The high persister phenotype of *Pseudomonas aeruginosa* is associated with increased fitness and persistence in cystic fibrosis airways

Authors

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Abstract

Despite intensive antibiotic treatment of cystic fibrosis (CF) patients, *Pseudomonas aeruginosa* often persists in patient airways for decades, and can do so without the development of antibiotic resistance. Using a high-throughput screening assay of survival after treatment with high concentrations of ciprofloxacin, we have determined the prevalence of high-persister variants (Hip) in a large patient cohort. In a screen of 467 longitudinal clinical isolates of *P. aeruginosa* from 40 CF patients, we classified 25.7% as Hip. Hip were identified in 26 patients, but only a few bacterial lineages were dominated by Hip. Instead, the emergence of Hip increased over time, suggesting that CF airways treated with ciprofloxacin select for Hip with an increased fitness in this environment. We generally observed diverse genetic changes in the Hip isolate population (as many co-occurring routes to increased fitness exist), but interestingly elevated mutation counts in the RpoN gene of 18 Hip isolates suggest that this sigma factor plays a role in shaping levels of antibiotic tolerance. To probe the impact of the Hip phenotype in a CF-similar environment, we tested the fitness properties of otherwise genotypically and phenotypically similar low-persister (Lop) and Hip isolates in co-culture using a specialized flow-cell biofilm system mimicking pharmacokinetic/-dynamic antibiotic dosing. Hip survived ciprofloxacin treatment far better than Lop isolates. The results of this investigation provide novel insights into persister dynamics and fitness contributions to survival in the CF lung, and show that the Hip phenotype of antibiotic susceptible bacteria plays an important role in long-term infections.

Significance

Antibiotic resistance is emphasized as a rapidly increasing health threat, but antibiotic tolerance via the occurrence of persister cells in antibiotic-treated bacterial populations is clinically and publicly neglected. In 40 CF patients representing a well-established human infection model – long-term lung infections by *Pseudomonas aeruginosa* – we show the emergence and accumulation of persister variants in a clinical population heavily reliant on antibiotic therapy. We observe that the high-persister (Hip) phenotype is independent of resistance and likely the consequence of numerous genetic alterations, complicating surveillance and inhibition in the clinic. Furthermore, we find Hip are selected for over time, survive better than 'normal' bacteria, and can outcompete them in CF-similar conditions, ultimately affecting 65% of patients in an early disease cohort.

56 **Introduction**

57 Antibiotic-tolerant persister cells are suspected to be a significant clinical problem that has
58 been seriously neglected in favor of combating antibiotic-resistant bacteria, though persisters
59 were in fact described shortly after the clinical introduction of antibiotics (1). Persisters are
60 distinct from antibiotic-resistant mutants, as they do not grow in the presence of antibiotics.
61 Instead, they remain dormant during antibiotic exposure but retain the capacity to resuscitate
62 and restore the population when antibiotic concentrations drop (2–4). However, our
63 understanding of the physiology and clinical relevance of persister cells is limited, given the
64 difficulty in reliably isolating what is theorized to be a stochastic phenotype *in vitro*, much less
65 monitoring this phenotype in routine clinical care. Thus, few studies have assayed persister
66 formation in clinical scenarios (5, 6). One study of oral carriage (0-19 weeks) of *Candida*
67 *albicans* isolates from 22 cancer patients undergoing chemotherapy found that patients with
68 carriage of greater than 8 weeks had significantly higher persister levels than those with less
69 than 8 weeks carriage, but did not address the underlying genetic mechanisms of persistence
70 in this pathogen (5). To examine the genetic underpinnings of the persister phenotype in a
71 clinical scenario, both a large, aligned patient cohort that places the bacteria under similar
72 environmental stresses as well as isolate sampling at a resolution that captures the emergence
73 and longevity of the phenotype are needed.

74

75 *P. aeruginosa* is the most frequent cause of chronic airway infections in patients with CF (7,
76 8). Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene often
77 result in inefficient mucociliary clearance of bacteria from the airways, creating opportunities
78 for bacterial colonization (9, 10). Upon entering the host, environmental *P. aeruginosa* adapts
79 to the CF lung environment, ultimately establishing an incurable airway infection (11, 12).
80 Despite intensive antibiotic treatment from the first discovery of the bacterium in the lung,
81 resistance emergence in the first years of infection is surprisingly low (13, 14). In the absence
82 of clinically defined antibiotic resistance, survival of the bacteria is likely supported by small
83 fractions of antibiotic tolerant subpopulations, among which persister cells may be clinically
84 important (6, 15). While persister cells are stochastic phenotypic variants in any bacterial
85 population, genetic changes in bacterial populations can result in a high persister (Hip) state,
86 producing increased numbers of antibiotic tolerant cells following exposure to antibiotics (16).
87 A previous investigation of the persister phenotype in young CF patients showed an increase
88 in persister phenotype in early/late infection isolate pairs from 14 patients. In this study, 35
89 longitudinal *P. aeruginosa* isolates taken from one child over a 96-month period showed

90 increased levels of persister cells over time as well as an accumulation of 68 mutations between
91 the first and last isolate (6). However, the mutations in the single patient resembled those
92 known to accumulate in other CF patients over infection rather than any mutations previously
93 associated with the Hip phenotype in other, non-CF studies.
94
95 To acquire a high-resolution pan-cohort perspective of persister emergence, genetic
96 mechanism, and impact in long-term infections, we have screened for Hip variants over a 10-
97 year period in 40 young CF patients via 467 longitudinal isolates of *P. aeruginosa* collected
98 from initial colonization onward. This unique isolate collection allows us to determine Hip
99 prevalence and dynamics during each colonizing strain's transition from environmental isolate
100 to persistent pathogen. We describe relationships between the Hip phenotype and the age of
101 the isolate, as well as other adaptive traits in longitudinal infections. We show that the Hip
102 phenotype is an independent and widespread trait. We further search for genetic changes
103 associated with the Hip phenotype in independent clonal lineages within distinct patients,
104 which may suggest adaptive routes to producing this phenotype. Finally, we show that the Hip
105 phenotype generally accumulates over time in patients via several archetypal patterns, appears
106 to contribute to long-term persistence of lineages, and increases the fitness of colonizing
107 populations of *P. aeruginosa* in antibiotic-treated CF patient lungs.
108
109 **Results**
110 *The isolate collection*
111 We examined a collection of 467 *P. aeruginosa* airway isolates obtained from 40 young CF
112 patients over a 10 year period while they were treated at the Copenhagen CF Centre (17). These
113 patients represent a cohort aligned at the early infection stage and undergoing similar treatment
114 regimens per CF Centre guidelines, with repeated culture of *P. aeruginosa* from their monthly
115 sputum sampling within a time frame of 2-10 years (patient inclusion was on a rolling basis
116 over the study period in order to capture all early colonization cases). Early isolates therefore
117 represent bacteria that have not been exposed to substantial antibiotic treatment before the
118 study start excepting rare cases of strain transmission from another patient. The bacterial CF
119 isolates have been grouped into 53 genetically distinct clone types based on their genome
120 sequences (17), and while the majority of patients retained a monoclonal infection during the
121 entire course of infection, half (n=21, 53 %) were infected at least transiently with another
122 clone type. To effectively account for these multi-clonal infections, clinical isolates are
123 described by their patient-specific lineage, which is defined by the clone type and the patient

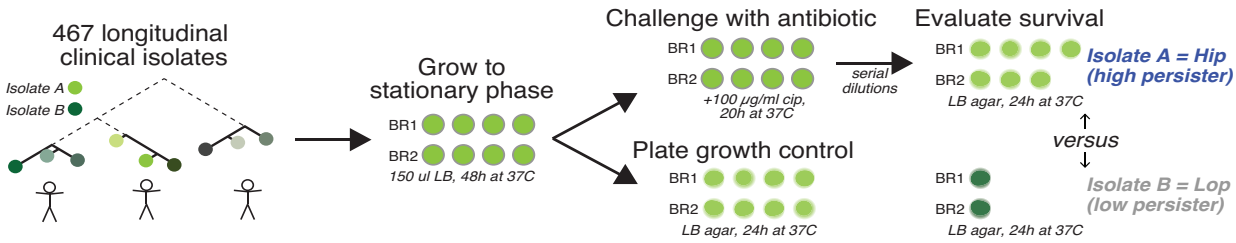

Figure 1. High-throughput screening approach for isolates with a high persister (Hip) phenotype. A large collection of *P. aeruginosa* clinical isolates were grown to stationary phase in quadruplicate wells for 2 experiments (4 technical replicates for each of 2 biological replicates - BRs). Each isolate was treated with 100 µg/ml of ciprofloxacin for 24 hours, while growth was assessed by plating on LB agar. Following antibiotic treatment, isolates were plated via serial dilution and survival was assessed. Each isolate was given a persister score based on consistent replicate survival following treatment. Isolates for which 2-4 replicates survived for each BR were given a score of 2-4, respectively, and were considered high persisters (Hip). Isolates with a respective score of 0-1 were considered low persisters (Lop).

124 of origin (75 lineages in total). Throughout this paper, we will also refer to 'Time since first
125 detection' for each isolate, which represents the length of time between first detection of *P.*
126 *aeruginosa* and subsequent isolations of the same clone type.
127
128 *Identification of Hip by high-throughput screening*
129 We screened the collection of *P. aeruginosa* isolates for the propensity to survive in the
130 presence of high concentrations of ciprofloxacin. This antibiotic was chosen because it is
131 commonly used to treat early *P. aeruginosa* infections in CF patients, and it is bactericidal
132 toward stationary phase *P. aeruginosa* (18, 19). Briefly, *P. aeruginosa* subcultures in micro-
133 titer plates were grown for 48 hours until they reached stationary phase, after which they were
134 challenged with a high concentration of ciprofloxacin (100 µg/ml) for 24 hours before survival
135 was assessed (Fig. 1). Each isolate was screened eight times (technical quadruplicates
136 performed in duplicate biological experiments) and scored based on the capacity to re-grow
137 after treatment. An isolate was given a score of 0 if it failed to re-grow in any replicate, a score
138 of 1 if it grew once in both biological duplicates, a score of 2 if it grew in half of the technical
139 replicates on each plate, a score of 3 if it grew in at least three replicates on each plate, and a
140 score of 4 if it grew in all replicates. We then defined high persister (Hip) isolates as those
141 scoring between 2-4, and low persister (Lop) isolates as those scoring between 0-1. Using this
142 scoring system, 120 isolates (25.7%) of the collection were defined as Hip (Fig. 2A). Isolates
143 with a score of four made up the largest Hip group, while most of the Lop isolates were scored
144 as zero, failing to grow in any replicate. To validate our high-throughput screening approach,
145 we randomly selected 14 Hip isolates and compared their persister phenotypes with 8 Lop

control isolates as well as the laboratory strain PAO1 using standard persister assays. The Hip variants on average produced 1000-fold-more CFU persisters than the Lop isolates (Fig. 2B).

As we were using ciprofloxacin as the antibiotic to identify persister isolates, and most of our patients have been treated with ciprofloxacin, we also wanted to check if we were simply selecting ciprofloxacin resistant isolates as opposed to high persisters. Ciprofloxacin MICs were determined using E-tests for isolates within the collection. Most of the isolates were characterized as susceptible based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (Fig. 2C). Forty-eight susceptible isolates were Hip variants while 92 Lop variants were resistant, confirming that our screening approach identified an antibiotic-resistant phenotype separate from the persister phenotype.

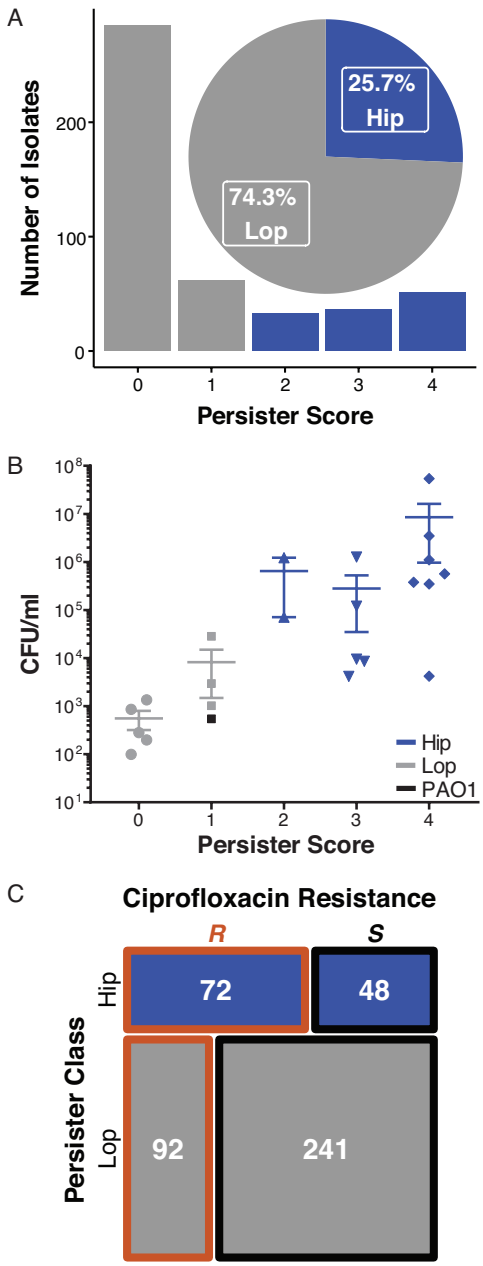

Figure 2. Persister screening results. (A) Distribution of *P. aeruginosa* Hip (blue) and Lop (grey) isolates. **(B)** Validation assays were performed for Hip and Lop isolates. Colony forming units (CFU) per ml were determined following overnight treatment with 100 µg/ml of ciprofloxacin. Each data point represents a single isolate and is the mean of 6 independent experiments. Bars represent SEM. **(C)** Hip and Lop persister isolates were classified as susceptible or resistant to ciprofloxacin according to EUCAST breakpoints (S≤0.5µg/ml, R>0.5µg/ml) based on their MIC obtained via E-test.

The persister phenotype is an independent trait

As illustrated by the comparison between persister class and response to ciprofloxacin, the persister phenotype in our collection is not arising in isolation from other adaptations. Adaptations accumulate based on a combinatorial response to both temporal and fixed environmental factors unique to a given patient. We and others have previously observed that CF isolates adapt towards slow growth rates, increased resistance to antibiotics, and preference for a biofilm lifestyle (20–22), and it has also been proposed that there is a specific association between slowing growth rate and the Hip phenotype (23). To probe interrelationships with other phenotypes and compare the adaptive background of the Hip and Lop variants, we used a principle component analysis to evaluate the distribution of Hip (blue diamonds) versus Lop (gray circles) variants by multiple traits under selection pressure in the CF lung. In Figure 3A, we see that Hip variants group with isolates exhibiting more adapted traits (increased antibiotic MICs and slowing growth), but they also appear across the full phenotypic space alongside Lop isolates. The data ellipses enclosing the approximated majority of each population (68% of the population, t distribution) show that Hip variants (solid blue ellipse) do not separate from Lop variants (solid gray ellipse). We also specifically identify the first Hip variant of each clone type infecting a given patient (FirstHip – blue triangles, dashed blue ellipse) in an attempt to assess these isolates' other traits and state of adaptation at first

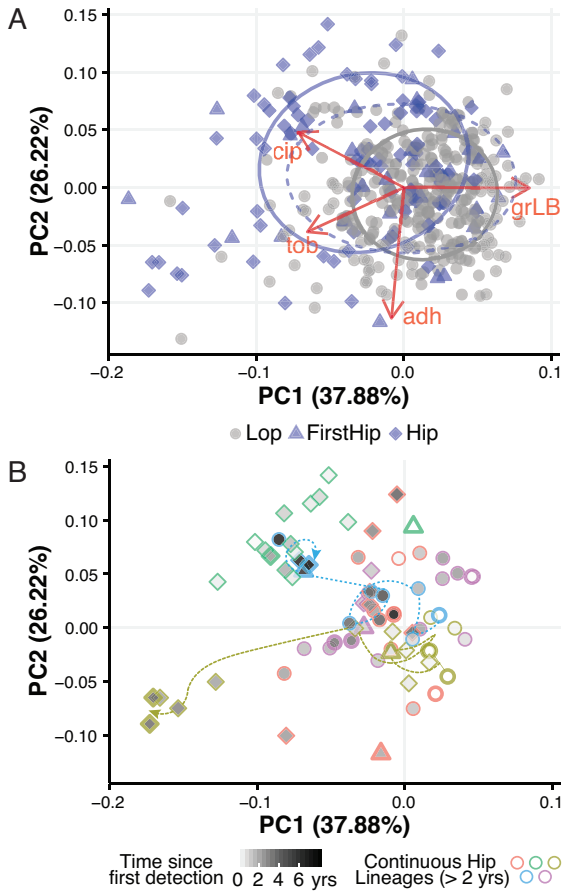

Figure 3. High persisters in the multi-trait landscape. Lop (grey circle), the earliest Hip (blue triangle) isolate(s) of each lineage, and the rest of the Hip isolates (blue diamond) were analyzed via principle component analysis with respect to their similarity with other infection-linked traits: growth rate (grLB), adhesion (adh), ciprofloxacin MIC (cip) and tobramycin MIC (tob)). FirstHip persisters were highlighted as Hip variants with mitigated effects of other accumulating mutations within the lineage to improve cross-lineage comparison. 438 isolates with complete trait sets are included. **(A)** Isolates exhibiting a persister phenotype do not consistently cluster with any other specific trait, while traits of Lop, FirstHip, and Hip isolates shift slightly from 'naïve' towards 'adapted' levels. We illustrate this using data ellipse enclosing samples approximately within the first standard deviation (t distribution, 68% of the set) for isolate sets characterized as Lop (gray ellipse), FirstHip (dashed blue ellipse), and Hip (blue ellipse). **(B)** Lop and Hip variants co-occur in time when evaluating five lengthy lineages characterized by periods of continuous Hip isolates (see Figure 3 – 'Continuous' set). First isolate (white fill), firstHip (triangle), and last isolate (dark fill) are demarcated by bolded marker outlines for all lineages.

202 appearance. These FirstHip isolates overlap substantially with both Lops and Hips, indicating
203 much variation in the adaptive state. This variation could be due to likely lapses of time
204 between the emergence of a Hip variant and its isolation as well as different adaptive
205 trajectories with patients.
206
207 We theorized that patients with increased numbers of clustered Hip variants might be easier to
208 interpret from a trait perspective, as Hip variants from the same patient-specific lineages would
209 be both closely related genetically and exposed to the same treatment and immune-related
210 selection pressures. We made a focused plot including only isolates associated with 5 patient-
211 specific lineages that exhibit continuous periods of Hip variants (as classified in association
212 with Figure 5) and have been sampled for more than 2 years since first detection (Figure 3B).
213 Two of 5 lineages evolve in a directed fashion (dashed directional lines) based on time since
214 detection (marker fill color). However, we do not see consistent separation of Hip variants from
215 Lop variants within each lineage by other phenotypes, even within directionally evolving
216 lineages – they co-occur across isolate age and direction of adaptation trajectory.
217
218 In a final related assessment, we contrast just the appearance of ciprofloxacin resistance versus
219 Hip variant for all study lineages. In seven lineages (17%), a Hip isolate was detected before
220 resistance emerged. In five lineages, resistance emerged in the absence of the Hip phenotype,
221 and in an additional seven cases, a resistant isolate was detected before a Hip isolate. This
222 supports that Hip and increased ciprofloxacin MIC are two independent phenotypes, which
223 may be concurrently selected for over time as patients undergo treatment.
224
225 *Genetic evolution of the persister phenotype is not convergent across patient-specific lineages*
226 Sequencing and identification of genetic variations accumulating within each clone type have
227 been previously performed for most of the isolates used in this study; furthermore, genes
228 targeted in convergent evolution were identified by the significant enrichment of observed
229 lineages with mutations in those genes compared to the number of lineages expected to have
230 mutations in the same genes according to genetic drift (derived from a simulated evolution
231 where lineages accumulate an equivalent number of mutations randomly for 1000 independent
232 evolution simulations) (17). We split our dataset into Hip and Lop variants, and then performed
233 this same observed versus expected lineage enrichment analysis for each population (see
234 Materials and Methods for further details). A comparison of the significantly enriched mutated
235 genes for Hip versus Lop populations allowed us to identify 9 candidate '*hip*' genes targeted

236 in Hip variants when compared to Lop variants (Figure 4A). However, a maximum of 4 (15%)
237 Hip-containing lineages accumulated non-synonymous mutations in the same enriched '*hip*'
238 gene, which is a weak signal of convergent evolution within our 26 lineages with Hip variants
239 (hereafter referred to as Hip$^+$ lineages). Moreover, there was a surprising lack of the most
240 prominent '*hip*' genes identified in *in vitro* studies and screens (Table S1).
241

A

Locus	Gene	Product	Hip-based Lineage Count	Lineage Enrichment Ratio
PA4462	***rpoN***	**RNA polymerase σ^{54} factor**	**4**	**5.4**
PA4069		Hypothetical protein	3	4.9
PA3257	*algO*	Periplasmic tail-specific protease	3	4.8
PA1380		Transcriptional regulator	3	2.4
PA2685	*vgrG4*		3	2.2
PA2403	*fpvG*	Hypothetical protein	3	2.1
PA0720		Helix destabilizing protein of Pf1	3	2.0
PA0888	*aotJ*	Arginine/ornithine binding protein	3	2.0
PA3467		Putative transporter	3	2.0

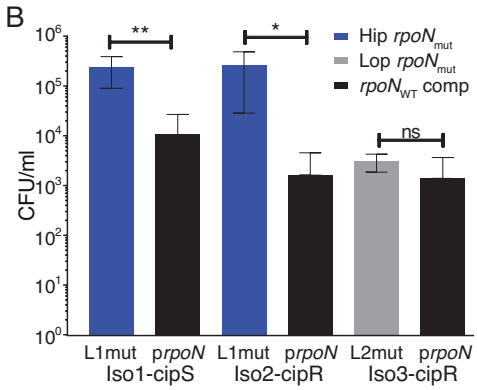

Figure 4. Survival following antibiotic treatment for clinical *P. aeruginosa* isolates harboring an intact and inducible copy of *rpoN*. (A) Mutated genes enriched in Hip versus Lop isolates as assessed from a convergent evolution perspective accounting for lineage adaptation. Lineage enrichment ratio was calculated by dividing lineage-based gene mutation enrichment within Hip variants by that within Lop variants for each gene. Top Hip-linked genes were selected via the following criteria: greater than 2 lineages presenting mutations in that gene in the Hip population and a lineage enrichment ratio greater than 2. **(B)** Hip (blue bars) and Lop (grey bar) isolates with *rpoN* null mutations were grown for 24 hours then treated with ciprofloxacin (100 µg/ml). Cultures were plated for survival following 24 hours of treatment, and colony forming units (CFU) were quantified. The expression of intact *rpoN* was induced via the addition of 0.02% arabinose (black bars). Data presented are the mean ± SD, n ≥ 3. Significance was determined using unpaired t-test (** $p<0.01$, * $p<0.05$, ns, not significant).

242 We experimentally evaluated the contribution of our top '*hip*' gene, the sigma factor encoding
243 gene *rpoN* (Figure 4B). The gene encodes the RNA polymerase σ^{54} subunit, and is among the
244 general pathoadaptive genes previously identified in a subset of our clinical isolate collection
245 (17). The gene was mutated in 19 isolates from five distinct lineages, and of these 18 isolates
246 were also Hip (of 4 Hip$^+$ lineages). The specific *rpoN* mutant alleles in these isolates differ, but
247 in all cases premature translational stop codons were identified. To evaluate the impact of *rpoN*
248 mutations in affected mutants, two representative Hip variants (from the DK06-P4405 lineage)
249 with differing ciprofloxacin sensitivities (an MIC of .25 and 4, respectively) and the one Lop
250 isolate (from the DK32-P8203 lineage, MIC of 4) were selected for further characterization
251 (see Materials and Methods for more details). Each isolate was transformed with a plasmid
252 carrying an intact and inducible copy of *rpoN* (p*rpoN*). When RpoN function was restored in
253 each of the Hip variants, the persister level was reduced for both (which also illustrated the

254 phenotype's independence from ciprofloxacin sensitivity level). In contrast, the Lop isolate did
255 not show any changes in persister class when complemented with an intact copy of *rpoN*.
256 Additionally, a PAO1 *rpoN* deletion mutant did not show a difference in persister phenotype
257 when compared to wild-type PAO1 (data not shown). This highlights the complex role of
258 lineage background on the persister phenotype – RpoN contributes to the persister phenotype,
259 but lineage-based factors such as potentiating mutations and/or background genotypic features
260 enable this contribution.
261
262 *Hip variants accumulate in patients over time (via diverse incidence patterns)*
263 The lack of strong genetic signatures differentiating Hip from Lop isolates motivated us to
264 examine the temporal dynamics of high persister incidence. Our isolate collection is unique
265 due to the coverage of each patient's initial colonization phase based on the patient's age and
266 clinical history. In half of the patients, the earliest bacterial isolate is also the first-ever
267 identified *P. aeruginosa* in the clinic. We can thus estimate the emergence of the Hip phenotype
268 as *P. aeruginosa* adapts from a wild type-similar naïve state into an adapted persistent
269 pathogen. Previous findings have indicated that the number of Hip variants from a lineage may
270 increase over time as the bacteria adapt to the antibiotic pressure in the host, and that once a
271 Hip isolate is observed, it is assumed to persist in the infecting population of the patient (5, 6).
272
273 To illustrate the range of persister dynamics we observe, we grouped each lineage by an array
274 of descriptors. The lineage descriptors include Hip presence versus absence (Hip^+ vs Hip^-),
275 transience of the lineage (whether it appears for less than 2 years and less than half the length
276 of a patient's infection and is afterwards replaced by another lineage), continuity of Hip
277 variants (whether Hip variants are consistently present for at least 3 sampling dates in a row),
278 dominance of Hip variants (whether Hip variants make up at least 2/3 of all collected isolates
279 of a lineage), and whether a Hip variant initiates the lineage. Figure 5A shows the ordered
280 distribution of the lineages in 10 different groups based on the distinct sets of descriptors,
281 illustrating both the complexity of our samples and the diversity of lineage Hip dynamics. An
282 assessment of the number of lineages in each group shows the following: 1) 29 of 75 lineages
283 are Hip^+, 2) 29 of 46 Hip^- lineages are transient, while 3 Hip^+ lineages are transient, 3) 20 Hip^+
284 lineages have Hip variants appear in a seemingly stochastic manner after colonization is
285 initiated versus 9 lineages that exhibit continuous periods and/or dominance of Hip variants,
286 and 4) 10 lineages have initiating Hip^+ variants. Thus, the fraction (25.7%, Fig. 2) of total
287 isolates with a Hip phenotype appears to be distributed over a subset of lineages (38.6%) in

both stable (continuous/dominant) and stochastic patterns of incidence, rather than present in every evolving lineage.

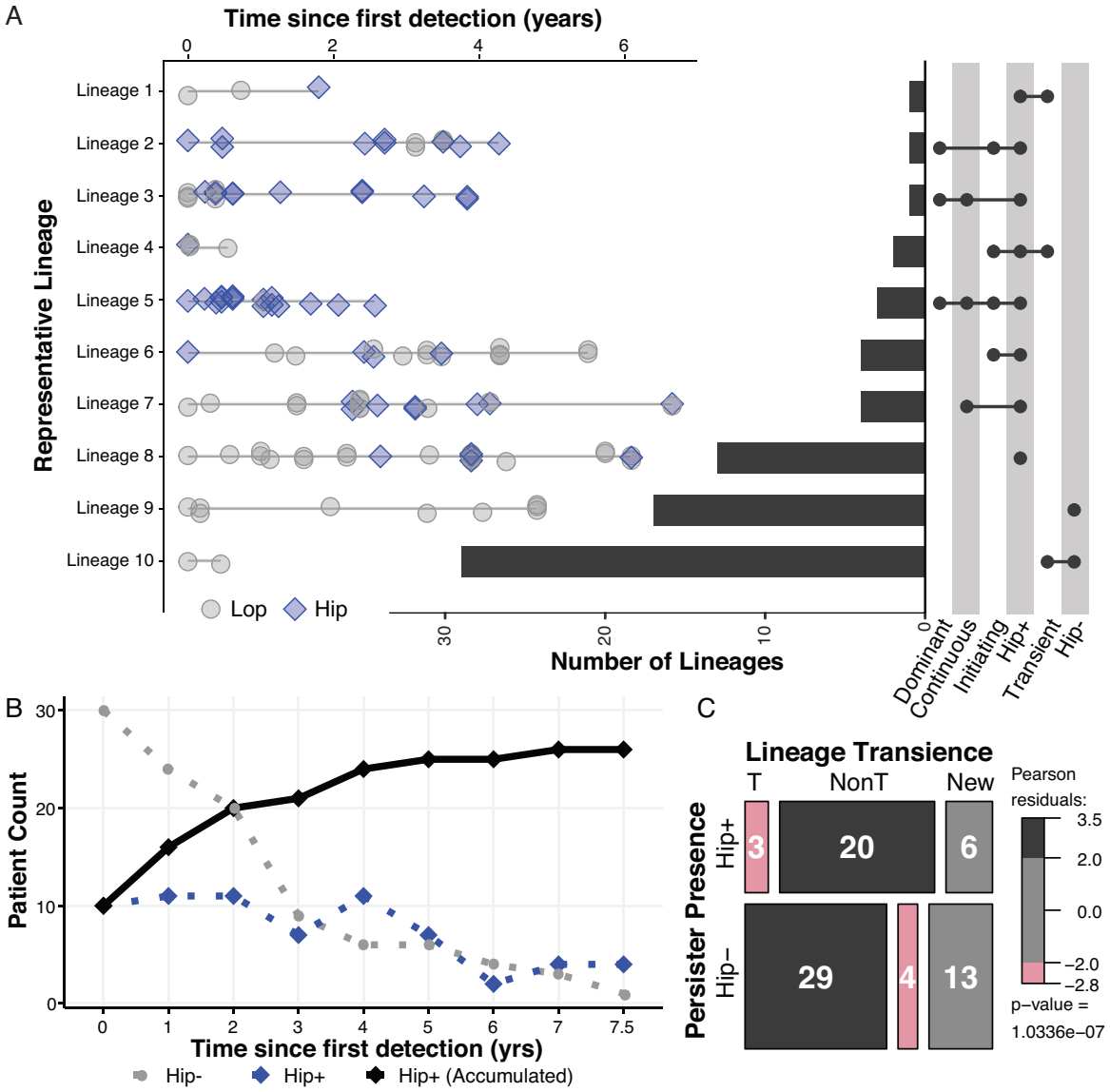

Figure 5. Persister incidence patterns from a lineage-based perspective. **(A)** Lineages were classed according to several nested characteristics: transient versus non-transient lineages, Hip presence, continuous periods of isolated Hips, lineage-initiating Hips, and Hips dominating a lineage. Lineages representing each combination of traits are shown on the left while characteristic sets are identified and enumerated for the entire collection on the right. **(B)** Hip$^-$ (gray circles) and Hip$^+$ (blue diamonds) show the continuous patient count of Hip$^-$ patients versus Hip$^+$ patients for the prior year of colonization, while the accumulating count of Hip$^+$ patients from time 0 is shown by black diamonds. **(C)** Transient lineages (lineages of shorter than 2 years duration, less than 50% of total patient infection length, and which are followed by a new lineage's appearance) are significantly associated with the Hip$^-$ phenotype, while non-transient lineages are associated with the Hip$^+$ phenotype based on Pearson's chi-squared test. Transience-unclassifiable lineages of shorter than 2 years' duration at the end of a patient's collection period are shown for context.

Given the apparent variability in Hip presence and pattern, we examined the incidence of Hip variants within patients over time as shown in Figure 5B. Here we plot the continuous counts

293 of patients exhibiting the Hip phenotype (Hip^+) versus no Hip presence (Hip^-) within the
294 previous year (dashed lines), which illustrates both the number of patients assessed at a given
295 colonization age and the increasing distribution of Hip^+ versus Hip^- phenotypes over time. An
296 inspection of the number of patients that have exhibited a Hip variant at least once by a certain
297 age of colonization (solid line) shows that the number of patients with Hip variants increases
298 over time, ultimately affecting 65% of the patients in our study cohort by 7 years of
299 colonization. Next, we evaluated the relationship between lineage transience and Hip presence.
300 In addition to our other classifications, we mark as 'New' the lineages present for less than 2
301 years in a patient at the end of our study period since we cannot classify them as transient or
302 non-transient without additional samples. Of the 56 remaining lineages, non-transient lineages
303 are significantly associated with the Hip^+ lineage status, while transient lineages are
304 significantly associated with the Hip^- lineage status (Fig. 5C). Thus, a given patient often has
305 multiple infecting lineages, but the Hip^- lineages are much more likely to disappear over the
306 course of infection. In summary, we find that despite variable incidence patterns, a clear
307 majority of patients are infected by Hip^+ lineages, and these lineages have a significant
308 persistence advantage in comparison to Hip^- lineages over time, suggesting that the Hip
309 phenotype carries a fitness increase in antibiotic-treated patients.
310
311 *Hip variants show increased fitness in patient-similar biofilms*
312 Having shown that Hip variants appear at high frequency in *P. aeruginosa* populations
313 infecting CF airways, and that the Hip phenotype is dissociated from other adapting traits such
314 as antibiotic resistance, we next asked if Hip variant bacteria are able to survive antibiotic
315 treatment better than Lop bacteria with similar antibiotic susceptibilities and growth properties.
316 This fitness test should be carried out in conditions as close as possible to those in which the
317 Hip variants had been selected. We therefore simulated antibiotic treatment of CF patients in a
318 recently developed biofilm Pharmacokinetic/Pharmacodynamic system, in which the bacteria
319 are challenged with antibiotics in much the same way as in patients (24). We chose this model
320 because *P. aeruginosa* often appears as biofilms in lungs of CF patients (25), because biofilms
321 have been shown to harbor increased levels of persister cells (26), and because our model
322 mimics the bacterial exposure to ciprofloxacin treatment as described for CF patients (24, 27).
323 The isolates were chosen based on the criteria that 1) the respective times of isolation since
324 their first detection in the CF lungs were similar, 2) they had similar MIC values for
325 ciprofloxacin, 3) similar growth rates, and 4) belong to the same clone type. Two independent
326 Hip/Lop pairs were differentially tagged with YFP (Hip) or CFP (Lop). All strains formed

327 biofilms with comparable biomasses in the flow-cell system (data not shown). Hip and Lop
328 cells were then mixed 1:1 and allowed to form a biofilm. Representative images of one of the
329 Hip/Lop biofilms are shown before (Fig. 6A) and after treatment with ciprofloxacin (Fig. 6B).
330

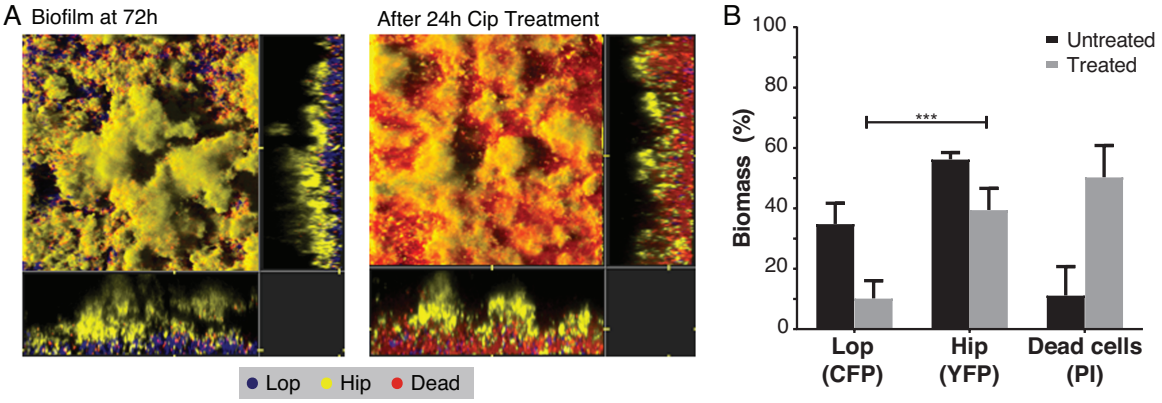

Figure 6. Fitness comparison of Lop and Hip isolates in biofilm conditions. **(A)** Lop (tagged with CFP) and Hip (tagged with YFP) isolates were cocultured and allowed to form biofilms in a flow-cell model for 72 hours. Mixed biofilms were treated for 24 hours with ciprofloxacin (4 µg/ml). Propidium iodine (PI) was added to visualise dead cells (red). **(B)** Biomass was quantified for each population. Significant differences in biomass following treatment were determined using unpaired t-test (*** $p < 0.001$).

331 The majority of Lop bacteria were located close to the glass substratum with the Hip population
332 proliferating at the external surface of the biofilm, facing the liquid flow. The addition of
333 ciprofloxacin preferentially killed the Lop population leaving the Hip population relatively
334 unaffected by the antibiotic. COMSTAT analysis confirmed this changed population structure
335 after ciprofloxacin addition (Fig. 5C). Similar results were observed for the second Hip/Lop
336 pairing (data not shown). This documentation of a Hip associated fitness increase in an
337 antibiotic containing environment is all the more striking, as it has been shown previously that
338 ciprofloxacin treatment of flow-cell biofilms preferentially kills the surface sub-populations of
339 micro-colonies (28) – yet the Hip cells on the colony surfaces survive much better than the
340 internal Lop bacteria under treatment with ciprofloxacin.
341

342 **Discussion**

343 In this communication, we focus our interest on the clinical significance of the persister
344 phenotype, mapping the prevalence of persisters in a large, aligned cohort of patients under
345 intensive antibiotic treatment in a 10 year period (17, 27). Of over 450 *P. aeruginosa* isolates
346 from the airways of 40 young CF patients (75 lineages in total), 26% of the isolates were scored
347 as Hip using a high-throughput screening approach (Fig. 1). The validity of the screen was
348 confirmed using traditional persister assays, suggesting our results provide a reliable reflection

349 of the frequency of occurrence of Hip isolates infecting CF patients (Fig. 2). We show that the
350 isolates display different levels of persisters, in accordance with the variance of persister levels
351 previously found between species and within strains (29–31). We have previously shown that
352 most adaptive changes occur during the first few years of colonization, which matches our
353 objective of searching for signs of increased fitness of Hip variants in patients treated
354 continuously with antibiotics (20, 32). Here, we show that in a young CF patient cohort
355 impacted by early longitudinal colonization by *P. aeruginosa* strains, Hip variants were
356 sampled from 65% of the patients (N=26) during a 10-year observation window. Our analysis
357 is a new and important comparative baseline for moving examinations of this clinically
358 important phenotype towards effective surveillance, impact assessment, and eventual control
359 in actual clinics.

360 In the early years of infection since the first detection of *P. aeruginosa*, the Hip phenotype
361 appeared and disappeared over time (Fig. 5A). However, the number of patients that exhibit
362 Hip variants increases over time (Fig. 5B), suggesting a selective advantage of this phenotype
363 during the continuation of antibiotic therapy. In support of this suggestion, the majority of
364 lineages that showed short-term colonization were made up of only Lop variants, which may
365 in fact explain why they were unable to establish a persistent infection (Fig. 5C). These in-
366 patient data support the hypothesis that the Hip phenotype may generally have increased fitness
367 in the antibiotic containing lung environment. It is, however, very important to note that neither
368 dominance nor continuous presence of Hip variants is observed very frequently (Fig. 5A). It is
369 likely that fitness trade-offs and clonal interference impact on the fitness properties and the
370 persistence level of Hip variants, including other fitness increasing mutations associated with
371 antibiotics in the lung environment which accumulate in the bacterial populations (13).

372

373 Multiple relationships between the Hip phenotype and other phenotypic traits such as growth
374 rate and antibiotic resistance have been suggested in the literature. Reduced growth rates have
375 been associated with high persister phenotypes in *E. coli* (23), but other studies point out that
376 there is no correlation between the mean growth rates of isolates and the observed Hip
377 phenotype (33–35). In our data, the ratio of Hip to Lop variants increases as traits adapt over
378 time, and therefore the average growth rate of all Hip variants is reduced versus Lop variants;
379 however, we did not observe a consistent link between reduced growth rates and Hip phenotype
380 among the clinical isolates of *P. aeruginosa*. Drug-tolerant cells have also been proposed to
381 facilitate evolution of true antibiotic resistance in *E. coli in vitro* (36). Intermittent antibiotic

382 exposure of a batch culture of *E. coli* first selected for mutant clones harboring tolerance
383 mutations that increased the growth lag-time, during which tolerance to killing by ampicillin
384 favored a subsequent selection for mutations that increased the MIC. Though *P. aeruginosa* in
385 the CF lung is also exposed to fluctuating concentrations of antibiotic over the course of
386 infection, we did not observe any obvious contingency in which Hip variants preceed
387 antibiotic-resistant mutants. In contrast, our data suggest that Hip variants and resistant clones
388 evolve independently in patients under antibiotic selection pressure as has been suggested by
389 comparative studies of lab strains (37) and that each of the phenotypes contributes to a
390 continued population survival and hence establishment of long term infection. In general, the
391 results described here suggest that Hip variants arise stochastically in infected patients treated
392 with antibiotics, and that this phenotype is one of several making it possible for the bacteria to
393 persist for extended periods of time. As Hip variants are present in the majority of our young
394 patients by the end of the study period, the persister phenotype may be an early advantageous
395 adaptation in contrast to the adaptive timeline of other traits (20); effective comparisons
396 between stochastic persistence versus more fixed changes such as adjustment of growth rate or
397 antibiotic susceptibility should be pursued in the future.

398 In many ways, persisters have been investigated in line with previous investigations of
399 antibiotic resistance, which has resulted in a similar parallel search for genetic explanations for
400 large variations in persister levels in bacterial populations treated with antibiotics. In a study
401 of clinical isolates from patients with urinary tract infection, it was shown that a gain-of-
402 function mutation in the HipA toxin is common among *E. coli* Hip isolates (38). The most
403 common mutated genetic target we identified among *P. aeruginosa* Hip in the present study is
404 the RpoN sigma factor. Restoring a functional RpoN in these clinical isolates caused a
405 reduction in persisters, but knocking out this gene in a laboratory strain had no effect. This
406 suggests that the RpoN mutation only confers a persister phenotype in the context of additional
407 mutations acquired by the *P. aeruginosa* Hip strains, underscoring the complexity of evolution
408 occurring in the lungs of CF patients. It is also noteworthy that we do not see the appearance
409 of mutations, which previously have been associated with Hip phenotypes *in vitro*. As the
410 RpoN mutation was present in only 15% of the Hip isolates in our large, longitudinal collection,
411 we conclude that a Hip phenotype may derive from a diverse array of genetic changes, and it
412 is likely that more than one mutation often determines the persister level in the respective
413 bacterial populations. It is possible that this difference reflects the multiple and dynamic
414 selection pressures *in vivo*, which challenge Hip variants in antibiotic-treated populations very

415 differently from what is the situation in steady state *in vitro* conditions with only one selective
416 force. Since nearly all published data on persister cells derive from simple *in vitro* experiments,
417 our results further suggest that extrapolations from such *in vitro* experiments to clinical
418 scenarios of infection cannot be made.

419 Many studies have shown the increased survival of persister cells under antibiotic treatment
420 (23, 39) and leveraged this in screens for genetic determinants of persistence, but few have
421 evaluated the fitness of Hip versus Lop variants in direct competition experiments. Stepanyan
422 et al. assessed the fitness tradeoffs of two Hip strains (one hospital strain and one *pilH* mutant)
423 and two Lop *P. aeruginosa* strains via competitive growth of paired Hip-Lop strains at a 1:1
424 ratio under periodic treatment with ofloxacin in Mueller-Hinton broth; Hip strains showed
425 higher fitness under treatment conditions and the reverse in control conditions (29). In our
426 study, we were able to test genotypically similar isolates from a similar point in infection with
427 other selected traits in alignment in order to characterize the selective advantage of the Hip
428 phenotype in a biofilm under clinically-replicating antibiotic exposure (24). In two independent
429 Lop/Hip strain pairs assessed in a PD/PK flow chamber, we show that Hip cells survived
430 ciprofloxacin treatment far better than Lop isolates, and we further find that this survival is
431 potentially reliant on biofilm architecture. While homogeneous monoclonal *P. aeruginosa*
432 biofilms treated with ciprofloxacin show preferential killing of bacteria in the top layers (13,
433 28, 40), Lop bacteria are preferentially killed in the deeper layers of the biofilm. This further
434 supports that the Hip phenotype is associated with increased survival in the presence of
435 ciprofloxacin under conditions simulating the in-patient environment during antibiotic
436 treatment. It is a striking observation that the *in vitro* biofilm fitness assessment shows efficient
437 elimination of the Lop strain in presence of ciprofloxacin, whereas in the patient lungs Hip
438 variants rarely outcompete Lop strains despite frequent treatments with ciprofloxacin as
439 indicated by Fig. 3A. This suggests that in the patient, direct competition is limited by the many
440 separate niches, the influence of the host and the large lung volume, which contrasts the flow-
441 cell biofilm system. This slow competition explains how fully adapted strains like the DK01
442 and DK02 strains simultaneously present in a single patient may require many months or even
443 years to finally outcompete the other (32, 41, 42).
444

445 In summary, we have shown that Hip variants of *P. aeruginosa* emerge frequently in younger
446 CF patients. The Hip phenotype provides a selective advantage for *P. aeruginosa* populations
447 treated with antibiotics, which may explain how persisters contribute to the establishment of

448 chronic infection. Ultimately, our results provide the first window into the evolving landscape
449 of persistence across a whole patient cohort; as pathogens increase their fitness in patients over
450 time, they clearly deploy the high persister phenotype as an important component in their
451 survival repertoire and can do so from the earliest stages of infection. It is still premature to
452 conclude that the high-persister phenotype described here differs from what has been identified
453 as Hip in *in vitro* experimental conditions, but we find a much broader bacterial repertoire for
454 survival in antibiotic containing environments than so far anticipated. Hip variants do not seem
455 to be mutated in genes previously found from *in vitro* experiments to associate with Hip or in
456 any strongly conserved genetic route. *In vitro*, antibiotic-mediated selection is the only
457 selective force, whereas, in the patients, antibiotics constitute one among several parallel
458 selective forces. In support of this concept, Hip variants emerge alongside other beneficial trait
459 adaptations but association with any single trait is inconsistent. We suggest that the difference
460 in selection pressure when comparing *in vitro* and *in vivo* environmental conditions including
461 antibiotics results in highly different evolutionary trajectories. With our investigation, we
462 provide an important platform for broader clinically based studies and contribute important
463 new context for monitoring and one day hopefully preventing the high persister phenotype in
464 the clinic.

465

466 **Materials and Methods**

467 *Strain collection.* In total, we analyzed 467 *P. aeruginosa* airway isolates from young CF
468 patients followed at the Copenhagen CF-clinic after excluding twenty-six isolates that had
469 acquired mutS and/or mutL mutations resulting in hypermutability. Of these 467 isolates, 388
470 isolates from 34 patients were described previously in Marvig et. al. (17) and the remaining
471 isolates were taken from six previously undescribed patients. The isolates were collected and
472 stored at the Department of Clinical Microbiology at Rigshospitalet, Copenhagen, Denmark,
473 between 2002 and 2014. Of the patients included in this study, 35% were diagnosed as
474 chronically infected with *P. aeruginosa* by the end of the study period. We defined chronicity
475 based on the Copenhagen CF Centre definition, whereby either *P. aeruginosa* has been
476 detected in six consecutive monthly sputum samples or fewer consecutive sputum samples
477 combined with observation of two or more *P. aeruginosa*-specific precipitating antibodies (27,
478 43). Intermittently colonized patients were defined as patients where at least one isolate of *P.*
479 *aeruginosa* is detected, and normal levels of precipitating antibiotics against *P. aeruginosa*
480 were observed. Phenotyping data for 407 isolates of this strain collection (growth rate in LB,

481 adhesion in LB, and ciprofloxacin MIC) have been previously published (20). We include trait
482 measurements for 60 additional isolates from the same phenotypic screens conducted as
483 described below, and all available trait data is provided in Supplementary Dataset 1 along with
484 the persister classification of each isolate and descriptive data such as patient origin, genotype,
485 date of sampling, and SRA number.
486
487 *Minimum Inhibitory Concentrations (MIC)* for ciprofloxacin and tobramycin were determined
488 using E-test methodology according to the manufacturer's recommendations (Liofilchem®,
489 Italy).
490
491 *Generation times.* Bacteria were grown overnight in LB, diluted to an OD of 0.05 in 96 well
492 plates containing 150 uL of LB medium, and incubated for 20 hours at 37°C with constant
493 shaking. OD 630 nm measurements were taken every 20 minutes using a microplate reader
494 (Holm & Halby, Copenhagen, Denmark/Synergy H1). Generation times (Td) were determined
495 on the best-fit line of a minimum of 3 points during exponential growth of the bacterial isolate.
496 Growth rates (hr^{-1}) were calculated using the formula log (2)/ Td x 60.
497
498 *Adhesion.* Adhesion was measured via attachment assays in 96-well plates using NUNC peg
499 lids and 96 well plates with 150µl Luria broth medium. OD_{600nm} was measured after incubation
500 for 20 hours at 37°C and subsequently, a "washing microtiter plate" with 180µl PBS was used
501 to wash the peg lids and remove non-adhering cells. After transfer of the peg lids to a microtiter
502 plate containing 160µl 0.01% crystal violet (CV), they were left to stain for 15 min. To remove
503 unbound crystal violet, the lids were then washed again three times in three individual
504 "washing microtiter plates" with 180µl PBS. Adhesion was measured by detaching adhering
505 CV stained cells through washing the peg lids in a microtiter plate containing 180µl 99%
506 ethanol. An ELISA reader was then used to measure the CV density at OD_{590nm}. (Microtiter
507 plates were bought at Fisher Scientific, NUNC Cat no. 167008, peg lids cat no. 445497).
508
509 *High-throughput screening for Hip mutants*. To determine the frequency at which *P.*
510 *aeruginosa* Hip mutants emerge in CF patients, we screened 467 isolates for ciprofloxacin
511 tolerance. Stock 96-well microtiter plates containing 4 technical replicates of each isolate
512 stored in glycerol (25 % v/v) were prepared and stored at -80°C. Using a 96-well spot
513 replicator, bacteria were transferred from the stock plates into sterile 96-well microtiter plates

514 containing 150 µl of Lysogeny Broth (LB) media. Plates were incubated statically for 48 hours
515 at 37°C until the bacteria reached the stationary phase of growth. To determine the initial
516 viability of bacteria in each well, the replicator was used to spot bacteria onto LB agar plates.
517 Subsequently, 100 µg/ml of ciprofloxacin was added to each well and the microtiter plates were
518 incubated statically for a further 20-24 hours at 37°C. Serial dilutions were performed in 96
519 well microtiter plates containing 0.9 % NaCl using an automated fluid handling robot
520 (Viaflo3844/ Integra Biosciences AG). Each dilution was spotted onto LB agar plates using
521 the replicator and plates were incubated at 37°C for at least 24 hours. The growth of the bacteria
522 was compared by counting colonies whenever possible and visually inspecting growth on the
523 plates before and after antibiotic treatment. The experiment was performed in duplicate.
524
525 *Persister assay validation.* Persister assays were performed on 23 isolates to validate the high
526 throughput persister screen. *P. aeruginosa* were inoculated in 3 ml of LB media in 10 ml tubes
527 and incubated for 48 hours at 37°C with shaking at 250 rpm. Following incubation, each culture
528 was serially diluted using sterile 0.9 % NaCl, plated onto LB agar and incubated at 37°C to
529 determine the initial colony forming units (CFU). The remaining culture was treated with 100
530 µg/ml of ciprofloxacin and incubated for a further 24 hours at 37°C with shaking. Following
531 treatment, cells were washed and diluted in sterile 0.9 % NaCl, spot plated onto LB agar and
532 incubated for 24 hours at 37°C. Bacteria survival was measured by counting (CFU) per ml.
533
534 *Pharmacokinetic/Pharmacodynamic (PK/PD) flow chamber biofilm model.* For fitness
535 experiments, we used a PK/PD biofilm model system combined with confocal laser-scanning
536 microscopy. This system simulates the changing antibiotic concentrations in CF patients during
537 intravenous dosing in addition to retaining a similar profile of antibiotic decay as the one taking
538 place in CF patients (24). First, Hip and Lop isolates were differentially tagged with a yellow
539 fluorescent protein (YFP) or cyan fluorescent protein (CFP) respectively (13). Flow chambers
540 were inoculated with a 1:1 mixture of Hip and Lop bacteria (each isolate had an initial OD_{600}
541 of 0.5). Bacteria were incubated for one hour at 30 °C, then nutrient flow was applied to each
542 chamber (40x diluted LB at a rate of 20 ml/h using a Watson Marlow 205S peristaltic pump).
543 Biofilms were allowed to form for 72 hours, at which point flow was stopped and medium
544 containing ciprofloxacin was added. Peak ciprofloxacin concentrations were calculated to be 4
545 mg/L based on PK parameters generated from healthy patients and CF patients (44). The
546 medium was pumped from the dilution flask through the antibiotic flask to the flow chambers
547 at a constant rate calculated to mimic the elimination rate constant of the antibiotic for 24 hrs.

548 A confocal laser-scanning microscope (Zeiss LSM 510) equipped with an argon/krypton laser
549 and detectors was used to monitor YFP (excitation 514 nm, emission 530 nm), CFP (excitation
550 458 nm, emission 490 nm), and dead cells (propidium iodine, excitation 543 nm, emission 565
551 nm). Multichannel simulated fluorescent projections (SFPs) and sections through the biofilms
552 were generated using Imaris software (Bitplane AG, Switzerland). The images were later
553 analyzed using COMSTAT (45). The PK/PD biofilm experiments were performed using two
554 independent Hip/Lop isolate pairs. Pairs were taken from the same patient at a similar time
555 since first detection and had similar growth rates and ciprofloxacin MICs (Table S2). The data
556 presented are from 2 biological experiments with 4 independent images taken from each
557 experiment.
558

559 *Lineage-based genetic analysis*. To generate a list of mutated genes associated with the Hip
560 phenotype, we used previously generated whole-genome sequencing data and variant calling
561 filtered to obtain nonsynonymous mutations that had accumulated within a lineage after the
562 first isolate (17) to evaluate differential mutation patterns for Lop and Hip variants for 388
563 sequenced isolates. To identify genes that were mutated more than would have been expected
564 by drift/random mutation while accounting for lineage-based mutation accumulation over time,
565 we adapted a statistical analysis of the relative mutation enrichment by lineage. After
566 separating Lop and Hip variants, we compared the mutated-gene lineage enrichment ratios for
567 each group - the number of lineages with observed mutation(s) in a given gene divided by the
568 number of lineages expected to have mutations in that gene according to random mutation.
569 This enrichment metric was obtained as follows for each group: we determined the observed
570 number of lineages mutated (sum-obs) in each gene. Then we estimated the average number of
571 lineages (avg-exp) that would have been mutated in each gene if mutations were spread out
572 randomly over the PAO1 genome. Using a random-roulette algorithm, the number of genes
573 that were observed to be mutated in a given lineage was spread out over the PAO1 genome for
574 1000 iterations, providing a m_{gene} by $n_{\text{iteration}}$ matrix of randomly mutated gene profiles for each
575 lineage. For the same iteration n across all lineages, it was noted whether a given gene was
576 mutated. This allowed us to determine an average number of lineages expected to be mutated
577 over 1000 iterations. If a gene was hit by chance more than once in a single iteration, this would
578 still only be denoted as one hit; this is in alignment with our observed mutation assessment,
579 where multiple isolates could be hit in the same gene but we only noted whether or not the
580 lineage was hit by unique mutations in the specific gene. After obtaining the relative

581 enrichment by lineage, a Poisson distribution was used to calculate the probability of the
582 observed given random drift (expected). We also divided the lineage enrichment metric for
583 genes mutated in Hip variants by that for Lop variants to obtain a lineage enrichment ratio to
584 identify targeted genes particularly impactful in the evolution of the Hip population.
585
586 *Lineage set analysis and principle component analysis.* Analyses were conducted in RStudio
587 v. 1.0.143 and R v. 3.4.0. Lineage set analysis was performed using UpSetR v. 1.3.3 in R.
588 Principal component analysis was performed in R using 'prcomp' with centered and scaled
589 phenotype data (Supplementary Dataset 1).
590
591 *Genetic manipulation.* We selected two Hip variants and one Lop variant with mutations in
592 *rpoN* for validation of a *rpoN*-associated Hip phenotype. Each of the *rpoN* Hip variants had a
593 single nucleotide deletion (T, nucleotide position 4993951 on PAO1 reference genome), which
594 resulted in a frameshift following the 360th amino acid (aa) and premature truncation of the
595 protein resulting in a 381aa polypeptide (RpoN has 497aa in the PAO1 reference genome). The
596 Lop isolate had a single nucleotide polymorphism (C→T at nucleotide 112) producing a
597 premature stop codon at the 38th aa. An *rpoN* expression plasmid was constructed for gene
598 complementation experiments. The *rpoN* gene from PAO1 was PCR amplified using primers
599 *rpoN* forward (5' GAATTCATGAAACCATCGCTAGTCCTC 3') and *rpoN* reverse
600 (5'AAGCTTTCACACCAGTCGCTTGCGCTC 3'). The product was cloned into pHERD28T
601 (46), using EcoRI and HindIII restriction sites and the resulting plasmid (p*rpoN*) was used to
602 transform *E. coli* OneShot Top10 (Thermo Fisher Scientific). The presence of the plasmid was
603 verified by PCR and DNA sequencing then introduced into selected clinical *P. aeruginosa*
604 isolates using biparental conjugation. Trimethoprim (TMP, 300 µg/ml) was used for selection
605 and arabinose (0.02 %) was used for *rpoN* induction. Each isolate was transformed with empty
606 pHERD28T and isolates with and without arabinose induction were included as controls.
607

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Conflict of interest

The authors declare no competing financial interests.

Author Contributions

SM and KL designed the study. HKJ collected all the bacterial isolates. BM, DRC, and JH performed all experiments. JAB and LMS performed genetic and lineage analysis. All authors contributed to the writing of the manuscript. All authors approved the final version.

Ethics approval

The local ethics committee at the Capital Region of Denmark (Region Hovedstaden) approved the use of the stored *P. aeruginosa* isolates: registration number H-4-2015-FSP.

Supplementary information

Table S1. Comparison of persister genes identified in previous *P. aeruginosa* studies to mutated genes highlighted by our lineage analysis.

Table S2. Characteristics of Hip/Lop pairs analyzed using the PK/PD biofilm model.

Supplementary Dataset 1. Phenotypic dataset for all isolates.

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