Polymicrobial infections can select against *Pseudomonas aeruginosa* mutators because of quorum sensing tradeoffs

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Abstract

Bacteria with increased mutation rates (mutators) are common in chronic infections and are associated with poorer clinical outcomes; especially in the case of *Pseudomonas aeruginosa* infecting cystic fibrosis (CF) patients. There is however considerable between-patient variation in both *P. aeruginosa* mutator frequency and the composition of co-infecting pathogen communities, and we investigated whether community context might affect selection of mutators. Using an *in vitro* CF model community, we show that *P. aeruginosa* mutators were favoured in the absence of other species but not their presence. This was because there were tradeoffs between adaptation to the biotic and abiotic environments (for example, loss of quorum sensing and associated toxin production was beneficial in the latter but not the former in our *in vitro* model community) limiting the evolvability advantage of an elevated mutation rate. Consistent with a role of co-infecting pathogens selecting against *P. aeruginosa* mutators *in vivo*, we show that the mutation frequency of *P. aeruginosa* population was negatively correlated with the frequency and diversity of co-infecting bacteria in CF infections. Our results suggest that coinfecting taxa can select against *P. aeruginosa* mutators, which may have potentially beneficial clinical consequences.

Main

Interactions with other species can be a key determinant of the rate of evolution[1–3], as well as potentially imposing indirect selection for mechanisms that increase genetic variation, such as

increased recombination rates[4,5] and, in the case of bacteria, increased mutation rates[6,7]. These findings are largely based on coevolutionary interactions between host-parasite pairs. It is unclear how being embedded in a multi-species microbial community affects selection for mutation rates in focal bacterial species. Fluctuations in the densities of community members, as well as coevolutionary changes, may result in continually changing selection pressures[1,2,8], potentially selecting for bacteria with elevated mutation rates (mutators). Alternatively, mutators may be selected against if interspecific competitors constrain adaptation to other components of the environment, including other community members[6,9]. Furthermore, changes in population size of the focal species caused by interactions with other species could result in both selection for or against mutators[10,11].

Despite most mutations being deleterious or neutral, mutators can occur at high frequencies in natural populations of bacteria[12–20]. They can be found at particularly high frequencies in chronic infections[15–20], where they are associated with treatment failure and patient morbidity [21–27], especially in cystic fibrosis (CF) chronic lung infections by the opportunistic pathogen *P. aeruginosa*[24–27]. The success of mutators in this context is attributed to the ability of mutator alleles to generate, and hitchhike with, beneficial mutations that allow adaptation to stressful environments in the host, such as those created by immunity and antibiotics. However, it is notable that there is large and unexplained between-patient variation in mutator frequency [15,16,28]. We investigated whether this variation in mutator frequency could be explained by the composition of the infecting pathogen community, which can range from highly diverse to almost exclusively *P. aeruginosa*[29,30]. Answering this question may provide opportunities for developing interventions

that could minimize the severity of infections, as well as providing fundamental insights into how communities affect selection for evolvability.

Results

Co-occurring microbes reduce selection for *P. aeruginosa* mutators.

We conducted an experiment to determine if the presence of other bacterial taxa imposed selection on *P. aeruginosa* mutation rates (Extended Data Fig. 1). We focused on purely ecological rather than coevolutionary interactions. We competed *P. aeruginosa* PAO1 wildtype and an isogenic *mutS* mutator (PAOMS) strain, initiated at approximately 1:1 ratio, in artificial CF sputum medium in the presence or absence of other typical CF-associated pathogens (*Staphylococcus aureus* strain 13 S44 S9, *Acinetobacter baumannii* clinical isolate FZ21 and *Burkholderia cenocepacia*, all isolated from patients at Queen Astrid Military Hospital, Brussels, Belgium[31]; referred to as the bacterial community). We propagated *P. aeruginosa* by transferring 5% of each population to fresh medium every week for 4 weeks. *P. aeruginosa* outcompeted the community over the course of 7 days, and we inoculated the ancestral strains of the other community members with the evolving *P. aeruginosa* at each transfer to maintain community-imposed selection pressures. We determined relative mutator fitness (v) by comparing the initial frequency of mutators to their final frequency at each weekly transfer, using: $v = x_2(1\text{-}x_1)/x_1(1\text{-}x_2)$, where x_1 is the initial frequency of mutators (i.e. proportion at t_0) and x_2 their final frequency at transfer i (weeks 1-4)[32]. Mutators had a higher fitness (relative to the wildtype) in the absence of the community than in the presence (Fig. 1a; LMM: main effect of community on $\log_2$-transformed v: $\chi^2_{(1)} = 5.28$, df =1, $p = 0.02$, see Extended Fig. 2 for parameter estimates and diagnostics plots). Note that mutators displayed consistently greater fitness in the absence of the community (estimated $\bar{v} \pm$ 95% confidence

intervals for monocultures = 10.56 [4.48, 24.88] and co-cultures = 2.71 [1.15, 6.39]; Fig. 1a) across all time points (LMM: main effect of transfer on $\log_2$-transformed v: $\chi^2_{(1)} = 0.91$, df =1, $p < 0.34$).

Tradeoffs between abiotic and biotic adaptation can explain reduced mutator fitness in the community.

We next considered what might be driving reduced mutator success in the presence of the community. An increased mutation rate can have direct fitness consequences, for example by reducing susceptibility to oxidative stress, with the latter potentially affected by the community context[33]. To investigate possible direct fitness effects, we estimated relative mutator fitness between each consecutive time point. Mutator frequencies only increased significantly in the first week (Extended Data Fig. 3). This rule out a major direct effect of selection on the mutator, which would be expected to resulted in increased mutator frequencies between all time points in the absence of the community (see Extended Fig. 4 for parameter estimates and diagnostic plots). This suggests that changes in the frequency of the mutator genotype were the result of indirect selection, whereby mutator alleles hitchhike with the mutations they generate[34]. The increased mutation rate appeared to give the mutator genotype an evolutionary head start over the wildtype, especially in the absence of the community.

Indirect selection for mutators tends to be greatest when adaptation is strongly mutation limited, i.e. when there are many large effect mutations that can increase fitness[6]. The presence of the community may have reduced the net fitness effects of beneficial mutations if mutations that confer an advantage (or are neutral) in the absence of the community are costly in the presence, i.e if there is a tradeoff between biotic and abiotic adaptation. The net benefit of a mutation that

increases abiotic adaptation will then be reduced because of associated costs to biotic adaptation, and vice versa. Note that population size can also affect indirect selection for mutators, by altering mutation supply rates (and hence the extent to which adaptation is mutation limited) and the efficacy of selection[10,11]. However, *P. aeruginosa* population sizes – at least at the end of each growth cycle – were not affected by the presence of the community (Extended Data Fig. 5).

To test the tradeoff hypothesis, we evolved PAO1 populations in the presence and in the absence of competitors for 4 weeks, and then measured the fitness of the evolved populations in both the presence and absence of the community. Rather than use the populations of competing mutator and wildtype bacteria from the previous experiment, we instead repeated the experiment using only wildtype bacteria (Extended Data Fig. 1). This was because the large number of mutations that typically accumulate in mutator genomes during adaptive evolution make it extremely difficult to identify the mutations that underpin fitness differences. Fitness was affected by an interaction between the environment fitness was measured and the environment populations were evolved in, indicative of a tradeoff (Fig. 1b, LMM on the relative fitness (v) of evolved PAO1 populations: interaction between selection × competition environment: $\chi^2_{(1)}= 7.07$, $p = 0.008$). Specifically, monoculture-evolved populations had significantly greater fitness in the absence versus presence of the community (contrast: t-value = 3.0, p-value = 0.01, df = 10), while community-evolved populations were equally fit in the presence and in the absence of the community (contrast: t-value = -1.0, p-value = 0.34, df = 10; Fig. 1b; see Extended Data Fig. 6 for parameter estimates and diagnostic plots). This suggests mutations that were neutral or weakly beneficial in the absence of the community were costly in the presence of the community.

***P. aeruginosa* loses QS activity in the absence of the community.**

To determine what traits might be under differential selection in the presence versus absence of the community, we re-sequenced all the evolved wildtype PAO1 populations (Extended Data Fig. 1). This revealed a complex picture, with most mutations unique to single populations and at relatively low frequencies (Supplementary Table 1). For this reason, we focus on those variants present at a frequency of at least 10% (Supplementary Table 2). We found a total of 41 mutations (SNPs, insertions and deletions) with an average of 3.6 and 3.16 mutations per population for monoculture and community treatments, respectively. Non-synonymous mutations (45% of SNPs and short indels) were distributed over 9 genes and mutations localized in intergenic regions did not affect any regulatory binding site (Fig. 2a, Supplementary Table 2). There were no significant differences in the rates of evolution based on Euclidean distances (*t*-test, $t = -0.325$, $p = 0.752$) or in the number of mutated genes (*t*-test, $t = 0$, $p = 1.0$) between populations in the presence versus absence of the community. However, most of the mutations with known functions that had reached the highest frequencies in at least one population were linked to the regulation of QS activity and biofilm formation, and these showed a between-treatment pattern (Fig. 2a). Notably, five out of six PAO1 populations evolved in the absence of the community accumulated non-synonymous (loss of function) mutations in one or more QS regulators (*lasR*, *pqsR*) and/or in a gene involved in biofilm development (*dipA*) (Fig. 2a). However, mutations in these genes were only present in 2 out of 6 populations in populations evolved in the presence of the bacterial community (Fig. 2a, Supplementary Table 2). By contrast, *mvaU* - a PqsR QS-repressor[35]- mutants were at high frequencies in 2 populations evolved with the community but were absent in populations evolved without (Fig. 2a). Chromosomal structural variations were also found in the PA2228 gene and its upstream region, which can reduce QS activity[36]. However, these variants were present in 4 and 3

replicates in the presence and absence of the community, respectively, suggesting that these mutations are associated with adaptation to the general laboratory conditions (Extended Data Table 5).

These mutational distributions suggested that the loss of QS gene regulation may have been beneficial in the absence of other taxa in our model community, but costly in the presence. To test this hypothesis directly, we constructed two PAO1 knockout mutants in which QS was lost (Δ*pqsR*) or up-regulated (Δ*mvaU*) and competed each strain against the ancestral PAO1 strain in the presence and absence of the community. The results confirmed that the *mvaU* strain was relatively fitter than the *pqsR* mutant in the presence of the community, and vice versa (Fig. 2b; strain × community interaction effect on $\log_2$-transformed v: $F_{(1, 20)} = 13.21$, $p = 0.0016$; contrasts: $mvaU_{\text{monoculture}} - pqsR_{\text{monoculture}}$ = t-ratio (-2.40), p-value (0.013) and $mvaU_{\text{community}} - pqsR_{\text{community}}$ = z-ratio (2.74), p-value (0.006); see Extended Data Fig. 7 for parameter estimates and diagnostic plots). That the tradeoff pattern here is more pronounced than for the naturally evolved populations is unsurprising: QS mutations were not at fixation and evolution followed divergent trajectories within treatments in general in the latter.

The loss of *P. aeruginosa* QS during *in vitro* cultivation in the absence of other species has been reported in other studies[37–39], and is assumed to be because many QS-regulated gene products confer little benefit[40], or can be exploited by conspecifics[41,42]. Why might the QS system then be retained in the presence of other taxa? The QS system regulates many traits, but in the context of competition with other species, regulation of pyocyanin might be particularly relevant[43,44]. Pyocyanin is a powerful redox agent and effective anti-competitor toxin[45] and its production is

reduced if the PqsR and LasR system is knocked out by *pqsR* and *lasR* mutations[44] or increased by mutations in *mvaU* [46]. We therefore measured pyocyanin production in differentially evolved and ancestral populations of PAO1 as well as their toxicity to competitors by growing each community member in spend supernatants. We found that pyocyanin production was significantly reduced in PAO1 populations evolved without the community. Community-evolved populations showed a slight decrease in pyocyanin production, but levels were similar to that of the ancestral PAO1 populations (Fig. 2c; ANOVA with Tukey contrasts: Overall difference in pyocyanin production; $F_{(2,15)} = 18.33$, $p < 0.001$; contrast monoculture v PAO1 (ancestral): $t = -6.005$, $p < 0.001$, df = 15; bacterial community v PAO1 (ancestral), $t = -2.33$, $p = 0.08$, df = 15; monoculture v bacterial community; $t = 3.67$, $p < 0.01$, df = 15). Supernatants of evolved PAO1 populations showed different toxicity profiles depending on the evolution treatment (Fig 2d. Kruskal-Wallis on cumulative competitor density $\chi^2_{(2)} = 8.5$, $p = 0.01$). Monoculture-evolved populations showed a strong reduction in their capacity to inhibit growth of the bacterial community (ancestral versus monoculture-evolved, Wilcoxon rank sum exact test: p-value = 0.006). Community-evolved populations showed toxicity levels similar to that of the ancestor (ancestor versus community-evolved, Wilcoxon rank sum exact test: p-value = 1.0). These findings are consistent with a recent study showing that *lasR* mutants of *P. aeruginosa* were more likely to evolve in the absence compared with the presence of *S. aureus*, and the QS mutants had greatly reduced killing activity against *S. aureus*[37].

Pyocyanin is also linked to increased severity of infection[47], and we next compared *in vivo* virulence of ancestral PAO1 with monoculture and community-evolved PAO1 populations using a waxmoth, *Galleria mellonella*, larvae infection model. We found that the rate at which *P.*

aeruginosa infections killed waxmoth larvae to be greater for populations evolved with the community compared to those without (Fig. 2e; log-rank test comparing survival curves across evolution treatments: $\chi^2_{(2)} = 12.7$, p-value: 0.002, total $n = 387$; post-hoc pairwise comparisons, adjusted for multiple testing using 'BH' method: monoculture versus bacterial community, $p = 0.001$; monoculture versus PAO1 (ancestral), $p = 0.44$; bacterial community versus PAO1 (ancestral), $p = 0.50$).

CF bacterial diversity correlates with *P. aeruginosa* mutation rate.

While our *in vitro* results suggest that the presence of a co-infecting community of pathogens can select against mutators, it is unclear if this is a useful predictor of mutator frequency in clinical contexts, where there are many other potential sources of selection, including communities that differ markedly from our *in vitro* model community. We therefore determined how the community composition of infections correlated with the average spontaneous mutation frequency (a proxy for mutation rate) of *P. aeruginosa* across 24 chronically infected CF patients. We used shot-gun metagenomics to determine bacterial community composition in patient sputum samples (see Methods, Fig. 3a and Extended Data Fig. 1, Supplementary Tables 3 and 4). There was a median of 25 species detected per sample (range, 9 - 88 species), but communities were typically dominated by a small number of species: on average, only 4.4 ± 3.7 species per sample represented more than 1% of total bacterial reads, with dominant species typically representing more than 76% (Fig. 3a and Extended Data Table 7). *P. aeruginosa* was the most abundant species (74.08%; range from 19-99%), followed by *Stenotrophomonas maltophilia* (14.78%) and *Rothia mucilaginosa* (7.73%) (Fig. 3a and Extended Data Table 8). Mutation frequency of *P. aeruginosa* populations (estimated from ~ 30 clones per population, see Methods) showed a range spanning approximately

4 orders of magnitude (Fig. 3b, ~10^{-9} - 10^{-5}), with approximately 50% of populations containing mutator bacteria (showing at least 20-fold greater mutation frequencies than a normo-mutator wildtype strain PAO1). Crucially, mutation frequency was negatively correlated with the CF bacterial diversity (Shannon index; Fig. 3b Pearson's $r = -0.46$, $t = -2.42$, df = 22, $p = 0.023$) and positively correlated with the proportion of *P. aeruginosa* in the infection (Fig. 3c, Pearson's $r = 0.49$, $t = 2.67$, df = 22, $p = 0.013$). These data suggest that coinfecting pathogens reduces the benefit of having a high mutation rate in *P. aeruginosa* during CF infections.

***P. aeruginosa* mutators are associated with QS mutations *in vivo*.**

Finally, we determined if a tradeoff in the benefit of QS in the presence and absence of the community could have constrained adaptation, and hence potentially explain mutator frequency, *in vivo*. We re-sequenced the 24 *P. aeruginosa* CF populations to determine the extent to which QS genes had detectable frequencies of non-synonymous mutations. We found a positive correlation between the number of QS mutated genes and the average mutation rate of *P. aeruginosa* CF populations (Fig. 4, two-sided Pearson's $r = 0.43$, $t = 2.23$, df = 22, $p = 0.036$; Supplementary Table 5). This correlation could simply have arisen as a consequence of mutation rate: mutations in QS genes are more likely to accumulate in mutator than non-mutator genotypes. However, QS loss of function mutations can reach high frequencies over a matter of weeks in non-mutator populations *in vitro*[37,38], and the clinical isolates likely came from populations that had been evolving in patients for many years. Moreover, we analyzed the relation between *P. aeruginosa* average mutation frequency and another group of genes, those belonging to the CF resistome[48] and no significant correlation was found (two-sided Pearson's $t = 0.98$, df = 22, p-value = 0.3344, Extended Data Fig. 8). Also, no significant correlation was found between *P.*

aeruginosa mutation frequency and mutations in genes (*mvaU* and *dnaX)* that reached high frequencies during the evolution in the presence of the community (*Pearson's r =0.181, t* =0.86, df = 22*, p* =0.396). Taking into account our *in vitro* findings, these results therefore suggest that selection for QS in the presence of coinfecting pathogens may help to explain reduced selection for mutators.

Discussion

The costs and benefits of increased bacterial mutation rates have been extensively investigated[6,49,50], but there has been little investigation into the role of a crucial component of the environment: the wider microbial community. Our *in vitro* work shows that *P. aeruginosa* mutator genotypes are less strongly selected for in the presence of the model community. This reduced selection for mutators can be explained by a tradeoff between adapting to hetero-specifics and other components of the environment, constraining the potential for increased adaptation afforded by an elevated mutation rate[6]. Molecular characterization presented here revealed that genes regulating QS were in part responsible for this tradeoff *in vitro*, but other traits are also likely to be important.

Previous work has shown that coevolution between bacteria and an obligately killing (lytic) bacteriophage can select for elevated mutation rates, despite there also being growth rate costs associated with evolved phage resistance[7]. The reasons for this stark difference with the present study is likely in part due to phage-imposed selection for resistance being both greater than that imposed by competitors and bacteria and phage continually coevolving to be resistant and infectious [51]. Whether or not adaptive constraints imposed by interacting with multiple community

members, as opposed to a single species, also contributes to this difference remains to be explored.

The negative correlation between *P. aeruginosa* mutator frequency and frequency of coinfecting pathogens in the CF samples suggests there may also be a causal link between interspecific interactions and selection for mutators in natural populations. Moreover, the data are consistent with a role of QS-mediated tradeoffs *in vivo*. Despite the parallel *in vitro* and *in vivo* findings, it is important to emphasise that our findings may be specific to the particular *P. aeruginosa* isolate and model community[52]. Indeed, other processes might well play an important role in driving the in vivo patterns. In particular, different antibiotic protocols in CF patients may not only select for *P. aeruginosa* mutators but also reduce the diversity of coinfecting pathogens that are less resistant, which in turn creates a more favorable scenario for mutators. It may be useful to take this possibility into account when deciding on specific treatment protocols. More generally, our work highlights the key role that interspecific interactions within microbial communities can play in the evolution of bacteria[30].

Materials and Methods

Statistical analysis

All data were analysed using R (Version 4.0.3). No statistical methods were used to predetermine sample size. The experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment.

Ethical statement.

All collection was in accordance with the Danish National Regulations. The study was approved by the local ethics committee (registration numbers H-4-2015-FSP and H-19029688).

Mutator fitness determination in artificial CF communities.

Artificial CF communities were assembled in 24-well microtiter plates containing 1 ml of Artificial Sputum Media (ASM)[53]. This medium contains amino acids, mucin and free DNA as in the CF patient sputum. *P. aeruginosa* growth in ASM mimics growth during CF infections, with the formation of self-aggregating biofilm structures and population divergence[54]. PAO1 Gm^r*lacZ* and PAOMS (mutator) Gm^r strains were generated by integrating a gentamicin resistance cassette (Gm) with a *lacZ* gene (Tn7-Gm-*lacZ*) or the Gm alone (Tn7-Gm) at the *att*::Tn7 locus in *P. aeruginosa* PAO1 and PAOMS strains, respectively using the methods of Choi *et al* 2006[55]. The bacterial community was composed of other CF pathogens: *Staphylococcus aureus* strain 13 S44 S9, *Acinetobacter baumannii* clinical isolate FZ21 and *Burkholderia cenocepacia* which were all isolated from patients at Queen Astrid Military Hospital, Brussels, Belgium. These bacaterial species were chosen because they are important CF pathogens and frequently co-infect with *P. aeruginosa* the CF lung. All strains were grown overnight in 30 ml glass universals containing 6 ml Luria Bertani (LB) medium in an orbital shaker (180 rpm). Each artificial community was inoculated with 5 x 10^6 CFU´s (colony forming units) each of the *P. aeruginosa* PAO1 and

PAOMS strains (1 x 10^7 total CFU of *P. aeruginosa*). Communities were divided in two treatments: "monoculture", where no community was added; and "bacterial community" where 1 x10^7 total CFU´s of the bacterial community (3 x 10^6 CFU´s of each species) were co-inoculated. Six replicates per treatment were performed and the experiment was repeated twice (n = 24 in total). The communities were grown with aeration in a shaking incubator at 37°C, 50 rpm for 7 days. After 7 days, the biofilm was disrupted by adding 1 ml of Sputosol (Thermo Fisher SR0233) and 100 µl of culture were transferred to a new well that contained 1 ml of fresh ASM and incubated again for 7 days. This cycle was repeated three additional times. *P. aeruginosa* PAO1 and PAOMS densities were followed by plating serial dilutions of every weekly transfers of each treatment on LB-agar supplemented with gentamicin (30 µg/ml) and X-gal (90 µg/ml). Since *P. aeruginosa* outcompeted the bacterial community (*S. aureus, B. cenocepacia* and *A. baumanii*), the community was replaced with -80ºC stocks at similar inoculation densities in every transfer.

We determined relative mutator fitness (v) by comparing the initial frequency of mutators to their final frequency at each weekly transfer, using: $v = x_2(1\text{-}x_1)/x_1(1\text{-}x_2)$, , where x_1 is the initial frequency of mutators (i.e. proportion at t_0) and x_2 their final frequency at transfer i (weeks 1-4)[32]. The value of v, therefore, signifies whether mutators increase in frequency ($v > 1$), decrease in frequency ($v < 1$), or remain at the same frequency ($v = 1$). We assessed temporal changes in the effect of community presence on relative mutator fitness, using a linear mixed effects model (LMM) (lmer() function in the 'lme4' package[56]) with v as response variable and community (factor) and transfer (factor) as fixed effects, as well as their two-way interaction. We fitted random intercepts for each population to account for non-independency of observations over time. We checked residual behaviour of all models using the 'DHARMa' R package[57]. To improve residual behaviour, we used a natural log transformation on v in all subsequent analyses.

For all analyses, the most parsimonious model was arrived at by sequentially deleting terms from the model and comparing model fits using χ^2-tests or F-tests, where appropriate. Parameter estimates and their confidence intervals were computed using the 'parameters' package[58]. Post-hoc contrasts were computed using the 'emmeans' package[59]. To correct for multiple comparisons, *p*-values were adjusted using the 'tukey' method. All plots were produced using the 'ggplot2' package[60]. We used *R* Version 4.0.3 for all analyses (R Development Core Team; http://www.r-project.org).

PAO1 *in vitro* evolution in artificial CF communities.

We evolved PAO1 (PAO1 Gm-*lacZ*) under the same culture conditions that were used in the competition experiments. Briefly, 5 x 10^6 CFU´s of *P. aeruginosa* PAO1 strain were inoculated in each of the ASM CF artificial communities, half of which were co-inoculated with 5 x 10^6 CFU`s of the bacterial community (*S. aureus*, *B. cenocepacia* and *A. baumanii*). Communities were grown with aeration in a shaking incubator at 37°C, 50 rpm for 7 days. After this, the biofilm was disrupted by adding 1 ml of Sputosol (Thermo Fisher SR0233) and 100µl of this mix was transferred to a new well that contained 1 ml of fresh ASM and incubated again for 7 days. After three cycles, dilutions of the cultures were plated onto LB-agar supplemented with 30 µg/ml gentamicin. The plates were incubated at 37ºC for 24 h and a subpopulation of 96 *P. aeruginosa* colonies was randomly selected per community for further analyses. Six replicates were performed for each treatment.

Fitness determination of PAO1 evolved populations.

Fitness of each of our 12 PAO1 evolved populations was assessed relative to the ancestral strain in both selective environments: in the presence and in the absence of the bacterial community. To do this, the PAO1 ancestral strain was tagged with a Gm^r as described in Choi *et al* 2006[55]. The

ancestor PAO1 Gm^r and each of the twelve evolved subpopulations (the 96 colonies pooled) were grown overnight in 30 ml glass universals containing 6 ml LB medium in an orbital shaker (180 rpm). Competitions were established as previously described, where 24 wells were inoculated with 5 x 10^6 CFU's ancestral PAO1 Gm^r, 5 x 10^6 CFU's of the appropriate evolved population (Gm^r-*lacZ*). The bacterial community (-80ºC stocks of *S. aureus*, *B. cenocepacia* and *A. baumanii*, ~1 x 10^7 CFU in total) was added to the half of wells. Cultures were incubated for 48 h after which populations were diluted, plated on LB agar supplemented with 30 µg/ml gentamicin and 90 µg/ml of 5-Bromo-4chloro-3indoyl-ß-Dgalactopyranoside (X-gal), and colonies were counted as before. Evolved and ancestral PAO1 strains were distinguished by blue-white colony screening and an average of 5000 colonies was screened for each replicate. To determine whether there was a tradeoff between biotic and abiotic adaptation, we used a LMM with relative fitness of evolved populations (*v*, frequency changes relative to the ancestral strain) as a response variable, selection environment (evolved with/without community) and current competition regime (bacterial community present/absent) as fixed explanatory factors, as well as their interaction. We fitted random intercepts for individual populations to account for non-independency of observations. To determine whether populations performed better (i.e. displayed a higher *v*) when competing with the ancestor under conditions similar to those they had evolved in (i.e. with or without the community), we carried out pairwise contrasts between competition treatments *within* each of the two selection environments.

To determine the effect of the Gm^r and Gm^r*lacZ* markers in PAO1 fitness, the same evolution experiments were performed using the PAO1 strain tagged with either of the two markers. No significant differences in fitness were found (One sample t-test, *r* significantly different from 0, monoculture: $t_5 = 2.400$, $p = 0.083$).

Whole-genome re-sequencing of PAO1 evolved populations.

a. Sample pooling and DNA extraction.

Genome re-sequencing of our 12 evolved populations and PAO1 ancestor strain was performed to determine the genetic traits under selection in the presence and in the absence of the bacterial community. For the evolved populations, the 96 isolates from each population were pooled and sequenced together. Isolate pooling was performed by growing each isolate overnight in LB separately, and then pooling the equivalent numbers of cells from the 96 isolates. Genomic DNA from pooled populations and PAO1 ancestral strain was extracted using the Wizard Genomic DNA Purification Kit (Promega). DNA was quantified using the Qubit dsDNA BR assay (Invitrogen). Pooled DNA was submitted to Liverpool Centre for Genomic Research for library preparation (550-bp insert size) and sequencing on the Illumina HiSeq 2500 sequencing system (125-bp paired-end reads).

b. Sequence data preparation and variant calling.

Preparation of sequence data was performed as described previously [61]. Briefly, sequenced read data were trimmed using Cutadapt version 1.2.1[62] setting option -O 3 and Sickle version 1.2 (https://github.com/najoshi/sickle) setting minimum window quality score to 20 and omitting reads shorter than 10 bp after trimming but retaining single remaining reads from pairs. Reads were mapped with bwa mem (https://arxiv.org/abs/1303.3997) and duplicate reads were identified and removed with Picard (https://github.com/broadinstitute/picard). The Genome Analysis Toolkit (GATK) HaplotyeCaller was used to perform local de novo assembly of haplotypes and call SNPs and indels from the pooled isolate sequence data with sample ploidy n = 96. Standard conservative filtering parameters were used to provide high-quality variant calls. In addition, the ancestral PA01 clone was resequenced and sites prone to sequencing errors identified and used to filter SNPs in

the evolved clone populations. Protein coding effects were computed using SnpEff (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3679285/). Structural variants (SV) in each evolved populations were detected using the CLC Genomics Workbench 21.0.5 (QIAGEN Bioinformatics CLC bio, Aarhus, Denmark). All variants (SNPs, indels, SV) were confirmed visually by aligning trimmed reads to the NC_002516.2 reference genome using Integrated Genomics Viewer (IGV) software (http://www.broadinstitute.org/igv). Euclidean distances from the ancestor was estimated as the square root of the sum of allele frequencies in each evolved population.

Fitness determination of PAO1Δ*psqR* and PAO1Δ*mvaU* strains.

To investigate the fitness effect of PsqR and MvaU in the presence and in the absence of the bacterial community shown in Fig. 2b, we first constructed Δ*psqR* and Δ*mvaU* deletion mutant strains. For this purpose, two DNA fragments containing 300 bp flanking upstream and downstream regions of *psqR* and *mvaU* genes (Supplementary Table 6), respectively were synthesized at Gene Universal Inc (Newark, DE) and delivered into the pUC57 vector. Each DNA fragment was cloned into the suicide vector pKNG-101[63] for gene replacement in PAO1. Deletion of *psqR* and *mvaU* in Δ*psqR* and Δ*mvaU* strains, respectively was confirmed by PCR and gene sequencing. PAO1 ancestral strain was tagged with a Gm^r and Δ*psqR* and Δ*mvaU* strains with Gm^R-*lacZ* at the *att*::Tn7 locus using the methods of Choi *et al* 2006MENDELEY CITATION PLACEHOLDER 50. Fitness of the PAO1 Δ*psqR* and Δ*mvaU* relative to PAO1 was experimentally determined by competing both strains at a 1:1 starting ratio in ASM in both the presence and absence of the bacterial community. Competitions were established as previously, where 12 wells were inoculated with 5 x 10^6 CFU's ancestral PAO1 (Gm^r), 5 x 10^6 CFU's of Δ*psqR* and Δ*mvaU*

(Gm^r-*lacZ*). Equal numbers of the bacterial community (*S. aureus*, *B. cenocepacia* and *A. baumanii*, ~1 x 10^7 CFU) was added to the half of wells. Plates were incubated for 168 h after which populations were diluted, plated on LB agar and LB agar supplemented with 30 μg/ml gentamicin and 90 μg/ml X-gal, and colonies were counted as before. Ancestral PAO1 and QS mutant strains were distinguished by blue-withe colony screening and an average of 5000 colonies was screened for each replicate. Relative mutant fitness was calculated as described above (i.e. changes in mutant frequency relative to the ancestral strain, v). To determine whether strains performed differentially depending on the environment they were competing in, we performed a generalized linear model (GLM) on $\log_2$-trranformed v with strain × community treatment as fixed explanatory variables, as well as their interaction. We carried out pairwise contrasts between strains *within* each of the two competition environments.

Pyocyanin determination of PAO1 evolved populations.

Pyocyanin was assayed by measuring the absorbance at 695 nm of cell-free supernatants after cell growth for 48 h at 37ºC in King's A broth. For results shown in Fig. 2c, pyocyanin production was determined independently for each evolved *P. aeruginosa* population (n = 6 for the monoculture- and community-evolved treatments, n = 10 clones per population), as well as the PAO1 ancestral strain (n = 6). Significant differences in pyocyanin production were assessed by performing a one-way ANOVA with Tukey contrasts.

Determination of the capacity to kill the bacterial community.

In order to evaluate whether the evolved PAO1 populations retained the capacity to kill the bacterial community, the toxicity of cell-free supernatants from PAO1 evolved populations was determined on each community member (*S. aureus*, *B. cenocepacia* and *A. baumanii*). Briefly, overnight cultures of the ancestral strain and of the twelve evolved PAO1 populations were grown

(these ONs were inoculated with 10 isolated colonies from each population) in 5 mL of ASM in 30 mL glass universal at 37°C at 180 rpm. Cell-free supernatants of each sample were obtained by centrifuging 3 mL of the ONs at 12,000 rpm for 10 min. To assess the capacity of PAO1 populations to kill community bacteria, $1x10^7$ CFU's of each community member (*S. aureus*, *B. cenocepacia* and *A. baumanii*) were individually grown in 96-well plates containing in 100 µl of ASM plus 100 µl cell-free supernatant of each of the 18 overnight *P. aeruginosa* populations (6 PAO1 Ancestral, 6 PAO1 monoculture, 6 PAO1 bacterial community). The carrying capacity of each community member was determined by measuring absorbance at OD_{600} after 16 h. The boxplots in Fig. 2c depict cumulative community densities ($OD_{S.aureus}$ + $OD_{B.\ cenocepacia}$ +$OD_{A.\ baumanii}$) averaged across treatment-specific populations. Significant differences in community toxicity were assessed by performing a Kruskal-Wallis on cumulative community densities with treatment as fixed explanatory variable. We carried out pairwise contrasts using Wilcoxon rank sum exact test. *p* values were adjusted using the fdr method.

***G. mellonella* infection experiments with PAO1 evolved populations.**

All virulence assays were done by injecting 10µl of diluted sample into the rear proleg of individual *G. mellonella* larvae (UK WaxWorm Ltd) using a sterile syringe as described in Hernandez *et al* 2019[64]. All larvae were checked for mortality and melanisation prior to injection. For the experiment shown in Fig. 2e, 10 clones per evolved populations were pooled together (*n* = 6 replicate populations per community treatment) in 6 ml of ASM medium. Each population mixture was injected into ten larvae, in three independent repeats (total larvae per treatment: monoculture = 180, bacterial community = 180 and ancestor = 30), with time to death recorded as a measure of virulence (three repeats comprised only 9 larvae, yielding *n* = 387 larvae in total). Throughout the experiment, larvae were stored in 12-well plates, containing one larva per well.

Before injection, all bacterial inoculums were grown for 24h at 37ºC on an orbital shaker (180 rpm), followed by assaying cell density using OD_{600} absorbance measurements, where 0.1OD equals ~1 x 10^8 CFU/ml. The OD_{600} measurements were subsequently used to calculate further dilutions down to approximately 10^4 CFU/ml, which was used for infection. Following infection, larvae were incubated at 37ºC, with mortality monitored hourly for up to 40 hours. The ancestral PAO1 strain and M9 salts were included as positive and negative controls, respectively (n = 10 larvae per repeat, with n = 3 repeats per control treatment). None of the larvae died following injection with M9 salts; hence, these data were not included in our subsequent analyses. We compared *G. mellonella* survival curves using the 'survival' and 'survminer' R packages[65]. Survival curves were plotted using Kaplan-Meier survival estimates and the overall effect of evolution treatment on curves was tested using a log-rank test ('survdiff' function), after which we computed pairwise contrasts, adjusted for multiple comparisons ('pairwise_survdiff' function with method = 'BH'). All work conforms to ethical regulations regarding the use of invertebrates, with approval from The University of Exeter ethics committee.

CF Sputum sample collection.

Sputum (0.05–2 ml) was obtained from CF patients chronically infected with *P. aeruginosa* attending the Copenhagen CF Center, Rigshospitalet, DK. Sputum was collected by voluntary expectoration and the samples were processed immediately after that routine microbiological tests were performed and the presence of *P. aeruginosa* was confirmed (2-3 days from collection). Each sample was mixed with an equal volume of a sputum-homogenizing agent (Sputasol; Oxoid Ltd, Basingstoke, UK) and the mucus was disrupted by vortexing during 30 minutes at room temperature. Then, each sample was divided into aliquots for the metagenomic analysis, culturing and – 80ºC storage.

Shotgun metagenomics of CF sputum samples.

a. DNA extraction

Sputum aliquots were subjected to a differential lysis protocol modified of Lim *et al*[66]. Bacterial cells from homogenized sputum aliquots were pelleted by centrifugation at 1.900 g for 15 min at 4°C. The pellets were resuspended in 2 ml deionized water, held at room temperature for 15 min, and centrifuged as above. This step was repeated, and then the pellets were resuspended in 2 ml 1x DNase buffer (50 mM NaAc, 10mM MgCl2, 2mM CaCl2, pH 6.5) and 3 µl of DNase I (1000U/ml) were added and the suspensions were incubated at 37ºC for 2 h. After centrifugation, the pellets were resuspended in 2 ml SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.5) and centrifuged as above. This step was repeated, and the final pellet was resuspended in ~ 500 µl SE. DNA was extracted from this cell suspension with MoBIO PowerSoil® DNA isolation kit (MO BIO Laboratories, Inc, ID 47016).

b. DNA quantity and quality

The quantity of total DNA present in the samples was determined by using the Qubit 2.0 flourometer and the Qubit® dsDNA HS Assay kit according to manufacter's protocol (Invitrogen Ltd., Paisley, United Kingdom). The quality was determined by measuring the A260/280 and A260/230 ratios using the NanoDrop ND-1000 Spectrophotometer (Nucliber).

c. Library preparation and sequencing

DNA samples were sent to Novo Nordisk Foundation Center for Biosustainability (Technical University of Denmark, DK) where all further laboratory analyses were performed. Sequencing libraries were prepared in triplicated using a Nextera XT Sample Preparation kit according to manufacter's instructions (Illumina UK, Litle Chesterfold, United Kingdom) and pooled together prior sequencing. Approximately 10 pM of pooled samples was loaded onto an Illumina MiSeq®

bench-top sequencer (Illumina UK, Litle Chesterfold, United Kingdom) and 300-cycle MiSeq v.1 reagent cartridges (Illumina, Litle Chesterfold, United Kingdom) were used to sequence libraries with paired-end, dual-indexing cycles per read (2 × 151). Data was demultiplexed on the MiSeq instrument automatically, and zipped FASTQ files were generated per sample, per read.

d. Data Preprocessing

Illumina sequencing of the sputum samples yielded 48.6 million sequences. Overlapping paired-end reads were merged in a single FASTA file for further analyses. The merged reads were processed using DeconSeq [67] to remove all human-like sequences with at least 90% query length coverage and 97% identity. This was done using the web version available at http://edwards.sdsu.edu/deconseq. The human reference database was created using three assemblies of the human genome: GRCh38, obtained from the Genome Reference Consortium - Primary Assembly (ftp://ftp.ncbi.nih.gov/genomes/H_sapiens/Assembled_chromosomes/seq/hs_ref_GRCh38_chr$i.fa.gz), CHM1_1.1, obtained from the Washington University School of Medicine (ftp://ftp.ncbi.nih.gov/genomes/H_sapiens/Assembled_chromosomes/seq/hs_alt_CHM1_1.1_chr$i.fa.gz) and HuRef, obtained from the J. Craig Venter Institute (ftp://ftp.ncbi.nih.gov/genomes/H_sapiens/Assembled_chromosomes/seq/hs_alt_HuRef_chr$i.fa.gz).

e. Database search and taxonomy assignment

After removing human reads, 18.4 million of ~ 2.600 millon pb were retained for further analyses. The preprocessed metagenomes were subjected to similarity database search against a bacterial sub-database built from NCBI non-redundant protein database (ftp://ftp.ncbi.nlm.nih.gov/refseq/release/bacteria/bacteria.nonredundant_protein.$i.protein.faa.gz

) using RAPSearch (v2.19)[68]. The default command-line options were used. RAPSearch output files (in aln format) were uploaded in MEGAN (MEtaGenome ANalyzer, version 4.70.4)[69] to perform taxonomic analysis using the following lowest common ancestor (LCA) parameters: minimum support = 1, minimum score = 100, max expected = 1.0e-5 and top percent = 10.0.

Isolation of CF *P. aeruginosa* subpopulations.

Serial dilutions from the sputum:sputosol mix were plated in *P. aeruginosa* isolation agar and incubated at 37 ºC for 48 hours. Thirty colonies were randomly isolated and grown in vials containing 5 ml of Luria Bertani broth (LB) and incubated at 37ºC with agitation. After 48 hours of growth glycerol 20% was added and all *P. aeruginosa* isolates collected were frozen at -80°C. All *P. aeruginosa* isolated were confirmed to be oxidase-positive.

Mutation frequency determination of CF *P. aeruginosa* subpopulations.

Thirty *P. aeruginosa* isolates were used to inoculate 30 ml universal vials containing 6 ml of LB and incubated ON at 37ºC with agitation (180 rpm). After that, samples where centrifuged and suspended and 500 µl of M9 salts and serial dilutions were plated on LB agar plates to determinate viable cells and on LB agar plates supplemented with 300 ug/ml of Rifampicin. Rifampicin resistant colonies were scored after 48 h of incubation at 37ºC and the mutation frequency for each *P. aeruginosa* CF population was determined as the number of CFU resistant to rifampicin per 10^8 viable cells per ml. *P. aeruginosa* normo-mutator wildtype PAO1 and mutator PAOMS (Δ*mutS*) strainsMENDELEY CITATION PLACEHOLDER 57 were used as controls.

Whole-genome sequencing of CF *P. aeruginosa* populations.

a. Sample pooling and DNA extraction.

We performed genome re-sequencing of our 24 *P. aeruginosa* populations isolated from CF sputum samples. For each sample, the total isolates obtained from each population (~ 30 isolates

per sample) were pooled and sequenced together. Pooling of isolates was performed by growing each colony ON in LB separately, and then pooling the equivalent numbers of cells from the total number of isolates. Genomic DNA from pooled populations was extracted using the Wizard Genomic DNA Purification Kit (Promega). DNA was quantified using the Qubit dsDNA BR assay (Invitrogen). Pooled DNA was submitted to Liverpool Centre for Genomic Research for library preparation (550-bp insert size) and sequencing on the Illumina HiSeq 2500 sequencing system (125-bp paired-end reads).

b. Sequence data preparation and variant calling.

Preparation of sequence data was performed as described previously[61]. Briefly, sequenced read data were trimmed using Cutadapt version 1.2.1[62] setting option -O 3 and Sickle version 1.2 (https://github.com/najoshi/sickle) setting minimum window quality score to 20 and omitting reads shorter than 10 bp after trimming but retaining single remaining reads from pairs. Reads were mapped with bwa mem (https://arxiv.org/abs/1303.3997) and duplicate reads were identified and removed with Picard (https://github.com/broadinstitute/picard). The GATK[70] HaplotyeCaller was used to perform local de novo assembly of haplotypes and call SNPs and indels from the pooled isolate sequence data with sample ploidy n = nº of isolates. Standard conservative filtering parameters were used to provide high-quality variant calls[71]. SNPs and indels calling was done using the *P. aeruginosa* PA14 genome (GCF_000404265.1) as reference genome. Protein coding effects were computed using SnpEff (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3679285/). To identify mutations in the QS-related genes found to be mutated during the PAO1 *in vitro* evolution experiments (Fig. 4 and Extended Data Table 9), the respective PAO1 orthologous genes in the PA14 strain (*psqR*/CIA_00903, *lasR*/CIA_01347, *dipA*/CIA_05650) were analyzed and the presence of non-synonymous mutations in each gene was determined. Each gene was compared

using NCBI-BLAST with 382 genomes of *P. aeruginosa* and those point mutations present in the majority of the genomes (>80%) were considered as PA14 polymorphisms and discarded from the analysis. To identify mutations in the CF resistome[48], 164 genes were analyzed and de the presence of non-synonymous mutations in each gene was determined. Each gene was compared using NCBI-BLAST with 382 genomes of *P. aeruginosa* and those point mutations present in the majority of the genomes (>80%) were considered as PA14 polymorphisms and discarded from the analysis.

Data availability

All data used in this study are available on figshare at https:// 10.6084/m9.figshare.13739452. Genome sequencing reads from *P. aeruginosa* populations from *in vivo* and *in vitro* experiments have been deposited under accession no PRJEB35620. All other data used in this paper is available in supplementary data.

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Author contributions: AML and AB conceived and designed the study. AML, LMS and EO carried out the experiments. SP, SML RLM, and MDS analysed sequence data. AML & EH analysed experimental data. AML, OC and HKJ were responsible for the CF sputum sample collection. HKJ, AMS and SM contributed to the design and interpretation of the *in vivo* analysis. AML and AB wrote the manuscript. All authors provided feedback on the manuscript.

Competing interests: The authors declare no conflict of interest.

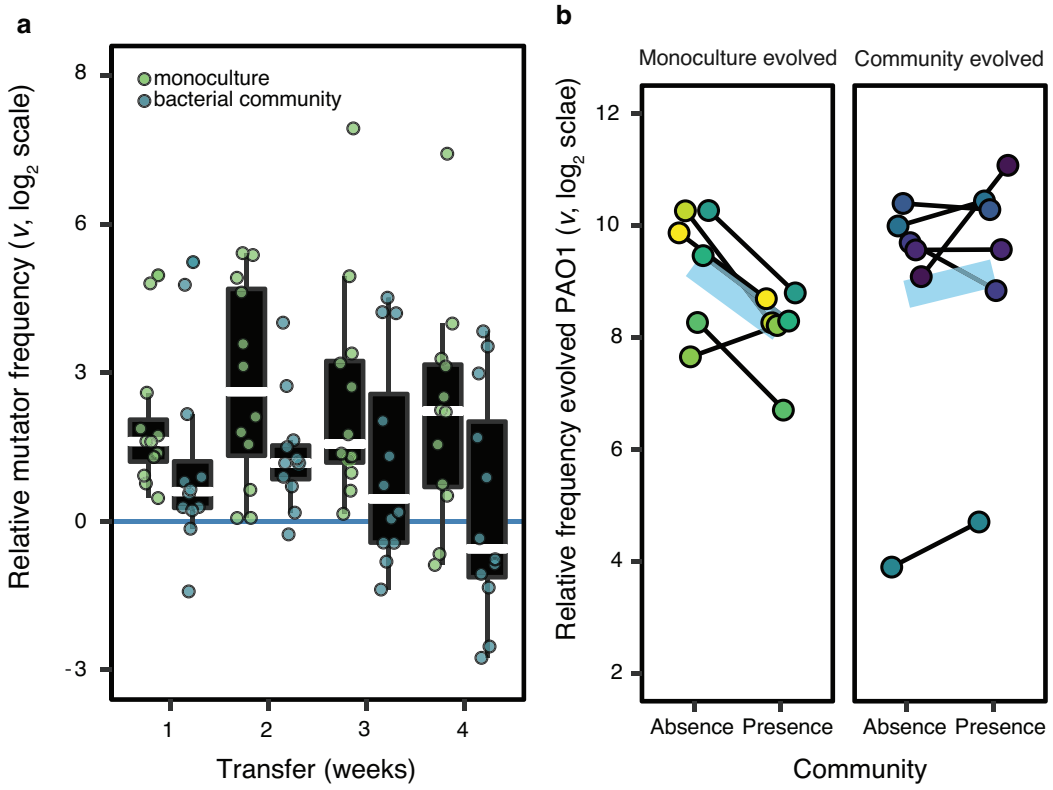

Fig. 1. Mutator fitness is reduced in the presence of the bacterial community. (**a**) Plot depicting temporal changes in relative mutator fitness (v) in the absence (green dots) and in the presence (blue dots) of the bacterial community. We determined relative mutator fitness (v) by comparing the initial frequency of mutators to the final frequency for each weekly transfer, using: $v = x_2(1\text{-}x_1)/x_1(1\text{-}x_2)$, where x_1 is the initial frequency of mutators at t_0 and x_2 their final frequency at transfer i. Boxes depict the upper and lower quartiles (the 75th and 25th percentiles) of the treatment-specific raw data with the center white line showing the median and the whiskers extending from the box to the largest and lowest value no further than 1.5x the interquartile range. Dots represent individual replicate populations (n = 12 per community treatment) (**b**) Plot depicting a trade-off between biotic and abiotic adaptation. Populations that had evolved as monocultures (n = 6) or in the presence of the bacterial community (n = 6) were

competed against the ancestral strain in both selective environments (i.e. in the absence and presence of the community). Fitness (v) of individual populations relative to the ancestral strain is depicted in different colors, and horizontal lines link the two different competition-treatments. Treatment-specific means are depicted in blue for each selective environment.

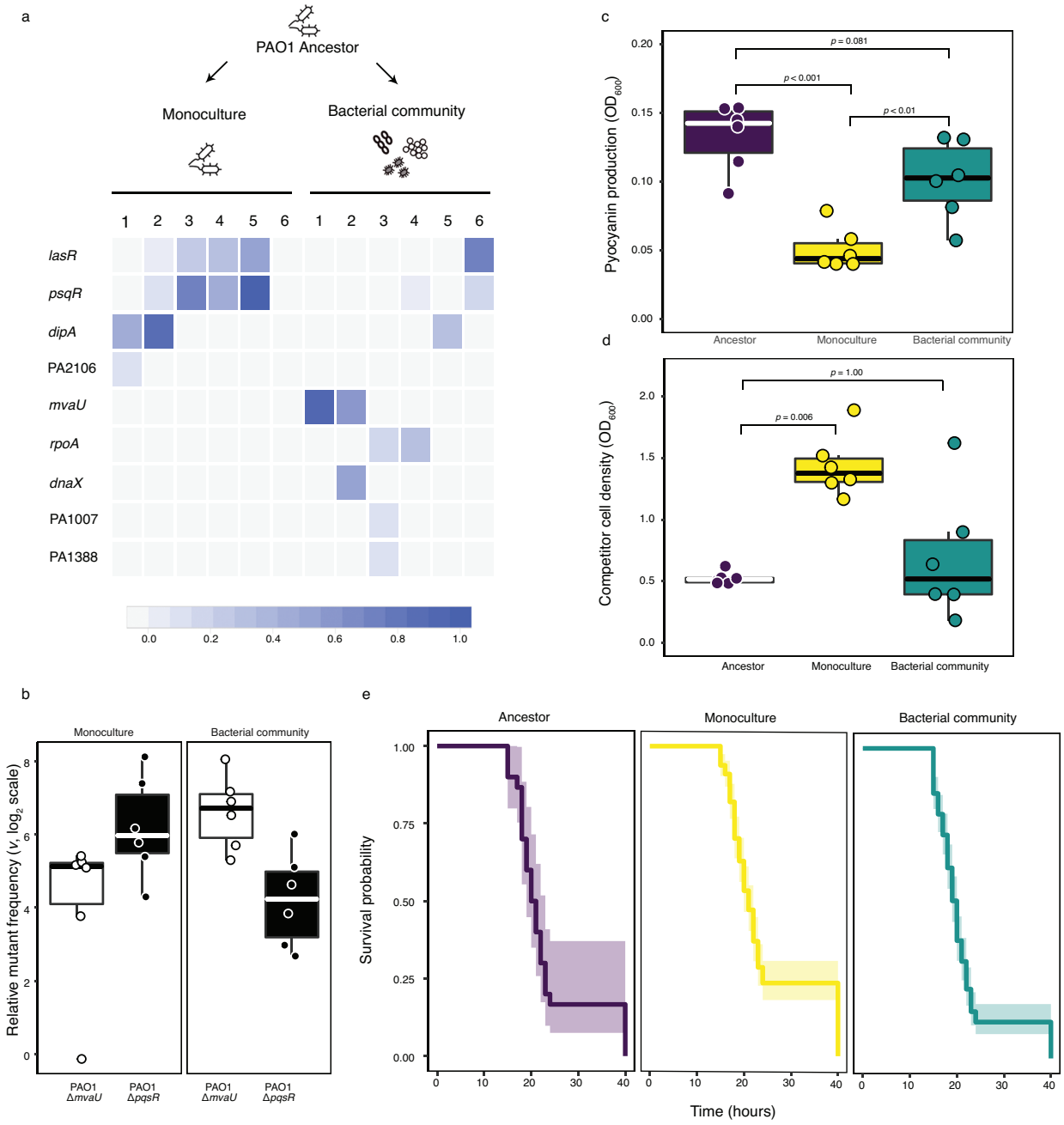

Fig. 2. Fitness tradeoff associated with interspecific interactions is related to QS regulation. **(a)** Heatmap of the frequency of mutated genes (>10%) obtained from the population genome sequencing of evolved PAO1 wildtype populations in the absence and in the presence of the bacterial community. The maximum non-synonymous SNPs and/or short indels frequency within a gene are shown; **(b)** Boxplot showing fitness (v, $\log_2$-scale) of PAO1 Δ*mvaU* and Δ*psqR* deletion

mutant strains relative to the ancestral strain in the absence and presence of the bacterial community. These data show that the loss of Quorum-Sensing is more beneficial in the absence (that is, Δ*psqR* higher *v*) than in the presence of the bacterial community (Δ*psqR* lower *v*). The virulence of PAO1 evolved populations was determined using conditioned media assays through the quantification of: (**c**) pyocyanin production (one-way ANOVA with Tukey contrasts: overall difference in pyocyanin production; $F_{(2,15)} = 18.33$, $p < 0.001$, $n = 6$ per community; significance of Tukey contrasts is shown on the top of boxplots (*p* values)), (**d**) the capacity to kill the bacterial community. Boxplots show the cumulative cell density of the three competitors forming the bacterial community (one-way Kruskal-Wallis on cumulative competitor density $\chi^2_{(2)} = 8.5$, $p = 0.01$, $n = 6$ per community treatment; significance of post hoc FDR corrected Wilcox rank sum test (*p* values) is shown in the top of boxplots), (**e**) survival curves for the different evolution treatments ($n = 387$, with 29-30 larvae per population, divided over 3 technical repeats), analysed using a log-rank test. Shaded areas represent the 95% confident intervals of the fitted curves. In all cases, boxplots depict the median, the lower and upper quartiles (the 25th and 75th percentiles), with whiskers extending to 1.5x the interquartile range.

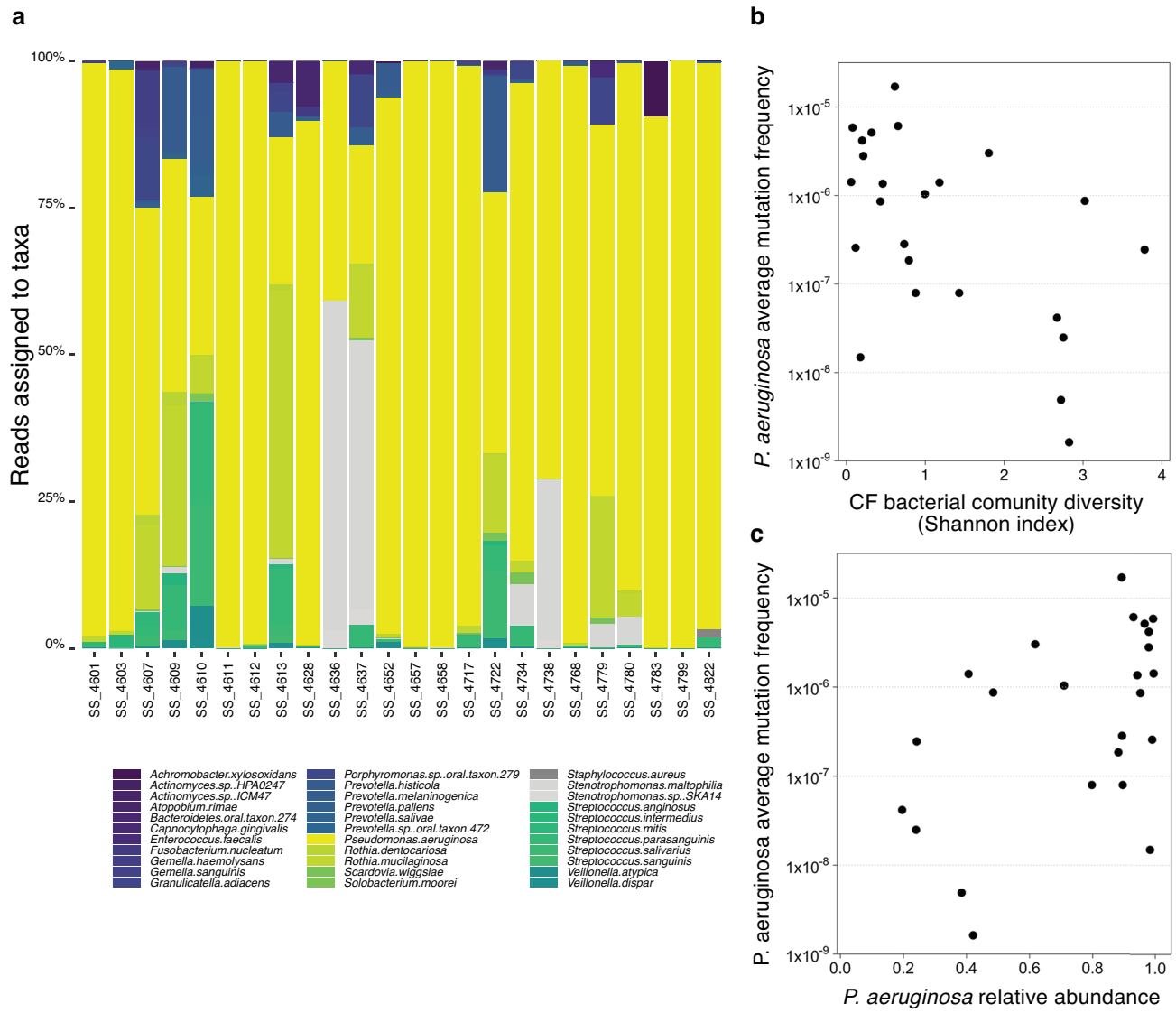

Fig. 3. Relationship between bacterial community diversity and *P. aeruginosa* mutation frequency in cystic fibrosis sputum samples. (**a**) Bacterial community composition of the twenty-four CF sputum samples. The coloured stacked bars represent the proportion of reads mapping to different bacterial taxa in each sample assessed by MEGAN. Only bacteria present in more than 1% are shown. (**b**) Correlation between CF bacterial community diversity (Shannon index) and average mutation frequency of *P. aeruginosa* populations obtained from each CF sputum sample (two-tailed Pearson's correlation $r = -0.46$, $t = -2.42$, df = 22, $p = 0.023$). (**c**) Correlation between the mutation frequencies of *P. aeruginosa*'s populations and it´s relative

abundance within each CF bacterial community (two-tailed Pearson's correlation $r = 0.49$, $t = 2.67$, df = 22, $p = 0.013$). Each point represents the mean of three independent replicates.

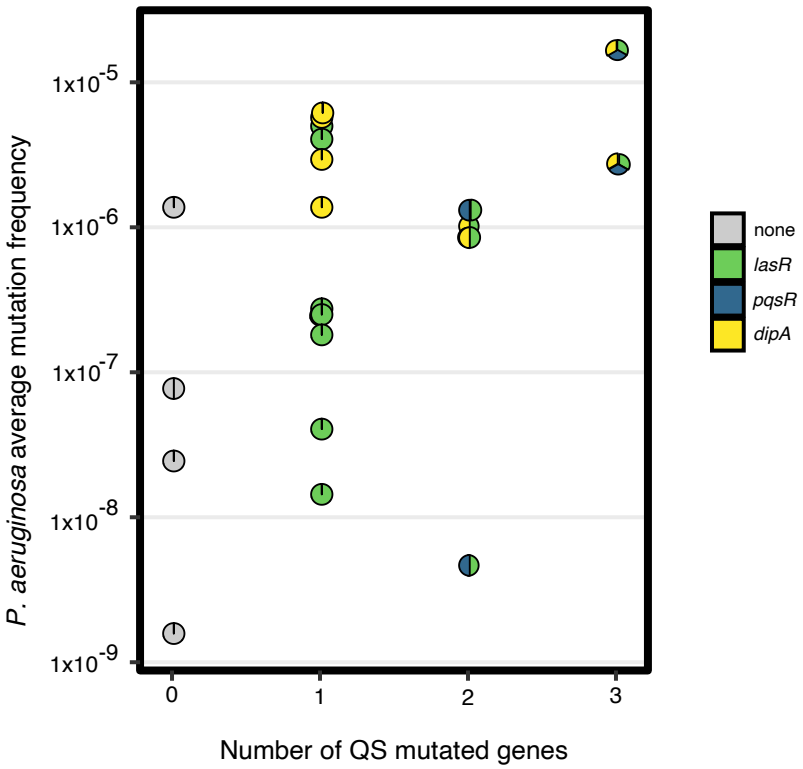

Fig. 4. *P. aeruginosa* CF populations showing high mutation frequencies accumulate more mutations in QS related genes. Relation between *P. aeruginosa* average mutation frequency and the number of QS transcriptional regulators mutated *in vivo* (two-tailed Pearson's correlation $r = 0.43$, $t = 2.23$, df = 22, $p = 0.036$). Each point represents data obtained for each *P. aeruginosa* population isolated from the twenty-four CF patients. Point color indicates mutations accumulated in QS transcriptional regulators shown to be altered during *in vitro* evolution in the absence of the bacterial community: *lasR*, *pqsR* and *dipA*. *P. aeruginosa* populations that did not show mutations in these genes are shown in grey. Note that correlations between QS genes and community properties (Shannon diversity, frequency of *P. aeruginosa*) were not significant.

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