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The phenotypic evolution of *Pseudomonas aeruginosa* populations changes in the presence of subinhibitory concentrations of ciprofloxacin

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Ciprofloxacin is a widely used antibiotic, in the class of quinolones, for treatment of *Pseudomonas aeruginosa* infections. The immediate response of *P. aeruginosa* to subinhibitory concentrations of ciprofloxacin has been investigated previously. However, the long-term phenotypic adaptation, which identifies the fitted phenotypes that have been selected during evolution with subinhibitory concentrations of ciprofloxacin, has not been studied. We chose an experimental evolution approach to investigate how exposure to subinhibitory concentrations of ciprofloxacin changes the evolution of *P. aeruginosa* populations compared to unexposed populations. Three replicate populations of *P. aeruginosa* PAO1 and its hypermutable mutant Δ*mutS* were cultured aerobically for approximately 940 generations by daily passages in LB medium with and without subinhibitory concentration of ciprofloxacin and aliquots of the bacterial populations were regularly sampled and kept at −80 °C for further investigations. We investigate here phenotypic changes between the ancestor (50 colonies) and evolved populations (120 colonies/strain). Decreased protease activity and swimming motility, higher levels of quorum-sensing signal molecules and occurrence of mutator subpopulations were observed in the ciprofloxacin-exposed populations compared to the ancestor and control populations. Transcriptomic analysis showed downregulation of the type III secretion system in evolved populations compared to the ancestor population and upregulation of denitrification genes in ciprofloxacin-evolved populations. In conclusion, the presence of antibiotics at subinhibitory concentration in the environment affects bacterial evolution and further studies are needed to obtain insight into the dynamics of the phenotypes and the mechanisms involved.

INTRODUCTION

Antibiotics, especially when present at sublethal concentrations, are responsible for increasing genetic variation (Blázquez *et al.*, 2012). Antibiotics may cause genetic changes by means of different pathways involving an increase of free radicals inside the cell or oxidative stress (Kohanski *et al.*, 2010), by inducing error-prone polymerases mediated by the SOS response (Cirz *et al.*, 2006), misbalancing nucleotide metabolism or acting directly on DNA (Blázquez *et al.*, 2012). In this respect, quinolones have been known for years to be mutagenic in bacteria (Gocke, 1991).

This implies that antibiotic therapy, in some cases, may have the detrimental side-effect of accelerating the adaptation of pathogens as well as of the commensal strains (suggested to be a major reservoir of resistance) to antibiotics and host defences (Blázquez *et al.*, 2012). The occurrence of resistant *Staphylococcus epidermidis* on the skin during treatment with ciprofloxacin has been demonstrated in healthy volunteers (Høiby *et al.*, 1997) as well as enrichment in the commensal gut flora of bacteria with increased mutation frequency and antibiotic resistance in

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Abbreviations: CF, cystic fibrosis; HSL, homoserine lactone; QS, quorum sensing.

Two supplementary tables and two supplementary figures are available with the online Supplementary Material.

cystic fibrosis (CF) patients with high antibiotic usage (Gustafsson *et al.*, 2003).

Fluoroquinolones, and particularly ciprofloxacin, are commonly used to treat *Pseudomonas aeruginosa* lung infection in patients with CF and some patients receive life-long treatment with this antibiotic (Hurley & Smyth, 2012).

This class of antibiotics interacts with complexes composed of DNA and either of the two target enzymes, DNA gyrase and/or topoisomerase IV (Fàbrega *et al.*, 2009). Primary intrinsic resistance of the wild-type *P. aeruginosa* to fluoroquinolones is due to MexAB-OprM and MexXY-OprM efflux pumps (Masuda *et al.*, 1999; Morita *et al.*, 2001). Increased expression of four RND-type multidrug efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) together with mutation in the target genes, *gyrA*, *gyrB*, *parC* and *parB* are well recognized as significant determinants of acquired fluoroquinolone resistance in laboratory and clinical isolates, although differences in the mechanisms of resistance have been observed between *P. aeruginosa* from acute and chronic infections, underlining the role of environmental factors in evolution (Poole 2011; Wong & Kassen, 2011). We have recently shown in an experimental evolution study conducted on more than 900 generations of *P. aeruginosa* that high-level ciprofloxacin-resistant mutants with co-resistance to beta-lactams (meropenem) developed quickly in the presence of a subinhibitory concentration of ciprofloxacin (Jørgensen *et al.* 2013).

The immediate responses of *P. aeruginosa* to subinhibitory concentrations of ciprofloxacin have been investigated in several studies that reported enhancement in the frequency of mutation leading to carbapenem (especially meropenem) resistance (Tanimoto *et al.*, 2008), reduction of swimming and swarming motilities (Linares *et al.*, 2006) and increase in spontaneous mutation frequencies (Nair *et al.*, 2013). Transcriptional responses of *P. aeruginosa* to subinhibitory and inhibitory concentrations of ciprofloxacin demonstrated the induction or repression of hundreds of genes (Brazas *et al.*, 2007; Brazas & Hancock, 2005; Cirz *et al.*, 2006). The downregulated genes appear to involve virtually every facet of cellular metabolism (Brazas *et al.*, 2007; Cirz *et al.*, 2006). At least one-third of the upregulated genes are found in regulons that are involved in the SOS response (which is a classic bacterial stress-response pathway that is induced by DNA damage), and fluoroquinolones, which cause DNA breaks, are potent inducers of the SOS response (Cirz *et al.*, 2006).

Although the immediate response of *P. aeruginosa* to quinolones has been studied, the long-term effect of exposure to subinhibitory concentrations of ciprofloxacin on phenotypic evolution has not previously been investigated. Experimental evolutionary approaches, such as the one adopted here, differ from traditional short-term studies in that competition occurs among the emerging phenotypes and selection acts as an extra sieve which will weed out phenotypes caused by mutations that have a fitness cost and are outcompeted on the way to fixation by other phenotypes caused by mutations conferring better fitness. This effect of natural selection may be more clinically relevant (Wong *et al.*, 2012).

Recently, an evolution study of *P. aeruginosa* exposed to subinhibitory concentrations of ciprofloxacin was conducted to determine whether the SOS-mediated stress-induced mutagenesis provides a long-term advantage by accelerating the evolution of resistance. The authors present evidence that SOS pathways increase competitive fitness in the presence of ciprofloxacin in the beginning of an evolution study (the first 50 generations) but has no effect on long-term adaptation to ciprofloxacin, which occurs by evolving mutations causing high-level ciprofloxacin resistance (Torres-Barceló *et al.*, 2015).

In the present study, ciprofloxacin at a subinhibitory concentration of 0.05 μg ml^{-1} (0.25 MIC) was the selective force imposed on *P. aeruginosa*, in daily passages of cultures in LB medium over 3 months. We have previously reported the fast occurrence of high-level ciprofloxacin-resistant subpopulations with low fitness cost during evolution at subinhibitory levels of ciprofloxacin (Jørgensen *et al.*, 2013). In the present study, we analysed the evolved bacterial populations for changes in their phenotypes and investigated the influence of a sub-MIC concentration of ciprofloxacin on the phenotypic evolution of *P. aeruginosa* bacterial populations.

For this, we compared various phenotypes of the PAO1 and its hypermutable mutant Δ*mutS* populations evolved in triplicate in an environment with and without a subinhibitory concentration of ciprofloxacin at the end of the experiment, after 940 generations (day 94), and compared them to the ancestor populations.

We show here that 94 days of evolution of *P. aeruginosa* in the presence of subinhibitory levels of ciprofloxacin select for phenotypes that differ from the control populations and no fitness cost of the phenotypic shift was observed in the PAO1 populations.

METHODS

Strains and growth conditions. For this study, *P. aeruginosa* reference strain PAO1 (Holloway, 1955) and its mismatch mutator Δ*mutS* were used (Jørgensen *et al.*, 2013). The strains were grown in LB medium (100 ml LB in 250 ml flasks) under shaking conditions of 170 r.p.m., which ensure aeration through the whole culture media, in triplicate (lineages A, B and C) with and without 0.05 μg ciprofloxacin ml^{-1}, with daily passages of 100 μl in 100 ml LB for 94 days, as previously described (Jørgensen *et al.*, 2013). At this concentration of ciprofloxacin, the doubling time (mean ± SD) of PAO1 and Δ*mutS* is 32 ± 1.25 and 31.4 ± 0.5 min, respectively, in ciprofloxacin compared to 24.5 ± 1.94 and 26.2 ± 0.6 min, respectively, in the absence of antibiotic.

For the phenotypic analysis, we investigated the protease activity, swimming motility, colony morphology, antibiotic sensitivity and auxotrophy of 170 colonies/strain representing 20 colonies from each of the three lineages in each of the two conditions and 50 colonies from the ancestor populations. Quorum sensing (QS) signal molecule production was investigated in the 170 colonies of the PAO1 lineage.

All the analysed colonies were passaged three times on antibiotic-free media.

The three lineages of PAO1 evolved in the presence or absence of ciprofloxacin were investigated for spontaneous mutation rates by fluctuation experiments and for fitness compared to the ancestor population by competition studies.

The catabolic profiles of the evolved populations were analysed using Phenotype MicroArrays (Biolog) and gene expression profiles were investigated by transcriptomic studies using *P. aeruginosa* GeneChip (Affymetrix).

Colony morphology on LB plates. Overnight cultures of 20 colonies from each of the three lineages/strain evolved with and without ciprofloxacin were streaked onto LB agar plates (1 % NaCl) and images of the colonies were taken at 2.5 × magnification using a Leitz Aristoplan light microscope. Colony morphology was classified into five different morphotypes based on features such as colony size, colour (dark or light), surface shape (mountain or flat) and sharp edge or fuzzy edge, according to Hansen *et al.* (2012). Pictures of the five morphotypes are shown in Fig. S1 (available in the online Supplementary Material).

Antibiotic sensitivity. Antibiotic sensitivity to ciprofloxacin, piperacillin/tazobactam, ceftazidime, meropenem, tobramycin and colistin of the 170 colonies/strain from evolved and ancestor populations was investigated by an antibiotic diffusion test using Neosensitabs tablets (Rosco, Diagnostica A/S) and expressed as width (mm) of the inhibition zones.

Determination of proteolytic activity. Supernatant from an overnight culture, filtered through 0.22 µm pore filters (50 µl), was applied to skimmed milk plates (40 % homogenized skimmed milk in demineralized water, 1.1 % agar) followed by overnight incubation at 37 °C. The diameter of the clearing zone surrounding the inoculation spot was measured at 48 h, indicating the ability of isolates to produce proteases that hydrolyse casein (Kessler & Safrin, 2014).

Swimming motility assay. Cells from one colony were inoculated by use of a sterile toothpick on tryptone plates containing 1 % tryptone, 0.5 % NaCl and 0.3 % Bacto agar. The swimming zone was measured after 24 h of incubation at 30 °C (Ha *et al.*, 2014).

Nutritional status determination. The nutritional status of the ancestor and evolved populations was determined by replica plating overnight cultures of the 170 colonies/strain (5 µl) on LB or M9 minimal glucose media (glucose minimal medium containing 0.4 % glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 2 % agar) plates. The colonies that grew only on LB after 48 h at 37 °C and not on minimal media were considered possible auxotrophs.

Measurement of QS signal molecules. The measurements for QS signal molecules were performed in PAO1 populations in 'black 96-welled microtitre plates' (black PolyBase; Nunc), using GFP-based, specific QS reporter strains. The reporter strains used were previously described: MH205 [C_4-homoserine lactone (C_4-HSL)] and MH155 (3oxo-C_{12}-HSL) (Bjarnsholt *et al.*, 2010). ABT minimal medium (0.2 % $(NH_4)_2SO_4$, 0.6 % $Na_2HPO_4 . 2H_2O$, 0.3 % KH_2PO_4, 0.3 % NaCl, 1 mM $MgCl_2$, 0.1 mM CaCl, 0.01 mM $FeCl_3$) supplemented with thiamin (25 µg ml^{-1}), 0.5 % glucose and 0.5 % Casamino acids was used to grow the reporter strains. For controls, 10 mM *N*-butanoyl-L-homoserine lactone (BHL) and 10 mM *N*-dodecanoyl-DL-homoserine lactone (DDHL) were used. BHL and DDHL are *P. aeruginosa* autoinducer molecules encoded by the *rhlR*/*rhlI* and *lasR*/*lasI* systems, respectively (Schuster & Greenberg, 2006) Overnight cultures of 50 colonies from the PAO1 ancestor and 20 colonies from each of the three PAO1 lineages evolved with and without ciprofloxacin at day 94 were prepared in 5 ml ABT supplemented with 0.5 % glucose and 0.5 % Casamino acids. For QS signal molecule measurements a final volume of 150 µl was used. To each well 150 µl of the appropriate monitor strain diluted to an OD_{450} of 0.1 was added.

The microtitre plates were then incubated and read in a Multi Label reader (wallac 1320Viktor; Perkin Elmer). OD_{450} and fluorescence (excitation 485 nm and emission 535 nm) for 17 h were measured every 15 min. The temperature was kept at 37 °C.

The amount of QS signal molecules produced by each colony was expressed as percentage of the signal produced by the positive controls of C4-HSL and 3-oxo-C12-HSL molecules.

Estimation of population mutation rates. Mutation rates were estimated using a fluctuation experiment, as previously described (Mandsberg *et al.*, 2009), where a culture of the three PAO1 lineages evolved in the presence or absence of subinhibitory concentrations of ciprofloxacin was diluted to 2×10^4 cells in 280 µl of LB and grown in 27 microtitre wells for 7 h (to approx. 10^7 cells in 1 ml), then plated on 120 mg rifampicin l^{-1} LB agar plates to count the number of mutants. Three wells for each strain were used to estimate the c.f.u. per well. The expected number of mutations per well was then estimated using the MSS maximum-likelihood method using a web-based fluctuation analysis calculator tool (Hall *et al.*, 2009).

Fitness studies. The fitness of the evolved PAO1 populations compared to the ancestor population was investigated in competition experiments, where blue and white screening on X-Gal plates was used to distinguish colonies of the evolved PAO1 population from the ancestor. In short, two similar plasmids, mini-CTX2-PBAD : : MCS and mini-CTX-PBAD : : *lacZ*, were constructed by cloning a 1210 bp fragment containing *araC* and the arabinose inducible PBAD promoter originating from pJN105 (Newman & Fuqua, 1999). The fragment was generated by PCR and cloned into the multiple cloning sites of mini-CTX2 (Hoang *et al.*, 2000) and mini-CTX-*lacZ* vectors (Becher & Schweizer, 2000) using *Pst*I and *Hin*dIII restriction sites. Plasmids and plasmid sequences are available upon request. The constructs were electroporated into *E. coli* CC118λpir (Herrero *et al.*, 1990) and selected on LB agar plates with 8 µg tetracycline ml^{-1}. The plasmids were mobilized by conjugation into evolved and ancestor populations of PAO1 integrating at the *attB* chromosomal site (Hoang *et al.*, 2000). The PBAD promoter was inactive in the absence of arabinose, which eliminated expression-related effects on fitness during the competitive growth experiments.

Growth experiments between ancestor PAO1 and evolved populations at a ratio of 1 : 1 were followed for 7 h in shake flasks containing LB without arabinose. To quantify the c.f.u. of each strain at the start and end of competitions, cultures were serially diluted at up to 10^{-4} after 30 min and up to 10^{-7} after 7 h. The dilutions were plated on 80 µg X-Gal ml^{-1} and 0.3 % arabinose LB plates and incubated at 37 °C until blue and white colonies appeared. The fitness index was calculated as the ratio between the numbers of generations of the two strains. The experiments were performed in triplicate.

Genetic background of hypermutability. The genetic background of the mutator colonies in the PAO1 population was investigated by sequencing the mutator genes *mutS* and *mutL*, as previously described (Ciofu *et al.*, 2010).

Metabolic profiling by Phenotype MicroArrays. Phenotype MicroArrays were applied to create metabolic profiles of the evolved and ancestor *P. aeruginosa* populations, as previously described (Jørgensen *et al.*, 2013). The populations were tested on two different microtitre plates (PM1 and PM2) containing 95 different carbon sources each.

Transcriptomic profiling by DNA microarray. The gene expression profile was investigated in the three lineages (A, B and C) of the evolved populations of PAO1 and Δ*mutS* in the absence or presence of ciprofloxacin at day 94 and compared to the transcription profile of the ancestor populations. A 10^{-6} dilution of the bacterial populations was plated on LB plates and three independent colonies were selected to start cultures in LB medium. At an OD_{600} of 1.0, 4 ml of each culture was harvested and RNA isolation and purification were performed using RNA Protect Bacteria Reagent and RNeasy Mini kit (Qiagen). RQ1 RNase-free DNase (Promega) was added to remove contaminating DNA.

Processing of the *P. aeruginosa* GeneChip (Affymetrix) was performed at the Department of Clinical Biochemistry, Microarray Core unit, Rigshospitalet, University of Copenhagen, Denmark.

Statistical analysis. Statistical analysis of the various phenotypes in the two population types (with and without ciprofloxacin) was performed by GraphPad Prism version 6.04. for Windows (GraphPad Software; www.graphpad.com).

The normal distribution of the values was tested by the D'Agostino and Pearson omnibus normality test. On values that did not pass the normality test, the non-parametric Mann–Whitney test was used to compare the changes of the measured parameters between populations evolved with or without sub-MIC concentration of ciprofloxacin and the ancestor population. The level of significance was 5 %. A one-tailed *t*-test on unpaired samples was used for values that followed a normal distribution.

Gene expression analysis was done using ArrayStar v.12.3.1 software (DNASTAR). The datasets were normalized using the robust multi-array average algorithm. This is an algorithm used to create an expression matrix from Affymetrix data. The raw intensity values are background corrected, $\log_2$ transformed and then quantile normalized. Next a linear model is fit to the normalized data to obtain an expression measure for each probe set on each array.

Differences in gene expression between ancestor and evolved bacterial populations in the presence or absence of sub-MIC ciprofloxacin were tested using Student's *t*-test. Genes were considered differently expressed if the fold-change was higher than twofold with a confidence of $P<0.05$.

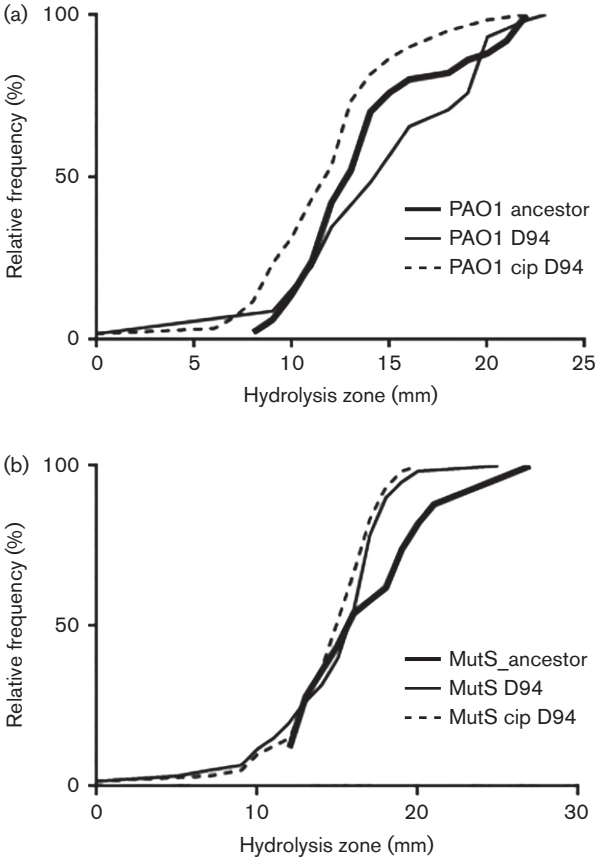

Fig. 1. Cumulative frequency distribution of protease activity of colonies of PAO1 (a) and Δ*mutS* (MutS) (b) populations. The colonies of the ancestor population ($n=50$) are shown with a thick line, those of the evolved population with ciprofloxacin ($n=60$) are shown with a stippled line and those of the control populations ($n=60$) with a filled line.

RESULTS

Protease activity

Significant decreases in protease activity of colonies from ciprofloxacin-evolved populations of PAO1 ($P=0.01$) and Δ*mutS* ($P=0.04$) compared to ancestor populations were seen at day 94, while no significant changes in protease activity between the ancestor and the control populations were observed (Fig. 1).

Swimming motility

A significant decrease in swimming motility of colonies from the ciprofloxacin-evolved populations of PAO1 ($P<0.0001$) and Δ*mutS* ($P<0.0001$) compared to the ancestor population was seen. A significant decrease in swimming motility was also found in control populations compared to the ancestor population for both PAO1 ($P=0.0002$) and Δ*mutS* ($P<0.0001$) (Fig. 2).

QS molecules

The day 94 (D94) bacterial populations of PAO1 that evolved in the presence of ciprofloxacin had significantly higher levels of C4-HSL ($P=0.02$) and especially 3-oxo-C12-HSL ($P<0.0001$) compared to the ancestor populations while the differences were not significant for the control populations (Fig. 3), suggesting that the ciprofloxacin treatment was a selective pressure for the maintenance of higher levels of C_4-HSL and 3-oxo-C_{12}-HSL during this evolution experiment.

Colony morphology

The distribution of five different colony morphologies encountered in the different bacterial populations is shown in Table 1 (PAO1 and Δ*mutS*). Images of the different morphotypes are presented in Fig. S1.

A higher heterogeneity of colony morphology was observed in the mutator population (Δ*mutS*) compared to PAO1

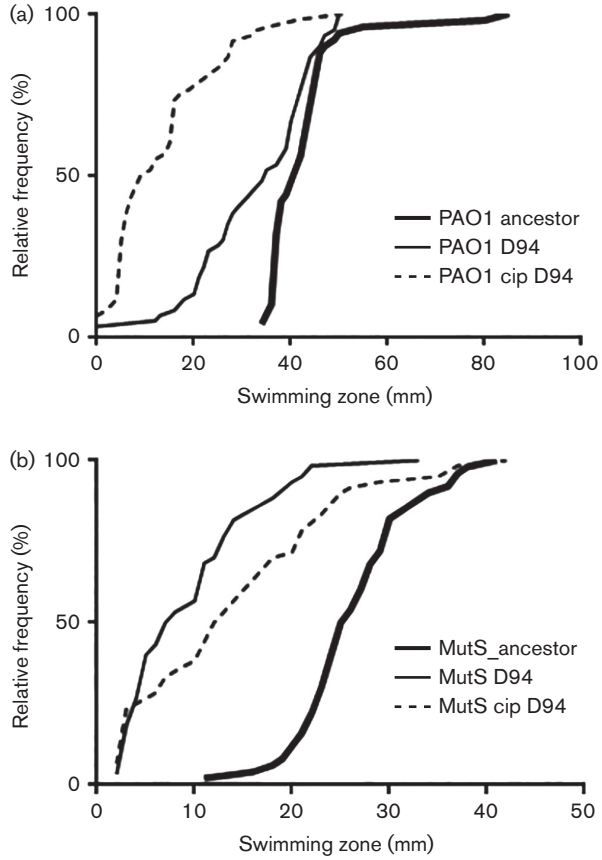

Fig. 2. Cumulative frequency distribution of swimming motility of colonies of PAO1 (a) and Δ*mutS* (MutS) (b) populations. The colonies of the ancestor population (n=50) are shown with a thick line, those of the evolved population with ciprofloxacin (n=60) are shown with a stippled line and those of the control populations (n=60) with a filled line.

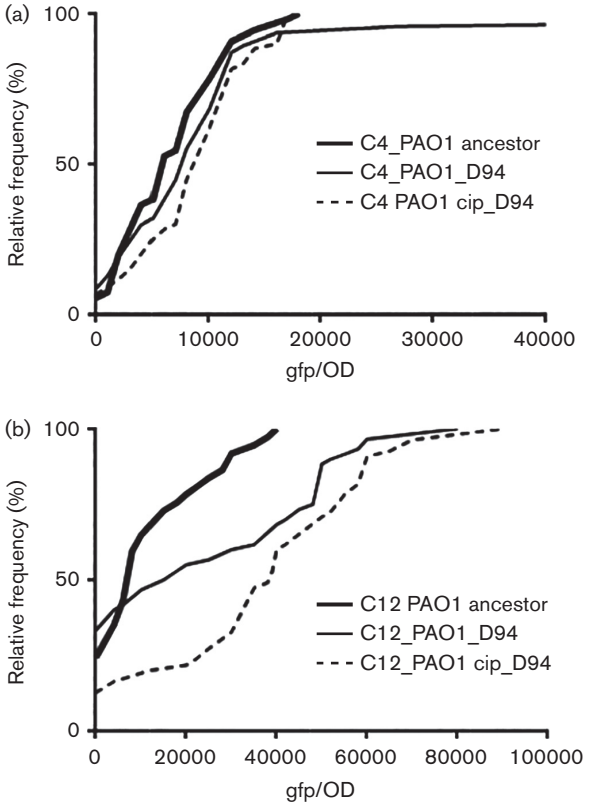

Fig. 3. Cumulative frequency distribution of the levels of QS molecules C_4-HSL (a) and 3-oxo-C_{12}-HSL (b) in PAO1 populations. The colonies of the ancestor populations (n=50) are shown with a thick line, those of the evolved populations with ciprofloxacin (n=60) are shown with a stippled line and those of the control populations (n=60) with a filled line.

and this was maintained during the experiment. Two morphotypes, A and G, appeared to dominate the different bacterial populations throughout the experiment, while morphotype D appeared to occur especially in the populations of both PAO1 ancestor populations and Δ*mutS* in the early stages of the evolution experiment and did not occur at day 94.

Similar morphotypes were observed in the bacterial populations that evolved in the presence or absence of ciprofloxacin, suggesting that ciprofloxacin did not select for a specific morphotype in the laboratory conditions of our experiment.

Antibiotic sensitivity

The PAO1 and Δ*mutS* populations evolved with subinhibitory concentrations of ciprofloxacin had significantly lower sensitivity to ciprofloxacin ($P<0.0001$) (Mann–Whitney t-test unpaired, two-tailed) (Table 2) compared to the ancestor population and the control populations. No decrease in susceptibility to ceftazidime, meropenem, tobramycin and colistin were observed in the populations evolved in the presence of subinhibitory concentration of ciprofloxacin compared to control populations.

Nutritional status

Few auxotroph colonies were found in the control (7 %) and ciprofloxacin-evolved (5 %) hypermutator Δ*mutS* populations while no auxotrophs were observed among the colonies from the evolved PAO1 populations.

The ciprofloxacin-exposed PAO1 populations and the Δ*mutS* populations showed improved catabolism on D-glycerol 1-phosphate and L-threonine, while the control populations showed unchanged or loss of catabolic capacity. Few biologically significant changes [defined in accordance with Jørgensen *et al.* (2015) as differences in areas of the two respiration curves of more than 20 000 software units] in substrate catabolism of PAO1 and Δ*mutS* evolved populations compared to ancestor populations were observed (Fig. S2).

Table 1. Distribution of four different colony morphologies in the bacterial populations presented as percentage of the total number of colonies analysed

Bacterial populations analysed: PAO1 ancestor and Δ*mutS*, control day 94 (D94) and ciprofloxacin evolved day 94 (D94 cip). Colony type is identified in accordance with the CF *P. aeruginosa* colony morphologies described by Hansen *et al.* (2012).

Bacterial populations (no. of colonies analysed)	Colony morphotypes (% of colonies from bacterial population)				
	A	D	E	F	G
PAO1 ancestor (n=50)	32 (64 %)	15 (30 %)			3 (6 %)
PAO1 D94 (n=60)	43 (72 %)		3 (5 %)		14 (23 %)
PAO1 D94 cip (n=60)	45 (75 %)				15 (25 %)
Δ*mutS* ancestor (n=50)	18 (36 %)	20 (40 %)	1 (2 %)		11 (22 %)
Δ*mutS* D94 (n=60)	31 (52 %)	5 (12 %)	2 (3 %)	2 (3 %)	15 (25 %)
Δ*mutS* D94 cip (n=60)	26 (43 %)	3 (2 %)	11 (18 %)	4 (7 %)	16 (27 %)

Mutation rates

The mutation rates of the evolved populations are presented in Table 3. All three lineages evolved in the presence of subinhibitory concentrations of antibiotics showed increased mutation rates compared to control lineages at day 94. The genetic background of the mutator subpopulation was identified in lineage B as insertion of a G after position 46 in *mutL* leading to a frame shift. This mutation was already present in the ciprofloxacin-evolved population of lineage B at day 59 (data not shown).

Fitness cost

The fitness index of the evolved populations from day 94 compared to the ancestor (PAO1) showed no or modest reduction in fitness of the selected phenotypes, when grown in LB. The fitness indices are presented in Table 3.

Gene expression analysis

Common differentially expressed genes found in evolved PAO1 populations (A, B, C) with and without ciprofloxacin at day 94 compared to the ancestor population are presented in Table 4.

Downregulation of type III secretion systems (Hauser, 2009) and of two-component and phosphorelay regulatory systems (Stephenson & Hoch, 2002) compared to the ancestor population was observed in all the evolved populations, with and without ciprofloxacin, as an adaptive response to the planktonic cultures in rich LB media (Table 4).

Upregulation of the *nir* and *nos* operons involved in denitrification (Zumft, 1997) were found in ciprofloxacin-evolved bacterial populations of both PAO1 and Δ*mutS* at day 94 (Table 5).

Among the common downregulated genes, the chemotactic operon PA0176-PA0179 is mentioned as it seems to be involved in aerotaxis (movement of bacteria towards oxygen) (Hong *et al.*, 2004), therefore supporting the change to anaerobic respiration in aerobic growth in the presence of ciprofloxacin. Downregulation of several other genes involved in chemotaxis was also observed, such as the *pct* operon which is responsible for sensing

Table 2. Inhibition zones (mm, mean ± SD) of 170 colonies/strain to six different antibiotics

CIP, ciprofloxacin; TZP, piperacillin-tazobactam; CAZ, ceftazidime; MEM, meropenem; TOB, tobramycin; CST, colistin. Significantly lower inhibition zones in PAO1 D94 cip compared to the PAO1 ancestor and Δ*mutS* D94 cip compared to Δ*mutS* ancestor populations were measured (P<0.0001) (Mann–Whitney t-test unpaired, two-tailed).

P. aeruginosa population	CIP (5 μg)	TZP (100 μg + 10 μg)	CAZ (30 μg)	MEM (10 μg)	TOB (10 μg)	CST (150 μg)
PAO1 ancestor (n=50)	30 ± 0.3	31 ± 0.8	27 ± 0.6	38 ± 1.3	17 ± 0.8	24 ± 0.5
PAO1 D94 (n=60)	30 ± 0.5	31 ± 1.1	27 ± 0.9	39.5 ± 0.9	17 ± 0.9	24 ± 0.8
PAO1 D94 cip (n=60)	18 ± 3.1	30 ± 1.9	26 ± 1.5	38.5 ± 2.6	17 ± 0.8	24 ± 0.7
Δ*mutS* ancestor (n=50)	30 ± 1.4*	31 ± 0.7*	27 ± 0.8*	39 ± 0.9*	16 ± 1	24 ± 0.5
Δ*mutS* D94 (n=60)	30 ± 7*	33 ± 1.4*	29 ± 1.6*	39 ± 2.3*	16 ± 1.1	24 ± 0.7
Δ*mutS* D94 cip (n=60)	10 ± 9.5*	32 ± 1.7*	28 ± 1.5*	39 ± 1.9*	16 ± 1.3	24 ± 0.8

*Colonies in the inhibition zone.

Table 3. Mutation rates and fitness index of PAO1 evolved populations in the presence or absence of subinhibitory concentrations of ciprofloxacin in comparison to the ancestor population

PAO1 population	Mutation rates (mutations per cell division)	Fitness index (mean ± SD)
Ancestral	9×10^{-9}	1
PAO1 A D94	3.5×10^{-9}	0.91 ± 0.062
PAO1 B D94	5×10^{-9}	0.92 ± 0.049
PAO1 C D94	3.9×10^{-9}	0.94 ± 0.125
PAO1 A cip D94	1.4×10^{-8}	0.98 ± 0.1
PAO1 B cip D94	1.26×10^{-7}	0.93 ± 0.058
PAO1 C cip D94	1.83×10^{-8}	0.86 ± 0.281

amino acids (Taguchi *et al.*, 1997). In addition, strong downregulation of *fliC* and *flaG* involved in bacterial motility was also observed.

Transcriptomic data of Δ*mutS* showed, not surprisingly, a higher number of differentially expressed genes in both the control and the ciprofloxacin-evolved population compared to the number of genes observed in PAO1 lineages (Tables S1 and S2).

The transcriptomic data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE78255 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE78255).

DISCUSSION

Evolution of *P. aeruginosa* with subinhibitory concentrations of ciprofloxacin changed the frequency distribution of the phenotypes observed in the *P. aeruginosa* populations compared to control populations, underlining the importance of the environmental conditions for the evolution of a bacterial population. Loss of virulence factors as shown by the protease assay (Fig. 1) and the downregulation of type III secretion at day 94 was a parallel phenotypic change in the six PAO1 (Table 4) and Δ*mutS* lineages (Table S2), suggesting that virulence traits can be lost in evolution without selection by the immune system. This is in accordance with recently published data for experimental evolution in *Caenorhabditis elegans* (Jansen *et al.*, 2015). Interestingly, no fitness costs of the PAO1 evolved populations compared to the ancestor populations were observed in our evolution study, suggesting the selection of phenotypes caused by low-cost mutations or the occurrence of compensatory mutations to reduce the fitness burden. The occurrence of compensatory mutations has been previously shown in an experimental evolution study by whole-genome sequencing of ciprofloxacin-resistant subpopulations (Wong *et al.*, 2012).

Table 4. Operons differentially expressed at day 94, common in both control and ciprofloxacin (cip) evolved PAO1 populations compared to ancestor PAO1 populations

Fold changes represent downregulation. For a complete list of genes and expression levels see Tables S1 and S2. Numbers in parentheses represent differences that did not reach the level of statistical significance.

Functional class: pathway	Gene (operon) PAO1 annotation	Fold changes lineage A control/cip	Fold changes lineage B control/cip	Fold changes lineage C control/cip
Protein secretion/ export apparatus: type III secretion	PA1694_pscQ	4.0/4.8	3.4/3	3.1/3.3
	PA1700_at	2.3/2.6	2.4/2.3	2.1/2
	PA1701_at	4.3/4.8	3.9/3.1	3.5/3.8
	PA1703_pcrD	2.9/3	2.4/2	2.2/2.2
	PA1706_pcrV	7.2/5.8	3.5/(2.7)	(3.1)/4.3
	PA1707_pcrH	11.0/8.7	4.5/3.5	5.0/5.5
	PA1708_popB	21.3/9.2	4.2/4	5.0/6
	PA1709_popD	16.1/9	5.0/4.4	4.4/4.7
	PA1710_exsC	5.1/3.5	2.8/2.7	2.2/4
	PA1711	5.7/4	3.4/3.8	3.1/4.3
	PA1712_exsB	5.2/3.7	4.6/5.1	2.9/4.1
	PA1713_exsA	6.1/5.2	5.7/5.4	(3.7)/3.6
	PA1714	5.4/4.7	4.1/3.4	3.3/3.8
	PA1715_pscB	6.3/6.4	6.0/5	3.8/4.6
	PA1716_pscC	7.9/7.4	5.5/4.8	4.0/4
	PA1717_pscD	5.8/6.2	5.0/3.6	4.5/3.8
	PA1718_pscE	31.5/12	6.1/(4.5)	6.0/4.4
	PA1719_pscF	10.2/8.3	5.5/4.3	4.8/4.1
	PA1720_pscG	4.7/4.6	3.5/(2.6)	3.1/3
	PA1721_pscH	5.7/5.3	4.0/2.8	3.7/3.6
	PA1722_pscI	11.4/7.7	5.9/(3.7)	4.7/4.4
	PA1723_pscJ	4.5/4.3	4.0/2.8	3.2/3.3
	PA1724_pscK	3.3/3.8	3.5/2.4	2.9/2.7
	PAO044_exoT	10.3/9.7	4.0/3.4	3.5/4
	PA3841_exoS	14.4/9.2	3.1/3.8	3.6/4.1
Two-component regulatory system: phosphorelay signal transduction	PA1179_phoP	10.0/5.2	(3.6)/6.2	(3.1)/3.6
	PA1180_phoQ	6.8/4	4.4/5	(3.0)/4.1
	PA2656	10.5/10.5	9.2/14.1	3.9/4.5
	PA2657	16.0/14	12.3/21	(3.9)/(3.7)
	PA2658	14.8/14	6.2/15	(3.7)/(3.3)
	PA2659	21.3/14	3.9/15	(3.2)/(2.5)

The occurrence and maintenance of a mutator subpopulation in lineage B of PAO1 populations during evolution in the presence of subinhibitory levels of ciprofloxacin might have important clinical consequences. The occurrence of mutator subpopulations due to a TTCCC insertion sequence after position 130 of *mutL* was also observed in lineage C of PAO1 at day 59 and day 66 of the evolution experiment but these subpopulations were not found at day 94 (data not shown).

It has been hypothesized that sub-MIC selection enriches for mutator bacteria as this environment favours accumulation of small-step mutations. Bacteria that have high mutation rates are enriched, as they accumulate mutations at an increased rate and thus experience more rapid

Table 5. Operons differentially expressed at day 94 in ciprofloxacin evolved populations of both PAO1 and Δ*mutS* populations compared to ancestor populations

Numbers in parentheses represent differences that did not reach the level of statistical significance.

Functional class: pathway	Genes (operon) PAO1 annotation	Fold change PAO1 A-cip-D94	Fold change PAO1 B-cip-D94	Fold change PAO1 C-cip-D94	Fold change Δ*mutS* A-cip-D94	Fold change Δ*mutS* B-cip-D94	Fold change Δ*mutS* C-cip-D94
Upregulation							
Energy metabolism:	PA511_nirJ	2.0	(2.6)	3.4	3.6	8.1	2
denitrification	PA0512	2.4	(2.9)	4	5	11	2.2
	PA3392_nosZ	5.9	(4.9)	8.6	10.7	28.3	3.2
	PA3393_nosD	4.7	(4.4)	6.9	8.2	20.8	2.2
	PA3394_nosF	6.3	(3.4)	8.4	12.6	22	2.6
	PA3395_nosY	5.8	(4.3)	6.7	10.8	24.5	2.6
Downregulation							
Motility:	PA1092_fliC	103	34	15	5	18	71.2
chemotaxis	PA1093_flaG	35.6	(14.3)	11	4	15.6	26.7
	PA0174	3.9	(2.6)	2.8	2.8	3.2	2.8
	PA0175	5.5	(3.0)	3.0	4.5	4.9	4.2
	PA0176_aer2	8.8	(3.8)	3.6	7	4.4	4.4
	PA0177	4.8	(2.9)	3.1	4	3.7	4.1
	PA0178	4.1	(2.4)	2.3	8.7	7	7.3
	PA0179	3.9	(2.4)	3.3	12	8.7	11.4
	PA4309_pctA	7.2	(3.7)	(3.8)	9.5	12.1	11.9
	PA4310_pctB	23.8	(8.3)	12.1	20	24.2	37.5

adaptation to the growth-inhibitory environment (Andersson & Hughes, 2014). Our *in vitro* study confirms the role of antibiotic exposure at subinhibitory concentration as an important factor that influences the dynamics of the mutator populations.

An unexpected finding was the upregulation of denitrification genes in the PAO1 and Δ*mutS* populations evolved in the presence of ciprofloxacin compared to controls (Table 5), probably as a metabolic response to improve the fitness cost of ciprofloxacin resistance mutations. Transcriptomic data showed an increased expression of several operons involved in denitrification, suggesting a metabolic shift of the bacteria under ciprofloxacin exposure in LB aerobic cultures.

LB is a rich medium which was found to contain ~20 μM NO_3^- (Line *et al.*, 2014), thus supporting denitrification. It has been shown in *P. aeruginosa* that overexpressing the efflux pump MexEF-OprN is associated with activation of the nitrate respiratory chain under aerobic conditions as a mechanism to compensate for fitness costs (Olivares *et al.*, 2014). Similarly, upregulation of *nor* and *nir* genes has been found in *nfxB* mutants, overexpressing the MexCD-OprJ efflux pump (Mulet *et al.*, 2011). Isolates with *nfxB* mutations have been found during our evolution experiment (Jørgensen *et al.*, 2013). Although we do not have an explanation for this metabolic shift during evolution at subinhibitory levels of ciprofloxacin, we might speculate that upregulation of denitrification probably occurs to compensate for the metabolic burden of acquired mutations causing ciprofloxacin resistance. Alternatively, the shift from aerobic to anaerobic metabolism might be a metabolic adaptation to reduce the formation of reactive oxygen species and thus the oxidative stress burden during continuous exposure to ciprofloxacin (Brochmann *et al.*, 2014; Dwyer *et al.*, 2009).

Different morphotypes were observed even in the ancestor populations of both PAO1 and Δ*mutS*. The evolved populations of Δ*mutS* showed, not surprisingly, a higher heterogeneity of morphotypes than the wild-type strain, although no difference between control and ciprofloxacin-exposed populations was observed (Table 1). Changes in colony morphology of *P. aeruginosa* have been shown to have a heterogeneous genetic background. Up to 55 different genes have been shown to influence colony morphology, including *mexR* (MexAB OprM efflux pump repressor), *pqsL*, *pqsD* (*Pseudomonas* quinolone signal), *mvfR* (transcription regulator), *pilW* and *pilY1* (type IV fimbrial proteins), and *chpA* and *chpC* (chemotaxis proteins) (Rakhimova *et al.*, 2008). Selection of specific phenotypes has been seen as an adaptive process in various environments with spatial compartmentalization or niche formation (Rainey *et al.*, 2000).

In this study of planktonic, shaking cultures of *P. aeruginosa*, a relatively homogeneous environment is expected and the populations selected by subinhibitory levels of ciprofloxacin do not seem to have a specific morphotype.

The relatively constant environment encountered by *P. aeruginosa* during daily passages in LB probably explains the lack of changes in the catabolic profiles of the evolved

populations compared to the ancestor (with the exception of improved catabolic capacity on D-glycerol).

A decrease in the protease activity of the bacterial populations was observed in the ciprofloxacin-treated populations compared to control populations (Fig. 1). *P. aeruginosa* secretes into its environment at least seven extracellular proteases but pseudolysin (LasB protease; elastase encoded by *lasB*) is the most abundant and most potent endopeptidase secreted by *P. aeruginosa* and is a classic virulence factor (Kessler & Safrin, 2014). Its activity on common protein substrates such as casein or azocasein is five- to tenfold higher than that of other *Pseudomonas* endopeptidases. This implies that assays of general proteolytic activity (such as the skimmed milk plate employed in this study) in crude enzyme preparations from *P. aeruginosa* largely reflect LasB activity (Kessler & Safrin, 2014).

As QS is involved in the regulation of protease production (Gambello & Iglewski, 1991; Toder *et al.*, 1991), the decrease in protease activity of the bacterial populations in the ciprofloxacin-treated populations compared to control populations (Fig. 1) could suggest an inactivation of the QS system in antibiotic evolved populations However, this could not be found in our study by measuring the QS molecules in the PAO1 lineages. In contrast, higher levels of C_4-HSL and especially 3-oxo-C_{12}-HSL molecules were observed at the end of the experiment in ciprofloxacin-evolved PAO1 populations compared to ancestor populations (Fig. 3). Phenotypes of protease-negative and QS-positive colonies have been previously reported in CF *P. aeruginosa* isolates (Bjarnsholt *et al.*, 2010; Markussen *et al.*, 2014).

Our data suggest that QS-regulated factors are required for survival of the PAO1 planktonic population under the continuous selective pressure imposed by sub-MIC ciprofloxacin. It has previously been shown that ciprofloxacin at a concentration of 0.04 μg ml^{-1}, very similar to the concentration used in the present study (0.05 μg ml^{-1}), inhibits QS in an overnight culture (Skindersoe *et al.*, 2008) and evolution towards maintenance of QS under treatment with QS inhibitors such as azitromycin has been shown before (Köhler *et al.*, 2010).

Catalase and superoxide dismutase, which are enzymes required for bacterial survival during oxidative stress, could be examples of QS-regulated factors required for adaptation at subinhibitory levels of ciprofloxacin in planktonic culture (Hassett *et al.*, 1999). We have shown increased production of reactive oxygen species in bacterial cells exposed to sub-MIC ciprofloxacin (Jørgensen *et al.*, 2013) and that a catalase mutant survived poorly in the presence of ciprofloxacin compared to the wild-type (Brochmann *et al.*, 2014). Our data suggest that the decreased protease activity in the ciprofloxacin-evolved population cannot be explained by QS-dependent regulation.

Motile bacteria have a number of potential benefits, including efficiency of nutrient acquisition and access to optimal colonization sites. *P. aeruginosa* possess three types of motility: swimming, swarming and twitching. The last two types of motility are surface-related and have not been investigated in this study, where *P. aeruginosa* has been grown by several passages in shaking liquid cultures. Swimming motility is driven by a single uni-polar flagellum which in *P. aeruginosa* PAO1 is dependent on 41 genes encoding structural/assembly and regulatory components of the flagellar organelle (Jyet & Ramphal, 2008). Swimming motility was reduced in ciprofloxacin-evolved populations compared to ancestor populations (Fig. 2). Previous studies showed that exposure to subinhibitory concentrations of ciprofloxacin causes a decrease in *P. aeruginosa* motility (Linares *et al.*, 2006) and our findings indicate that this phenotype has been selected during the evolution experiment. However, a decrease in motility was also observed in the control populations, suggesting that in conditions of good oxygenation and sufficient nutrition, swimming motility is not a required phenotype.

In accordance with this phenotype, the gene expression analysis showed downregulation of the flagellin type B *fliC* in the ciprofloxacin-evolved lineages compared to ancestor populations in PAO1 and Δ*mutS* populations (Table 5).

There are some limitations to our study, such as the use of LB medium, which although a rich medium is not similar to the *in vivo* environment of *P. aeruginosa* growing in the CF lung. To mimic the *in vivo* conditions, artificial sputum media (ASM) (Palmer *et al.* 2005;Wong *et al.* 2012) would have been more appropriate. In addition, *P. aeruginosa* grows as biofilms in the CF lung and the present data obtained from an evolution study in planktonic growth cannot be extrapolated to evolution in a biofilm. Such evolution studies in biofilms are in progress in our lab and comparison with the results obtained in planktonic growth will be possible in the future.

In conclusion, our study shows that exposure to constant subinhibitory concentrations of ciprofloxacin influences phenotypic evolution in *P. aeruginosa* bacterial populations associated with decreased virulence factors, increased spontaneous mutability and changes in bacterial metabolism, which might have clinical implications for the treatment of CF patients.

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REFERENCES

Andersson, D. I. & Hughes, D. (2014). Microbiological effects of sublethal levels of antibiotics. *Nat Rev Microbiol* **12**, 465–478.

Becher, A. & Schweizer, H. P. (2000). Integration-proficient *Pseudomonas aeruginosa* vectors for isolation of single-copy chromosomal *lacZ* and *lux* gene fusions. *Biotechniques* **29**, 948–950, 952.

Bjarnsholt, T., Jensen, P. O., Jakobsen, T. H., Phipps, R., Nielsen, A. K., Rybtke, M. T., Tolker-Nielsen, T., Givskov, M., Høiby, N., Ciofu, O. & Scandinavian Cystic Fibrosis Study Consortium (2010). Quorum sensing and virulence of *Pseudomonas aeruginosa* during lung infection of cystic fibrosis patients. *PLoS One* **5**, e10115.

Blázquez, J., Couce, A., Rodríguez-Beltrán, J. & Rodríguez-Rojas, A. (2012). Antimicrobials as promoters of genetic variation. *Curr Opin Microbiol* **15**, 561–569.

Brazas, M. D. & Hancock, R. E. (2005). Ciprofloxacin induction of a susceptibility determinant in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **49**, 3222–3227.

Brazas, M. D., Breidenstein, E. B., Overhage, J. & Hancock, R. E. (2007). Role of *lon*, an ATP-dependent protease homolog, in resistance of *Pseudomonas aeruginosa* to ciprofloxacin. *Antimicrob Agents Chemother* **51**, 4276–4283.

Brochmann, R. P., Toft, A., Ciofu, O., Briales, A., Kolpen, M., Hempel, C., Bjarnsholt, T., Høiby, N. & Jensen, P. O. (2014). Bactericidal effect of colistin on planktonic *Pseudomonas aeruginosa* is independent of hydroxyl radical formation. *Int J Antimicrob Agents* **43**, 140–147.

Ciofu, O., Mandsberg, L. F., Bjarnsholt, T., Wassermann, T. & Høiby, N. (2010). Genetic adaptation of *Pseudomonas aeruginosa* during chronic lung infection of patients with cystic fibrosis: strong and weak mutators with heterogeneous genetic backgrounds emerge in *mucA* and/or *lasR* mutants. *Microbiology* **156**, 1108–1119.

Cirz, R. T., O'Neill, B. M., Hammond, J. A., Head, S. R. & Romesberg, F. E. (2006). Defining the *Pseudomonas aeruginosa* SOS response and its role in the global response to the antibiotic ciprofloxacin. *J Bacteriol* **188**, 7101–7110.

Dwyer, D. J., Kohanski, M. A. & Collins, J. J. (2009). Role of reactive oxygen species in antibiotic action and resistance. *Curr Opin Microbiol* **12**, 482–489.

Edgar, R., Domrachev, M. & Lash, A. E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* **30**, 207–210.

Fàbrega, A., Madurga, S., Giralt, E. & Vila, J. (2009). Mechanism of action of and resistance to quinolones. *Microb Biotechnol* **2**, 40–61.

Gambello, M. J. & Iglewski, B. H. (1991). Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. *J Bacteriol* **173**, 3000–3009.

Gocke, E. (1991). Mechanism of quinolone mutagenicity in bacteria. *Mutat Res* **248**, 135–143.

Gustafsson, I., Sjölund, M., Torell, E., Johannesson, M., Engstrand, L., Cars, O. & Andersson, D. I. (2003). Bacteria with increased mutation frequency and antibiotic resistance are enriched in the commensal flora of patients with high antibiotic usage. *J Antimicrob Chemother* **52**, 645–650.

Ha, D. G., Kuchma, S. L. & O'Toole, G. A. (2014). Plate-based assay for swimming motility in *Pseudomonas aeruginosa*. *Methods Mol Biol* **1149**, 59–65.

Hall, B. M., Ma, C. X., Liang, P. & Singh, K. K. (2009). Fluctuation analysis CalculatOR: a web tool for the determination of mutation rate using Luria-Delbruck fluctuation analysis. *Bioinformatics* **25**, 1564–1565.

Hansen, S. K., Rau, M. H., Johansen, H. K., Ciofu, O., Jelsbak, L., Yang, L., Folkesson, A., Jarmer, H. O., Aanæs, K. & other authors (2012). Evolution and diversification of *Pseudomonas aeruginosa* in the paranasal sinuses of cystic fibrosis children have implications for chronic lung infection. *ISME J* **6**, 31–45.

Hassett, D. J., Ma, J. F., Elkins, J. G., McDermott, T. R., Ochsner, U. A., West, S. E., Huang, C. T., Fredericks, J., Burnett, S. & other authors (1999). Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. *Mol Microbiol* **34**, 1082–1093.

Hauser, A. R. (2009). The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nat Rev Microbiol* **7**, 654–665.

Herrero, M., de Lorenzo, V. & Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J Bacteriol* **172**, 6557–6567.

Hoang, T. T., Kutchma, A. J., Becher, A. & Schweizer, H. P. (2000). Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. *Plasmid* **43**, 59–72.

Høiby, N., Jarløv, J. O., Kemp, M., Tvede, M., Bangsborg, J. M., Kjerulf, A., Pers, C. & Hansen, H. (1997). Excretion of ciprofloxacin in sweat and multiresistant *Staphylococcus epidermidis*. *Lancet* **349**, 167–169.

Holloway, B. W. (1955). Genetic recombination in *Pseudomonas aeruginosa*. *J Gen Microbiol* **13**, 572–581.

Hong, C. S., Shitashiro, M., Kuroda, A., Ikeda, T., Takiguchi, N., Ohtake, H. & Kato, J. (2004). Chemotaxis proteins and transducers for aerotaxis in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **231**, 247–252.

Hurley, M. & Smyth, A. (2012). Fluoroquinolones in the treatment of bronchopulmonary disease in cystic fibrosis. *Ther Adv Respir Dis* **6**, 363–373.

Jansen, G., Crummenerl, L. L., Gilbert, F., Mohr, T., Pfefferkorn, R., Thänert, R., Rosenstiel, P. & Schulenburg, H. (2015). Evolutionary transition from pathogenicity to commensalism: global regulator mutations mediate fitness gains through virulence attenuation. *Mol Biol Evol* **32**, 2883–2896.

Jørgensen, K. M., Wassermann, T., Jensen, P. O., Hengzuang, W., Molin, S., Høiby, N. & Ciofu, O. (2013). Sublethal ciprofloxacin treatment leads to rapid development of high-level ciprofloxacin resistance during long-term experimental evolution of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **57**, 4215–4221.

Jørgensen, K. M., Wassermann, T., Johansen, H. K., Christiansen, L. E., Molin, S., Høiby, N. & Ciofu, O. (2015). Diversity of metabolic profiles of cystic fibrosis *Pseudomonas aeruginosa* during the early stages of lung infection. *Microbiology* **161**, 1447–1462.

Jyet, J. & Ramphal, R. (2008). Flagella and pili of *Pseudomonas aeruginosa*. In *Pseudomonas: Model Organism, Pathogen and Cell Factory*, pp. 85–108. Edited by B. H. A. Rehm. Oxford: Wiley-Blackwell.

Kessler, E. & Safrin, M. (2014). Elastinolytic and proteolytic enzymes. *Methods Mol Biol* **1149**, 135–169.

Kohanski, M. A., DePristo, M. A. & Collins, J. J. (2010). Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Mol Cell* **37**, 311–320.

Köhler, T., Perron, G. G., Buckling, A. & van Delden, C. (2010). Quorum sensing inhibition selects for virulence and cooperation in *Pseudomonas aeruginosa*. *PLoS Pathog* **6**, e1000883.

Linares, J. F., Gustafsson, I., Baquero, F. & Martinez, J. L. (2006). Antibiotics as intermicrobial signaling agents instead of weapons. *Proc Natl Acad Sci U S A* **103**, 19484–19489.

Line, L., Alhede, M., Kolpen, M., Kühl, M., Ciofu, O., Bjarnsholt, T., Moser, C., Toyofuku, M., Nomura, N. & other authors (2014). Physiological levels of nitrate support anoxic growth by denitrification of *Pseudomonas aeruginosa* at growth rates reported in cystic fibrosis lungs and sputum. *Front Microbiol* **5**, 554.

Mandsberg, L. F., Ciofu, O., Kirkby, N., Christiansen, L. E., Poulsen, H. E. & Høiby, N. (2009). Antibiotic resistance in *Pseudomonas aeruginosa* strains with increased mutation frequency due to inactivation of the DNA oxidative repair system. *Antimicrob Agents Chemother* **53**, 2483–2491.

Markussen, T., Marvig, R. L., Gómez-Lozano, M., Aanæs, K., Burleigh, A. E., Høiby, N., Johansen, H. K., Molin, S. & Jelsbak, L. (2014). Environmental heterogeneity drives within-host diversification and evolution of *Pseudomonas aeruginosa*. *MBio* **5**, e01592–e01514.

Masuda, N., Gotoh, N., Ishii, C., Sakagawa, E., Ohya, S. & Nishino, T. (1999). Interplay between chromosomal beta-lactamase and the MexAB-OprM efflux system in intrinsic resistance to beta-lactams in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **43**, 400–402.

Morita, Y., Kimura, N., Mima, T., Mizushima, T. & Tsuchiya, T. (2001). Roles of MexXY- and MexAB-multidrug efflux pumps in intrinsic multidrug resistance of *Pseudomonas aeruginosa* PAO1. *J Gen Appl Microbiol* **47**, 27–32.

Mulet, X., Moyá, B., Juan, C., Macià, M. D., Pérez, J. L., Blázquez, J. & Oliver, A. (2011). Antagonistic interactions of *Pseudomonas aeruginosa* antibiotic resistance mechanisms in planktonic but not biofilm growth. *Antimicrob Agents Chemother* **55**, 4560–4568.

Nair, C. G., Chao, C., Ryall, B. & Williams, H. D. (2013). Sub-lethal concentrations of antibiotics increase mutation frequency in the cystic fibrosis pathogen *Pseudomonas aeruginosa*. *Lett Appl Microbiol* **56**, 149–154.

Newman, J. R. & Fuqua, C. (1999). Broad-host-range expression vectors that carry the L-arabinose-inducible *Escherichia coli araBAD* promoter and the *araC* regulator. *Gene* **227**, 197–203.

Olivares, J., Álvarez-Ortega, C. & Martinez, J. L. (2014). Metabolic compensation of fitness costs associated with overexpression of the multidrug efflux pump MexEF-OprN in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **58**, 3904–3913.

Palmer, K. L., Mashburn, L. M., Singh, P. K. & Whiteley, M. (2005). Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. *J Bacteriol* **187**, 5267–5277.

Poole, K. (2011). *Pseudomonas aeruginosa*: resistance to the max. *Front Microbiol* **2**, 65.

Rainey, P. B., Buckling, A., Kassen, R. & Travisano, M. (2000). The emergence and maintenance of diversity: insights from experimental bacterial populations. *Trends Ecol Evol* **15**, 243–247.

Rakhimova, E., Munder, A., Wiehlmann, L., Bredenbruch, F. & Tümmler, B. (2008). Fitness of isogenic colony morphology variants of *Pseudomonas aeruginosa* in murine airway infection. *PLoS One* **3**, e1685.

Schuster, M. & Greenberg, E. P. (2006). A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. *Int J Med Microbiol* **296**, 73–81.

Skindersoe, M. E., Alhede, M., Phipps, R., Yang, L., Jensen, P. O., Rasmussen, T. B., Bjarnsholt, T., Tolker-Nielsen, T., Høiby, N. & Givskov, M. (2008). Effects of antibiotics on quorum sensing in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **52**, 3648–3663.

Stephenson, K. & Hoch, J. A. (2002). Two-component and phosphorelay signal-transduction systems as therapeutic targets. *Curr Opin Pharmacol* **2**, 507–512.

Taguchi, K., Fukutomi, H., Kuroda, A., Kato, J. & Ohtake, H. (1997). Genetic identification of chemotactic transducers for amino acids in *Pseudomonas aeruginosa*. *Microbiology* **143**, 3223–3229.

Tanimoto, K., Tomita, H., Fujimoto, S., Okuzumi, K. & Ike, Y. (2008). Fluoroquinolone enhances the mutation frequency for meropenem-selected carbapenem resistance in *Pseudomonas aeruginosa*, but use of the high-potency drug doripenem inhibits mutant formation. *Antimicrob Agents Chemother* **52**, 3795–3800.

Toder, D. S., Gambello, M. J. & Iglewski, B. H. (1991). *Pseudomonas aeruginosa* LasA: a second elastase under the transcriptional control of *lasR*. *Mol Microbiol* **5**, 2003–2010.

Torres-Barceló, C., Kojadinovic, M., Moxon, R. & MacLean, R. C. (2015). The SOS response increases bacterial fitness, but not evolvability, under a sublethal dose of antibiotic. *Proc Biol Sci* **282**, 20150885.

Wong, A. & Kassen, R. (2011). Parallel evolution and local differentiation in quinolone resistance in *Pseudomonas aeruginosa*. *Microbiology* **157**, 937–944.

Wong, A., Rodrigue, N. & Kassen, R. (2012). Genomics of adaptation during experimental evolution of the opportunistic pathogen *Pseudomonas aeruginosa*. *PLoS Genet* **8**, e1002928.

Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev* **61**, 533–616.

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