MOLECULAR ECOLOGY

Molecular Ecology (2015) **24**, 1572–1583 doi: 10.1111/mec.13127

Fitness trade-offs explain low levels of persister cells in the opportunistic pathogen *Pseudomonas aeruginosa*

KRISTINE STEPANYAN,*[1] TOM WENSELEERS,†[1] EDGAR A. DUÉÑEZ-GUZMÁN,† FRÉDÉRIC MURATORI,† BRAM VAN DEN BERGH,* NATALIE VERSTRAETEN,* LUC DE MEESTER,‡ KEVIN J. VERSTREPEN,*§ MAARTEN FAUVART*[2] and JAN MICHIELS*[2]

*Centre of Microbial and Plant Genetics, KU Leuven – University of Leuven, Kasteelpark Arenberg 20 bus 2460, 3001 Leuven, Belgium, †Laboratory of Socio-Ecology and Social Evolution, KU Leuven – University of Leuven, Zoological Institute, Naamsestraat 59, 3000 Leuven, Belgium, ‡Laboratory of Aquatic Ecology, Evolution and Conservation, KU Leuven – University of Leuven, Charles Deberiotstraat 32 bus 2439, 3000 Leuven, Belgium, §VIB Laboratory for Systems Biology, Vlaams Instituut voor Biotechnologie (VIB), Bioincubator Leuven, Gaston Geenslaan 1, 3001 Leuven, Belgium

Abstract

Microbial populations often contain a fraction of slow-growing persister cells that withstand antibiotics and other stress factors. Current theoretical models predict that persistence levels should reflect a stable state in which the survival advantage of persisters under adverse conditions is balanced with the direct growth cost impaired under favourable growth conditions, caused by the nonreplication of persister cells. Based on this direct growth cost alone, however, it remains challenging to explain the observed low levels of persistence (<<1%) seen in the populations of many species. Here, we present data from the opportunistic human pathogen *Pseudomonas aeruginosa* that can explain this discrepancy by revealing various previously unknown costs of persistence. In particular, we show that in the absence of antibiotic stress, increased persistence is traded off against a lengthened lag phase as well as a reduced survival ability during stationary phase. We argue that these pleiotropic costs contribute to the very low proportions of persister cells observed among natural *P. aeruginosa* isolates (3×10^{-8}–3×10^{-4}) and that they can explain why strains with higher proportions of persister cells lose out very quickly in competition assays under favourable growth conditions, despite a negligible difference in maximal growth rate. We discuss how incorporating these trade-offs could lead to models that can better explain the evolution of persistence in nature and facilitate the rational design of alternative therapeutic strategies for treating infectious diseases.

Keywords: evolutionarily stable strategy, persistence, pleiotropy, *Pseudomonas aeruginosa*

Received 19 October 2014; revision received 9 January 2015; accepted 13 February 2015

Introduction

Development of antibiotic resistance in bacteria poses a serious threat to both human and animal health (Bush *et al.* 2011). Apart from genetic adaptation, however, additional mechanisms enable micro-organisms to transiently tolerate antibiotics (Stewart 2002; Fux *et al.* 2005; Levin & Rozen 2006; Dhar & McKinney 2007). One such mechanism is known as persistence and involves a small fraction of cells making a phenotypic switch to a quiescent state. This switch results in slow or zero growth and confers tolerance to high doses of antibiotics (Balaban *et al.* 2004; Keren *et al.* 2004; Levin & Rozen 2006; Gefen & Balaban 2009; Kint *et al.* 2012; Maisonneuve & Gerdes 2014). In contrast to resistance, antibiotic tolerance of persister cells is a reversible, noninherited phenotype. Consequently, following

Correspondence: Jan Michiels, Fax: +32 16 32 19 63;
E-mail: Jan.Michiels@biw.kuleuven.be
[1]These two authors contributed equally as first authors.
[2]These two authors contributed equally as senior authors.

antibiotic treatment, the surviving persisters resume growth and give rise to a new population that is as sensitive to antibiotics as the ancestral population (Keren *et al.* 2004; Levin & Rozen 2006; Lewis 2007).

Persistence is of great medical importance, as it occurs in numerous pathogens, including *Acinetobacter baumannii*, *Burkholderia cepacia*, *Escherichia coli*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Staphylococcus aureus* and *Streptococcus suis* (Willenborg *et al.* 2014) (Stewart *et al.* 2003; Levin & Rozen 2006; Dhar & McKinney 2007; Lewis 2010; Fauvart *et al.* 2011; Barth *et al.* 2013; Knudsen *et al.* 2013; Slattery *et al.* 2013; Van Acker *et al.* 2013; Maisonneuve & Gerdes 2014; Willenborg *et al.* 2014). In addition, persistence has been identified as a key cause of failure of antibiotic treatments, particularly in recalcitrant chronic infections (LaFleur *et al.* 2010; Lewis 2010; Mulcahy *et al.* 2010; Fauvart *et al.* 2011; Orman & Brynildsen 2013). Given this huge medical importance, the past decade has seen rapidly growing efforts to unravel the biology and mechanisms underlying persistence (Mulcahy *et al.* 2010; Kint *et al.* 2012; Stewart & Rozen 2012; Barraud *et al.* 2013; Hofsteenge *et al.* 2013; Knudsen *et al.* 2013; Van Acker *et al.* 2013; Maisonneuve & Gerdes 2014; Willenborg *et al.* 2014). Despite this active ongoing research, many questions remain, particularly regarding the mechanisms and evolutionary forces that drive variation in persistence in natural populations (Balaban *et al.* 2013). Empirically, it has been observed that persisters can be formed either by random switching (Balaban *et al.* 2004; Keren *et al.* 2004) or in response to specific environmental cues (Dörr *et al.* 2009; Vega *et al.* 2012; Maisonneuve & Gerdes 2014). These findings have inspired several theoretical models aimed at untangling the selective forces that determine the development and level of persistence. For example, Kussell & Leibler (2005) showed that stochastic switching is favoured most in environments that change relatively infrequently, whereas responsive switching was found to be most beneficial in highly unpredictable environments. Other studies have analysed persistence as a bet-hedging mechanism, in which bacterial lineages trade instantaneous growth for long-term survival (Kussell *et al.* 2005; Veening *et al.* 2008). Similar to models describing dormancy and diapause in plant seeds and aquatic invertebrates (Cohen 1966; Philippi & Seger 1989; Patra & Klumpp 2013; Baskin & Baskin 2014), these models of persistence predict that the optimal rate of entering the persister state should be inversely proportional to the duration of favourable environmental conditions, while the optimal rate of waking-up from the persister state should be inversely proportional to the duration of stressful environmental conditions (Lachmann & Jablonka 1996; Thattai & van Oudenaarden 2004; Kussell & Leibler 2005; Kussell *et al.* 2005; Patra & Klumpp 2013). Similarly, Gardner *et al.* (2007) predicted that the unbeatable or 'evolutionarily stable' allocation to persister cells should be inversely proportional to the interval between antibiotic strikes and argued that the commonly observed increase in the proportion of persisters under nutrient-limiting conditions (Keren *et al.* 2004; Moker *et al.* 2010; Luidalepp *et al.* 2011; Knudsen *et al.* 2013) could be an adaptive strategy to reduce competition among genetically related lineages.

Despite the importance of models of bacterial persistence, few studies have tried to validate such models in detail. More problematically, there appears to be a large discrepancy between typical model predictions and empirical observations, limiting the practical use of available models. For example, Kussell *et al.* (2005) calculated that the percentage of persister cells typically found in *E. coli* (approximately 0.0001–0.001 %, Lewis 2010) would match optimal switching rates if the bacteria were exposed to antibiotics for just 1 day every 100 years. Clearly, this prediction appears highly unrealistic and hints at low levels of persistence being maintained by other factors than just a direct growth cost. Indeed, several studies have hinted at the possibility that the main cost of persistence might not stem from the nonreplication of persister cells but rather from fitness trade-offs with other growth parameters, such as the length of the lag phase before growth commences upon new colonizations (Balaban *et al.* 2004) or survival ability in resource-deprived environments (Gardner *et al.* 2007). In tentative support of this 'pleiotropic cost' hypothesis, it was found that in *E. coli*, the high-persister mutant strain *hipA7* had an extended lag phase when inoculated into fresh medium (Balaban *et al.* 2004; Gefen *et al.* 2008) and that wild-type cultures displayed lengthened lag phases when persistence was increased through starvation (Levin-Reisman *et al.* 2010; Luidalepp *et al.* 2011). In addition, a similar lengthening of the lag phase has been observed in the high-persister mutant *shp* in *Salmonella enterica* (Slattery *et al.* 2013). The full extent of such fitness trade-offs and antagonistic pleiotropy, however, has not been examined in detail, and it remains unknown whether also in other species, persistence negatively affects other growth parameters.

The aim of the present study was to study the factors that drive the evolution of persistence in the opportunistic human pathogen *P. aeruginosa* and document the pleiotropic costs and fitness trade-offs that could help explain the observed low levels of persistence (Moker *et al.* 2010; Mulcahy *et al.* 2010; Fauvart *et al.* 2011). In particular, we present competition assays among strains and mutants with variable levels of persisters under contrasting antibiotic treatment schedules (cf. Balaban *et al.* 2004; Stewart & Rozen 2012) and provide data on persistence

levels among 16 environmental and clinical *Pseudomonas* isolates. Subsequently, we show that the competitive advantage of low-persister strains under favourable growth conditions as well as the extremely low observed proportions of persistence in natural isolates cannot adequately be explained based on a direct growth cost alone. Subsequently, this hypothesis is confirmed by experiments that show that increased persistence in *P. aeruginosa* is traded off against a lengthened lag phase under favourable growth conditions and a reduced survival ability in resource-deprived environments. We conclude with a discussion about the impact that our results could have for the rational design of alternative therapeutic strategies to treat infectious diseases.

Materials and methods

Strains and culture conditions

We used the representative *P. aeruginosa* wild-type strain PA14 (*wt*) and the isogenic high-persister (*hip*) mutant CMPG13407 (De Groote *et al.* 2009), as well as a natural low-persister strain Br257 and the high-persister strain Br642 (Pirnay *et al.* 2002a). The CMPG13407 *hip* mutant has a mutation in *pilH*, which encodes a CheY-like response regulator that controls type IV pilus expression, and produces almost 50 times more persister cells than the *wt* (0.03 % in the *hip* mutant vs. $7 \times 10^{-4\%}$ in the *wt*, Table S1, Supporting information; De Groote *et al.* 2009). The environmental isolate Br257 and the clinical isolate Br642 were chosen because they vary almost 200-fold in their respective proportion of persister cells ($1 \times 10^{-5\%}$ in Br257 vs. $2 \times 10^{-3\%}$ in Br642, Table S1, Supporting information), but otherwise have similar growth characteristics (i.e. no significant differences in growth rate and carrying capacity, Fig. S5, Supporting information). Natural levels of persistence were determined in 16 environmental and clinical isolates of *P. aeruginosa* (Pirnay *et al.* 2002a, Table S1, Supporting information).

All strains were cultured in Mueller Hinton Broth (MHB, BD Difco) at 37 °C, shaking at 200 rpm, unless stated otherwise. Serial dilutions of samples were plated on TSB agar plates and incubated at 37 °C for 24 h to quantify the number of colony-forming units (CFU).

Competition assays between high- and low-persister strains

To determine whether persistence levels can be interpreted as evolutionarily stable strategies that are attuned to particular frequencies of antibiotic attack (Kussell *et al.* 2005; Gardner *et al.* 2007; Patra & Klumpp 2013), we carried out competition assays between high- and low-persister strains either in the absence or presence of periodic antibiotic exposure (Balaban *et al.* 2004; Stewart & Rozen 2012). We refer to the latter as frequent antibiotic treatment. The expectation was that in the absence of antibiotic exposure, the low-persister strains would win out, whereas the high-persister strains would do better in its presence (Balaban *et al.* 2004; Kussell *et al.* 2005; Stewart & Rozen 2012).

To test this prediction, we compared the growth of pairs of high- and low-persister strains in competition, either when they were regrown repeatedly in fresh medium, or when the strains were intermittently treated with the fluoroquinolone antibiotic ofloxacin, which is widely used for the treatment of *P. aeruginosa* infections (Mulcahy *et al.* 2010). Importantly, the high-persister strains or mutants that we used for these experiments had much lower absolute proportions of persisters (0.002% for the high-persister strain Br642 and 0.03% for the PA14 *hip* mutant, Table S1, Supporting information) than those used in comparable experiments carried out in *E. coli* (e.g. up to 4% in the competition assays among three natural pairs of strains carried out by Stewart & Rozen 2012). This meant that in our experiments, in contrast to previous studies (Balaban *et al.* 2004; Stewart & Rozen 2012), there was a negligibly small direct growth cost due to nonreplication of persister cells under favourable growth conditions. This in turn implied that any advantage of low-persister strains under such conditions was expected to be caused by the presence of additional, pleiotropic costs of persistence.

Competition experiments were carried out by first adjusting overnight cultures of the competing strains to an optical density of 0.5 at 595 nm (OD_{595}), mixing equal volumes of both, diluting them 100-fold in 100 mL of fresh medium and growing them together for 19 h until stationary phase (approximately 5×10^9 CFU per ml). Subsequently, persistence levels were quantified as described below, using colony morphology after plating to visually distinguish competing strains, and samples were subjected either to antibiotic or control treatments, after which 100 mL of fresh medium was re-inoculated via 100- or 100 000-fold dilution, respectively. These 24-h cycles were repeated five times, and each treatment combination was replicated at least three times. In the antibiotic treatment conditions, ofloxacin was administered for 5 h at approximately 10× the minimum inhibitory concentration (MIC), which was 5 μg/mL for the *wt* strain and *hip* mutant, and 100 μg/mL for rhizosphere isolate Br257 and clinical isolate Br642 (Table S1, Supporting information). The MIC was determined by broth microdilution as described previously (Liebens *et al.* 2014). To exclude the emergence of antibiotic resistance, MIC values were measured in triplicate for the different strains after each

treatment round, and it was checked that there were no significant correlations between MIC values and treatment round. An increase or decrease in the proportional representation of one strain relative to the other over subsequent growth or treatment cycles was tested by fitting a mixed model on a logit scale and including cycle as a fixed factor and replicate as a random factor. This was performed using *lme4* in *R*. Statistical significance was assessed using likelihood ratio tests. Analogous control experiments were carried out using pure cultures of each strain (Fig. S1, Supporting information).

Variation in persistence levels among natural isolates

To determine natural levels of persistence, and to be able to make a comparison to theoretically expected levels of persistence under plausible antibiotic treatment frequencies (see Appendix S1, Supporting information), we measured persistence among 16 environmental and clinical isolates of *P. aeruginosa* (Pirnay *et al.* 2002a; Table S1, Supporting information). Persister fractions were measured as the ratio of the number of CFU per mL obtained after treatment with ofloxacin (Sigma-Aldrich) relative to the number of CFU per mL obtained after control treatment with sterile water. Measurements were made by diluting overnight cultures 100-fold in 100 mL of fresh MHB medium and culturing them for 19 h. Next, stationary phase cultures were treated for 5 h either with ofloxacin (at 10× the MIC) or with sterile water. Surviving cells were quantified by plate counting. Each experiment was performed with at least three biological replicates. The MIC of each strain was determined by broth microdilution in triplicate, as described previously (De Groote *et al.* 2009). To exclude the possibility of confounding persistence with antibiotic resistance, we tested for a correlation between persistence and the MIC across our strains using Spearman rank correlation and showed it to be nonsignificant (Table S1, Supporting information). In addition, we confirmed that antibiotic exposure of an isogenic PA14 *wt* culture resulted in characteristic biphasic killing, expected by the fast killing of normal antibiotic-sensitive cells followed by the slow killing of persister cells (Hofsteenge *et al.* 2013; Kint *et al.* 2012; Patra & Klumpp 2013; Fig. S2, Supporting information). Average levels of persistence per strain were calculated using a binomial mixed model fitted using *R* package *lme4*, and 95% confidence limits on the estimates were calculated using the *effects* package. In this model, strain and replicate were coded as fixed and random factors, respectively. As a measure of the genetic heritability of persistence levels, we calculated the repeatability of replicate measurements of persistence in the same strains based on the intraclass correlation coefficient (ICC) (Lynch & Walsh 1998). This ICC and the 95% confidence limits were calculated on a logit scale using function ICCest in R package ICC. To provide a reference point of known historical antibiotic exposure, observed persistence levels of strains from various sampling sites were compared with the persistence levels documented by Mulcahy *et al.* (2010) in *P. aeruginosa* collected from patients with cystic fibrosis either before or after extended ofloxacin treatment schedules. Methodologically, this data set can be compared to ours, given that persistence levels were measured using a nearly identical method (i.e. in stationary phase using an overnight treatment with ofloxacin and using the same growth medium). Finally, observed persistence levels were compared to previously formulated theoretical models of persisters (Gardner *et al.* 2007; Kussell *et al.* 2005, Appendix S1, Supporting information) to demonstrate that under plausible antibiotic exposure schedules, the observed low persistence levels are challenging to explain without invoking fitness trade-offs with other growth parameters.

Costs and trade-offs of persistence

To directly measure both direct and indirect, pleiotropic costs of persistence, we correlated observed levels of persistence with the growth parameters of nine different strain derived from the Pirnay *et al.* (2002b) type collection, as well as those of the PA14 *wt* and isogenic CMPG13407 *hip* high-persister mutant (De Groote *et al.* 2009). Growth parameters were determined by measuring OD_{595} values in liquid culture every 15 min over a period of 43.25 h using an automated plate reader (Bioscreen C, Oy Growth Curves Ab, Finland), after which OD values were converted back to cell numbers (CFU) using an independently derived nonlinear calibration curve (given by the equation $\mathrm{Log_{10}(CFU)} = 9.066(1.174 + \mathrm{Log_{10}(OD)})^{0.144}$, $R^2 = 0.9997$). Subsequently, a modified Gompertz growth model (Zwietering *et al.* 1990) was fitted using *Wolfram Mathematica v9* (code available from the authors on request), which enabled the simultaneous estimation of initial cell number, lag time, maximal growth rate and carrying capacity. This growth model was chosen on the basis that it resulted in robust fits with very high explanatory power (average $R^2 = 0.9990$, SD = 0.0026, $n = 377$). In all cases, samples were first adjusted to near single-cell level by dilution based on the estimated number of viable cells present in the sample. Next, single-cell inoculation was achieved through serial twofold dilution of the adjusted samples. For each sample, the well with the largest dilution where growth was still observed was considered to yield a growth curve originating from a single cell and this growth curve was selected for further analysis. Growth curves were determined after dilution in fresh

medium either with or without intermittent treatment with ofloxacin (cf. section above). To obtain accurate estimates, 15–20 (avg. 17) replicate growth curves were measured per strain under each condition.

Finally, to measure possible costs of persistence in terms of affecting cellular mortality and turnover in the stationary phase, we diluted overnight cultures of each strain 100-fold in 100 mL of fresh medium and monitored them for 5 days after reaching stationary phase. During this period, we determined the number of persisters and nonpersisters at 24-h intervals as described above. Subsequently, we calculated the net rate of decline of normal and persister cells in stationary phase cultures as the average decline in the log-transformed number of normal and persister cells (expressed in CFU) over time and correlated these with the average proportion of persisters observed in stationary phase in each strain using Spearman rank correlations. This was performed in triplicate. Net rates of decline were calculated from the slope of the logarithm of the number of normal or persister cells as a function of time using linear mixed models (LMM), in which the three replicates were coded as a random factor. We should note that this net rate of decline could be affected by several processes, including direct mortality, switching between nonpersisters and persisters as well as direct growth of nonpersister cells (Patra & Klumpp 2013). However, for our purposes, which was to document the costs of persistence, the net rate of decline of normal and persister cells in function of persistence was a good measure of overall cost, and hence, it did not matter that this estimate could potentially be affected by several distinct processes.

Results

Low-persister strains have a competitive benefit in the absence of antibiotic treatment

As expected if persistence is an evolutionarily stable strategy adapted to a particular frequency of antibiotic attack (Kussell *et al.* 2005; Gardner *et al.* 2007; Patra & Klumpp 2013), the results of our competition experiments demonstrate that in the absence of antibiotic treatments, the low-persister strains (*wt* and Br257) were more fit than the high-persister strains (*hip* and Br642), but that the reverse was true in the presence of periodic ofloxacin antibiotic treatment (Fig. 1). In fact, in the absence of antibiotic treatment, five cycles of dilution and regrowth caused the *wt* strain to outcompete the *hip* mutant by a factor of 1.75 ± 0.39 (mean $\pm$ standard deviation (SD), $n = 3$) (Fig. 1a, significance of increase in *wt* representation after 5 dilution and growth cycles: LMM on logit-transformed proportions, $\beta = 0.006$, $\chi^2 = 98.11$, $P < 10^{-15}$). Similarly, over the course of our experiment, the low-persister strain Br257 outcompeted the high-persister strain Br642 by a factor of 1.89 ± 0.56 (Fig. 1c, significance of increase in Br257 representation over 5 dilution and growth cycles: LMM, $\beta = 0.007$, $\chi^2 = 52.67$, $P = 4 \times 10^{-13}$). The large fitness benefit of the low-persister strains under

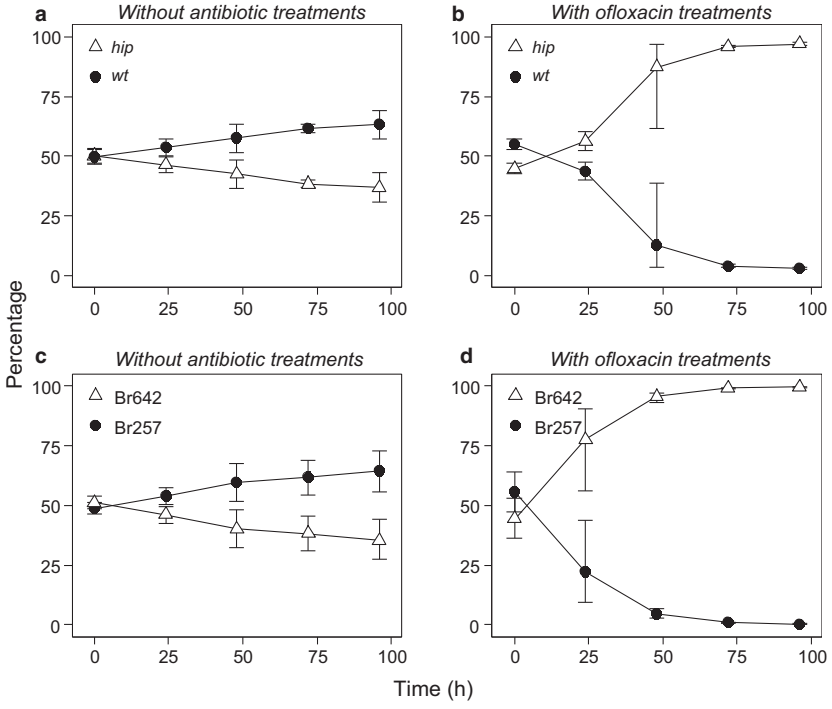


Fig. 1 Relative fitness of high- and low-persister strains over subsequent growth cycles in the presence or absence of intermittent antibiotic treatments. Strains with high and low levels of persister cells (laboratory-adapted *hip* mutant and PA14 *wt*, panels a and b; or the Br642 and Br257 strains, panels c and d) were grown together in a mixture to stationary phase. Before each growth cycle, cultures were exposed to either a control treatment (panels a and c) or to the antibiotic ofloxacin (panels b and d). Low-persister strains took over in the absence of intermittent antibiotic treatments, whereas the high-persister ones took over if each growth cycle was preceded by an antibiotic treatment. Error bars represent 95% confidence limits ($n = 3$ replicates per treatment combination).

favourable growth conditions was surprising, given the expected negligibly small direct growth cost of persistence in the competing high-persister strains, owing to their overall low fractions of nonreplicating persister cells (0.002–0.03%). Hence, these results imply the likely existence of pleiotropically linked costs of persistence.

In the presence of periodic antibiotic treatments, the reverse pattern could be observed, with 5 cycles of antibiotic treatment and regrowth then causing the *hip* mutant to outcompete the *wt* strain by a factor of 31.4 ± 2.5 (Fig. 1b, significance of decrease in *hip* representation over five antibiotic treatment cycles: LMM, $\beta = 0.04$, $\chi^2 = 190.65$, $P < 10^{-15}$) and the high-persister strain Br642 to outcompete the Br257 strain by a factor of 328 ± 162 (Fig. 1d, significance of increase in Br642 representation over five treatment cycles: LMM, $\beta = 0.06$, $\chi^2 = 709.24$, $P < 10^{-15}$). In control experiments, the cell numbers (Log_{10}(CFU)) obtained for the *hip* mutant or *wt* after five growth or treatment cycles were not significantly different from each other (in the absence of antibiotics: paired *t*-test on logit-transformed proportions: $t = 0.94$, $P = 0.45$, $n = 3$, in the presence of antibiotic treatment: $t = -2.69$, $P = 0.15$, $n = 3$, Fig. S1, Supporting information). Likewise, the numbers of cells obtained from the Br257 and Br642 strains after 5 dilution and growth cycles were not significantly different from each other (paired *t*-test: $t = 2.64$, $P = 0.12$, $n = 3$), while a marginal difference in cell numbers was observed over 5 antibiotic treatment cycles ($t = -5.93$, $P = 0.03$, $n = 3$, Fig. S1, Supporting information). This difference, however, was negligible compared to the difference in representation seen in the actual competition experiments (Fig. 1d). Antibiotic resistance did not emerge in any of our competition assays, as shown by the fact that MIC values remained unchanged over the course of our experiments and that we never detected a significant correlation between MIC and treatment round (Pearson correlations, all $P >> 0.05$). In addition, ofloxacin treatment induced a biphasic killing pattern, characteristic for the presence of a normal and persister cell subpopulations with differing antibiotic tolerances (Fig. S2, Supporting information).

Low levels of persistence among natural P. aeruginosa isolates

Persister fractions in 16 natural strain isolates derived from the Pirnay *et al.* (2002) type collection varied by five orders of magnitude, but were very low, ranging from 3×10^{-8} to 3×10^{-4} (Table S1, Supporting information). Interstrain variation in persistence was highly significant (fixed effect ANOVA on logit-transformed proportions of persisters: $F_{17,36} = 76.9$, $P < 2 \times 10^{-16}$), and persistence levels were highly repeatable among replicate measurements of the same strains (intraclass correlation 0.96, 95% C.L.: 0.92–0.98). This implies that variation in persistence levels had a very high genetic heritability. With a proportion of 7×10^{-6} persister cells, the PA14 *wt* strain was representative for the average level of persistence observed among the typed strains, whereas the PA14 *hip* mutant, with a proportion of 3×10^{-4} persister cells, was more similar to natural isolates with high persistence levels (Table S1, Supporting information). Proportions of persisters were highest among lung and hospital infection isolates, intermediate in wound, throat or blood infection isolates and lowest in one environmental isolate (Table S1, Supporting information). Importantly, persistence levels were not confounded with antibiotic resistance, as shown by the nonsignificant correlation between persistence and the MIC for the antibiotic ofloxacin, used to monitor persistence (Spearman rank $R = 0.20$, $P = 0.42$, $n = 18$).

Comparison of observed persistence levels to those obtained by Mulcahy *et al.* (2010) in *P. aeruginosa* isolated from cystic fibrosis patients with known historical records of antibiotic exposure shows that the persistence levels of our clinical strains were not significantly different from the average persistence levels of *P. aeruginosa* strains isolated from patients with cystic fibrosis early on in the infections (1×10^{-5}, $n = 15$; *t*-test on Log_{10}-transformed persistence levels, $t = -1.16$, d.f. = 29, $P = 0.25$), but that they were significantly lower than the persistence levels found in the *P. aeruginosa* strains in those same patients with CF 6–16 years later, after having been exposed to extended periods of antibiotic treatment (2×10^{-4}, $n = 15$; $t = -4.2$, d.f. = 29, $P = 0.0002$) (Fig. 2). Furthermore, the single environmental isolate had the second lowest level of persistence of all strains examined (Fig. 2). Finally, comparison of the observed levels of persistence to values predicted by previously formulated models (Kussell *et al.* 2005; Gardner *et al.* 2007) shows that the observed low levels of persistence are challenging to explain without either invoking additional, pleiotropically linked costs of persistence aside from a direct growth cost (Appendix S1, Fig. S3, Supporting information), or assuming that also many normal cells would survive antibiotic attacks (Gardner *et al.* 2007). Survival of normal cells, however, was estimated to be very low after longer durations of antibiotic treatment (Fig. S4, Supporting information). Consequently, we decided to test whether the existence of pleiotropic costs and fitness trade-offs could be an alternative explanation for the observed low levels of persistence in *P. aeruginosa*.

Complex fitness trade-offs explain low levels of persistence

Theoretical models of persistence dynamics have proposed that a reduction in the population-level growth

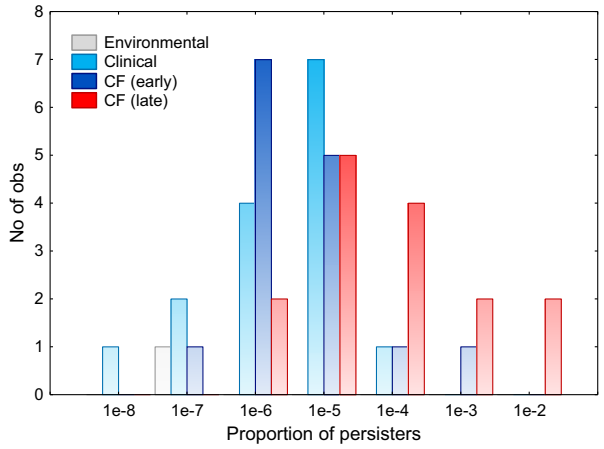


Fig. 2 Persistence levels in clinical and environmental isolates compared to persistence levels in patients with cystic fibrosis. Persistence levels of 15 clinical strains were comparable to the average persistence levels of the early cystic fibrosis *P. aeruginosa* isolates of Mulcahy *et al.* (2010), but lower than the persistence levels found in those same patients with CF 6–16 years later, after having been exposed to extended periods of antibiotic treatment. The single environmental isolate had the second lowest level of persistence of all strains examined.

rate in populations with high-persister fractions would represent the main competitive cost of persistence under favourable growth conditions (Kussell *et al.* 2005; Gardner *et al.* 2007; Patra & Klumpp 2013). Nevertheless, given the observed low levels of persistence in our study strains (Table S1, Supporting information), this direct growth cost was expected to be negligibly small. Indeed, the maximal growth rate did not correlate with the level of persistence across the strains examined (following dilution only: Pearson $R = -0.50$, $t = -1.72$, d.f. = 9, $P = 0.12$; following ofloxacin treatment and dilution: Pearson $R = 0.27$, $t = 0.83$, d.f. = 9, $P = 0.43$; Fig. S2a,b, Supporting information). The maximal growth rate of the PA14 *wt* was slightly higher than that of the high-persister *hip* mutant when measured following dilution in fresh medium (2.058 h^{-1} in the *wt* vs. 1.992 h^{-1} in the *hip* mutant, $t = -4.77$, d.f. = 167, $P = 4 \times 10^{-6}$). However, no significant difference was observed when growth rate was measured following antibiotic treatment and redilution (1.914 h^{-1} in the *wt* vs. 1.902 h^{-1} in the *hip* mutant, respectively, $t = -0.30$, d.f. = 52, $P = 0.76$). These results suggest that there is either no or a negligibly small direct growth cost of persistence. In addition, this growth cost would definitely not be large enough to explain the large competitive advantage of the low-persister PA14 wild type under favourable growth conditions (Fig. 1). Persistence levels also did not show a strong correlation with carrying capacity (Fig. S2c,d, Supporting information).

In contrast to growth rate or carrying capacity, lag phase duration was strongly correlated with persistence. In particular, strains with higher persister fractions showed significantly longer lag phases following redilution in fresh medium in the absence of antibiotic treatment, reflecting mainly the lag phase duration of nonpersisters (Fig. 3a, correlation between lag phase duration and the Log_{10}-transformed proportion of persisters: Pearson $R = 0.81$, $t = 4.11$, d.f. = 9, $P = 0.003$). In contrast, a reverse correlation was observed when cultures were regrown after antibiotic treatment, reflecting lag phase duration of persisters. In this case, strains with higher persister fractions displayed a shorter lag phase (Fig. 3b, correlation between lag phase duration and the Log_{10}-transformed proportion of persisters: Pearson $R = -0.84$, $t = -4.73$, d.f. = 9, $P = 0.001$). These correlations were not only observed for the set of natural strains, but also for the PA14 *wt* and isogenic *hip* mutant, with the *hip* mutant showing a significantly longer lag phase duration than the *wt* following redilution in fresh medium (lag phase durations: 10.993 h in *hip* vs. 10.447 h in *wt*, $t = 7.57$, d.f. = 167, $P = 2 \times 10^{-12}$) and a reverse pattern following antibiotic treatment (lag phase durations: 16.476 h in *hip* vs. 24.583 h in *wt*, $t = -4.96$, d.f. = 56, $P = 6 \times 10^{-6}$). This suggests that the observed correlations between lag phase duration and persistence were not caused by varying selection pressure in environments characterized by different levels of antibiotic exposure, but instead are directly or indirectly linked to the persister phenotype. Taken together, these data imply that in environments with favourable growth conditions, increased persistence does not only carry a direct growth cost, but also an extra cost related to the increased lag phase duration. The reverse, however, would be true in environments where periods of growth would be frequently interrupted by stress. Under these conditions, high persistence levels combined with short lag phases would provide a clear competitive advantage.

Finally, the net rate of cellular decline in stationary phase showed a positive correlation with persistence levels and therefore represented another previously undocumented cost of persistence (Fig. 4a,b). Surprisingly, this correlation was seen both for persister cells (Spearman $R = 0.81$, d.f. = 9, $P = 0.004$) as well as for normal cells (Spearman $R = 0.81$, d.f. = 9, $P = 0.004$). This correlation implies that the more a strain invests in persister cells in stationary phase, the faster persister cells and normal cells decline under growth-limiting conditions in the absence of antibiotics. This conclusion is further supported by data from the *hip* mutant, which shows a significantly higher rate of decline (0.07 h^{-1}, 95% C.L. from mixed model: 0.06–0.08) compared to the PA14 *wt* strain it was derived from (0.04, 95% C.L.: 0.03–0.05).

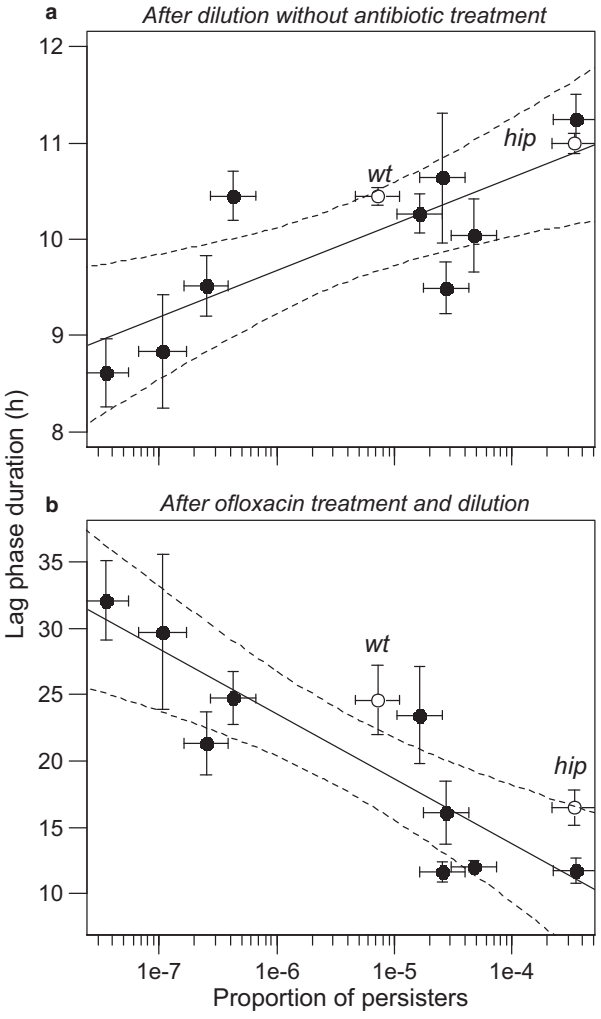


Fig. 3 Pleiotropic effects of persistence on lag phase duration. Correlation between lag phase duration and level of persistence upon regrowth, either in the absence (a) or presence of a prior antibiotic attack (b) (black circles = 9 natural isolates, open circles: PA14 *wt* and *hip* mutant). (a) In the absence of antibiotic treatment, the lag phase duration upon dilution in fresh medium is positively correlated with the level of persistence (Pearson $R = 0.81$, $t = 4.11$, d.f. = 9, $P = 0.003$). The same pattern is seen when the lag phase of the PA14 *hip* mutant is compared with that of the *wt*. Under predominantly favourable growth conditions, this effect would cause persistence to carry an additional cost, on top of a small direct growth cost. (b) Following ofloxacin treatment, however, the situation reverses so that lag phase duration upon dilution in fresh medium is then negatively correlated with the level of persistence (Pearson $R = -0.84$, $t = -4.73$, d.f. = 9, $P = 0.001$), and again the same trend is seen when the lag phase of the PA14 *hip* mutant is compared with that of the *wt*. Error bars indicate 95% confidence limits ($n = 3$ replicates per treatment combination).

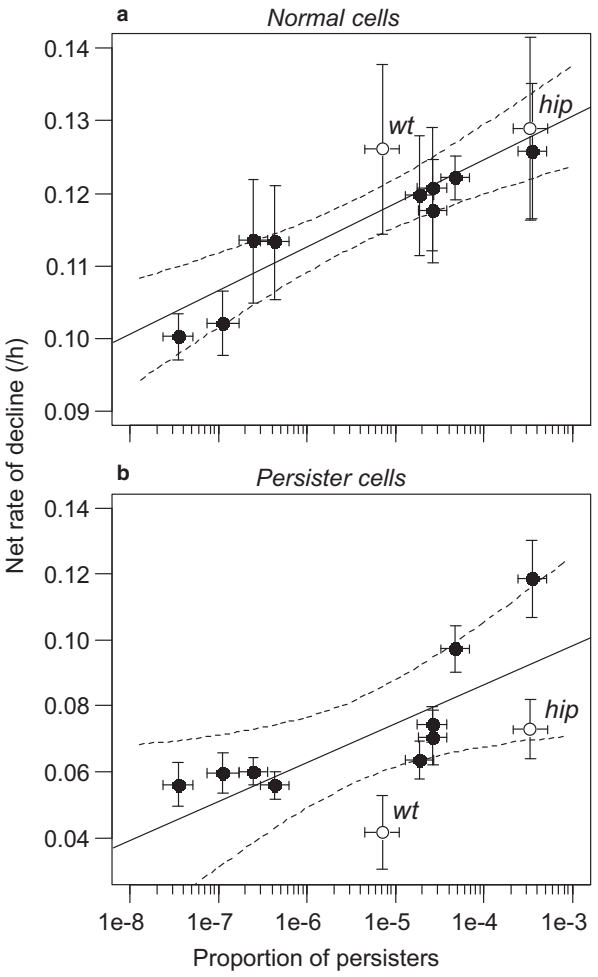


Fig. 4 Costs of persistence in terms of increased rates of cellular decline in stationary phase culture. Net rates of cellular decline in stationary phase of both normal (a) and persister cells (b) are both positively correlated with persistence levels and therefore represent another previously undocumented cost of persistence (Spearman rank correlation of net rate of decline of persister and normal cells both equal to $R = 0.81$, d.f. = 9, $P = 0.004$) (black circles = 9 natural isolates, open circles: PA14 *wt* and *hip* mutant; error bars indicate 95% confidence limits calculated from the three replicates).

Discussion

Our results provide several lines of evidence that show that the fraction of antibiotic-tolerant persister cells in *P. aeruginosa* is kept at a low level through the existence of fitness trade-offs with other traits, that is through antagonistic pleiotropy. Initial support for this hypothesis came from our competition assays, which not only demonstrated that high-persister strains or mutants were strongly selected for under environmental regimes with periodic antibiotic exposure, as had also been shown in *E. coli* (Balaban *et al.* 2004; Stewart & Rozen 2012) and in *P. aeruginosa* (Mulcahy *et al.* 2010), but also that in the absence of antibiotics, the reverse was true, so that low-persister strains then experienced a large competitive advantage. This large fitness benefit of

low-persister strains under favourable growth conditions was surprising, given that the direct growth cost of persistence in our competing high-persister strains was expected to be negligibly small, owing to their overall low fraction of nonreplicating persister cells (0.002–0.03%, Table S1, Supporting information). Hence, these experiments clearly suggest the presence of additional, pleiotropically linked costs of persistence. More generally, these competitions assays confirm the theoretical prediction that higher persistence should be favoured when antibiotic strikes are frequent, whereas lower persistence levels should be selected for in environments where antibiotic attacks are infrequent or absent (Gardner *et al.* 2007; Kussell *et al.* 2005, Figs S3–S4, Supporting information).

Aside from the large fitness benefits experienced by low-persister strains under favourable growth conditions, the very low and repeatable levels of persistence we documented among natural isolates (3×10^{-8}–3×10^{-4}, Fig. 2, Table S1, Supporting information) were also clearly suggestive for the existence of additional, pleiotropically linked costs of persistence. In fact, according to a model in which persistence was assumed to only carry a direct growth cost (Gardner *et al.* 2007), we calculated that observed persistence levels would be stable if antibiotics struck only once every 200 days for the highest persister fractions, or once every 5500 years for the lowest fractions (Appendix S1, Fig. S3, Supporting information), figures that are evidently unrealistic. Previous studies have also documented levels of persistence in *P. aeruginosa* stationary phase cultures that are very low (Moker *et al.* 2010; Mulcahy *et al.* 2010) and many orders of magnitude lower than in *Escherichia coli*, *Staphylococcus aureus* or *Acinetobacter baumannii*, where fractions of persisters can sometimes represent 1% or more of the total population (Barth *et al.* 2013; reviewed in Lewis 2007, 2010). Despite these low levels of persistence, we found interstrain variation in the proportion of persisters to be highly repeatable (intraclass correlation = 0.96), implying that persistence levels are genetically heritable and can respond to natural selection. Recently, similarly, large interstrain variation in persistence has also been documented in *P. aeruginosa* (Mulcahy *et al.* 2010), *E. coli* (e.g. Stewart & Rozen 2012; Hofsteenge *et al.* 2013) and *A. baumannii* (Barth *et al.* 2013).

In a final set of experiments, we found direct support for the existence of pleiotropically linked costs of persistence and fitness trade-offs with other growth parameters. First, we found that in *P. aeruginosa*, increased persistence was not correlated with a reduction in growth rate, unlike what would be expected if the nonreplication of persister cells represented the main cost of persistence (Kussell *et al.* 2005; Gardner *et al.* 2007). Second, we found that increased persistence did strongly correlate with an increased duration of the lag phase following new colonization without intermittent antibiotic attack (Fig. 3). This implies that, in the absence of antibiotics, high-persister strains suffer an additional delay before growth is resumed. Third, there was a positive correlation between the level of persistence in a given strain and the population-level rate of cellular decline in stationary phase (Fig. 4). This suggests that populations with a relatively large fraction of persister cells may suffer increased mortality in resource-deprived environments. Significantly, both of these effects were found not only in a comparison of 9 natural strains, but also in the contrasting growth patterns of a high-persister mutant and the wild type from which it was derived. This suggests that the patterns we observe are general and are caused by intrinsic, pleiotropically linked fitness effects as opposed to by varying selection pressure in environments characterized by different levels of antibiotic exposure (which is known to be capable of affecting lag time, Fridman *et al.* 2014; and growth rate, e.g. Gilbert *et al.* 1990). Surprisingly, our results also demonstrated that persistence and lag phase showed a reverse correlation when cultures were regrown after antibiotic treatment. In that case, strains with higher persister fractions displayed a shorter lag phase. Nevertheless, given that following antibiotic attack, persistence is already expected to be highly advantageous without any further benefits, and we expect that the pleiotropic costs, caused by the lengthening of the lag phase under favourable growth conditions and the increased mortality under resource-deprived conditions, are more important and more strongly affect the evolutionary dynamics of persistence. A detailed theoretical analysis of the effect of antagonistic pleiotropy on optimal levels of persistence, however, remains to be performed.

Taken together, our results provide strong support for the hypothesis that variation in persistence in bacteria is not only shaped by differences in the frequency of antibiotic exposure, but also by fitness trade-offs with other growth parameters, including the length of the lag phase and stationary phase survival. Earlier, tentative evidence for effects of persistence on lag phase had also been collected in *E. coli*, where the high-persister mutant strain *hipA7* was shown to have an extended lag phase when inoculated into fresh medium (Balaban *et al.* 2004; Gefen *et al.* 2008) and wild-type cultures displayed extended lag phases when persistence levels were increased through starvation (Levin-Reisman *et al.* 2010; Luidalepp *et al.* 2011). In addition, a similar lengthening of the lag phase has been observed in the high-persister mutant *shp* in *Salmonella enterica* (Slattery *et al.* 2013). This suggests that the trade-off between increased persistence and a lengthening of the lag phase

duration may well be a general feature of bacterial persistence. In fact, Geisel *et al.* (2011) reviewed evidence that this trade-off may be intrinsic to many bacterial stress responses and dormancy-type phenomena. On the other hand, it could be that the magnitude of these trade-offs would depend on the type of antibiotic the bacteria are exposed to. For example, beta-lactam antibiotics are known to kill only growing cells, whereas fluoroquinolones of the type we used also kill nongrowing cells (Keren *et al.* 2004), and it might be expected that this could impact the expression of particular trade-offs. In addition, there is growing evidence that the tolerance of persisters may be antibiotic specific in some species, including *E. coli* and *Acinetobacter baumannii* (Luidalepp *et al.* 2011; Stewart & Rozen 2012; Barth *et al.* 2013; Hofsteenge *et al.* 2013), and this also appears to be the case in *P. aeruginosa* (Pan *et al.* 2012).

The trade-off between persistence level and cellular decline in stationary phase that we document has not been reported before. One explanation for the existence of this type of antagonistic pleiotropy might be linked to the fact that in *P. aeruginosa* and *E. coli*, increased persister formation after treatment with fluoroquinolone antibiotics is increased in strains lacking the stationary phase sigma factor RpoS (Murakami *et al.* 2005; Hong *et al.* 2012). In gamma-proteobacteria, this same factor is also essential for stationary phase survival (Potvin *et al.* 2008; Battesti *et al.* 2011), explaining the observed negative correlation. Alternatively, increased mortality in stationary phase cultures of high-persister strains could be linked to increased release of the toxin and virulence factor pyocyanin, which in *P. aeruginosa* is released at high densities as a quorum sensing molecule and has been shown to induce persister formation (Moker *et al.* 2010). Pyocyanin production, however, is under control of the *Pseudomonas* quinolone signal (PQS), which if highly upregulated can cause partial autolysis (D'Argenio *et al.* 2002). Indeed, such autolysis is most frequently observed in *P. aeruginosa* isolates from patients with chronic cystic fibrosis (D'Argenio *et al.* 2002), which are known to display high levels of persistence (Mulcahy *et al.* 2010).

Overall, we believe that our results may not only help explain natural persistence levels, but also boost the development of more refined mathematical models of persistence, which could be important to optimize treatment strategies. Persistence levels, for example, could be kept at a low level by shortening the duration of antimicrobial therapy in the case of acute infections (Levin & Rozen 2006), or optimizing the timing of periodically dosed antibiotic treatments in the case of chronic infections (Fauvart *et al.* 2011; Cogan *et al.* 2012, 2013; Cogan 2013; Orman & Brynildsen 2013). Keeping persistence at a low level in clinical infections is of great importance, given that persister cells have been shown to act as a reservoir for the emergence of antibiotic resistance (Levin & Rozen 2006; Cohen *et al.* 2013). Building on the results obtained in this study, we therefore believe that important improvements can be made to existing models of persistence as well as to optimal treatment schedules of infectious diseases.

Acknowledgements

This work was supported by grants from the KU Leuven Research Council (IDO/09/010 and DBOF/12/035), the Fund for Scientific Research – Flanders (FWO G.0413.10), KU Leuven Excellence Center Financing (PF/2010/07) and the Interuniversity Attraction Poles Programme initiated by the Belgian Science Policy Office. BVDB received a fellowship from the Fund for Scientific Research – Flanders (FWO). Research in the laboratory of KJV is supported by ERC Starting Grant 241426, HFSP programme grant RGP0050/2013, VIB, EMBO YIP programme, FWO, and IWT. Environmental and clinical isolates of *P. aeruginosa* were kindly provided by Prof. Pierre Cornelis (VUB, Belgium).

References

Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S (2004) Bacterial persistence as a phenotypic switch. *Science*, **305**, 1622–1625.

Balaban NQ, Gerdes K, Lewis K, McKinney JD (2013) A problem of persistence: still more questions than answers? *Nature Reviews Microbiology*, **11**, 587–591.

Barraud N, Buson A, Jarolimek W, Rice SA (2013) Mannitol enhances antibiotic sensitivity of persister bacteria in *Pseudomonas aeruginosa* biofilms. *PLoS One*, **8**, e84220.

Barth VC Jr, Rodrigues BÁ, Bonatto GD *et al.* (2013) Heterogeneous persister cells formation in *Acinetobacter baumannii*. *PLoS One*, **8**, e84361.

Baskin CC, Baskin JM (2014) *Ecology, Biogeography, and Evolution of Dormancy and Germination*, 2nd edn. Academic Press, Elsevier, Waltham, Massachusetts.

Battesti A, Majdalani N, Gottesman S (2011) The *RpoS*-mediated general stress response in *Escherichia coli*. *Annual Review of Microbiology*, **65**, 189–213.

Bush K, Courvalin P, Dantas G *et al.* (2011) Tackling antibiotic resistance. *Nature Reviews Microbiology*, **9**, 894–896.

Cogan N (2013) Concepts in disinfection of bacterial populations. *Mathematical Biosciences*, **245**, 111–125.

Cogan N, Brown J, Darres K, Petty K (2012) Optimal control strategies for disinfection of bacterial populations with persister and susceptible dynamics. *Antimicrobial Agents and Chemotherapy*, **56**, 4816–4826.

Cogan N, Szomolay B, Dindos M (2013) Effect of periodic disinfection on persisters in a one-dimensional biofilm model. *Bulletin of mathematical biology*, **75**, 94–123.

Cohen D (1966) Optimizing reproduction in a randomly varying environment. *Journal of Theoretical Biology*, **12**, 119–129.

Cohen NR, Lobritz MA, Collins JJ (2013) Microbial persistence and the road to drug resistance. *Cell Host & Microbe*, **13**, 632–642.

D'Argenio DA, Calfee MW, Rainey PB, Pesci EC (2002) Autolysis and autoaggregation in *Pseudomonas aeruginosa* colony morphology mutants. *Journal of Bacteriology*, **184**, 6481–6489.

De Groote VN, Verstraeten N, Fauvart M *et al.* (2009) Novel persistence genes in *Pseudomonas aeruginosa* identified by high-throughput screening. *Fems Microbiology Letters*, **297**, 73–79.

Dhar N, McKinney JD (2007) Microbial phenotypic heterogeneity and antibiotic tolerance. *Current Opinion in Microbiology*, **10**, 30–38.

Dörr T, Lewis K, Vulic M (2009) SOS response induces persistence to fluoroquinolones in *Escherichia coli*. *Plos Genetics*, **5**, e1000760.

Fauvart M, De Groote VN, Michiels J (2011) Role of persister cells in chronic infections: clinical relevance and perspectives on anti-persister therapies. *Journal of Medical Microbiology*, **60**, 699–709.

Fridman O, Goldberg A, Ronin I, Shoresh N, Balaban NQ (2014) Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature*, **513**, 418–421.

Fux C, Costerton J, Stewart P, Stoodley P (2005) Survival strategies of infectious biofilms. *Trends in Microbiology*, **13**, 34–40.

Gardner A, West S, Griffin A (2007) Is bacterial persistence a social trait? *PLoS One*, **8**, e752.

Gefen O, Balaban NQ (2009) The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress. *Fems Microbiology Reviews*, **33**, 704–717.

Gefen O, Gabay C, Mumcuoglu M, Engel G, Balaban NQ (2008) Single-cell protein induction dynamics reveals a period of vulnerability to antibiotics in persister bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 6145–6149.

Geisel N, Vilar JMG, Rubi JM (2011) Optimal resting-growth strategies of microbial populations in fluctuating environments. *PLoS One*, e18622.

Gilbert P, Collier PJ, Brown M (1990) Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. *Antimicrobial Agents and Chemotherapy*, **34**, 1865–1868.

Hofsteenge N, van Nimwegen E, Silander OK (2013) Quantitative analysis of persister fractions suggests different mechanisms of formation among environmental isolates of *E. coli*. *BMC Microbiology*, **13**, 25.

Hong SH, Wang XX, O'Connor HF, Benedik MJ, Wood TK (2012) Bacterial persistence increases as environmental fitness decreases. *Microbial Biotechnology*, **5**, 509–522.

Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K (2004) Persister cells and tolerance to antimicrobials. *Fems Microbiology Letters*, **230**, 13–18.

Kint CI, Verstraeten N, Fauvart M, Michiels J (2012) New-found fundamentals of bacterial persistence. *Trends in Microbiology*, **20**, 577–585.

Knudsen GM, Ng Y, Gram L (2013) Survival of bactericidal antibiotic treatment by a persister subpopulation of *Listeria monocytogenes*. *Applied and Environmental Microbiology*, **79**, 7390–7397.

Kussell E, Leibler S (2005) Phenotypic diversity, population growth, and information in fluctuating environments. *Science*, **309**, 2075.

Kussell E, Kishony R, Balaban N, Leibler S (2005) Bacterial persistence: a model of survival in changing environments. *Genetics*, **169**, 1807–1814.

Lachmann M, Jablonka E (1996) The inheritance of phenotypes: an adaptation to fluctuating environments. *Journal of Theoretical Biology*, **181**, 1–9.

LaFleur MD, Qi QG, Lewis K (2010) Patients with long-term oral carriage harbor high-persister mutants of *Candida albicans*. *Antimicrobial Agents and Chemotherapy*, **54**, 39–44.

Levin BR, Rozen DE (2006) Non-inherited antibiotic resistance. *Nature Reviews Microbiology*, **4**, 556–562.

Levin-Reisman I, Gefen O, Fridman O *et al.* (2010) Automated imaging with ScanLag reveals previously undetectable bacterial growth phenotypes. *Nature Methods*, **7**, 737–739.

Lewis K (2007) Persister cells, dormancy and infectious disease. *Nature Reviews Microbiology*, **5**, 48–56.

Lewis K (2010) Persister cells. *Annual Review of Microbiology*, **64**, 357–372.

Liebens V, Defraine V, Van der Leyden A *et al.* (2014) A putative de-N-acetylase of the PIG-L superfamily affects fluoroquinolone tolerance in Pseudomonas aeruginosa. *Pathogens and Disease*, **71**, 39–54.

Luidalepp H, Joers A, Kaldalu N, Tenson T (2011) Age of inoculum strongly influences persister frequency and can mask effects of mutations implicated in altered persistence. *Journal of Bacteriology*, **193**, 3598–3605.

Lynch M, Walsh B (1998) *Genetics and Analysis of Quantitative Traits*. Sinauer, Sunderland, Massachusetts.

Maisonneuve E, Gerdes K (2014) Molecular mechanisms underlying bacterial persisters. *Cell*, **157**, 539–548.

Moker N, Dean CR, Tao JS (2010) *Pseudomonas aeruginosa* increases formation of multidrug-tolerant persister cells in response to quorum-sensing signaling molecules. *Journal of Bacteriology*, **192**, 1946–1955.

Mulcahy LR, Burns JL, Lory S, Lewis K (2010) Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. *Journal of Bacteriology*, **192**, 6191–6199.

Murakami K, Ono T, Viducic D *et al.* (2005) Role for *rpoS* gene of *Pseudomonas aeruginosa* in antibiotic tolerance. *Fems Microbiology Letters*, **242**, 161–167.

Orman MA, Brynildsen MP (2013) Establishment of a method to rapidly assay bacterial persister metabolism. *Antimicrobial Agents and Chemotherapy*, **57**, 4398–4409.

Pan JC, Bahar AA, Syed H, Ren DC (2012) Reverting antibiotic tolerance of *Pseudomonas aeruginosa* PAO1 persister cells by (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one. *PLoS One*, **7**, e45778.

Patra P, Klumpp S (2013) Population dynamics of bacterial persistence. *PLoS One*, **8**, e62814.

Philippi T, Seger J (1989) Hedging ones evolutionary bets, revisited. *Trends in Ecology & Evolution*, **4**, 41–44.

Pirnay JP, De Vos D, Cochez C *et al.* (2002a) *Pseudomonas aeruginosa* displays an epidemic population structure. *Environmental Microbiology*, **4**, 898–911.

Pirnay JP, De Vos D, Mossialos D *et al.* (2002b) Analysis of the *Pseudomonas aeruginosa oprD* gene from clinical and environmental isolates. *Environmental Microbiology*, **4**, 872–882.

Potvin E, Sanschagrin F, Levesque RC (2008) Sigma factors in *Pseudomonas aeruginosa*. *Fems Microbiology Reviews*, **32**, 38–55.

Slattery A, Victorsen AH, Brown A, Hillman K, Phillips GJ (2013) Isolation of highly persistent mutants of salmonella enterica serovar typhimurium reveals a new toxin-antitoxin module. *Journal of Bacteriology*, **195**, 647–657.

Stewart PS (2002) Mechanisms of antibiotic resistance in bacterial biofilms. *International Journal of Medical Microbiology*, **292**, 107–113.

Stewart B, Rozen DE (2012) Genetic variation for antibiotic persistence in *Escherichia coli*. *Evolution*, **66**, 933–939.

Stewart GR, Robertson BD, Young DB (2003) Tuberculosis: a problem with persistence. *Nature Reviews Microbiology*, **1**, 97–105.

Thattai M, van Oudenaarden A (2004) Stochastic gene expression in fluctuating environments. *Genetics*, **167**, 523–530.

Van Acker H, Sass A, Bazzini S *et al*. (2013) Biofilm-grown *Burkholderia cepacia* complex cells survive antibiotic treatment by avoiding production of reactive oxygen species. *PLoS One*, **8**, e58943.

Veening J, Smits W, Kuipers O (2008) Bistability, epigenetics, and bet-hedging in bacteria. *Annual Review of Microbiology*, **62**, 193–210.

Vega NM, Allison KR, Khalil AS, Collins JJ (2012) Signaling-mediated bacterial persister formation. *Nature Chemical Biology*, **8**, 431–433.

Willenborg J, Willms D, Bertram R, Goethe R, Valentin-Weigand P (2014) Characterization of multi-drug tolerant persister cells in *Streptococcus suis*. *Bmc Microbiology*, **14**, 120.

Zwietering MH, Jongenburger I, Rombouts FM, Vantriet K (1990) Modeling of the bacterial-growth curve. *Applied and Environmental Microbiology*, **56**, 1875–1881.

K.S. helped design all the experiments and performed all the laboratory work and helped analyse the data and write the study. T.W., F.M. and E.D.G. helped design the experiments, analyse the data and write the study. B.V.D.B., N.V., L.D.M., K.J.V., M.F. and J.M. helped design the experiments and write the study.

Data accessibility

Primary data are available on Dryad: doi:10.5061/dryad.7k130.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Evolutionarily stable levels of persistence.

Table S1 Persistence levels as a function of strain origin among natural isolates of *P. aeruginosa*.

Fig. S1 Control experiments for competition assays with single strains in pure cultures.

Fig. S2 Biphasic killing curve of the total bacterial population after exposure to antibiotics.

Fig. S3 Expected evolutionarily stable proportion of persister cells as a function of the duration between antibiotics attack.

Fig. S4 Expected evolutionarily stable proportion of persister cells as a function of the duration between antibiotics attack if some non-persister cells survive antibiotic attacks.

Fig. S5 Persistence does not significantly affect the maximal growth rate and is not pleiotropically linked to carrying capacity.

Supplementary Online Material

Appendix S1. Evolutionarily stable levels of persistence.

Derivation of evolutionarily stable levels of persistence

To assess whether additional costs of persistence, aside from a direct growth cost, are required to explain the observed low levels of persistence in *P. aeruginosa*, we compared our empirical estimates of persistence to the values expected by previously published evolutionary models of persistence (Gardner *et al.* 2007; Kussell *et al.* 2005; Kussell & Leibler 2005). These models have consistently found that allocation to non-growing persisters or other dormant life stages should be inversely proportional to the duration of favorable environmental conditions (Gardner *et al.* 2007; Kussell *et al.* 2005; Kussell & Leibler 2005; Lachmann & Jablonka 1996; Thattai & van Oudenaarden 2004). Under the assumption that bacterial populations are locally founded by small numbers of cells, the model of Gardner *et al*. (2007), for example, predicted that the time-averaged optimal or "evolutionarily stable" investment in persister cells π^* should equal $1/\mathrm{T}$, where T is the time between antibiotic strikes or other environmental catastrophes (Gardner *et al*. 2007, eqn. (1) with $z_0 \to 0$). The timescale in this model was normalized so that the maximum intrinsic rate of growth of non-persister cells and carrying capacity were both equal to 1. To map these predictions onto a regular timescale, we substituted T for $\mu.t$ where $\mu.t$ is the ratio of the time to the inflection point in a normal logistic growth curve with maximal growth rate μ relative to that in the rescaled logistic growth model of Gardner *et al*. (2007) and t is the time between antibiotic attacks. Further taking into account lag time λ as a shift in time, the predicted average ESS proportion of persister cells is therefore

$$\pi^* = \frac{1}{\mu.(t-\lambda)} \qquad \text{eqn. (S1)}$$

Similarly, Kussell *et al*. (2005) found that the optimal rate for cells to switch to the persister state a is equal to approximately $1/t$, where t is the average duration of favorable growth conditions (i.e. the time between antibiotic strikes). Since the proportion of persister cells during exponential growth is approximately equal to $\pi = a/\mu$ (Patra & Klumpp 2013), this leads to an ESS allocation to persister cells that is identical as in the Gardner *et al*.

(2007) model, $\pi^* = 1/\mu t$, or $1/(\mu.(t-\lambda))$ if we take into account the single-cell lage time λ as a shift in time. In an extended version of his model, Gardner *et al.* (2004) also considered a situation in which some non-persister cells would be able to survive antibiotic attacks (eqn. (A.16). This was done by including a parameter *s* for the survival probability of non-persister cells relative to persister cells following an antibiotic attack, which using substitutions as above resulted in a somewhat lower ESS proportion of persister cells of

$$\pi^* = \frac{1}{\mu.(t-\lambda)} - \frac{s}{1-s} \qquad \text{eqn. (S2)}$$

which is zero when the time between antibiotic attacks t exceeds $\lambda + (1-s)/\mu.s$.

Other limiting cases of the model of Gardner *et al*. (2007) were not considered, given that the predictions above are most realistic with respect to the observed low levels of persistence, and including other factors such as kin competition ($z_0 > 0$), or increased persister cell formation during stationary phase (Gardner *et al*. 2007; Keren *et al.* 2004; Knudsen *et al.* 2013; Luidalepp *et al.* 2011; Patra & Klumpp 2013), would lead to much higher ESS persister allocations, which are not consistent with our actual observations. In other words, the predictions we considered were evolutionary stable proportions of persister cells under average growth conditions, for which our early stationary phase culture conditions during which persistence was measured were probably quite representative. We should also note that all predictions assume that persister-formation is not antibiotic-specific. If it would be (cf. Barth Jr *et al.* 2013; Hofsteenge *et al.* 2013; Luidalepp *et al.* 2011; Stewart & Rozen 2012), we would expect a total ESS allocation to persisters as given but with subpopulations within the persisters corresponding to the probability of each antibiotic being used. At present, little is known, however, about whether in *P. aeruginosa*, persister fractions differ according to the antibiotic that is used. In addition, fluoroquinolone antibiotics such as ofloxacin are among the most common antibiotics used to treat *P. aeruginosa* infections (Mulcahy *et al.* 2010).

To estimate how long the time between antibiotic strikes would have to be to make the observed persistence levels in our *P. aeruginosa* strains consistent with the predicted ESS values, we plotted equations (1) and (2) as a function of the time between antibiotic strikes

t (Figs. S3 and S4). For equation (2), parameter *s* was estimated from a biphasic killing curve, obtained by treating the *wt* strain PA14 with ofloxacin at 10x the MIC and measuring the number of surviving cells after 1, 2, 3, 4, 5, 12 and 24h of ofloxaxin exposure using plate counting (*n*=3 replicates) (for details see Figs. S2 and S4).

Comparison of observed and theoretically predicted perister levels

Based on the basic model of Gardner *et al*. (2007) (eqn. (S1)), and using our estimates for the growth parameters of our study strains, we calculate that a proportion of 10^{-4} persisters would be expected to be the ESS if antibiotics were encountered every ca. 200 days, whereas the lowest levels of persistence of 10^{-8} would be the ESS if antibiotics struck only every ca. 5500 year (Fig. S3). These estimates are clearly highly unrealistic, as the high-persister strains are typically derived from chronic infections in hospital environments that would be exposed to intensive antibiotic treatment schedules, and that many of the low-persister strains were also derived from clinical environments. If a small fraction of non-persister cells would be able to survive antibiotic attack (eqn. (S2)), predictions become slightly more realistic in that very low levels of persistence could also become evolutionarily stable under a narrow parameter range (Fig. S4). Whether antibiotic tolerance of normal, replicating cells could explain the observed low levels of persistence, however, critically depends on the average length of each round of antibiotic exposure. From a biphasic killing curve, characteristic for the fast killing of normal cells followed by the slower killing of persister cells (Fig. S2), we estimate that if an average antibiotic treatment episode lasted longer than ca. 10 hours, so few normal cells would survive that predictions would approach those of the basic model (Fig. S4). In addition, even with shorter treatment times, where some normal cells could still survive, predicted persistence levels in the extended ESS model are either much higher (for short periods between antibiotic attacks) or lower (zero, for long periods between antibiotic attacks) than observed (Fig. S3). From this, we conclude that the observed low levels of persistence are challenging to explain based on existing models, thereby prompting us to investigate possible pleiotropic costs of persistence on other growth parameters.

Supplementary Table S1. Persistence levels as a function of strain origin among natural isolates of *P. aeruginosa*. Observed proportions of persister cells in stationary phase cultures varied by 5 orders of magnitude among 16 natural isolates, and were highest among lung and hospital infections, intermediate in wound, throat or blood infections and lowest in one environmental isolate. Persistence levels were not confounded with antibiotic resistance, as shown by the nonsignificant correlation between persistence and the minimum inhibitory concentration (MIC) for the antibiotic ofloxacin, used to monitor persistence (Spearman rank R=0.20, p=0.42, n=18).

Strain	*Geographical origin*	*Sampling site*	*Reference*	*Mean prop. of persister cells*	*95 % confidence limits (n = 3)*		$MIC_{ofloxacin}$ *(µg/ml)*
Bu004	Budapest (Hungary)	Throat	Pirnay et al. 2002	3.E-08	2.E-08	5.E-08	0.625
Br257	Brussels (Belgium)	Plant rhizosphere	Pirnay et al. 2002	1.E-07	7.E-08	2.E-07	1.25
Br735	Brussels (Belgium)	Burn wound	Pirnay et al. 2002	2.E-07	2.E-07	4.E-07	0.625
Mi162	Michigan (USA)	Burn wound	Pirnay et al. 2002	4.E-07	3.E-07	7.E-07	10
Mi159	Michigan (USA)	Pressure sore	Pirnay et al. 2002	2.E-06	1.E-06	3.E-06	1.25
Ro124	Rotterdam (The Netherlands)	Burn wound	Pirnay et al. 2002	3.E-06	2.E-06	5.E-06	2.5
Bo546	Boston (USA)	Burn wound	Pirnay et al. 2002	4.E-06	3.E-06	6.E-06	2.5
PAO1 Krylov	Melbourne (Australia)	Wound	Pirnay et al. 2002	5.E-06	3.E-06	8.E-06	1.25
PA14 *wt*	Wild type lab strain	Burn wound	De Groote et al. 2009	7.E-06	5.E-06	1.E-05	0.2
ATCC 27853	Boston (USA)	Blood	Pirnay et al. 2002	1.E-05	7.E-06	2.E-05	10
Br680	Brussels (Belgium)	Burn wound	Pirnay et al. 2002	1.E-05	8.E-06	2.E-05	0.625
Br642	Brussels (Belgium)	Hospital environment	Pirnay et al. 2002	2.E-05	1.E-05	3.E-05	2.5
PAO1 Pirnay	Melbourne (Australia)	Wound	Pirnay et al. 2002	2.E-05	1.E-05	3.E-05	1.25
Be128	Beverwijk (The Netherlands)	Bronchus	Pirnay et al. 2002	3.E-05	2.E-05	4.E-05	1.25
Bo548	Boston (USA)	Burn wound	Pirnay et al. 2002	3.E-05	2.E-05	4.E-05	2.5
Be136	Beverwijk (The Netherlands)	Bronchus	Pirnay et al. 2002	5.E-05	3.E-05	7.E-05	2.5
PA14 *hip*	Mutant lab strain		De Groote et al. 2009	0.0003	0.0002	0.0005	0.4
Aa249	Aachen (Germany)	Burn wound	Pirnay et al. 2002	0.0003	0.0002	0.0005	40

Supplementary Figures

Figure S1. Control experiments for competition assays with single strains in pure cultures. Strains were grown until stationary phase and treated with either sterile water (a, c) or ofloxacin (b, d) before being diluted in fresh medium. Data shown in panels (a) and (b) represent results for the PA14 laboratory strains (*wt* and *hip* mutant), whereas panels (c) and (d) show results for clinical isolates Br257 and Br642. At each stage, the number of CFU was determined by plate counting. Data points show the values before treatment. Error bars represent 95 % confidence limits calculated from the 3 replicates.

Figure S2. Biphasic killing curve of the total bacterial population after exposure to antibiotics. As expected when a subpopulation of antibiotic-tolerant perister cells are present, an isogenic *wt* culture shows distinctly biphasic killing dynamics following treatment with the antibiotic ofloxacin (at 10x the MIC), corresponding with a fast decay of normal, antibiotic-sensitive cells and a slow decay of antibiotic-tolerant persisters (Kint *et al.* 2012; Patra & Klumpp 2013). Data points and error bars are means and 95% confidence limits of 3 replicates. The fitted curve represents the nonlinear best-fit biphasic killing curve, which was fitted to the Log_{10} transformed numbers of cells (CFU/ml) based on the equation $Log_{10}(CFU)=Log_{10}((N(0)-P(0)).Exp(-k_n.\tau)+P(0).Exp(-k_p.\tau))$, where τ was treatment time (in hours), $N(0)$ and $P(0)$ were the number of normal and persister cells at treatment time 0 and k_n and k_p were the rate at which normal and persister cells were killed (per hour). The best-fit curve is shown for the best fit parameters $N(0)= 84055552$, $P(0)= 20232$, $k_n=1.54$ (h^{-1}) and $k_p=0.023$ (h^{-1}).

Figure S3. Expected evolutionarily stable proportion of persister cells as a function of the duration between antibiotics attack. Based on the model of Gardner et al. (2007) (eqn. (S1)), the evolutionarily stable (ESS) proportions of persister cells are plotted as a function of the duration between antibiotic attacks for the average growth parameters as measured in our *P. aeruginosa* study strains (average maximal growth rate μ = 0.0345 min^{-1} = 49.68 day^{-1} and lag phase duration λ = 517 mins, minimum value, dotted curve; 911 mins, average value, dashed curve; 1923 mins, maximum value, full curve). The influence of the observed variation in growth rate across strains was very small and therefore not shown. Predictions are shown for the case where persistence levels do not facultatively respond to local environmental conditions and bacterial populations are locally founded by small numbers of cells ($z_0 \to 0$).

Figure S4. Expected evolutionarily stable proportion of persister cells as a function of the duration between antibiotics attack if some non-persister cells survive antibiotic attacks. Based on the extended model of Gardner et al. (2007) (eqn. (2)), the evolutionarily stable (ESS) proportions of persister cells are plotted as a function of the duration between antibiotic attacks and the survival probability of non-persister relative to persister cells *s* for the average growth parameters as measured in our *P. aeruginosa* study strains (average maximal growth rate μ = 0.0345 min^{-1} = 49.68 day^{-1} and average lag phase duration λ = 911 mins) and with *s*=0.00052 (dotted line), 5.5 x 10^{-6} (dashed line) or 1.3 x 10^{-8} (full line), corresponding to a situation where antibiotic attacks would last for an average of 5h (the treatment duration using in our competition experiments), 8h or 12h (given that $s=\text{Exp}(-k_n.\tau)/\text{Exp}(-k_p.\tau$ and with best-fit parameters given in Fig. S1).

Figure S5. Persistence does not significantly affect the maximal growth rate and is not pleiotropically linked to carrying capacity. The maximal growth rate (h^{-1}) does not correlate with the level of persistence across the 9 natural isolates (following dilution only, (a): Pearson R = -0.50, t = -1.72, df = 9, p = 0.12; following ofloxacin treatment and dilution, (b): Pearson R = 0.27, t = 0.83, df = 9, p = 0.43). Similarly, persistence also does not or only very weakly correlate with the carrying capacity reached in stationary-phase cultures (following dilution only, (c): Pearson R = -0.04, t = -0.14, df = 9, p = 0.89; following ofloxacin treatment and dilution, (d): Pearson R = 0.61, t = 2.32, df = 9, p = 0.04), and no differences were observed in the carrying capacity reached by the PA14 *wt* and isogenic *hip* mutant (t-tests, $p > 0.05$).

Figure S1

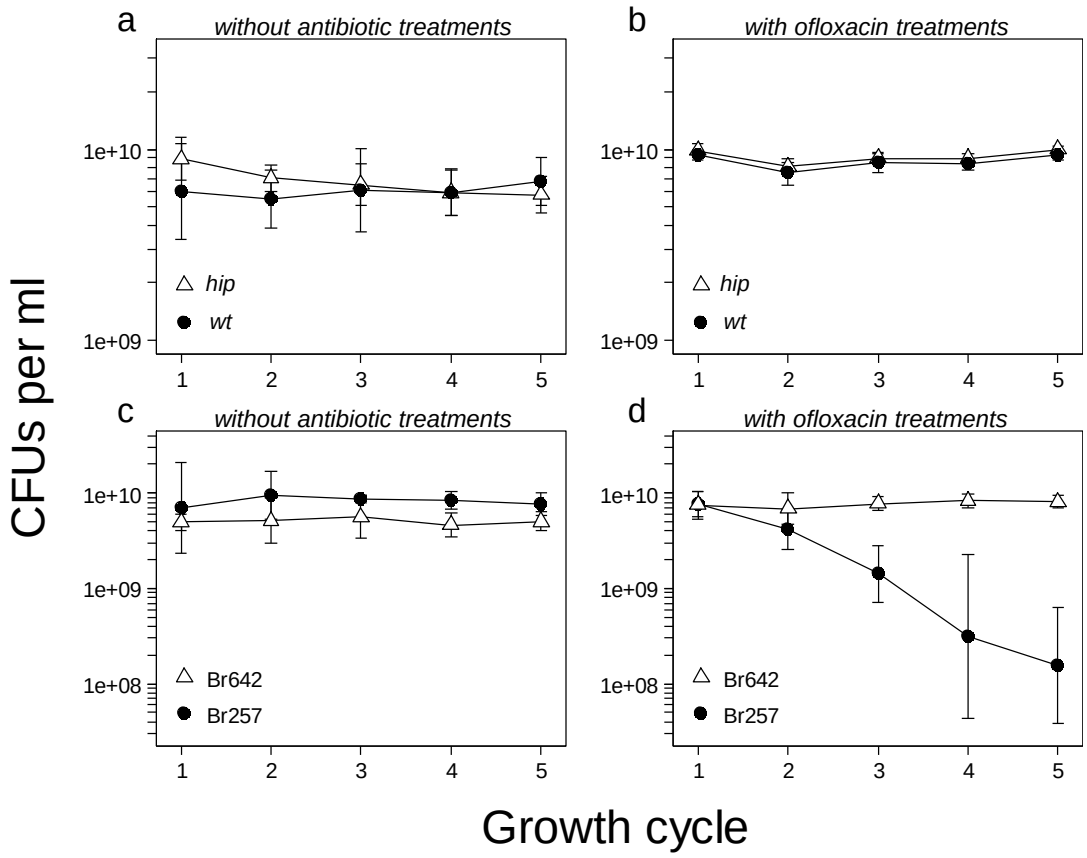

a
without antibiotic treatments
b
with ofloxacin treatments
c
without antibiotic treatments
d
with ofloxacin treatments
CFUs per ml
1e+10
1e+09
1e+08
hip
wt
Br642
Br257
1
2
3
4
5
Growth cycle

Figure S2

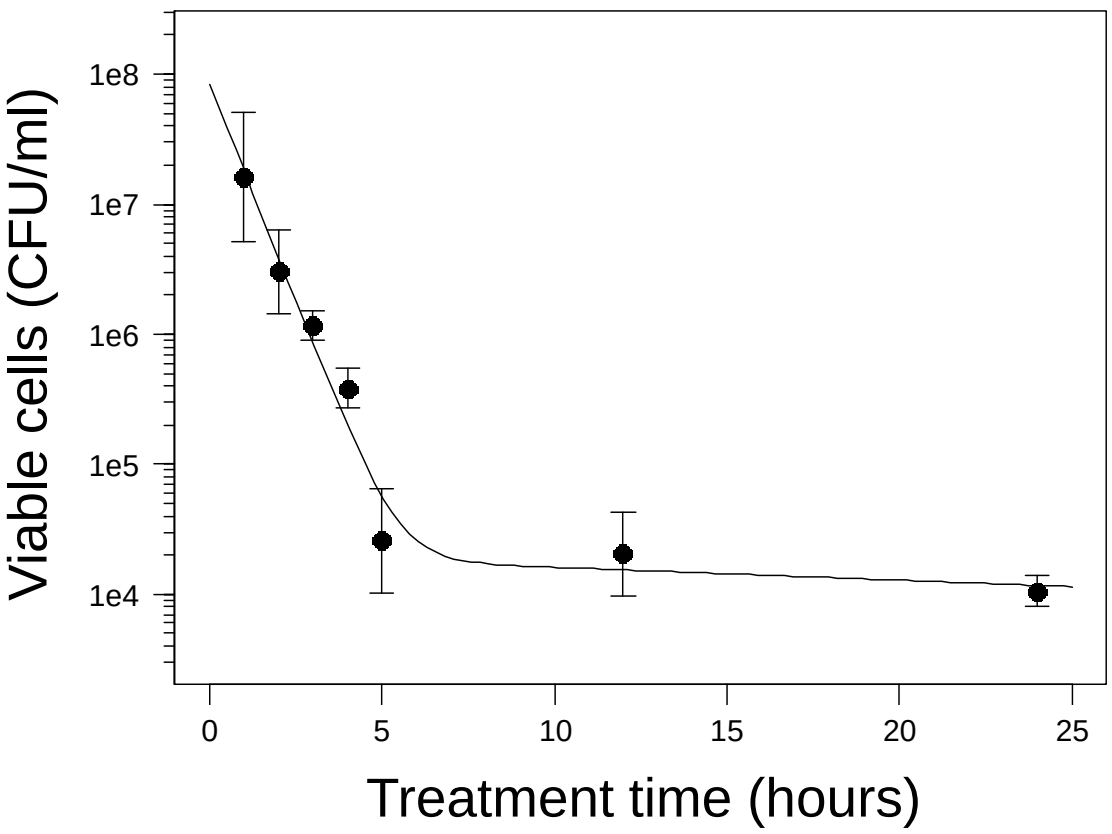

Viable cells (CFU/ml)
1e8
1e7
1e6
1e5
1e4
0
5
10
15
20
25
Treatment time (hours)

Figure S3

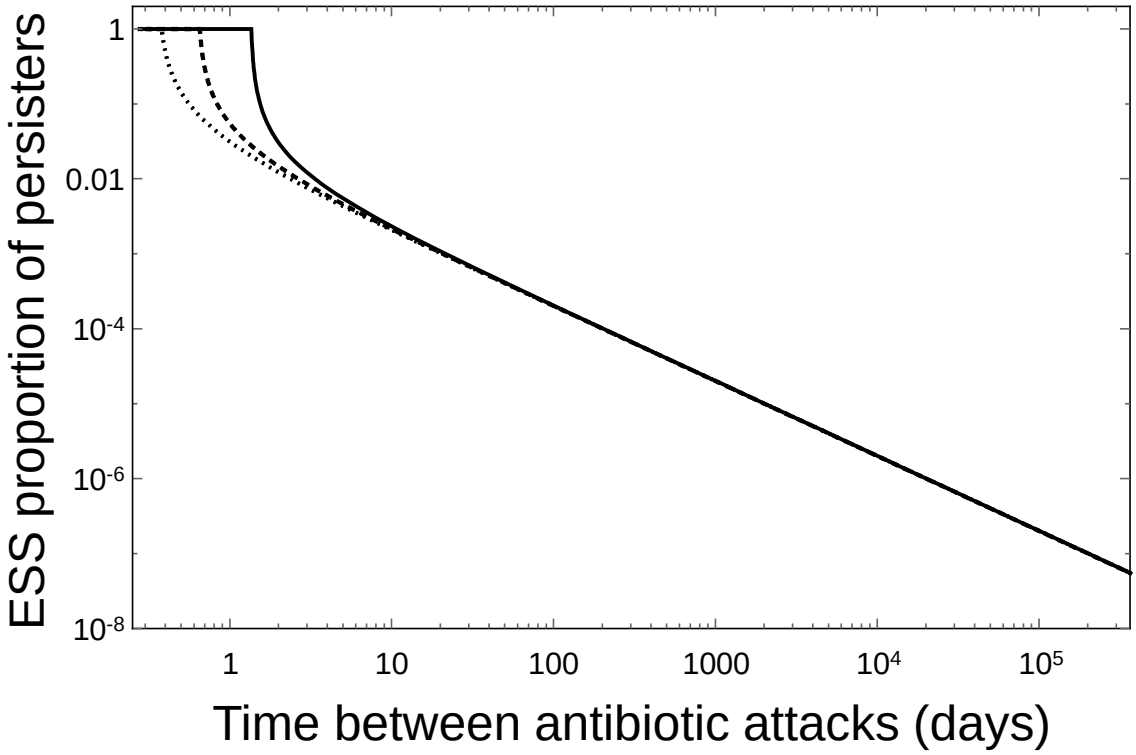

ESS proportion of persisters
1
0.01
10-4
10-6
10-8
1
10
100
1000
10^4
10^5
Time between antibiotic attacks (days)

Figure S4

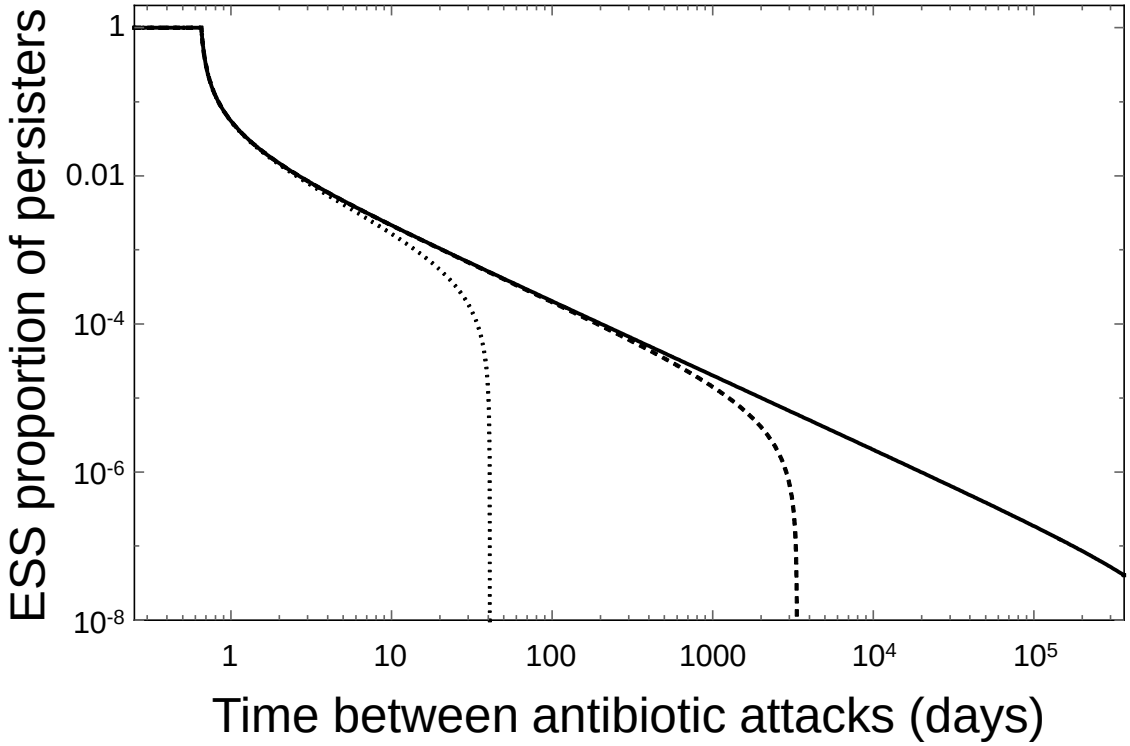
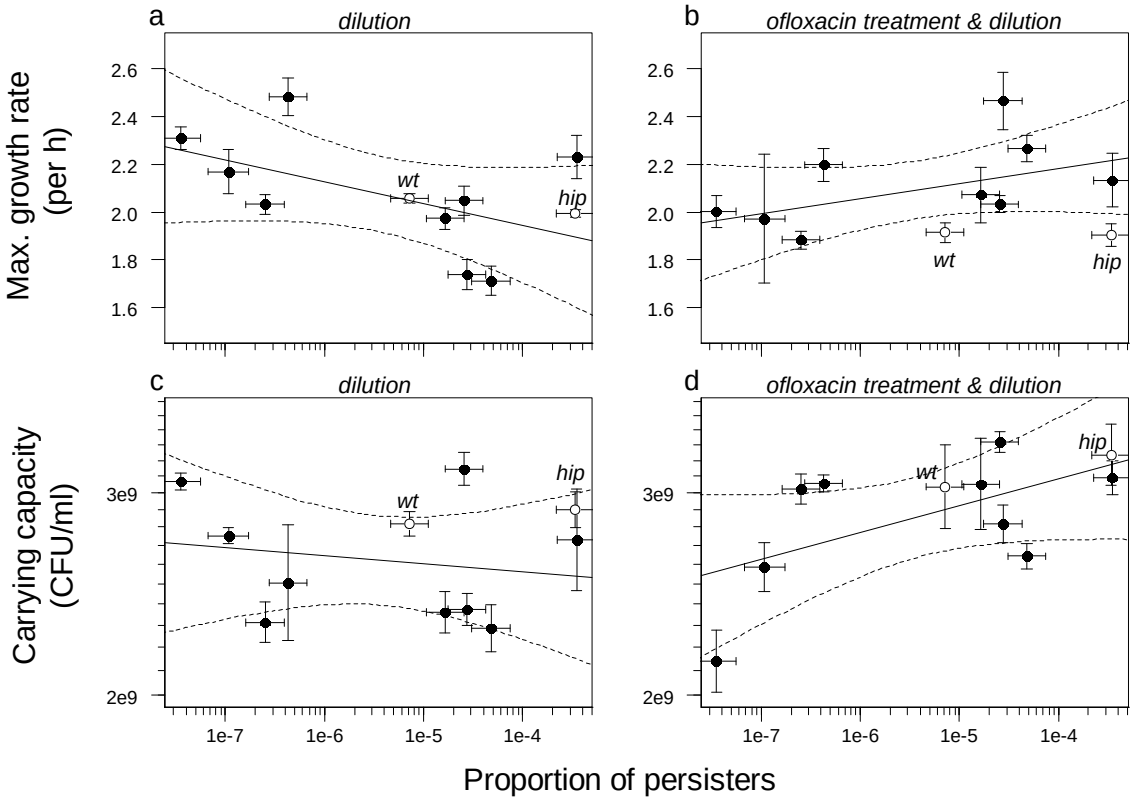

ESS proportion of persisters
1
0.01
10-4
10-6
10-8
1
10
100
1000
10^4
10^5
Time between antibiotic attacks (days)

Figure S5


a
dilution
b
ofloxacin treatment & dilution
c
dilution
d
ofloxacin treatment & dilution
Max. growth rate (per h)
Carrying capacity (CFU/ml)
wt
hip
2.6
2.4
2.2
2.0
1.8
1.6
3e9
2e9
1e-7
1e-6
1e-5
1e-4
Proportion of persisters

Supplemental references

Barth Jr VC, Rodrigues BÁ, Bonatto GD*, et al.* (2013) Heterogeneous persister cells formation in *Acinetobacter baumannii*. *PLoS ONE* **8**, e84361.

Gardner A, West S, Griffin A (2007) Is bacterial persistence a social trait? *PLoS ONE* **8**, e752.

Hofsteenge N, van Nimwegen E, Silander OK (2013) Quantitative analysis of persister fractions suggests different mechanisms of formation among environmental isolates of E. coli. *Bmc Microbiology* **13**, 25.

Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K (2004) Persister cells and tolerance to antimicrobials. *Fems Microbiology Letters* **230**, 13-18.

Knudsen GM, Ng Y, Gram L (2013) Survival of bactericidal antibiotic treatment by a persister subpopulation of *Listeria monocytogenes*. *Applied and Environmental Microbiology* **79**, 7390-7397.

Kussell E, Kishony R, Balaban N, Leibler S (2005) Bacterial persistence: A model of survival in changing environments. *Genetics* **169**, 1807-1814.

Kussell E, Leibler S (2005) Phenotypic diversity, population growth, and information in fluctuating environments. *Science* **309**, 2075.

Lachmann M, Jablonka E (1996) The inheritance of phenotypes: An adaptation to fluctuating environments. *Journal of Theoretical Biology* **181**, 1-9.

Luidalepp H, Joers A, Kaldalu N, Tenson T (2011) Age of Inoculum Strongly Influences Persister Frequency and Can Mask Effects of Mutations Implicated in Altered Persistence. *Journal of Bacteriology* **193**, 3598-3605.

Mulcahy LR, Burns JL, Lory S, Lewis K (2010) Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. *Journal of Bacteriology* **192**, 6191-6199.

Patra P, Klumpp S (2013) Population dynamics of bacterial persistence. *PLoS ONE* **8**, e62814.

Stewart B, Rozen DE (2012) Genetic variation for antibiotic persistence in Escherichia coli. *Evolution* **66**, 933-939.

Thattai M, van Oudenaarden A (2004) Stochastic gene expression in fluctuating environments. *Genetics* **167**, 523-530.