Polygenic adaptation, clonal interference, and the evolution of mutators in experimental *Pseudomonas aeruginosa* populations

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Abstract:

In bacterial populations, switches in lifestyle from motile, planktonic growth to surface-associated biofilm is associated with persistence both in the environment and infections. Studies have identified the first steps of adaptation to biofilm growth but have yet to replicate the extensive genetic diversity observed in chronic infections or in the natural environment. We conducted an evolution experiment with *Pseudomonas aeruginosa* PA14 in growth media that promotes biofilm formation for 90 days in either planktonic culture or in a biofilm bead model. Surprisingly, all populations evolved extensive genetic diversity with hundreds of mutations maintained at intermediate frequencies, while fixation events were rare. Instead of the expected few beneficial mutations rising in frequency through successive sweeps, we observe a remarkable 40 genes with parallel mutations spanning both environments and often on coexisting genotypes within a population. Additionally, the evolution of mutator genotypes (*mutS* or *mutL* mutator alleles) that rise to high frequencies in as little as 25 days contribute to the extensive genetic variation and strong clonal interference. Parallelism in several transporters (including *pitA, pntB, nosD,* and *pchF*) indicate probable adaptation to the arginine media that becomes highly alkaline during growth, whereas genes involved in signal transduction (including *gacS*, *aer2, bdlA, and* PA14_71750) reflect likely adaptations to biofilm-inducing conditions. This experiment shows how extensive genetic and phenotypic diversity can arise and persist in microbial populations despite strong selection that would normally purge diversity.

Importance:

How biodiversity arises and is maintained in clonally reproducing organisms like microbes remains unclear. Many models presume that beneficial genotypes will outgrow others and purge variation via selective sweeps. Environmental structure like biofilms may oppose this process and preserve variation. We tested this hypothesis by evolving *P. aeruginosa* populations in biofilm-promoting media for three months and found that adaptation and diversification were coupled and mostly uninterrupted by fixation events that eliminate diversity. Genetic variation tended to be greater in lines grown using a bead model of biofilm growth but many lineages also persisted in planktonic lines. Convergent evolution affecting dozens of genes indicates that selection acted on a wide variety of traits to improve fitness, causing many adapting lineages to co-occur and persist. This result demonstrates that some environments may expose a large fraction of the genome to selection and select for many adaptations at once, causing enduring diversity.

48 **Introduction:**

49 Bacterial populations inhabit countless environments along a continuum of
50 spatial structure, ranging from well-mixed liquid to clusters bound to surfaces. Growth
51 on surfaces is associated with biofilm production that in turn generates nutrient and
52 oxygen gradients[1], but so too does the metabolic activities of neighboring cells. Life in
53 confined spaces results in varied levels of nutrients, wastes, and signaling molecules
54 that may alter selective forces on parts of the population and create novel ecological
55 opportunities[2]. These physical differences are hypothesized to select for genetic
56 differences in biofilm populations distinct from well mixed cultures. Experimental
57 microbial evolution (EME) studies have found that biofilm populations select for different
58 mutations than those seen in planktonic growth, and the greater environmental structure
59 of the biofilm produces increased genetic diversity[3,4,5,6,7,8,5,8,4,9]. Despite this process of
60 diversification, replicate populations propagated in both biofilm and planktonic
61 conditions exhibit high levels of both phenotypic and genetic parallelism, suggesting
62 some measure of predictability within the same environment[10,7,11,12,13,14,15]. However, we
63 still have much to learn about how the biofilm life cycle influences evolutionary
64 dynamics and processes, including the relative roles of mutation and selection, and
65 whether biofilm growth becomes the dominant selective force relative to other stresses
66 like nutrient limitation or external toxins.

67 *Pseudomonas aeruginosa* is an opportunistic pathogen found in soil and water
68 and is known for its ability to thrive in numerous environments. *P. aeruginosa* is a highly
69 studied pathogen due to its association with poor outcomes in clinical settings when it
70 forms biofilm infections within patients[16,17,18,19,4,20] Adaptation to biofilm growth has been

71 indicated as a cause of infection persistence in numerous studies[21,22,23] and manifests
72 as important colony phenotypes such as mucoidy and rugose small colony variants
73 (RSCVs) as well as the loss of virulence factor production and altered cell surface
74 virulence determinants[24,25]. Several studies have been performed to identify some of the
75 first steps of adaptation to a biofilm environment but these studies have yet to replicate
76 the high levels of genetic diversity often seen in chronic biofilm-associated infections,
77 such as those of the airways of cystic fibrosis patients[26,19,18,27,17,28].
78 We designed a long-term evolution experiment over 90 days of propagation
79 (~600 generations) to explore how the population-genetic dynamics of *P. aeruginosa*
80 adaptation differ in a model of the biofilm life cycle compared to a well-mixed
81 environment. We previously reported findings of greater phenotypic and ecological
82 diversity in biofilm populations in comparison with those passaged by serial dilution,
83 where planktonic growth predominates. This diversity was linked to traits that enhance
84 biofilm growth and thus likely adaptations. Further, the relative fitness of each
85 population was greater when all colony types were present, suggesting a process of
86 niche partitioning and facilitation among lineages[6]. Surprisingly, these putatively
87 adaptive phenotypes differed among replicate populations and changed over time as
88 opposed to the parallelism observed in previous studies[5,3]. A preliminary survey of the
89 genomes of these lineages revealed that this diversity was in part caused by the
90 evolution of *mutS* and *mutL* genotypes that increased mutation rate, a genotype also
91 observed in many chronic infections of *P. aeruginosa*[29,30,31,32]. Here, we use longitudinal
92 whole-population genome sequencing (WPGS) to study the underlying evolutionary
93 dynamics of this experiment at high resolution. Some genes that experienced

94 mutational parallelism contribute to arginine metabolism, the sole carbon and nitrogen
95 source in these experiments. More surprising, in light of the duration of the experiment
96 and the strength of selection in these populations, few mutations actually fixed in any of
97 the six populations and much more genetic diversity was preserved than has been seen
98 in previous evolution experiments. We discuss potential evolutionary and phenotypic
99 causes for this maintenance of such levels of high genetic diversity.

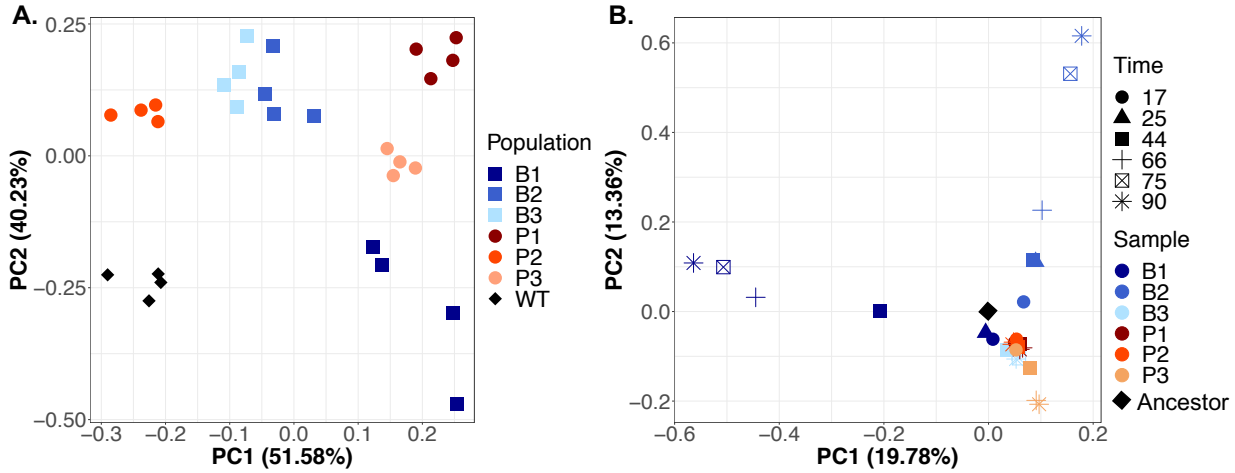
100 **Results:**

101 **Experimental evolution**

102 Six populations of *P. aeruginosa* strain PA14 (PA), a strain already proficient in
103 forming biofilm, were propagated for 90 days (approximately 600 generations, or ~6.6
104 generations per day) to examine the population-genetic dynamics of prolonged growth
105 in a biofilm life cycle, where bacteria that attach to a polystyrene bead are transferred
106 daily to a new tube containing a fresh bead (populations B1, B2, B3), or during serial
107 1:100 dilutions (planktonic populations P1, P2, P3) as described previously[2,6] (Figure
108 S1). The culture medium was M63 media containing arginine as the sole carbon and
109 nitrogen source and supplemented with 25 μM iron (see methods), a combination which
110 has been shown to promote biofilm production in *Pseudomonas* species[33]. We also
111 sought to identify adaptations to these environments at both phenotypic and genetic
112 levels.

113 After 90 days, all six populations became vastly more fit than ancestral PA14,
114 with selective coefficients 10-fold greater (*r* between 3 and 5) than the fitness gains
115 observed in other evolution experiments of similar duration[34,35,3,36,7] (Figure S2, Table
116 S1). Five populations increased their maximum growth rate (Vmax; Figure S3a) and five

117 populations evolved to become less motile than the ancestor (Figure S3b). Interestingly,
118 despite selection to attach to a plastic bead each day presumably by producing biofilm,
119 only the B3 population evolved higher biofilm production than the ancestor as measured
120 by the standard crystal violet assay, while four populations, including bead populations
121 B1 and B2, evolved lower biofilm production (Figure S3c). This result may reflect the
122 high starting biofilm production of PA14 in these conditions being difficult to improve. To
123 merge these three phenotypes, we performed a principal component analysis (Figure
124 1a) and found that evolved populations were distinguished from the ancestor but not
125 clearly separated by treatment.



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128 **Figure 1. Divergent phenotypes linked to fitness and genotypes distinguish each**
129 **evolved population after 90 days of passage**. (A) PCA of evolved phenotypes
130 including biofilm production, maximum growth rate (Vmax), and swimming motility. Blue
131 squares are biofilm populations, red circles are planktonic populations, and the ancestor
132 is indicated by black diamond(s). The first three components explain 51.58%, 40.23%,
133 and 8.19% of variance. (B) PCA of mutation identities and frequencies at Days 17, 25,

44, 66, 75, and 90. Replicate data points for each population are indicated in color. Colors are the same as in A, but shape indicates time through the experiment. The top four components explain 19.78%, 13.36%, 9.31%, and 6.5% of the variance.

High genetic diversity arose and persisted in all populations

We used longitudinal WPGS to characterize the genetic diversity and evolutionary dynamics of each lineage with an average depth of 617.1± 142.3 reads per sample. A total of 145.7 ± 55 mutations were detected per population over the course of the 90-day time period (see methods for filtering criteria). This number of mutations is remarkable in comparison to previous evolution experiments including those performed on *Burkholderia cenocepacia* using the same bead model, in which 37 mutations were identified in the best-studied population[5] over twice the transfer days. Biofilm populations accumulated more mutations (535, with 382 found at day 90) over the 90 days than planktonic populations (339, with 201 found at day 90; Table 1).

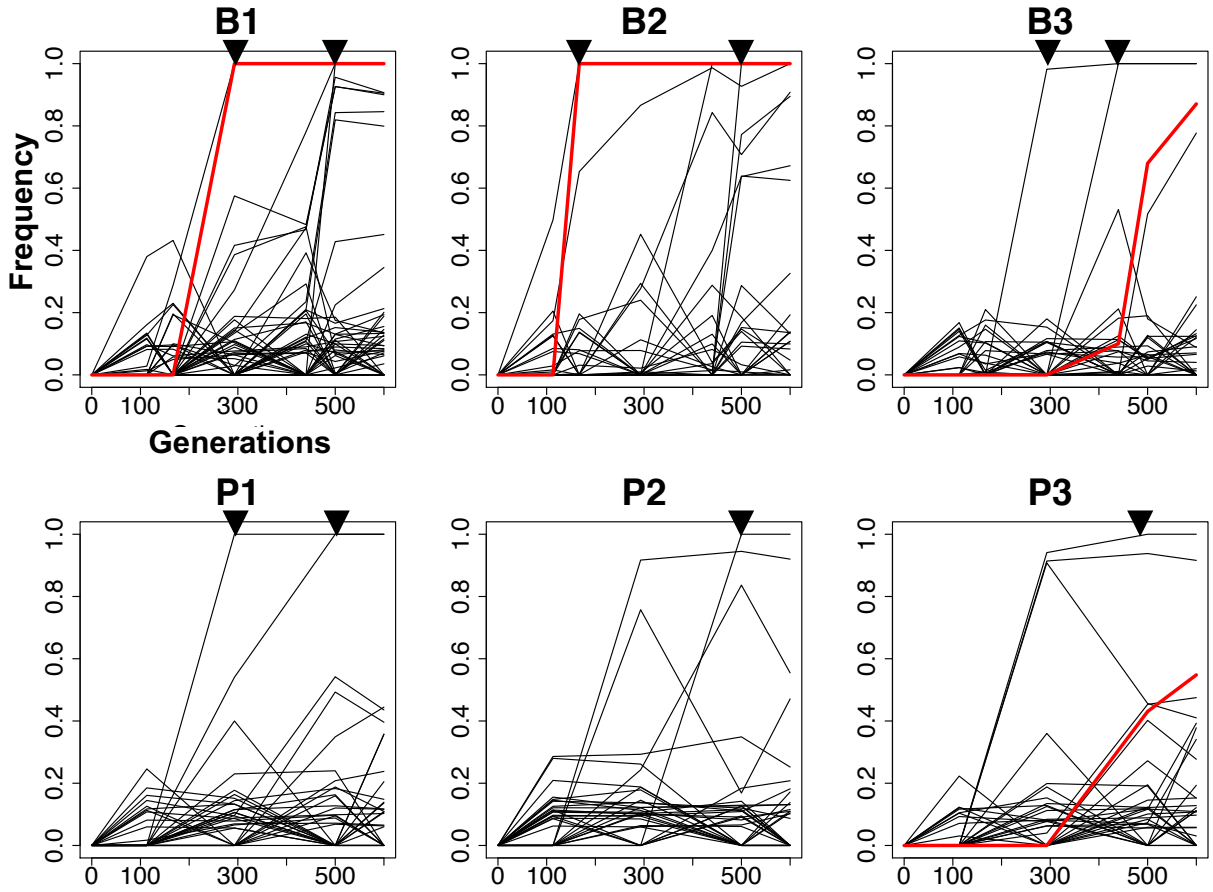
Total cumulative mutations	**874**
Biofilm only	535
Planktonic only	339
Total mutations at day 90	**583**
Biofilm only	382
Planktonic only	201
Total fixed mutations	**53**
Biofilm only	48
Planktonic only	5
Total fixation events	**10**

Table 1. Summarized mutation statistics from all six populations following 90 days of experimental evolution.

150 A characteristic known to be associated with positive selection is the increase in
151 nonsynonymous (NS) mutations over synonymous (S) mutations[37,27]. In following with
152 this trend, the normalized NS/S ratio for planktonic populations averaged 1.73 and for
153 biofilm populations 2.34. A total of 31 indels were also identified in the six evolved
154 populations (Table S2, Table S3) while, interestingly, no large structural variants were
155 detected. A large number of intergenic mutations were also identified (233 mutations, or
156 27.5%), many in likely promoter or terminator regions. The importance of intergenic
157 mutations still remains unclear, however recent work has implicated intergenic
158 regulatory regions as critical in *Pseudomonas aeruginosa* evolution in the laboratory
159 and during infections[20,38].
160 We hypothesized that the structured biofilm environment would maintain greater
161 genetic diversity than mixed liquid culture, but this ecological process is potentially at
162 odds with the diversity-purging effect of selection on beneficial mutations. For example,
163 a mutation that generally improves growth would be expected to sweep and eliminate
164 genetic variation, even though the biofilm phase of growth is predicted to increase the
165 probability that mutations become established within various subpopulations[5,39,40].
166 Planktonic populations would also be subject to similar effects of selective sweeps but
167 their admixture might limit the cooccurrence of contending mutations[41]. To summarize,
168 comparing populations at a given time point might not adequately capture the
169 longitudinal ecological and evolutionary dynamics affecting diversity. For this reason, we
170 sampled biofilm populations at six time points (17, 25, 44, 66, 75, and 90 days) and
171 planktonic populations at four time points (17, 44, 66, and 90 days) and focused on
172 temporal changes in all mutations supported by three reads on each of both strands.

173 As predicted, the nucleotide diversity of biofilm populations tended to be greater
174 at the end of the experiment than after only 17 transfers (day 17 vs day 90: $p = 0.0478$, t
175 $= 2.821$, $df = 4$ via two tailed t-test). Planktonic populations continued to select on new
176 mutations throughout the experiment, but they did not become more diverse by day 90
177 than at day 17 ($p = 0.239$, $t = 1.384$, $df = 4$ via two tailed t-test), indicating a process
178 where new mutations were displacing older ones. Biofilm populations also tended
179 toward greater diversity than planktonic populations later in the experiment, albeit not
180 significantly (planktonic vs biofilm, day 90, $p = 0.0749$, $t = 2.394$, $df = 4$ via two tailed t-
181 test; Figure S4a). These findings are consistent with the greater morphological variation
182 in biofilm populations than planktonic ones that we reported previously[6].
183 A major cause of the high genetic diversity is the prevalence of mutator alleles,
184 mutations that cause a genome-wide increase in mutation rate, in four populations. In all
185 biofilm populations and one planktonic population (P3) NS mutations in the DNA
186 mismatch repair (MMR) genes *mutS* and *mutL* became frequent or fixed. Two
187 populations, B1 and B2, independently evolved the same *mutS* mutation (T112P) that
188 has been shown to lower affinity for heteroduplex DNA in *E. coli*[42] and results in a 116-
189 fold increase in mutation rate in isogenic mutants (Figure S5). Genotypes containing
190 these mutations fix in populations B1 and B2 by days 44 and 25, respectively (Figure 2,
191 red trajectories). A premature stop mutation (W307*) in *mutS* also rose to intermediate
192 frequency (53%) in the P3 population by day 90. While we did not create an isogenic
193 mutant of this mutator allele, we predict that it acts as other *mutS* mutations with a
194 roughly 100-fold increase in mutation rate. The rise of *mutS* alleles caused these
195 populations to diverge genetically from the others, as seen from PCA analysis of

196 mutational frequency data (Figure 1B). A fourth mutator genotype evolved in the B3
197 population by a mutation in *mutL* (D467G) that caused a 16-fold increase in mutation
198 rate and rose to 83% frequency by day 90 (Figure 2; Figure S5). Consequently, each of
199 these mutator populations became enriched for transition and single-base indel
200 mutations, signatures typical of defects in mismatch repair[43,44,45] (Table S2).
201 .



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203 **Figure 2.** Evolutionary trajectories of inferred genotypes within six PA populations under
204 biofilm (B) or planktonic (P) selection. Genotypes containing mutator alleles are colored
205 red. Triangles indicate timing of the ten fixation events observed throughout the study.
206
207

208 **High diversity is a byproduct of clonal interference**

209 While mutator alleles are not beneficial themselves[44] and their dominant effect is
210 to introduce neutral or deleterious mutations, they can facilitate the acquisition of
211 combinations of beneficial mutations. A more beneficial genotype that has acquired
212 several beneficial mutations may be able to outcompete less fit lineages with fewer
213 mutations, overcoming competition among adaptive lineages that slows their rate of
214 increase. This dynamic is known as clonal interference or more generally as the Hill-
215 Robertson effect[46,47,48]. The ability to acquire many beneficial mutations and escape
216 clonal interference may be particularly advantageous when beneficial genetic variation
217 is limiting or, more likely in these populations, saturating[49,50].

218 As described above, hundreds of mutations rose to detectable frequencies in
219 these populations within ~200 generations and persisted. We used software recently
220 developed by our lab to group mutations into genotypes on the basis of shared, nested
221 frequency-trajectories[51]. Remarkably, between 30 and 47 genotypes each composed of
222 multiple mutations accumulated within each population throughout the experiment
223 (figure 2). The number of genotypes present at a given sampled timepoint continuously
224 increase in biofilm populations, whereas the maximum diversity in planktonic
225 populations occurred at day 44 (supplemental figure 4). Yet despite extensive variation
226 at both nucleotide and genotype levels, only 10 fixation events were observed involving
227 a total of 53 mutations. These selective sweeps involved genotypes with more
228 mutations in biofilm populations than in planktonic lines (Table 1), and two sweeps were
229 linked to mutator alleles in which 24 mutations fixed (Table S3). These results suggest
230 that the biofilm environment selected larger mutational cohorts, which could involve

more differentiated genotypes, to fix, whereas planktonic populations selected one or few mutations to fix. One cause of this pattern was the superiority of mutator genotypes containing more linked mutations, which were more common in biofilm lines. Combined, these findings suggest that the rapid rise of mutator alleles may have enabled escape from clonal interference, especially in biofilms.

To better understand the evolutionary processes within these populations, we visualized genotype frequencies over time by constructing Muller plots[5,52]. These figures demonstrate, for example, how new mutations within genotypes enable the spread of that lineage by outcompeting others and giving rise to new daughter lineages. The most conspicuous genotype invasions were the mutator genotypes in populations B1 and B2 containing 11 or 13 mutations (Figure 3; mutator ancestry depicted in color and fixation events depicted by vertical lines). These figures also illustrate effects of particularly consequential mutations that overtake others or give rise to further diversity as well as genotypes that arise simultaneously and coexist for hundreds of generations. This latter dynamic is consistent with either clonal interference or genotypes adapting to inhabit discrete niches, possibilities we evaluate below.

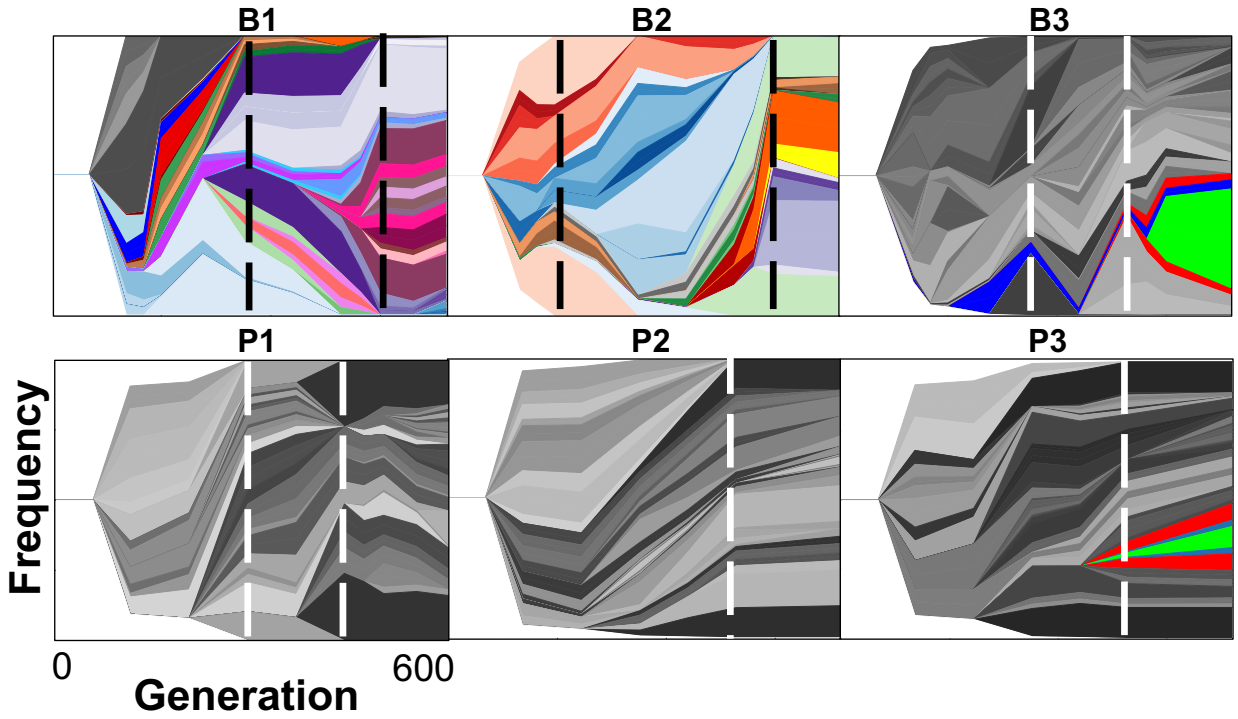
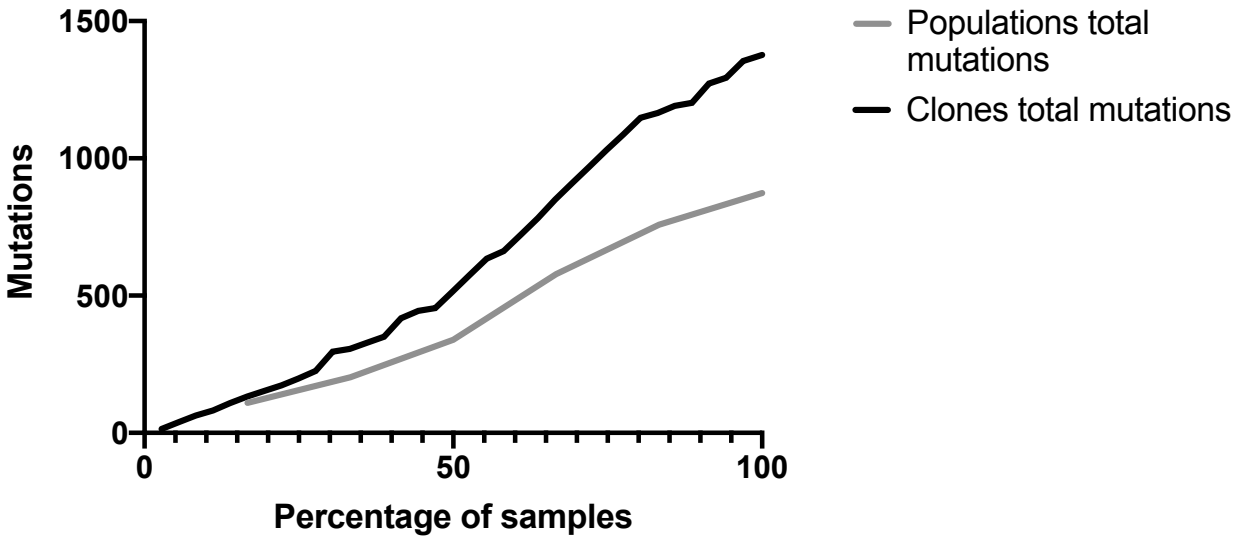


Figure 3. Genealogy and genotype frequencies over time. Each shade or color represents a different genotype and vertical area corresponds to genotype frequency, inferred by LOLIPop[51]. Shades of grey indicate genotypes not on a mutator background, whereas colors indicate genotypes on a mutator background. Dashed lines indicate timing of fixation events predicted by genotype trajectories (Figure 2). Biofilm populations are on the top row and planktonic populations are on the bottom row.

To test the genotype prediction algorithm producing these Muller plots and to identify other mutations undetected due to their low frequencies or sequencing coverage, we sequenced 26 clones from population B1 and 10 from population P1. B1 clones contained 48- 138 mutations per clone whereas clones from the P1 population only contained between 14- 28 mutations, results consistent with population sequencing results. However, many of these mutations were previously undetected in the metagenomes, indicating much greater genetic variation at frequencies below our detection limits from population sequencing (Figure 4) [53]. The 26 B1 clones belonged to a total of nine competing genotypes at day 90 (Figure S6), whereas the 10 planktonic

265 clones only belonged to two distinct genotypes (Figure S7). This finding confirms that
266 the biofilm population maintained greater genetic diversity than the planktonic
267 population at the end of the experiment.



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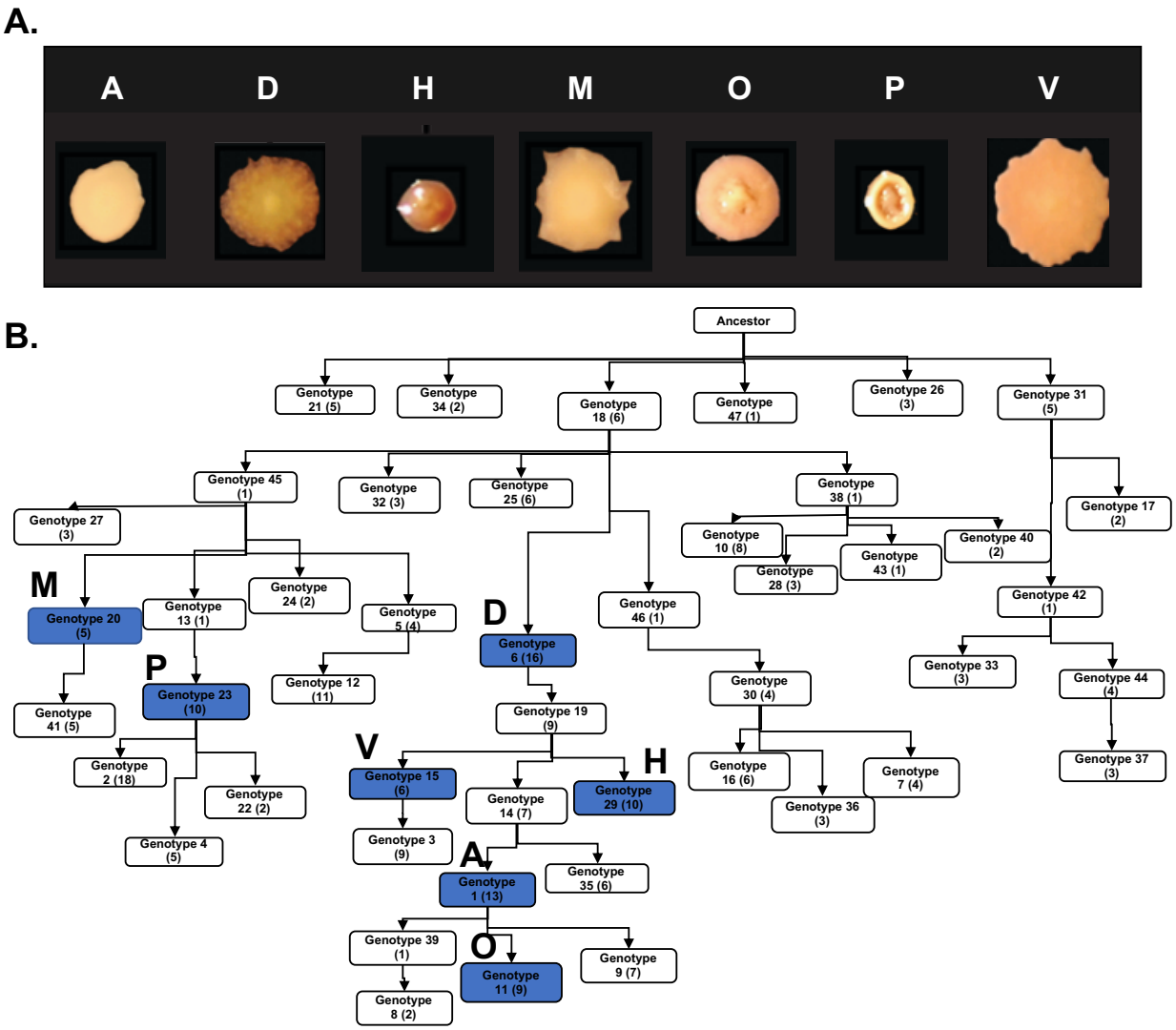
269 **Figure 4.** Clonal sequencing identifies more mutational variation than population level
270 sequencing. Collector's curve of total mutations identified by both population and clonal
271 sequencing. To enable comparison results are plotted by the percentage of samples
272 obtained (6 populations or 36 clones).
273

274 Another goal of sequencing these clones was to determine the genetic causes of
275 the distinct colony morphologies that we previously showed to associate with distinct
276 niches and phenotypes within the B1 biofilm population.[6] Each of the seven
277 morphotypes (Figure 5a) represented a different long-lived genetic lineage (Figure 5b)
278 that was separated by at least five mutations. This indicates the previous study that was
279 based solely on colony morphotypes captured a good representation of the population
280 diversity as it included seven of nine identified genotypes. We examined mutations
281 unique to each genotype in an effort to identify those responsible for their unique

282 phenotypes, which included the ability to form more productive biofilm communities in

283 mixture (Table 2).



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285 **Figure 5.** Clones representing different colony morphologies from the B1 population are
286 all at different points on the evolutionary trajectory of the B1 population. (A) Seven
287 colony morphologies were identified at day 90 and their phenotypes and ecological
288 interactions were characterized previously[6]. (B) Ancestry was inferred using LOLIPop[51].
289 Genotypes of named are shown in blue.
290

291 For five morphotypes we can predict a genetic cause of the high biofilm formation

292 and the related high c-di-GMP levels. Morphotypes A and H each independently

293 acquired mutations in *gacS*, a sensor kinase known to be associated with high biofilm

294 production[54], and which will be discussed more below. The additional three evolved
295 mutations in separate genes that have all been linked to *Pseudomonas* biofilm
296 production previously: mutant D acquired a mutation in the sensor histidine kinase
297 *rcsC*[55], M acquired a mutation in the type III secretion system gene *pscQ*[56], and V
298 acquired a mutation in the two component response regulator PA14_52250[57,58] (Table
299 2). While these are not the only mutations that distinguish the morphotypes from one
300 another, these are leading candidates that could produce their varied biofilm
301 phenotypes.

Clone	Gene	PA01 ortholog	Pseudocap function
A	bkdA1	bdkA1	
	flgB	flgB	Motility & Attachment
	oprC	oprC	Transport of small molecules
	oprC	oprC	Transport of small molecules
	PA14_06960	PA0534	Putative enzymes
	PA14_10830	no PA01 ortholog	Transcriptional regulators
	PA14_14530>/> PA14_14540		
	gacS	gacS	Two-component regulatory systems
	PA14_52760	PA0891	Transport of small molecules
	PA14_72320	PA5478	Membrane proteins
	ppc	ppc	Central intermediary metabolism
	thrB>/<PA14_72 520		
	TrpB	TrpB	Amino acid biosynthesis and metabolism
D	oprH</>napE	oprH</>napE	
	PA14_04050	PA0227	Biosynthesis of cofactors, prosthetic groups and carriers
	PA14_06030	PA0461	Fatty acid and phospholipid metabolism
	PA14_10910	PA4096	Transport of small molecules
	PA14_19450	PA3453	Hypothetical, unclassified, unknown
	PA14_23670	PA3127	Antibiotic resistance and susceptibility
	PA14_29260	PA2696	Transcriptional regulators
	PA14_31470	PA2557	Fatty acid and phospholipid metabolism
	PA14_48210	PA1239	Putative enzymes
	PA14_55380</< PA14_55390		
	magD	magD	Adaptation, Protection
	PA14_66380	PA5021	Membrane proteins
	pauR	pauR	Carbon compound catabolism
	PA14_71750	PA5437	Transcriptional regulators
	pvdJ	pvdJ	Adaptation, Protection
H	algU</>nadB	algU</>nadB	
	dsbA2	dsbA2	Translation, post-translational modification, degradation
	gbuR	gbuR	Transcriptional regulators
	PA14_19190	PA3471	Central intermediary metabolism
	prkA	prkA	
	fliT	fliT	Motility & Attachment
	gacS	gacS	Two-component regulatory systems
	PA14_58960	no PA01 ortholog	Hypothetical, unclassified, unknown
	PA14_71750	PA5437	Transcriptional regulators
	rpoB	rpoB	Transcription, RNA processing and degradation
M	flhA	flhA	Motility & Attachment
	PA14_07810	PA0599	Hypothetical, unclassified, unknown
	PA14_13150	PA3921	Transcriptional regulators
	PA14_43150</> PA14_43160		
	pscQ	pscQ	Protein secretion/export apparatus
O	PA14_12160	PA3992	Putative enzymes
	PA14_14390</> PA14_14400		
	PA14_27610>/> PA14_27620		
	PA14_27770	PA2812	Transport of small molecules
	PA14_51510	PA0988	Hypothetical, unclassified, unknown
	pstA	pstA	Transport of small molecules
	spuF	spuF	Transport of small molecules
	str	str	Antibiotic resistance and susceptibility
	trbL	trbL	
P	lpxC	lpxC	Cell wall / LPS / capsule
	mdoH	mdoH	Cell wall / LPS / capsule
	PA14_03000	PA0242	Putative enzymes
	PA14_13140>/> PA14_13150		
	PA14_1340>/<P A14_13350		
	PA14_18760	PA3523	Transport of small molecules
	PA14_43150	PA1652	Membrane proteins
	pilF	pilF	Motility & Attachment
	purB	purB	Amino acid biosynthesis and metabolism
	rep	rep	DNA replication, recombination, modification and repair
V	rsmE	rsmE	Translation, post-translational modification, degradation
	pqiB	no PA01 ortholog	Hypothetical, unclassified, unknown
	PA14_52250	PA0929	Two-component regulatory systems
	rsmI	rsmI	Translation, post-translational modification, degradation
	thrB>/<PA14_72 520		
	til5a>/>pldA		

303 Table 2. Unique mutations found in seven clones from an experimentally evolved biofilm
304 populations with distinct ecological roles[6].

305 **Targets of selection**

306 Evolution in large populations (>10^8) over a few hundred generations greatly
307 empowers selection relative to the effect of drift, even when mutation rates increase.
308 This allows us to predict that genotypes that rise to a detectable frequency in an evolve-
309 and-resequence experiment like this one are fitter than their ancestor. Further, parallel,
310 nonsynonymous mutations affecting the same gene provides strong evidence of an
311 adaptation[59]. Across all six populations, 53 mutations reach 100% frequency, as part of
312 the 10 genotype sweeps (Table 1), and each affected a unique gene except for two
313 each in *mutS*, *pitA*, and *rpoB* (Table S3). However, the population dynamics depicted in
314 Figure 3 and the clonal genomes summarized in Figure 5 demonstrate substantial
315 genetic variation that did not fix, so a more appropriate screen for parallelism might
316 involve a lower frequency threshold. At 80% frequency, eight genes were mutated more
317 than once: *lysC, mutS*, the intergenic region *fabI/ppiD*, PA14_23670*, pitA*, PA14_69980,
318 PA14_71750, and *rpoB*, and all six populations are represented. Mutations affecting
319 these genes were either small indels or nonsynonymous substitutions predicted to alter
320 or eliminate function. Because these genes were mutated in both planktonic and biofilm
321 environments, they likely enable adaptation to the growth medium, temperature, and
322 dilution timing. However, these represent only 17 of the 116 mutations that reach 80%
323 frequency, a remarkably flat distribution of high-frequency genotypes that suggests
324 many loci may provide a benefit under these conditions.

325 We next analyzed the complete set of parallel mutations found at all frequencies
326 for those passing a statistical threshold of expected random co-occurrence and
327 identified 153 genes or intergenic regions. Forty genes were mutated three or more
328 times, more than would be expected by chance, accounting for 179 mutations (21.1% of
329 all mutations; Table 3). Most mutations are nonsynonymous, as expected from positive
330 selection, and many suggest possible adaptive phenotypes.

331 The gene with the most independent mutations, *rluB* (PA14_23110) with 10 (8
332 nonsynonymous; Table 3, Table S3) found in four populations at frequencies of 12.1-
333 17.7%, is difficult to understand as an adaptation. *RluB* encodes a 23s rRNA
334 pseudouridine synthase responsible for modifying this rRNA at position 2605 during
335 maturation[60]. This gene was mutated in both biofilm and planktonic populations,
336 indicating it is not specific to lifestyle, but all mutations clustered in residues 313 and
337 323 found in the disordered C-terminus of this protein. In addition, one mutation in *rluC*,
338 another pseudouridine synthase, was detected. The literature remains vague about the
339 specific phenotypic roles of these 23S rRNA modifications and effects of disrupting
340 these genes are difficult to detect *in vitro*, but a recent study implicated these
341 modifications as important for growth under anoxia[61,62].

342 Fortunately, a wealth of literature has identified genes involved in the transition
343 from planktonic to biofilm growth in *P. aeruginosa*[16,9]. These genes are often well
344 annotated at http://pseudomonas.com for the PA14 genome or in related strains[63]. For
345 example, five mutations in PA14_02220, encoding the aerotaxis transducer Aer2, were
346 selected in two biofilm and one planktonic population, and may alter the response to low
347 oxygen that is common in biofilms[64]. In addition, three independent V334G mutations

348 were selected in each biofilm population in PA14_46030, encoding the ortholog of
349 biofilm dispersion locus BdlA[65],[66]. This cytoplasmic protein contains two sensory PAS
350 domains and a chemoreceptor domain that has been shown to sense and mediate
351 responses to oxygen levels[67]. Two-component regulatory systems are also commonly
352 implicated in this lifestyle transition, and in agreement with this model, 19 mutations in
353 14 genes encoding sensor kinases, response regulators, or hybrid complexes were
354 identified (Table 3, Table S3). Likewise, nine different nonsynonymous or indel
355 mutations affecting motility and attachment (e.g. *flgB/G, fliC/H/M, pilB/pilQ,* and *cupB3*)
356 were selected in both biofilm and planktonic populations, indicating advantages of
357 disrupting these processes in both lifestyles.

358 More surprising, the most common functional classification of mutated genes was
359 transporters. Nine transporters were mutated repeatedly including: *pitA, pntB, nosD,*
360 *pchF*, PA14_09300, PA14_22650, PA14_45060, PA14_46110, and PA14_47900, and
361 account for 24% (43) cases of parallelism (Table3, Table S3). These transporters
362 include genes with known function including inorganic phosphate transporter *pitA* and
363 copper transporter *nosD*, as well as those with unknown substrates including
364 PA14_46110 and PA14_45060. Of all transporters identified, *pitA* was the only one to
365 reach frequencies above 50% in any population. This led us to identify an additional
366 stressor acting on these populations as a byproduct of growth on arginine as a sole
367 carbon source, which is an alkaline pH (pH = 9.2 at 24 hr) caused by the release of
368 ammonia through deamination[68]. It remains unclear how these mutated transporters
369 produce adaptations, but they could be acting to balance pH or compensate for redox
370 imbalances or changes in proton motive force. In addition, 18 mutations affected genes

371 involved in various metabolic processes, including *argJ*, a key component of arginine
372 biosynthesis expected to be no longer required due to abundant arginine in the growth
373 media, *soxA*, encoding sarcosine oxidase[69], and *cobG*, involved in the aerobic pathway
374 of cobalmin (Vitamin B12) biosynthesis[70]. Further, three mutations are observed in each
375 of *napF*, *nqrE*, and *nuoG* potentially altering the energy conserving respiratory NADH
376 dehydrogenase chain[71]. We suspect that many of these nonsynonymous or indel
377 mutations are partial loss of function mutations in metabolic pathways that are
378 extraneous in the M63+arginine medium, though an exact mechanism is unknown.
379 There are four notable cases of mutated genes that are lifestyle specific. Four
380 genes (*rpoB, gacS*, PA14_71750, and PA14_13150) are only mutated in biofilm
381 populations, and in all cases, mutations rise to greater than 90% in at least one
382 population (Table 3, Table S3). Mutations in *rpoB* and other RNA polymerase genes
383 (*rpoACD*) have been frequently identified as adaptations during evolution
384 experiments[72],[73]. One explanation in *Escherichia coli* suggests they are adaptive for
385 growth in minimal media by redistributing RNA polymerase from small RNA promoters
386 (i.e. rRNA) to rate-limited promoters required for anabolic processes from a limited set
387 of carbon sources, such as arginine in the case of this study[74]. GacS is a
388 sensor/regulator hybrid known to govern a broad range of traits involved in virulence,
389 secondary metabolism and biofilm formation through the regulation of small rRNA's[75].
390 Further, *gacS* has been shown to be involved in the switch from a hyperadherent small
391 colony phenotype back to a wild type phenotype indicating that loss of GacS function is
392 beneficial in biofilms[54]. The additional two biofilm specific loci, PA14_13150 and

393 PA14_71750, are less understood transcriptional regulators, however PA14_71750 has
394 been previously associated with biofilm adaptation in *Pseudomonas*[76].
395 The rarity of fixation events and the high numbers of genes with parallelism
396 throughout the experiment provides further evidence of competition between adaptive
397 lineages in a population, or clonal interference[41]. Given a set of genes in which
398 mutations are adaptive, we would expect each competing genotype to contain different
399 combinations of these mutations, with the most beneficial of them repeatedly evolving
400 on different genetic backgrounds. As predicted, we identified 55 cases of within-
401 population gene level parallelism (4 – 16 cases per population), with most (87%)
402 predicted to be on different genotypes. This result also demonstrates the polygenic
403 capacity for adaptation by *P. aeruginosa* growing in minimal arginine medium.
404

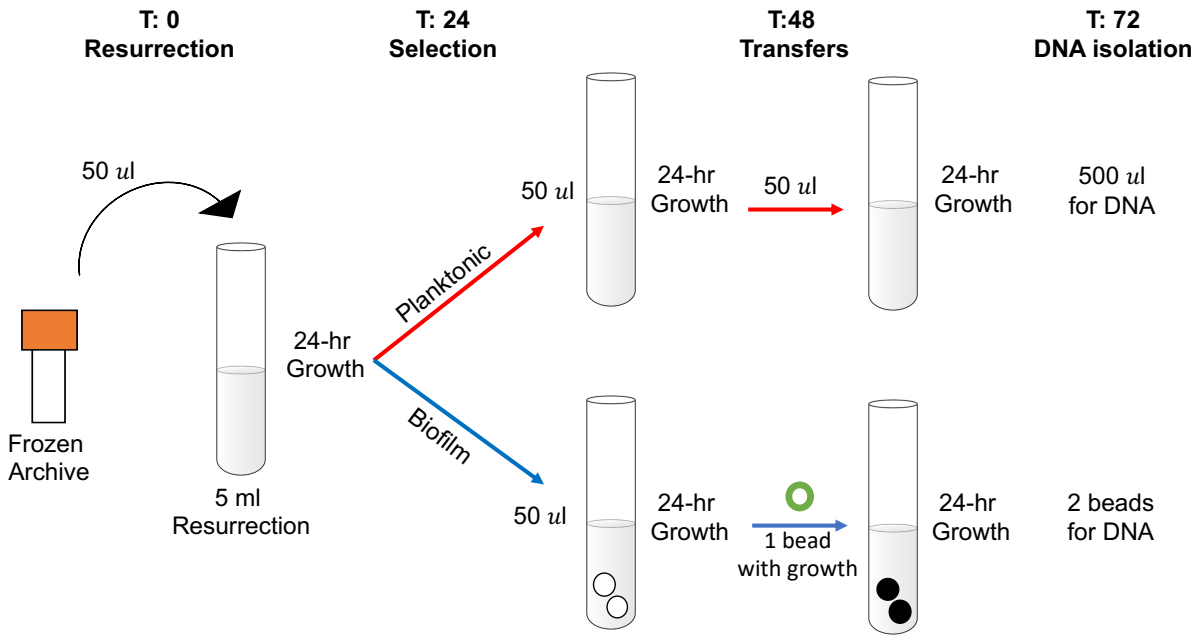
Gene	Total cases	Type			Biofilm	Planktonic	Highest frequency						Function
		NS	S	Indel			B1	B2	B3	P1	P2	P3	
argJ	3	3	0	0	2	1		13.5	10.5			11.4	Arginine biosynthesis
soxA	3	2	1	0	1	2			10.6		14.7	11.4	Carbon compound catabolism
aer2	5	3	2	0	3	2		9.6	10.9	10.2		14.2	Chemotaxis
bdlA	3	3	0	0	3	0	14.4	14.7	13.5				Chemotaxis
cobG	3	2	0	1	1	2		61.8			13.6	13.1	Cofactor biosynthesis
mutS	3	3	0	0	2	1	100	100				52.6	DNA replication
napF	3	3	0	0	2	1	13.8		10.9			10.7	Energy metabolism
nqrE	3	3	0	0	1	2		11.7			10.4	11.2	Energy metabolism
nuoG	3	3	0	0	1	2	12.9			12.1	13.9		Energy metabolism
PA14_53110	7	7	0	0	2	5		14.8	12.8	12.9	14.4	12.1	Putative enzymes
pslI	7	7	0	0	3	4	16.7	16.4	14.6	16	14.7		Putative enzymes
algF	3	3	0	0	2	1	13.1			10.6			Secreted Factors
fha1	4	4	0	0	3	1	27.6		25.1	35.8			Secreted Factors
rpoB	4	4	0	0	4	0	100	66	100				Transcription
PA14_13150	3	3	0	0	3	0	46.3	17.8	100				Transcriptional regulators
PA14_18200	6	6	0	0	5	1	22.3	22.1		12.8			Transcriptional regulators
PA14_51840	3	3	0	0	0	3					12	16.6	Transcriptional regulators
PA14_58510	5	3	2	0	4	1	23	15.7		15.5			Transcriptional regulators
PA14_71750	3	3	0	0	3	0	93.9	89.7					Transcriptional regulators
dsbA2	4	2	2	0	3	1	17.1	11.4	20.8			10.5	Translation
rluB	10	8	2	0	4	6	10.8	17.7			14.7	13.2	Translation
nosD	5	4	1	0	1	4	14.1				15.3	12.9	Transporter
PA14_09300	3	3	0	0	2	1	16		15.2	18.6			Transporter
PA14_22650	7	7	0	0	3	4	43.2	24		40	28	37.8	Transporter
PA14_45060	6	6	0	0	2	4	24.4	19.1		19.5	14.1	13.6	Transporter
PA14_46110	7	7	0	0	1	6			15.5	13.7	13.6	10.7	Transporter
PA14_47900	3	3	0	0	0	3						19.9	Transporter
pchF	3	3	0	0	3	0	18.4		19				Transporter
pitA	4	2	0	2	2	3		91.1		100	75.8	100	Transporter
pntB	5	3	2	0	1	4	11.3				13.9	15.6	Transporter
PA14_10770	3	2	1	0	1	2		100		12.2		26.3	Two-component regulatory systems
PA14_32300	3	3	0	0	2	1	17.1					15.3	Two-component regulatory systems
gacS	3	3	0	0	3	0	85.2	92.2					Two-component regulatory systems
PA14_01160	3	3	0	0	1	2	14.3				13.8		Unknown
PA14_20510	6	6	0	0	3	3	9.6	11.5		11	11.9	11.1	Unknown
PA14_31070	10	5	5	0	4	6	19.3	16.9		16.2	16.9		Unknown
PA14_32830	6	6	0	0	2	4		13.8	12.7	12.1	13.5	12.1	Unknown
PA14_54810	3	3	0	0	3	0	11.7		7.5				Unknown
PA14_58070	6	6	0	0	5	1	13.6	17.5	12.9		20.8		Unknown
PA14_69010	5	5	0	0	3	2	20.1	12.2	13	12.5		12.4	Unknown

Table 2. Genes (n=40) with 3 or more evolved mutations. NS: nonsynonymous, S: synonymous, indel: insertion/deletion. Shading corresponds to frequency. Function from PseudoCap via pseudomonas.com.

Selected genotypes are adapted to both growth conditions and lifestyle

Most cases of gene-level parallelism were shared between biofilm and planktonic conditions and indicate adaptations to the common growth media. However, minority variants could include environment specific adaptations. For example, our bead transfer model simulates the complete life cycle of the biofilm – attachment, assembly, dispersal, and reattachment – each of which could select for discrete phenotypes. To distinguish

416 genotypes adapted to either biofilm or planktonic growth, we grew 90-day samples of
417 evolved populations B1, B2, P1, and P2 for two days in each of these conditions
418 independently (Figure 6). We hypothesized that these treatments would enrich
419 genotypes adapted to either condition that we could resolve by resequencing treated
420 populations and correlating their relative frequencies. Mutation frequencies that depart
421 from the diagonal of equivalency could represent genotypes that are adapted to biofilm
422 or planktonic growth.



423

424 **Figure 6**. **Methods for isolating DNA to identify genotype by environment**
425 **interactions**. 90 day populations were resurrected from frozen archives in planktonic
426 environments for 24 hours. Each population was then split and subjected to two days of
427 either planktonic selection, via 1:100 daily transfers, or biofilm selection, via the bead
428 model. After the second day of selection 0.5 ml of planktonic culture and two biofilm
429 beads were used to obtain the planktonic and biofilm DNA samples, respectively (see
430 methods for more detail).
431
432 In all four populations tested we found significant enrichment in both biofilm and
433 planktonic environments (Figure S8). As expected, the two biofilm populations tested
434 had more biofilm enriched mutations than planktonic enriched (37 and 55 biofilm

435 enriched compared to 4 and 2 planktonic enriched), though planktonic populations had
436 roughly equal numbers of biofilm and planktonic enriched mutations (8 and 10 biofilm
437 enriched versus 10 and 11 planktonic enriched; Table S4). Upon closer examination,
438 the B2 population appears to contain a set of 36 mutations belonging to 4 genotypes
439 enriched for biofilm fitness (Table S4). These genotypes also rose in frequency between
440 days 75 to 90 in the evolution environment and appear to be generally adaptive.
441 However, these experiments did not generally cause large (>10%) shifts in genotype
442 frequency, which suggests that they correspond to adaptations to the overall
443 experimental conditions and not just these growth phases.
444
445 **Discussion:**
446 We used experimental evolution to examine how the opportunistic pathogen
447 *Pseudomonas aeruginosa* (PA) adapts in laboratory culture in a medium known to
448 promote biofilm formation[68], comparing simple serial dilution with a model simulating the
449 biofilm life cycle[2]. After 90 propagations (~600 generations) we observe more
450 mutations, fewer fixation events, and earlier prevalence of mutator genotypes than
451 observed in other evolution experiments. These findings are consistent with two
452 potentially overlapping processes: 1) the existence of many different niches in the form
453 of metabolic or ecological strategies within the laboratory environment that select for a
454 variety of specialized genotypes or 2) high clonal interference, involving many
455 genotypes with roughly the same fitness competing against one another in the same
456 niche. The repeated evolution of mutations in the same gene in the same population
457 provides the clearest evidence of this clonal interference, because such mutants are

458 likely to share similar phenotypes. For example, within the B1 population mutations in
459 *gacS* evolve independently in two genotypes (Table S3). We confirmed this finding
460 through clonal sequencing, finding clones H and A to each contain a *gacS* mutation in
461 their distinguishing genotypes (Table 2), and further, these clones were previously
462 shown to compete for a common niche[6]. While clonal interference likely contributes to
463 the high genetic variation observed in these populations, we also predict that multiple
464 niches exist within each population as our previous study indicated[6]. As further
465 evidence, most cases of gene-level parallel mutations reached only 20-30% frequency
466 within their respective populations (Table 3). We hypothesize that this results from
467 multiple coexisting ecotypes within each population, a phenomenon that has been
468 shown in previous evolution studies, particularly with biofilms[5,50,77].

469 To identify selected traits during evolution in a minimal medium containing
470 arginine as sole energy source, we focused on the 40 loci mutated most frequently
471 (Table 2). This medium was selected because it had previously been shown to induce
472 high biofilm production by *Pseudomonas*,[68] more specifically by increasing intracellular
473 levels of the secondary messenger molecule c-di-GMP through the two diguanylate
474 cyclase proteins RoeA and SadC. Further, arginine was the only amino acid to
475 completely repress swarming motility, indicating this carbon source could be a cue to
476 enter an attached lifestyle[33]. As we were interested in seeing how *P. aeruginosa*
477 continues to evolve under conditions where it is already proficient for biofilm, this media
478 choice was suitable. However, it is a naturally stressful environment because arginine
479 catabolism by arginine deiminase produces ammonia as a byproduct and hence an
480 alkaline environment[78,79]. These conditions are opposed to previous evolution

481 experiments with *P. aeruginosa* in which media with multiple carbon sources are used
482 to mimic an infection, and the medium often becomes acidic[7,68,33,52]. The exact effects
483 of arginine as the sole carbon and nitrogen source on the metabolic evolution of *P.*
484 *aeruginosa* are unclear and the topic of a future study, however it is likely that arginine
485 as a sole carbon and nitrogen source produced strong selection and may have been the
486 dominant pressure.

487 The remarkable, unexpected consequence of this experimental design was a
488 lack of convergent evolution affecting a small subset of mutated genes in replicate
489 populations. Rather, dozens or even hundreds of mutations in nearly as many genes
490 appear to have been selected, suggesting polygenic adaptation involving many
491 metabolic and regulatory systems (Figure 3). Such polygenic selection in large
492 populations increases the probability of clonal interference, whereby each lineage
493 acquires its own combination of adaptations, but any given genotype would struggle to
494 become dominant. Consequently, according to this model, fixation events were rare,
495 with only ten across all six replicate populations over 600 generations. Further, one of
496 the most commonly mutated pathways was DNA mismatch repair, which increased
497 mutation rates of linked genotypes in the four populations in which they evolved. We
498 hypothesize these mutator genotypes provided the means to escape clonal interference
499 because they had a higher probability of producing multiple, linked beneficial mutations
500 that could outcompete other genotypes.

501 Our previous study of these populations predicted that the different colony
502 morphotypes represented lineages that had evolved to occupy distinct ecological niches
503 within a biofilm population and interact with one another[6]. This genomic study confirms

504 that these genotypes indeed represent different long-lived lineages within the B1
505 population. Each clone possesses different mutations that are candidates for the causes
506 of their varied fitness versus the ancestral PA14 strain, levels of cyclic-di-GMP, and
507 biofilm-associated traits[6]. However, considerably more variation was detected by
508 population sequencing than these lineages could explain, and likewise, sequencing
509 additional clones led to an ever-increasing census of new mutations (Figure 4). These
510 results reinforce the polygenic model of adaptation to the biofilm-inducing arginine
511 medium and the diversity of genotypes that can generally improve growth and survival
512 or inhabit the niches illustrated in our prior study[80,6]. More generally, this work has
513 implications for understanding processes underpinning the evolution and maintenance
514 of high levels of genetic diversity, including how different nutrient sources influence this
515 process. Although differences in the mode of growth have been shown to influence
516 genetic diversity[81,82,9,83], the metabolic environment may be equally or even more
517 important by exposing varied fractions of the genome to selection. Further, this study
518 provides a concrete example of how microbial populations evolving in a new
519 environment can adapt and diversify without the periodic losses of genetic variation
520 caused by selective sweeps. This dynamic was demonstrated in the Long-Term
521 Evolution Experiment with *E. coli* after many thousands of generations had passed and
522 the rate of adaptation had slowed[43,77]. Yet here, populations amassed extreme fitness
523 gains over only hundreds of generations while maintaining many lineages, suggesting
524 that the *P. aeruginosa* genome encodes vast potential to meet new environmental
525 challenges.
526

527 **Materials and methods**

528 **Experimental evolution**

529 Replicate populations were experimentally evolved as previously reported (Flynn
530 et al, 2016). Briefly, the ancestral *Pseudomonas aeruginosa* (PA14; NC_008463) strain
531 was reconstituted from a freezer stock in Luria-Bertani broth (LB; 1.0% w/v tryptone,
532 0.5% w/v yeast extract, 1.0% w/v NaCl). Six replicate populations were started with
533 1:100 dilutions of an overnight growth into 5 mL M63 media (15 mM $(NH_4)_2SO_4$, 22 mM
534 KH_2PO_4, 40 mM K_2HPO_4, 40 mM galactose, 1 mM $MgSO_4$, 25 μM $FeCl_2$, and 0.4% w/v
535 L-arginine[33]). Three populations were propagated under liquid grown, planktonic,
536 selection (P1, P2, P3), while three populations were propagated, concurrently, under
537 constant biofilm selection[5,2,6] (B1, B2, B3). Planktonic selection consisted of daily 1:100
538 liquid dilutions into 5 mL M63 media, while biofilm selection consisted of the transfer of
539 one 7 mm polystyrene bead every 24 hours to a new tube of 5mL M63 media containing
540 one clean bead. This biofilm selection method requires the entire biofilm lifecycle of
541 dispersal, attachment, and growth between every transfer. All six populations were
542 propagated for 90 days (~600 generations) in 18x150 mm test tubes at 37 °C on a roller
543 drum at 30 rpm.

544 Archives were made for all populations at days 17, 25, 33, 45, 66, 75, and 90 in
545 8% dimethyl sulfoxide (DMSO) at -80 °C. Planktonic populations were archived by
546 freezing a 1 ml aliquot of the 24-hour culture, whereas biofilm populations were archived
547 by sonicating 48 hr beads in 1 ml PBS, and then freezing the PBS with 8% DMSO.

548 **Phenotypic characterization of evolved populations**

549 All populations were revived from freezer stocks for characterization by adding
550 50 µl of freezer stock to a culture of 4:1 M63 media to LB media, with trace elements to
551 recreate previous water mineral content (110.764 g/L $CaCl_2$, 0.824 g/L $MnSO_4$, 33.48
552 g/L KBr, 132.737 g/L Na_2SiO_3, 123.485 $ZnSO_4$, 0.178 g/L $CoCl_2$ *$6H_2O$, 1.458 g/L
553 $CuSO_4$). This media combination was used to minimize selection effects of growing in a
554 fully complex medium, while providing enough nutrients for the frozen cells to revive.
555 Resurrected populations were grown for 24 hr at 37 °C on a roller drum before being
556 vortexed for 10 seconds, twice, to ensure biofilm growth was disrupted from the sides of
557 the glass tube.

558 Growth curves were measured for resurrected populations in 96 well plates, at 37
559 °C. All growth curves were started at an OD_{600} of 0.01 and growth was measured every
560 10 min following 9 min of shaking for 24 hr in M63 media with trace elements. Maximum
561 growth rate was determined for the average of all replicates, by using the following
562 equation:

563 $$\mu = \frac{\Delta \log (OD600)}{\Delta t}$$

564 Census population size was calculated by diluting resurrected populations 1:100
565 in 5 mL M63 media with trace elements. One 7 mm polystyrene bead was added to
566 each biofilm culture at time zero. After 24 hr growth, all populations were vortexed for 30
567 seconds and then diluted and plated in triplicate on tryptic soy agar and grown at 37 °C
568 for 48 hrs. Average colony counts of the three technical replicates were reported for
569 each population. Additionally, populations plated for census population size were also
570 used for pH measurements as they were complete replications of experimental
571 conditions. Average pH of triplicate readings are reported.

572 Biofilm assays were performed on resurrected populations diluted to an OD_{600} of
573 0.01 in M63 media and grown in a 96 well plate under static conditions for 24 hr at 37
574 °C. Media was discarded and the plate was washed twice with deionized H_2O, which
575 was also discarded. Wells were then stained with the addition of 250 μl 0.1% crystal
576 violet solution for 15 minutes. Plates were rinsed to remove excess dye and the plate
577 was allowed to dry for 24 hours. A de-stain solution (95% ethanol, 4.95% dH_2O, and
578 0.05% Triton X-100 (fisher bioreagents)) was added to wells (250 μl per well), and, after
579 a 15-minute incubation, was transferred to a new 96 well plate. Crystal violet
580 absorbance readings were measured at 590 nm. Reported values are the average of 21
581 replicates, done across three plates (7 technical replicates per plate). Data were
582 analyzed with a one-way ANOVA with post hoc Tukey test.
583 Motility assays were performed as described previously (Ha, et al 2014). Briefly,
584 pipet tips were dipped into resurrected populations and stabbed into motility agar (0.3%
585 agar, 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 0.2% glucose, 0.5% casamino acids,
586 1 mM $MgSO_4$). Increased or decreased motility was determined for all day 90 evolved
587 populations as the diameter of bacterial growth after 24 hr growth at 37 °C compared to
588 the clonal ancestor. Reported values are the average of 5 replicates. Results were
589 analyzed by one-way ANOVA with post hoc Tukey test.
590 **Mutation rate estimate**
591 Isogenic mutants of biofilm evolved mutator alleles *mutS* T112P and *mutL*
592 D467G were used as reported previously[6]. Briefly, the two mutator strains and the PA14
593 ancestor were revived from freezer stocks by streaking on ½ trypic soy agar. After 24
594 hrs growth individual colonies were used to start 30 replicate 5-ml cultures for each

595 strain. After 24 hrs growth at 37° F on a rollerdrum, populations were diluted and plated
596 on ½ tryptic soy agar both with and without 1mg/ml Ciprofloxacin as a selective agent.
597 Colonies were counted after a 24 hour incubation period on both the antibiotic and non-
598 antibiotic plate. We then calculated the fold change in mutation rate using the maximum
599 likelihood ethod of Gerrish (2008). We performed measurements for all strains
600 simultaneously to minimize variation. Reported values are the average fold change of all
601 replicates.

602 **Genomic sequencing of evolved metagenomes**

603 DNA was isolated from biofilm populations at 113, 167, 293, 440, 500, and 600
604 generations (17, 25, 44, 66, 75, and 90 days) and from planktonic populations at 113,
605 293, 500, and 600 generations (17, 44, 66, and 90 days). Culture media for DNA
606 isolation was composed of 4:1 M63 to LB media. DNA was isolated from 1 mL
607 resurrected populations using Qiagen's DNeasy Blood & Tissue Kit. Library construction
608 was done using the Illumina Nextera kit as described previously (Baym 2015). Libraries
609 were sequenced on Illumina's NextSeq 500 platform by the Microbial Genome
610 Sequencing Center at the University of Pittsburgh. Between 13,545,344 and 46,890,487
611 reads were obtained for each sample, resulting in coverage of 265x - 860x per sample.

612 **Determining mutational frequencies from sequencing data**

613 Initial metagenomics sequencing efforts of the six populations resulted in 121.2
614 gbps of 2x151 bp sequencing reads, that we trimmed and quality filtered with
615 trimmomatic v0.36 using default parameters[84]. The breseq software package v0.31.0
616 was used to align the filtered reads to the reference PA14 genome (NCBI's RefSeq
617 database: NC_008463.1) and make polymorphism calls[85]. All populations were run

618 using the polymorphism mode of breseq with a sliding window of 5 bp and a quality
619 score cutoff of 10. This analysis was done for every population and every time point
620 sequenced. The genome of the ancestral strain differed from the reference by 435
621 mutations, which were removed from all evolved population samples before any
622 downstream analysis to remove mutations that did not evolve over the course of the
623 evolution.

624 All 12,250 resulting mutation calls were filtered in R (v3.5.1, [5]) to require a
625 mutation to be called by at least 3 reads on each strand (positive and negative). Finally,
626 mutations were filtered to remove regions of high polymorphism. Due to the abundance
627 of repetitive regions in the PA14 genome the false positive rate is high. We therefore
628 examined each call using the alignments produced by breseq and excluded mutations
629 with high sequence variation within the 15 bases both upstream and downstream of the
630 called mutation. Mutations were also filtered for known regions of misalignment such as
631 repeat regions before consolidation into time-series tables containing all real mutated
632 loci for each evolved population (Table S1). The resulting 874 mutations were then used
633 as input in the software package LOLIPop Version 0.8.1. using –similarity-cutoff value of
634 0.1 for P1, P2, P3, and B1 and a --similarity-cutoff of 0.2 for populations B2 and B3.
635 Increased similarity cutoffs increased the amount of mutations that were grouped into a
636 genotype. Types of mutations were broken down by both nucleotide and amino acid
637 mutation for all evolved populations in R. All scripts for the R filtering and analysis are
638 available on github at: https://github.com/KatrinaHarris23/PALTEanalysis

639 **Calculating dN/dS ratios**

640 To determine the dN/dS ratios, first the dN/dS ratio for each of the 64 codons was
641 calculated. The codon usage for the ancestral, PA14, genome was obtained from
642 (http://www.kazusa.or.jp/codon/). The two resulting matrices were multiplied to obtain a
643 neutral ratio of nonsynonymous to synonymous substitutions (dN/dS ratio) of 2.96 for
644 the ancestral UCBPP-PA14 genome (script for calculating this can be found on github
645 here https://github.com/KatrinaHarris23/PALTEanalysis/blob/master/dnds_calculator).
646 All reported dN/dS ratios are standardized according to this number with the final
647 number reported being a result of the following equation:

648 $$dN/dS = \frac{\frac{\#nonsynonymous}{\#synonymous}}{2.96}$$

649 **Experimentally determining ecologically enriched mutations**

650 To determine if mutations were specific to the biofilm lifestyle steps of swimming
651 or attaching, we isolated DNA from evolved populations after additional strong selection
652 in two distinct environments (Figure 5). Populations were resurrected from day 90
653 archives for the B1, B2, P1, and P2 populations by using 50 ul of the frozen stock into 5
654 ml resurrection media. After 24 hr growth at 37°C each culture was used to inoculate 2
655 identical 5 ml M63 arginine media cultures. Two 8mm polystyrene beads were added to
656 one replicate, creating movable biofilm, before both inoculated cultures were placed at
657 37°C. One transfer was performed for all populations at 24 hr. Swimming was selected
658 for by transferring liquid phase growth form replicates with no beads to a new tube with
659 5 ml M63 arginine media. Additionally, one bead from replicates with beads was rinsed
660 with PBS and subsequently transferred to a fresh tube of 5 ml M63 media with two
661 differently colored beads. After a final 24 hr incubation at 37°C “planktonically enriched”

662 DNA samples were isolated from 0.5 ml liquid phase from replicates without beads.
663 Growth from the two colored beads, first rinsed in PBS, then sonicated in 1 ml PBS was
664 used in total for our “biofilm enriched” DNA sample. DNA was isolated using the
665 Quiagen DNeasy Blood and Tissue kit.

666 Between 328 - 410x coverage was obtained for all eight samples. Mutations were
667 filtered by removing all ancestral mutations, and only mutations detected by both biofilm
668 and planktonic samples for a given population were considered. This sequencing effort
669 resulted in 979, 1021, 879, and 876 mutations in the B1, B2, P1 and P2 populations
670 respectively. Relative frequencies of mutations in the two environments were plotted in
671 R using the ggplot2 package (Figure S8; code used in this analysis is found on github
672 https://github.com/KatrinaHarris23/PALTEanalysis/blob/master/201020_ecological_inter
673 actions.R).

674 **Clonal sequencing**

675 To acquire clonal DNA, populations were resurrected in 4ml M63 arginine media
676 with 1ml LB and grown for 24 hr at 37 on a roller drum. Populations were then plated on
677 ½ Tsoy plates down to the -6 dilution to isolate individual colonies. After 24 hr growth
678 and 37 followed by 48 hr room temperature growth clones were selected ensuring that
679 all visible colony morphologies were sampled. Colony growth was picked up and used
680 as inoculum for 5 ml LB cultures. After 24 hr at 37 on a roller drum 1 ml of culture was
681 used for archiving (9% DMSO) and 0.5 ml was used for DNA isolation using the DNeasy
682 Blood and tissue kit. Named clones that were reported previously[6] were already
683 archived and archives were used for resurrection directly into 5 ml LB.

684 DNA sequencing was performed as with population sequencing, but only
685 requiring 30x coverage per sample. All reads were trimmed with trimmomatic, as
686 populations were, and variant calling was performed using Breseq software default
687 settings. As clonal samples are only looking for mutations that fix in each sample,
688 filtering was only performed to remove ancestral mutations before analysis.

689 **Statistical testing**

690 Alpha diversity was calculated for all detected mutations in each population at
691 each time point. We used the “shannon”, “simpson” and “invsimpson”, modes of the
692 diversity function in R. Populations were grouped by growth environment, biofilm (n = 3)
693 and planktonic (n = 3), and by mutator allele presence (n = 4) or absence (n = 2). T-
694 tests were performed in Prism (version 8, www.graphpad.com, Figure S4).

695 To perform the Fisher’s exact test, a table of loci observed mutated 2 or more
696 times was generated from the final mutation calls. Loci lengths were determined using
697 http://pseudomonas.com as either gene lengths of the distance between surrounding
698 genes in intergenic cases. Fisher’s exact test was performed using this compiled list:
699 the numbers of mutations per loci, the total number of mutations observed (624), and
700 the PA14 genome length (6537648). Testing was performed using the fisher.test()
701 function in R and only loci with p-values lower than 0.05 were used for parallelism
702 analysis. 153 loci were tested for parallelism and then the number of false positives
703 were reduced using the Benjamini-Hochberg correction with a false discovery rate of
704 5%. The equation used was (i/m)Q where i is the p-value’s rank, m is the total number
705 of tests, and Q is the false discovery rate.

Finally, ecological enrichment significance was calculated in R by using cooks distance. First, a linear regression was calculated for each population using planktonic versus biofilm frequencies using the lm() function. Cooks distance was then calculated for each point, using cooks.distance() function. A mutation was considered significantly enriched if it's cooks distance was four times the mean distance or more.

Principal component analysis (PCA)

All PCA calculations and plotting were performed in R. Principal components were computed from the biofilm production, swimming motility, and maximum growth rate (Vmax) data, and from the mutational frequency data at each sampled day using the prcomp() function. Plotting was done using the autoplot() function that is part of the ggfortify package.

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