicles were cultivated for 48h at 30°C and visualized using stereomicroscope. Upper panels represent controls (two isogenic WT or Δ*rsiX* strains labelled with different fluorescent markers), middle panel represents pellicles formed by WT vs Δ*eps* and bottom panels represent pellicles formed by Δ*rsiX* vs Δ*eps,* each in two alternative combinations of fluorescent markers. Scale bar corresponds to 500 µm.

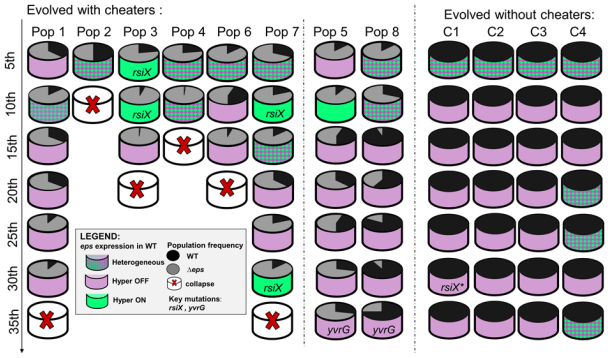

Fig. 6 Summary figure shows the population dynamics based on producer and cheater frequency per population from 5th transfer to 35th transfers for populations evolved with cheaters with collapse (Pop 1, 2, 3, 4, 6 and 7) and without collapse (Pop 5 and 8) and populations evolved without cheaters (C1, C2, C3, C4) with indications of phenotypes based on hyper ON, hyper OFF or heterogenous *eps*-expression. Key mutations on single clone level of evolved WT and evolved Δ*eps* or population level are specified. The **rsiX* mutation differs from mutation observed in strains evolved with cheaters.