Lack of the major multifunctional catalase KatA in P.aeruginosa accelerates evolution of antibiotic resistance in ciprofloxacin-treated biofilms

Ahmed, Marwa N; Porse, Andreas; Abdelsamad, Ahmed; Sommer, Morten Otto Alexander; Høiby, Niels; Ciofu, Oana

Published in:
Antimicrobial Agents and Chemotherapy

Link to article, DOI:
10.1128/AAC.00766-19

Publication date:
2019

Document Version
Peer reviewed version

Lack of the major multifunctional catalase KatA in *P.aeruginosa* accelerates evolution of antibiotic resistance in ciprofloxacin-treated biofilms

Marwa N.Ahmed[1,2], Andreas Porse[3], Ahmed Abdelsamad[4,5], Morten Sommer[3], Niels Høiby[1,6] and Oana Ciofu[1*]

1. Costerton Biofilm Center, Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark.

2. Department of Microbiology, Faculty of Agriculture, Cairo University, Giza, Egypt.

3. Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Lyngby, Denmark.

4. Department of Genetics, Faculty of Agriculture, Cairo University, Giza, Egypt

5. Department of Biology, University of Copenhagen, Copenhagen, Denmark

6. Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark

Key words: *P. aeruginosa*, biofilm, catalase mutant, oxidative stress, antibiotic resistance

Corresponding author*:

Oana Ciofu. MD. PhD. Dr.med.

Costerton Biofilm Center.

University of Copenhagen.

Panum Inst.. 24.1

Blegdamsvej 3

2200 N

Copenhagen N

Denmark

ociofu@sund.ku.dk

45 29217367

Abstract

During chronic biofilm infections, *Pseudomonas aeruginosa* are exposed to increased oxidative stress as a result of the inflammatory response. As reactive oxygen species (ROS) are mutagenic, the evolution of resistance to ciprofloxacin (CIP) in biofilms under oxidative stress conditions was investigated. We experimentally evolved six replicate populations of *P.aeruginosa* lacking the major catalase KatA in colony biofilms and stationary-phase cultures for seven passages in the presence of sub-inhibitory levels (0.1 mg/L) of CIP or without CIP (eight replicate lineages for controls) under aerobic conditions.

In CIP-evolved biofilms, a larger CIP resistant subpopulation was isolated in *ΔkatA* compared to WT PAO1 population suggesting oxidative stress as a promoter of antibiotic resistance development.

A higher number of mutations identified by population sequencing were observed in evolved *ΔkatA* biofilm populations (CIP and control) compared to WT PAO1 evolved under same conditions. Genes involved in iron assimilation were found to be exclusively mutated in CIP-evolved *ΔkatA* biofilm populations, probably as a defense mechanism against ROS formation resulting from Fenton reactions. Furthermore, a hypermutable lineage due to *mutL* inactivation developed in one CIP-evolved *ΔkatA* biofilm lineage.

In CIP-evolved biofilms of both *ΔkatA* and WT PAO1, mutations in *nfxB,* the negative regulator of the MexCD-OprJ efflux pump were observed while in CIP-evolved planktonic cultures of both *ΔkatA* and WT PAO1, mutations in *mexR* and *nalD*, regulators of MexAB-OprM efflux pump, were repeatedly found.

In conclusion, these results emphasize the role of oxidative stress as an environmental factor that might increase the development of antibiotic resistance in *in vivo* biofilms.

Introduction

Antibiotic resistance is considered a major threat to modern medicine, challenging the treatment of common bacterial infections. Evolution of antibiotic resistance has primarily been studied in liquid cultures; however, in most natural environments and in some chronic infections bacteria are growing in biofilms.

Biofilm-grown *P. aeruginosa* experience heterogeneous physiological and metabolic conditions in a compartmentalized environment, which are not encountered in planktonic liquid cultures. These biofilm-specific conditions cause differences in the mutation supply and in the selective pressures acting on biofilm forming cells compared to cells growing in planktonic cultures, which might influence the evolution of antimicrobial resistance.

Distinct bacterial subpopulations have been described in biofilms with a metabolically active population at the biofilm surface and a metabolically inactive population in the deeper layers of the biofilm (1). In addition, a steep oxygen gradient, with decreasing levels from the surface to the deeper layers is observed in the colony- biofilm model (2). The described endogenous oxidative stress in biofilms (3,4) is most probably occurring in the superficial layers of the biofilm, where enough oxygen is present and cells are metabolically active. In microaerofilic or anaerobic conditions, as inside microcolonies, *P. aeruginosa* is able to respire on nitrogen oxides in the presence of nitrate and bacterial cells may be exposed to nitrosative stress.

Among antibiotics that promote the evolution of resistance, fluoroquinolones such as ciprofloxacin (CIP) are of particular concern because they directly interfere with DNA replication by binding gyrases and topoisomerase IV and hence may encourage replication errors that are the major source of mutations. Gyrases bound with fluoroquinolones molecules result in cross-linked protein-DNA complexes containing broken DNA that induces an SOS response which can contribute through

activation of error-prone DNA polymerases to elevated mutation rates(5). In addition, CIP treatment causes increased intracellular reactive oxygen species (ROS) levels in planktonic cultures (6,7). In biofilms, antibiotic-induced ROS production have been described during treatment with quinolones in *P. aeruginosa* (8) and *Proteus mirabilis* (9) and for aminoglycosides in *Burkholderia cepacia* complex (10).

It is widely accepted that patients with cystic fibrosis (CF) and chronic *P. aeruginosa* lung infection are exposed to increased oxidative stress mainly driven by chronic inflammation with polymorphonuclear leukocytes, as a source of ROS and a CF-related deficit in the anti-oxidants, such as glutathione, which further increases the inflammatory response, as we have previously shown in animal models (11). Thus, the *P. aeruginosa* biofilms in the CF lungs are exposed to an oxidative stress environment and our study aims at reproducing *in vitro* the oxidative stress by investigating the evolution of antibiotic resistance in *P. aeruginosa* lacking the major catalase KatA, an important anti-oxidant defence system.

Given that ROS can damage DNA, membranes, lipids and proteins, *P.aeruginosa* possess different antioxidant defenses to survive during aerobic growth and under antibiotic attack, one of them being the *katA*- encoded major catalase. *P. aeruginosa* has three differentially evolved catalase genes, *katA*, *katB*, and *katE* (*katC*). KatA is the major, constitutively expressed catalase, which is highly produced in all phases of growth but increased upon the stationary growth phase (12). KatA is important for the resistance of *P. aeruginosa* growing in planktonic and biofilm states when exposed to H_2O_2 at high concentrations(12). KatB is induced in both planktonic and biofilm cells in response to H_2O_2 exposure, but plays a relatively small role in biofilm resistance (12,13).

It has been suggested that the bactericidal effect of beta-lactams, fluoroquinolones and aminoglycosides have a ROS-dependent component (14), although the significance of this has been

challenged (15,16)(17). Biofilms formed by mutants lacking anti-oxidants systems, such as catalases (*katA* for *P. aeruginosa* and *kat B* for *B. cepacia*) showed increased sensitivity to antibiotics (8,10) and this is regarded as solid indication for a contribution of ROS to the bactericidal effect of antibiotics.

In addition, It has been shown in biofilms that anti-oxidant systems, such as catalases and superoxide dismutases, are up regulated by the activation of the stringent response (18,19). This suggests that inhibiting oxidative stress is an important strategy used by biofilm forming bacteria to increase antibiotic tolerance. Moreover, the analysis of gene expression of P. aeruginosa biofilms revealed that antixoxidant enyzmes including katA are downregulated during the biofilm growth in comparison to planktonic culture (2).

It has been shown that KatA catalase, besides its role in protection against ROS-dependent mutagenesis, it also plays a critical role in nitric oxide buffering produced under anaerobic respiration in the presence of nitrate (20). Recently, two different promoters of *katA* have been identified in *P. aeruginosa*, one coping with ROS produced under aerobic respiration and the other with reactive nitrogen species (RNS) produced under anaerobic respiration (21).

As the antibiotic-tolerant biofilm-grown *P. aeruginosa* are using both aerobic and microaerophilic respiration, the hypothesized production of mutagenic ROS and RNS in the different biofilm layers might lead to increased mutagenesis and faster development of antibiotic resistance in a Δ*katA* mutant.

To investigate the role of KatA in biofilm mutagenesis and development of resistance to antibiotics, we experimentally evolved a catalase mutant (*katA*) of *P. aeruginosa* (22) in colony-biofilm in aerobic and anaerobic conditions in the presence and absence of sub-inhibitory concentrations of

CIP. In addition, evolution in the presence and absence of antibiotic was conducted in stationary-phase aerobic planktonic cultures

In the present study, we show that the CIP resistant population was larger in *ΔkatA* than in WT PAO1 aerobic biofilms. Population sequence showed higher number of mutations in *ΔkatA* populations compared to WT PAO1 suggesting that lack of catalase Kat A might promote development of antibiotic resistance in *P. aeruginosa* aerobic biofilms. However, CIP-resistant mutants occurred also during anaerobic evolution of WT PAO1 biofilms suggesting that development of resistance to CIP can occur even in the absence of oxygen. In addition, we show that similarly to an experimental evolution study of wild-type *P.aeruginosa* PAO1(23), a larger CIP resistant population developed in *Δkat A* biofilm compared to planktonic cultures confirming the biofilm mode of growth as a promoter of low-level resistance development.

Materials and methods

Bacterial strains, media and antibiotics

P. aeruginosa ΔkatA strain (22) (planktonic MIC ciprofloxacin=0.094 mg/L) was used to test the development of antibiotic resistance during experimental evolution in the colony-biofilm model (24,23) and in planktonic batch cultures. Both biofilms and planktonic cultures were grown in Luria Bertani (LB) media and exposed to 0.1 mg/L CIP (Ciprofloxacin hydrochloride; Bayer; Germany). The minimal inhibitory concentration for a 48h stationary *ΔkatA* was 0.2 mg/L and the minimal biofilm inhibitory concentration was 0.5 mg/L. The spontaneous mutation rate to 0.5 mg/L CIP was 1 E-8 for PAO1 and 1.4E-8 for *ΔkatA*, as measured by fluctuation test.

Experimental evolution of colony-biofilm and planktonic cultures.

The experimental evolution of colony-biofilms and planktonic cultures were conducted as previously described (23). In short, a single colony of *P. aeruginosa ΔkatA* was used to inoculate LB for an overnight culture. Five µl of the diluted overnight culture containing approximately 10^6 cells (OD600 adjusted to 0.05 and diluted 1:10) was spot-inoculated on the top of polycarbonate membrane filters (Whatman® Nuclepore Track-Etched Membranes, 25 mm diameter, 0.2 µm pore size) and incubated at 37^0C to form 48 h colony-biofilms on LB plates. Membranes bearing 48 h colony-biofilms were transferred to fresh LB plates with either 0.1 mg/L CIP (1/5 minimal biofilm inhibitory concentrations) (CIP) or without CIP for 48h (CTRL) (passage 0 consisted of a 4 days old biofilm; 2 days on LB followed by 2 days on LB with CIP). Every 48h the CIP and CTRL membranes were transferred in 10 ml tubes with saline and biofilms were dispersed by vortex and sonication. After adjusting the OD600 to 0.005 the bacterial suspension of CTRL biofilms was used to start new biofilms on LB plates without antibiotics (CTRL). The bacterial suspensions of CIP biofilms were used to start new biofilms on plates with 0.1 mg/L CIP (CIP).

A total of seven exposures (from passage 0 (P0) to passage 6 (P6)) to CIP with 6 independent lineages were performed.

Two parallel lineages of WT PAO1 and *P. aeruginosa ΔkatA* were also evolved in anaerobic chamber (Whitley A85 anaerobic workstation) for 7 passages (as described). To allow bacterial growth by denitrification, the LB plates were supplemented with 1mM KNO3.

After each 48h, the CFU counts for each biofilm population were measured and the disrupted biofilm populations were kept in 20 % glycerol at -80^0C until further analysis.

Planktonic experiments were conducted in aerobic conditions with the same experimental design as implemented with biofilm cultures. Briefly, 5 µl of an overnight culture of single colony of *P. aeruginosa ΔkatA* was used to inoculate 10 ml LB media and incubated for 48h with shaking on an

orbital shaker (180 rpm) at 37°C. From the 48 h culture two flasks were inoculated: one with 0.1 mg/L CIP (CIP) and one in LB (CTRL). From CIP stationary cultures, every 48h new planktonic cultures were established in flasks with CIP (CIP). From CTRL stationary cultures, every 48 h new planktonic cultures were established in flask with LB. This was repeated for 6 passages (P1 to P6).

Population analysis

Bacterial populations (100 µl of different dilutions) obtained after sonication and vortex of membranes containing colony-biofilms or of planktonic cultures from each passage and treatment group were plated on LB plates to estimate the size of the bacterial population and on LB plates containing 0.5, 1 and 2 mg/L CIP, to estimate the size of the resistant subpopulations (growing on CIP concentrations higher than the MIC of the strain *P. aeruginosa ΔkatA*). The size of the resistant population was expressed as % of the total bacterial population and calculated by dividing the CFU/ml on CIP by the CFU/ml on LB and multiplied by 100.

Three colonies were selected from the plates with the highest CIP concentrations allowing growth, passed twice in antibiotic free media and the minimum inhibitory concentrations (MIC) of CIP were determined by performing E-test (Biomerieux) according to the manufacturer.

Mutation frequencies and rates determination

The mutation frequencies of the evolved populations after each passage were investigated on LB plates containing rifampicin (300 mg/L), as previously described (25). A population was considered hypermutable when the mutation frequency was 20-fold higher than the mutation frequency of the reference strain PAO1 ($\geq 3 \times 10^{-7}$). The mutation rates were determined by a fluctuation test as previously described (26) and calculations were performed using bz-rates web-tool (http://www.lcqb.upmc.fr/bzrates).

Genome sequencing of aerobically evolved bacterial populations

The genome sequence was performed as previously described (23). In short, The CIP evolved populations (after 7 passages in the presence of 0.1 mg/L CIP) from four *P. aeruginosa ΔkatA* biofilm lineages and three planktonic lineages were grown on LB plates containing 1 mg/L CIP for 48 h to enrich for the resistant subpopulation. The CTRL evolved biofilm and planktonic populations (four lineages/condition) were grown on LB plates. All colonies for each population were collected in 3 ml saline (0.9% NaCl) for genomic DNA extraction using Gentra puregene yeast/bacteria DNA purification kit**.** The DNA was prepared for sequencing using the Illumina TruSeq DNA Nano kit and sequenced on an Illumina MiSeq yielding a coverage of approximately 5 million reads per sample. Sequencing reads were mapped to the reference genome of *P. aeruginosa* PAO1 (GenBank accession. NC_002516) and single and multiple nucleotide variants (SNVs and MNVs) were called using CLC genomic workbench (Qiagen). Mutations present in the evolved Δ*katA* CTRL populations were filtered out from the genome of the CIP evolved populations (biofilm and planktonic). R (version 3.2.5) was used for further statistical analysis of the mutations detected in each population and all mutations occurring in > 10% of the reads were included in the analysis.

The Pseudomonas Genome Database was used for gene function analysis. dN/dS, the ratio of the rate of nonsynonymous substitutions (dN) to the rate of the synonymous substitutions (dS), was calculated as a measure of the selection pressure acting on the protein-coding genome, as previously described, assuming that 25% of all single-nucleotide polymorphisms (SNP) result in synonymous changes (27). dN/dS is expected to be >1 if natural selection promotes changes in protein sequences and <1 if natural selection suppresses changes.

Sequencing of *nfxB* was performed on CIP resistant colonies selected from anaerobic experiments, as described previously (28).

Statistical analysis

Graphs and statistical analysis were done using GraphPad Prism 7 software and R (version 3.2.5). We conducted D´Agostino Pearson to check for normal distribution and Student t-test was used for comparisons among populations (comparing CIP populations to CTRL populations and CIP biofilm populations to CIP planktonic populations) and to compare the level of resistance at different exposure time points (passages). The differences were considered significant when the p-value was ≤ 0.05. Error bars in all graphs represent the standard error of the mean.

Results

Development of a larger CIP resistant subpopulation in aerobically evolved *Δ*katA *P. aeruginosa* biofilms compared to planktonic cultures

The development of CIP resistant subpopulations during the experimental evolution in the presence or absence of 0.1 mg/L CIP was investigated by plating the evolved biofilm and planktonic *ΔkatA* populations (six and three replicates, respectively), at different passages on LB plates containing 0.5, 1 and 2 mg/L CIP.

ΔkatA CIP-biofilm evolved populations showed a significantly higher resistant subpopulation in comparison to *ΔkatA* CIP-planktonically evolved population on 0.5 and 1 mg/L CIP (p=0.002 and 0.003 respectively, t-test) (Figure 1A). The dynamics of resistance development during passages showed that in comparison to *ΔkatA* CIP-planktonic populations, *ΔkatA* CIP-biofilm populations developed a significantly higher resistant subpopulation (on 1 mg/L CIP) at P1 (p=0.04), P2, P3

(p=0.001), P4 (p=0.003), P5 and P6 (p=0.002 and 0.003 respectively, t-test) (Figure1B). In *ΔkatA* CIP-biofilm, there was a significant increase in the size of the resistant subpopulation developed from P0 to P6 on 0.5 and 1 mg/L CIP (p=0.0001 and 0.001, t-test respectively).

Compared to the evolved CTRL populations (eight replicates), CIP-evolved biofilm *ΔkatA* populations developed a significantly larger resistant subpopulation on 0.5, 1 ($p<0.0001$) and 2 mg/L CIP (p=0.01) (though for planktonic *ΔkatA* only on 0.5 mg/L) (Figure 1A).

The ancestor *ΔkatA* strain, which was used for initiating the evolution experiments, was tested for the preexistence of resistant variants but no growth on 0.5, 1 and 2 mg/L CIP was observed; indicating that the CIP resistant colonies were not present before the initial CIP treatment but developed during the experimental evolution study.

Compared to WT PAO1(23), a higher CIP resistant subpopulation was observed in Δ*katA* at passage 3 (p= 0.01), passage 4 (p=0.02), passage 5 (p=0.02) and passage 6 (p=0.03)(figure S1).

Evolution of CIP resistance can occur in anaerobic evolved biofilms

In order to investigate the role of oxygen for the CIP resistant development, we evolved PAO1 and *ΔkatA* colony biofilms in anaerobic chamber in the presence and absence of 0.1 mg/L CIP on LB plates supplemented with 1 mM KNO3, a concentration resembling the concentrations in CF sputum(29). The size of the biofilm population was lower in anaerobic compared to aerobic conditions, probably due to the consumption of KNO3 during growth (table S1). We observed occurrence of CIP resistant colonies (MIC = 1 mg/L) during anaerobic evolution with CIP in PAO1 (one of the two lineages) but not in *ΔkatA* biofilm populations (not shown). The population analysis of the anaerobically evolved biofilms showed that the percentage of CIP resistant subpopulation on 1 mg/L CIP in one of the WT PAO1 lineages was 0, 0, 0.05, 0.26, 0.65, 0.33 from passage 1 to passage 6, respectively.

This suggests that mutagenic mechanisms independent of ROS and RNS formation, such as SOS response might play a role in mutagenesis towards ciprofloxacin in anaerobic conditions, as the ones described in the CF mucus (30)(31).

Hypermutators evolve in aerobic *ΔkatA* biofilm populations and attain high MICs

The mutation frequencies were measured in CIP biofilm and planktonic lineages throughout the passages.

In one out of six independent replicate lineages of *ΔkatA* biofilm, we observed increased mutation frequencies corresponding to hypermutable phenotypes in the end of the experiment (P6) (> 3 x 10^{-7}). Analysis of the mutation frequencies at the different passages in this lineage showed that already after the first 48h exposure to ciprofloxacin (passage 0), the bacterial population had a hypermutable phenotype and this was maintained during passages until the end of the evolution experiment. This was not observed in the other biofilm or planktonic lineages of *ΔkatA* or WT PAO1.

The MIC for ciprofloxacin of the resistant isolates collected at the first and last passage of the experimental evolution of *ΔkatA* showed that the isolates with the highest MICs were selected from the lineage with the hypermutable phenotype (Figure 2).

Genetic evolution of biofilm and planktonic *P. aeruginosa ΔkatA* under ciprofloxacin exposure in aerobic conditions

To get insight into the underlying genetic changes contributing to the accelerated development of antibiotic resistance in biofilms in the absence of catalase, we sequenced the *ΔkatA* populations at the endpoint of the experimental evolution. A complete list of mutations in the different conditions

is presented in table S3. The identified genetic changes, defined as minimum variant frequency of 10%, were also compared to those observed in WT PAO1(23).

The highest number of mutations was observed in CIP-evolved biofilm populations of *ΔkatA* (table 1).

According to gene functional categories, CIP treated *ΔkatA* biofilm and planktonic populations have a significantly higher number of mutations compared to CTRL populations in the category "secreted factors (toxins, enzymes)" p=0.044 and 0.021, respectively, t-test) (figure S2).

CIP-evolved *ΔkatA* populations contain the largest number of mutations encountered with high frequency compared to CTRL *ΔkatA* populations but also compared to the WT PAO1 evolved in the same conditions (Figure 3).

Large indels, inversions and duplications were present only in the CIP-evolved populations of *ΔkatA* and were not present in the populations of WT PAO1 evolved in the absence or presence of CIP. The ratio between nonsynonymous and synonymous mutations dN/dS >1 in *ΔkatA* evolved population suggest a positive selection for the described mutations which was not observed in the evolved WT PAO1 populations (dN/dS<1). Analysis of the mutational spectrum (table S2) revealed that the most frequent mutation in *ΔkatA* populations was transversion A:T-C:G. Mutations which are repeatedly observed after independent exposures to a condition provide strong evidence of adaptive evolution (Figure 3 and Table S3). This was the case for *nfxB,* a negative regulator of MexCD-OprJ (32), (33) which was frequently and repeatedly mutated in *ΔkatA* CIP biofilm evolved populations as well as *pil* genes encoding for type IV pili, compared to CTRL biofilms. Mutations in different other *mex* genes (*mexR*, *mexD*, *mexF, mexT*) or RND efflux pumps were also observed in several lineages with lower frequencies. A large replacement of a 39 nucleotides with 31 nucleotides was observed in *mexT* in one of the CIP biofilm lineages (figureS3).

The genetic background of the observed hypermutable phenotype in one of the *ΔkatA* CIP biofilm lineages was shown to be an insertion of 27 nucleotides in *mutL* gene (codes for DNA mismatch repair protein) (Figure S3) and as expected, the number of transitions in this lineage was higher compared to the non-hypermutable lineages.

Mutation C1397T in *gyrB* coding for DNA gyrase subunit B, leading to an amino acid change S466F was observed in the hypermutable lineages of the CIP-evolved *ΔkatA* biofilms correlating to the high CIP MIC of the resistant isolates from this lineage.

Mutations in either *mexR* or *nalD*, which are regulators of the MexAB-OprM efflux pump, were frequent in CIP-exposed planktonic *ΔkatA* lineages. Two different large indels were detected in *mexR* in two lineages. (Figure S3)

In CIP-evolved biofilm *ΔkatA* populations, genes related to iron acquisition and transport were mutated in several lineages such as genes encoding for the siderophores pyoverdin (*pvd*) and pyochelin (*pch*) ; iron transporters (iron transport system permease HitB, as well as in pyrroquinolone quinone biosynthesis genes (*pqq*) which are encoding a redox-sensing protein (Table S2). In evolved biofilm and planktonic *ΔkatA* populations, mutations in TonB-dependent receptors were found. TonB proteins are essential components in iron-siderophore uptake in bacteria (34) and mutations in these genes were not observed in the evolved populations of WT PAO1.

In *ΔkatA* CTRL biofilm compared to planktonic CTRL populations (evolved without CIP exposure), *minC* which acts an inhibitor for cell division by inhibiting Z-ring assembly was observed to be mutated in three different lineages.

Genetic basis of resistance in CIP-resistant colonies isolated from anaerobically-evolved biofilms

The genetic basis of the six CIP resistant colonies (MIC= 1 mg/L) isolated from the anaerobically evolved PAO1 biofilm was investigated by sequencing the *nfxB* gene and a CTTCT deletion at position 162 leading to frameshift was found in all isolates.

Discussion

Evolution of *P. aeruginosa* biofilms under exposure to sub-inhibitory concentrations of CIP resulted in a larger CIP-resistant subpopulation in Δ*katA* compared to WT PAO1 biofilm populations indicating that the lack of KatA catalase accelerates the evolution of antibiotic resistance in aerobic conditions. Given the role of KatA in the defense against oxidative and nitrosative stress (20), we propose that these stresses play a role in the biofilm-related increased mutagenesis in biofilms. Respiration by denitrification of biofilm-embedded *P. aeruginosa* exposed to low-oxygen tension could be supported by the nitrate present in LB media (approx. 20µM NO^{3-})(29). In addition, it has been shown by transcriptomic (26) and proteomic studies (35) that under CIP treatment *P. aeruginosa* switch to anaerobic respiration.

In support of the oxidative stress mechanism acting in Δ*katA* but not in WT PAO1 in aerobically-evolved populations are the repeatedly observed mutations in iron assimilation genes, such as mutations in genes encoding for TonB-dependent receptors which are essential for iron-siderophore uptake in *P. aeruginosa*, mutations in genes encoding the siderophores pyoverdine and pyochelin and in genes encoding various iron binding and redox-proteins (PQQ). Mutations in these genes might represent a protective mechanism against iron uptake probably as a defense mechanism against increased intracellular production of ROS by the Fenton reaction (36) as increased ROS production was measured in Δ*katA* biofilms compared to WT PAO1 (8). Interestingly, it has been reported that genes encoding TonB-dependent receptors were preferentially deleted in CF *P. aeruginosa* isolates during adaptation in the CF lungs (34).

Although important for occurrence of mutagenic resistance in biofilms, oxidative stress is not the only mechanism, as we have observed CIP resistant mutants also when PAO1 biofilms were evolved in anaerobic conditions. It is unclear at the present time which mutagenesis mechanisms occur in anaerobic conditions but one might consider the SOS stress response as a possible mechanism.

However, this accelerated evolution of the antibiotic resistance in biofilms under exposure to sub-inhibitory levels of ciprofloxacin in Δ*katA* compared to WT PAO1 aerobic biofilms was not observed during the evolution in planktonic cultures (Figure S1). Although we do not have an explanation for this, we can speculate that this might be due to accumulation of deleterious mutations during evolution in the planktonic Δ*katA* population compared to WT PAO1.

Compared to planktonic populations, larger CIP resistant subpopulations were observed in biofilms of both *ΔkatA* and PAO1 (23) confirming that biofilm mode of growth promotes development of mutational resistance in experimental evolution studies (Figure S1).

The genomic analysis of the aerobically evolved populations showed that the highest number of mutations were observed in the CIP-evolved Δ*katA* biofilm populations and this is in agreement with the hypothesis that ciprofloxacin exposure induces mutations either via SOS response or through increased ROS levels in the catalase deficient mutants compared to WT PAO1 (37,3). Analysis of the mutational spectra revealed that A:T-C:G transversion was the most frequent mutation type in *ΔkatA* populations and this has been previously shown to be related to unrepaired oxidized guanine in the nucleotide pool (38). The genes belonging to the functional category of secreted factors (toxins and enzymes) were also mutated in significantly higher number in CIP evolved *ΔkatA* biofilm and planktonic populations compared to CTRL biofilms and planktonic

372 populations and this is in accordance to our previous observations of loss of virulence factors during
373 evolution in the presence of CIP (26).

374 During the experimental evolution of Δ*katA* biofilms, *mutL* mutants with mutator phenotype and
375 high MICs of ciprofloxacin evolved corresponding with our previously study on the association
376 between bacterial hypermutability and chronic inflammation in chronically infected CF patients,
377 which is a source of chronic oxidative stress (25). The high MIC levels of ciprofloxacin in the
378 mutator lineage were found to be related to mutations in the CIP target gene, *gyrB*. Interestingly, a
379 mutation at the same site C1397T causing S466F has been previously found in planktonic
380 experimental evolution of a hypermutator strain due to impaired repair of oxidative lesions
381 (PAOMY-Mgm)(39) (40) and both amino acid changes have been described in levofloxacin non-
382 susceptible clinical *P. aeruginosa* isolates(41).

383 The genes found to be mutated in biofilms or planktonic evolution of both PAO1 and Δ*katA,*
384 strongly suggest parallel and distinct evolutionary trajectories in the different mode of growth of *P.*
385 *aeruginosa.* Confirming previous results, the experimental evolution of biofilm and planktonic
386 Δ*katA P. aeruginosa* populations revealed that low-level CIP resistance evolves readily in biofilms
387 (23). For example, in CIP evolved Δ*katA* and WT PAO1 biofilms, mutations in the negative
388 regulator, *nfxB*, of the MexCD-OprJ efflux pump were frequently found. *nfxB* mutations have
389 previously been identified in *P.aeruginosa* isolates from CF patients (33). Mutations in *pil* genes
390 encoding for Type IV pili were mutated frequently in CIP evolved biofilm populations of both
391 Δ*katA* and WT PAO1. While in CIP evolved planktonic populations of both Δ*katA* and WT PAO1,
392 mutations in the negative regulator *mexR* and *nalD* of MexAB-OprM were found. These biofilm-
393 related low-level resistant subpopulations may accumulate further mutations which can further
394 increase the MIC of the population. This dynamic of resistance development under exposure to sub-
395 inhibitory levels of ciprofloxacin emphasizes the importance of treatment of the infections caused

by biofilm-growing *P.aeruginosa* with doses ensuring high antibiotic concentration at the site of infection that can eliminate the first-step mutants or by combination therapy.

Mutations in the target genes of ciprofloxacin, *gyrA* and *B* were found only in the CIP-evolved planktonic populations of WT PAO1(23)but not of non hypermutable Δ*katA* lineages, which phenotypically correlated to high MIC levels in planktonic WT PAO1 but not Δ*katA* populations.

In conclusion, this study of the experimental evolution of Δ*katA* in biofilm and planktonic growth is complementing earlier findings of the evolutionary study with PAO1, emphasizing the role of environmental stresses such as oxidative, nitrosative stress and SOS responses for the mutational landscape and the development of antibiotic resistance, which might play an important role in vivo during chronic infections(42). A pitfall of all these correlative analysis between WGS and phenotypic susceptibility is that it fails to capture the contribution of gene expression, an important contributor to tolerance and resistance to antibiotics. Complementary analysis such as transcriptome sequencing (RNA-seq) is the next step for correlating the susceptibility phenotype with genetic content.

Acknowledgements

A.P. and M.O.A.S. acknowledge the Novo Nordisk Foundation under NFF grant number: NNF10CC1016517, the European Union H2020 (ERC-2014-STG) under Grant Agreement 638902, LimitMDR and the Danish Council for Independent Research, Sapere Aude Program DFF 4004-00213.

The financial support of M.N.A. from the Egyptian Ministry of Higher Education is acknowledged.

The technical assistance of Tina Wassermann and Janna Baker are highly appreciated.

Reference List

1. **Stewart PS.** 2015. Antimicrobial Tolerance in Biofilms. *Microbiol.Spectr.***3**:

2. **Borriello G, Werner E, Roe F, Kim AM, Ehrlich GD, and Stewart PS.** 2004. Oxygen limitation contributes to antibiotic tolerance of Pseudomonas aeruginosa in biofilms. *Antimicrob Agents Chemother.***48**: 2659-2664.

3. **Boles BR and Singh PK.** 2008. Endogenous oxidative stress produces diversity and adaptability in biofilm communities. *Proc Natl Acad Sci U S A.***105**: 12503-12508.

4. **Driffield K, Miller K, Bostock JM, O'Neill AJ, and Chopra I.** 2008. Increased mutability of Pseudomonas aeruginosa in biofilms. *J Antimicrob Chemother.***61**: 1053-1056.

5. **Blazquez J, Couce A, Rodriguez-Beltran J, and Rodriguez-Rojas A.** 2012. Antimicrobials as promoters of genetic variation. *Curr.Opin.Microbiol.***15**: 561-569.

6. **Dwyer DJ, Kohanski MA, and Collins JJ.** 2009. Role of reactive oxygen species in antibiotic action and resistance. *Curr.Opin.Microbiol.***12**: 482-489.

7. **Gutierrez A, Stokes JM, and Matic I.** 2018. Our Evolving Understanding of the Mechanism of Quinolones. *Antibiotics.(Basel).***7**:

8. **Jensen PO, Briales A, Brochmann RP, Wang H, Kragh KN, Kolpen M, Hempel C, Bjarnsholt T, Hoiby N, and Ciofu O.** 2014. Formation of hydroxyl radicals contributes to the bactericidal activity of ciprofloxacin against Pseudomonas aeruginosa biofilms. *Pathog.Dis.***70**: 440-443.

9. **Aiassa V, Barnes AI, and Albesa I.** 2010. Resistance to ciprofloxacin by enhancement of antioxidant defenses in biofilm and planktonic Proteus mirabilis. *Biochem.Biophys.Res.Commun.***393**: 84-88.

10. **Van AH, Sass A, Bazzini S, De RK, Udine C, Messiaen T, Riccardi G, Boon N, Nelis HJ, Mahenthiralingam E, and Coenye T.** 2013. Biofilm-grown Burkholderia cepacia complex cells survive antibiotic treatment by avoiding production of reactive oxygen species. *PLoS.One.***8**: e58943

11. **Jensen PO, Lykkesfeldt J, Bjarnsholt T, Hougen HP, Hoiby N, and Ciofu O.** 2012. Poor antioxidant status exacerbates oxidative stress and inflammatory response to Pseudomonas aeruginosa lung infection in guinea pigs. *Basic Clin.Pharmacol.Toxicol.***110**: 353-358.

12. **Elkins JG, Hassett DJ, Stewart PS, Schweizer HP, and McDermott TR.** 1999. Protective role of catalase in Pseudomonas aeruginosa biofilm resistance to hydrogen peroxide. *Appl.Environ.Microbiol.***65**: 4594-4600.

13. **Stewart PS, Roe F, Rayner J, Elkins JG, Lewandowski Z, Ochsner UA, and Hassett DJ.** 2000. Effect of catalase on hydrogen peroxide penetration into Pseudomonas aeruginosa biofilms. *Appl Environ Microbiol.***66**: 836-838.

14. **Kohanski MA, Dwyer DJ, and Collins JJ.** 2010. How antibiotics kill bacteria: from targets to networks. *Nat.Rev.Microbiol.***8**: 423-435.

15. **Liu Y and Imlay JA.** 2013. Cell death from antibiotics without the involvement of reactive oxygen species. *Science*.**339**: 1210-1213.

16. **Hassett DJ and Imlay JA.** 2007. Bactericidal antibiotics and oxidative stress: a radical proposal. *ACS Chem.Biol.***2**: 708-710.

17. **Keren I, Wu Y, Inocencio J, Mulcahy LR, and Lewis K.** 2013. Killing by bactericidal antibiotics does not depend on reactive oxygen species. *Science*.**339**: 1213-1216.

18. **Khakimova M, Ahlgren HG, Harrison JJ, English AM, and Nguyen D.** 2013. The stringent response controls catalases in Pseudomonas aeruginosa and is required for hydrogen peroxide and antibiotic tolerance. *J.Bacteriol.***195**: 2011-2020.

19. **Martins D, McKay G, Sampathkumar G, Khakimova M, English AM, and Nguyen D.** 2018. Superoxide dismutase activity confers (p)ppGpp-mediated antibiotic tolerance to stationary-phase Pseudomonas aeruginosa. *Proc.Natl.Acad.Sci.U.S.A.***115**: 9797-9802.

20. **Su S, Panmanee W, Wilson JJ, Mahtani HK, Li Q, Vanderwielen BD, Makris TM, Rogers M, McDaniel C, Lipscomb JD, Irvin RT, Schurr MJ, Lancaster JR, Jr., Kovall RA, and Hassett DJ.** 2014. Catalase (KatA) plays a role in protection against anaerobic nitric oxide in Pseudomonas aeruginosa. *PLoS.One.***9**: e91813

21. **Chung IY, Kim BO, Jang HJ, and Cho YH.** 2016. Dual promoters of the major catalase (KatA) govern distinct survival strategies of Pseudomonas aeruginosa. *Sci.Rep.***6**: 31185

22. **Hassett DJ, Ma JF, Elkins JG, McDermott TR, Ochsner UA, West SE, Huang CT, Fredericks J, Burnett S, Stewart PS, McFeters G, Passador L, and Iglewski BH.** 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.

23. **Ahmed MN, Porse A, Sommer MOA, Hoiby N, and Ciofu O.** 2018. Evolution of Antibiotic Resistance in Biofilm and Planktonic Pseudomonas aeruginosa Populations Exposed to Subinhibitory Levels of Ciprofloxacin. *Antimicrob.Agents Chemother.***62**:

24. **Walters MC, III, Roe F, Bugnicourt A, Franklin MJ, and Stewart PS.** 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of Pseudomonas aeruginosa biofilms to ciprofloxacin and tobramycin. *Antimicrob Agents Chemother*.**47**: 317-323.

25. **Ciofu O, Riis B, Pressler T, Poulsen HE, and Hoiby N.** 2005. Occurrence of hypermutable Pseudomonas aeruginosa in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. *Antimicrob Agents Chemother*.**49**: 2276-2282.

26. **Wassermann T, Meinike JK, Ivanyshyn K, Bjarnsholt T, Khademi SM, Jelsbak L, Hoiby N, and Ciofu O.** 2016. The phenotypic evolution of Pseudomonas aeruginosa populations changes in the presence of subinhibitory concentrations of ciprofloxacin. *Microbiology*.**162**: 865-875.

27. **Yang L, Jelsbak L, Marvig RL, Damkiaer S, Workman CT, Rau MH, Hansen SK, Folkesson A, Johansen HK, Ciofu O, Hoiby N, Sommer MO, and Molin S.** 2011. Evolutionary dynamics of bacteria in a human host environment. *Proc.Natl.Acad.Sci.U.S.A.***108**: 7481-7486.

Antimicrobial Agents and Chemotherapy

28. **Zaborskyte G, Andersen JB, Kragh KN, and Ciofu O.** 2016. Real-time monitoring of nfxB mutant occurrence and dynamics in P. aeruginosa biofilm exposed to sub-inhibitory concentrations of ciprofloxacin. *Antimicrob.Agents Chemother.*

29. **Line L, Alhede M, Kolpen M, Kuhl M, Ciofu O, Bjarnsholt T, Moser C, Toyofuku M, Nomura N, Hoiby N, and Jensen PO.** 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

30. **Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, Birrer P, Bellon G, Berger J, Weiss T, Botzenhart K, Yankaskas JR, Randell S, Boucher RC, and Doring G.** 2002. Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. *J Clin Invest*.**109**: 317-325.

31. **Kolpen M, Hansen CR, Bjarnsholt T, Moser C, Christensen LD, van GM, Ciofu O, Mandsberg L, Kharazmi A, Doring G, Givskov M, Hoiby N, and Jensen PO.** 2010. Polymorphonuclear leucocytes consume oxygen in sputum from chronic Pseudomonas aeruginosa pneumonia in cystic fibrosis. *Thorax*.**65**: 57-62.

32. **Poole K.** 2007. Efflux pumps as antimicrobial resistance mechanisms. *Ann.Med.***39**: 162-176.

33. **Imamovic L, Ellabaan MMH, Dantas Machado AM, Citterio L, Wulff T, Molin S, Krogh JH, and Sommer MOA.** 2018. Drug-Driven Phenotypic Convergence Supports Rational Treatment Strategies of Chronic Infections. *Cell*.**172**: 121-134.

34. **Dingemans J, Ye L, Hildebrand F, Tontodonati F, Craggs M, Bilocq F, De VD, Crabbe A, Van HR, Malfroot A, and Cornelis P.** 2014. The deletion of TonB-dependent receptor genes is part of the genome reduction process that occurs during adaptation of Pseudomonas aeruginosa to the cystic fibrosis lung. *Pathog.Dis.***71**: 26-38.

35. **Peng J, Cao J, Ng FM, and Hill J.** 2017. Pseudomonas aeruginosa develops Ciprofloxacin resistance from low to high level with distinctive proteome changes. *J.Proteomics.***152**: 75-87.

36. **Touati D.** 2000. Iron and oxidative stress in bacteria. *Arch.Biochem.Biophys.***373**: 1-6.

37. **Blazquez J, Rodriguez-Beltran J, and Matic I.** 2018. Antibiotic-Induced Genetic Variation: How It Arises and How It Can Be Prevented. *Annu.Rev.Microbiol.***72**: 209-230.

38. **Sanders LH, Sudhakaran J, and Sutton MD.** 2009. The GO system prevents ROS-induced mutagenesis and killing in Pseudomonas aeruginosa. *FEMS Microbiol.Lett.***294**: 89-96.

39. **Mandsberg LF, Macia MD, Bergmann KR, Christiansen LE, Alhede M, Kirkby N, Hoiby N, Oliver A, and Ciofu O.** 2011. Development of antibiotic resistance and up-regulation of the antimutator gene pfpI in mutator Pseudomonas aeruginosa due to inactivation of two DNA oxidative repair genes (mutY, mutM). *FEMS Microbiol Lett*.**324**: 28-37.

40. **Jørgensen KM, Wassermann T, Jensen PO, Hengzuang W, Molin S, Hoiby N, and 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.

41. **Kos VN, Deraspe M, McLaughlin RE, Whiteaker JD, Roy PH, Alm RA, Corbeil J, and Gardner H.** 2015. The resistome of Pseudomonas aeruginosa in relationship to phenotypic susceptibility. *Antimicrob.Agents Chemother.***59**: 427-436.

42. **Crabbe A, Jensen PO, Bjarnsholt T, and Coenye T.** 2019. Antimicrobial Tolerance and Metabolic Adaptations in Microbial Biofilms. *Trends Microbiol.*

	Insertions /deletions	Transitions	Transversions	Large indels	Inversions Duplications	Total number of mutations	dN/dS
PAO1 *							
CTRL biofilm	24 (28%)	13 (15%)	48 (56%)			85	0.35
CTRL planktonic	24 (26%)	22 (23%)	48 (51%)			94	0.25
CIP biofilm	60 (37%)	27 (16%)	77 (47%)			164	0.44
CIP planktonic	29 (22%)	44 (34%)	57 (44%)			130	0.45
ΔkatA							
CTRL biofilm	147 (22%)	58 (9%)	452 (69%)		1	658	1.36
CTRL planktonic	141 (24%)	70 (12%)	362 (63%)		5	578	1.24
CIP biofilm	301 (28%)	206 (19%)	557 (52%)	2	9	1075	0.90
CIP planktonic	150 (22%)	65 (10%)	469 (69%)	6	7	697	2.59

538 Table 1.

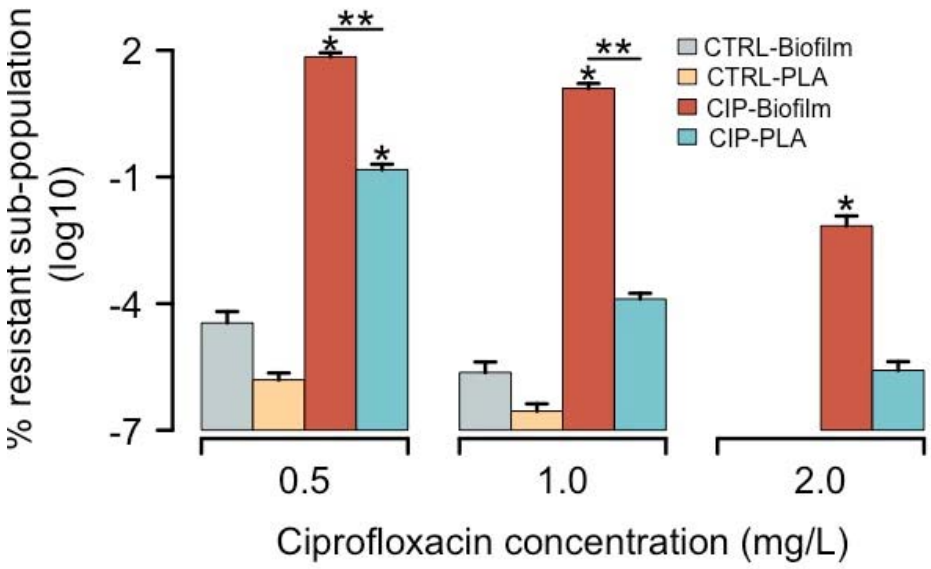
539

540 Table 1. The types of mutations (number and % of total number of mutations in the populations) in
541 WT PAO1 and *ΔkatA P. aeruginosa* evolved populations.The ratio between the
542 nonsynonymous/synonymous mutations (dN/dS) is presented. * the number and types of mutations
543 in PAO1 evolved populations has been re-analyzed including non-synonymous mutations in
544 hypothethical proteins (not included in (23)).

545

546

547 Figure 1A.

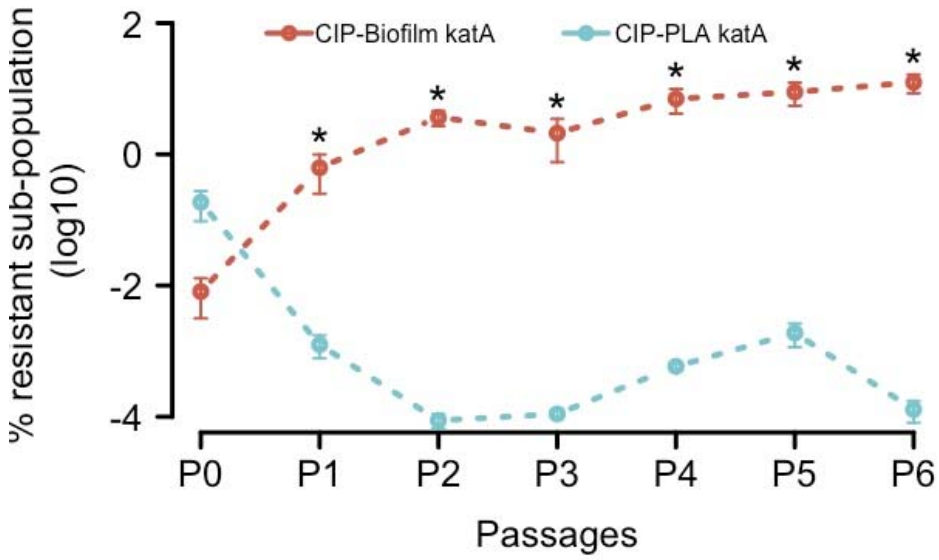


548

549 Figure 1A. The size of *P. aeruginosa Δkat* (KatA) biofilm(biofilm)and planktonic (PLA)
550 populations recovered from 0.5, 1 and 2 mg/L CIP after evolution in the presence of CIP (0.1 mg/l)
551 or in the absence of antibiotic (CTRL). The values represent the mean (SEM) of the replicates for
552 each growth condition. * significantly larger population compared to control populations, **
553 significantly larger population in biofilm compared to planktonic population.

554

555 Figure 1B.

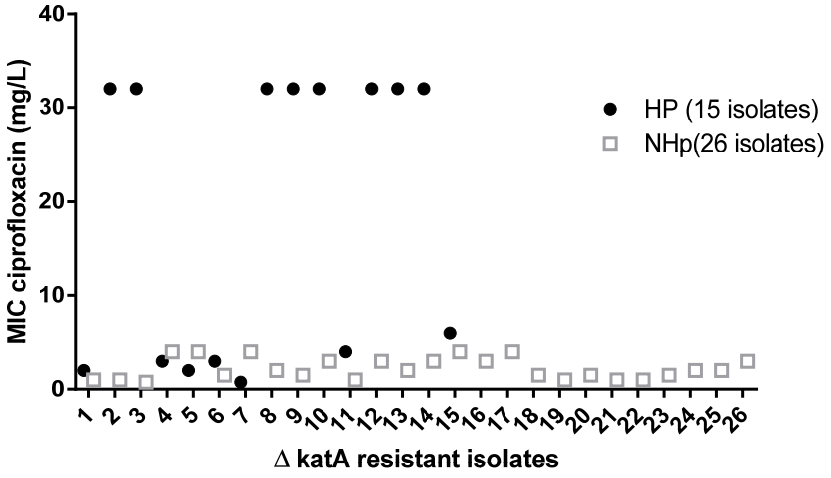


556

557 Figure 1B. The development of CIP resistance in *ΔkatA* biofilm (biofilm) and planktonic (PLA)
558 populations recovered from 1 mg/L CIP during passages (P0 to P6). Under exposure to
559 ciprofloxacin, *ΔkatA* CIP-biofilm populations developed a significantly higher resistant
560 subpopulation compared to planktonic populations at P1, P2, P3, P4, P5 and P6 (p=0.04, 0.001.
561 0.001, 0.003, 0.002 and 0.003 respectively). The values represent the mean (SEM) for replicates for
562 each growth condition.

563

564 Figure 2.



565

566 Figure 2. The MIC of ciprofloxacin (mg/L) of resistant colonies isolated from CIP plates of the
567 *ΔkatA P. aeruginosa* population analysis from the hypermutable lineage (HP) and the
568 nonhypermutable (NHp) lineages.

569

570

571

572

573

574

575

576

577

578

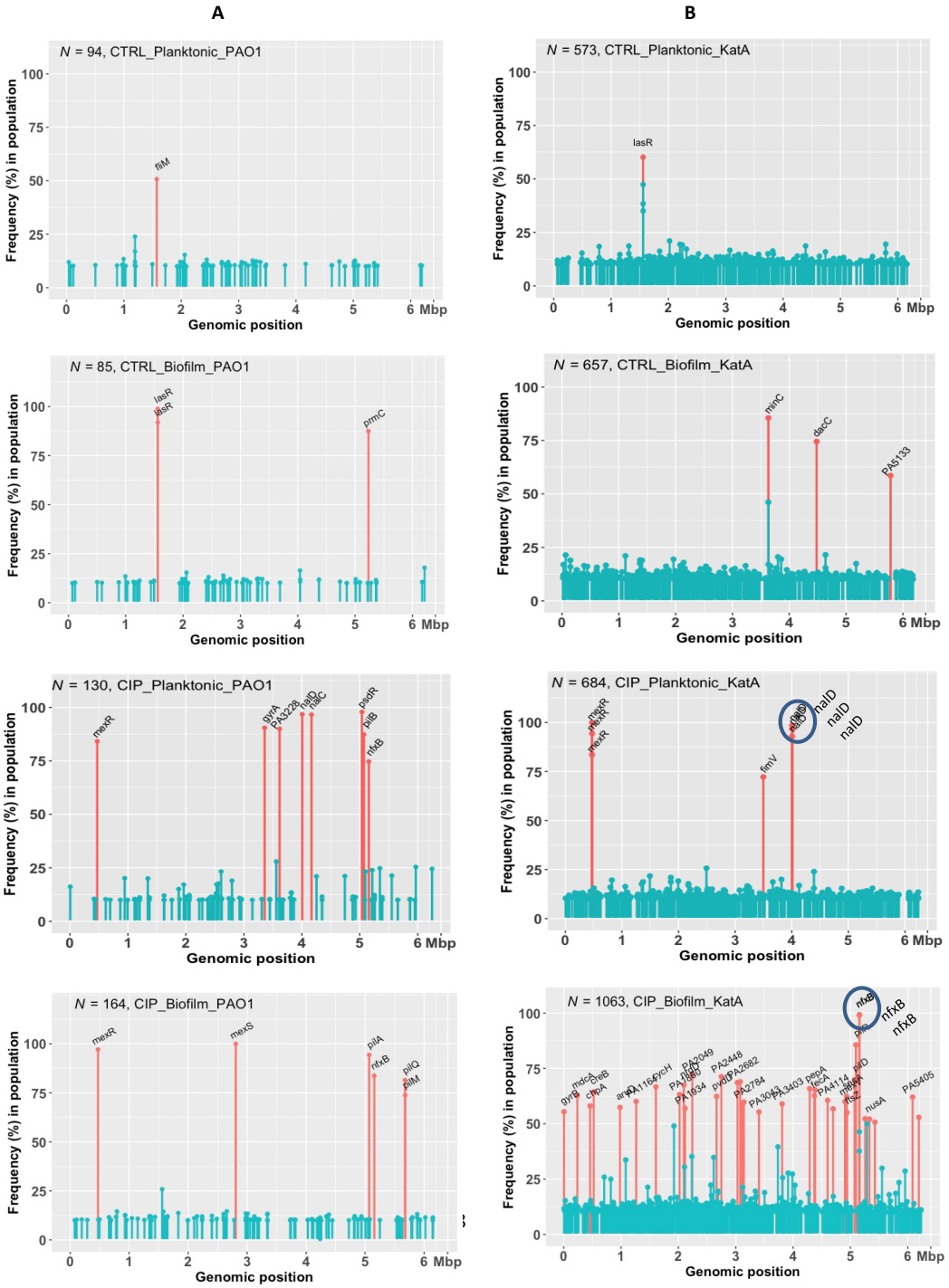


Figure 3. The frequency (%) of each mutation (y axis) and their genomic location (x axis) in the WT PAO1 (A) and *ΔkatA* (B) biofilm and planktonic populations in the different conditions. Red bars represent mutations with frequencies > 50% and green bars represent mutations with frequencies < 50%. The blue circles are marking genes with mutations at the same position occurring in several lineages with similar frequencies.

596