Cross-feeding maintains diversification in an experimentally evolved microbial community

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Competing interests

The authors declare that they have no conflict of interest.

Running title: Bacterial diversification persists only in a community.

Abstract

Microbial communities are incredibly diverse. Yet, the eco-evolutionary processes originating and maintaining this diversity remain understudied. Here, we investigate the effect of interspecies interactions on the generation and maintenance of diversity in microbial communities. We studied a commensal interaction in a community of *Acinetobacter johnsonii* and *Pseudomonas putida* where the latter species cross-feeds on leaked resources produced by *A. johnsonii*. By evolving four experimental replicates in monoculture and co-culture for 200 generations, we observed the diversification of *P. putida* into two distinct morphotypes that differed from their ancestor by single-point mutations. One of the most prominent mutations hit the *fleQ* gene encoding the master regulator of flagella and biofilm formation. We experimentally confirmed that *fleQ* mutants were unable to swim and formed less biofilm than their ancestor, but they also produced higher yields. Interestingly, the *fleQ* genotype swept to fixation in monocultures but not in co-cultures. In co-cultures, the two lineages stably coexisted for approximately 150 generations. We hypothesized that *A. johnsonii* modulates the coexistence of the two lineages through frequency-dependent selection. However, invasion experiments in monoculture and co-culture did not support this hypothesis, suggesting other mechanisms maintain coexistence. Overall, our study shows that interspecies interactions play an important role in maintaining the diversity of newly formed lineages in microbial communities.

Importance

In nature, bacteria live in microbial communities and interact with other species, for example, through the exchange of resources leaked into the external environment (i.e., cross-feeding interactions). The role that these cross-feeding interactions play in the formation of new bacterial

'species' remains unexplored. Using a simple bacterial system in which one species cross-feeds resources to a second species (commensal species), we showed that the commensal species diversified into two subpopulations that persisted only when the cross-feeder partner was present. We further observed loss-of-function mutations in flagellar genes in both monocultures and co-cultures. Instead, mutants that retained the ability to swim and form biofilms were exclusively observed in co-cultures. These findings suggest that motility and surface attachment traits are important during cross-feeding, even in a well-shaken environment. Given that nutrient leakage is pervasive in microbial communities, the findings from this study have the potential to extend beyond our specific bacterial system.

Keywords: interspecies interactions, cross-feeding, evolutionary tradeoffs, maintenance of diversity, experimental evolution, synthetic communities

Introduction

Long-term experimental evolution studies have demonstrated that microbial populations can undergo significant adaptation and diversification over thousands of generations (1). Such adaptation involves the acquisition of new genetic mutations that enhance survival and reproduction in specific environments. Often, these adaptive mutations sweep in the population, resulting in a loss of genetic polymorphism (2, 3). However, polymorphisms are sometimes maintained, leading to the formation of new lineages. For example, in the bacterium *Escherichia coli*, acetate specialists evolved from a clonal ancestor that uses glucose and releases acetate as a waste product, resulting in a polymorphic population (4, 5). Spatial structure can also drive microbial diversification by creating distinct ecological niches, which leads to more genetic polymorphism within populations (6–8). Additionally, ecological mechanisms such as localized dispersal and interactions can maintain diversity by decreasing negative interactions (9). In these previous examples of diversification, polymorphism is maintained by frequency-dependent selection, which is often linked to fitness tradeoffs (10). During frequency-dependent selection, a rare mutant rises in frequency in a resident population, but it loses its fitness advantage when it becomes common (11).

Despite the numerous studies on microbial diversification, the role that other species in a community play in diversification has been largely understudied. When interspecies interactions have been studied in the context of diversification, they have mostly focused on negative interactions such as those between predators and bacteria (12, 13). However, interspecific microbial interactions can also be positive. For example, during cross-feeding, microbes exchange metabolites that benefit each other. This phenomenon is prevalent across different

71 systems (14). However, it remains unclear how positive interspecific interactions such as cross-
72 feeding influence the generation and maintenance of biodiversity.

73 To address this question, we are using a synthetic microbial community composed of
74 *Acinetobacter johnsonii* C6 and *Pseudomonas putida* KT2440. These bacterial species have been
75 isolated from a creosote-polluted aquifer and have naturally evolved to biodegrade aromatic
76 compounds that make up a significant portion of environmental pollutants (15, 16). In addition to
77 its importance for bioremediation, this synthetic community is considered a model system for
78 studying metabolic and commensal interactions (17–19). The commensalism is based on a
79 unidirectional cross-feeding interaction between *A. johnsonii* and *P. putida*. *A. johnsonii*
80 consumes benzyl alcohol and produces benzoate – an intermediate by-product of benzyl alcohol
81 oxidation – which leaks into the external environment where *P. putida* uses it as a source of
82 carbon and energy. This cross-feeding interaction has been shown to be stable over ecological
83 and evolutionary timescales (20, 21).

84 In this study, we build on a previous evolution experiment in which *P. putida* evolved
85 independently and in conjunction with *A. johnsonii* over 200 generations (21). Over the course of
86 this experiment, we observed that populations of *P. putida* diversified into two morphotypes, but
87 this diversification was maintained exclusively in the presence of *A. johnsonii*. To understand
88 how diversity is maintained in the presence of a commensal interaction, we used both genotypic
89 and phenotypic data to address the following questions: How does trait evolution differ in the
90 presence and absence of *A. johnsonii*? What types of mutations underpin the observed
91 phenotypic diversity? How do these mutations affect physiology and tradeoffs? Finally, what
92 mechanism underlies the persistence of this diversity in the co-culture conditions?

Materials and Methods

Growth conditions

All strains were cultured in 10 mL FAB minimal medium (1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.003 mM $FeCl_3$, 15 mM $(NH_4)_2SO_4$, 33 mM Na_2HPO_4, 22 mM KH_2PO_4, and 50 mM NaCl) with either 0.6 mM of benzoate or 0.6 mM benzyl alcohol as the sole carbon source. The media was run through a 0.2 µm filter and stored in glass treated to remove assimilated organic carbon (AOC). AOC-free glassware was prepared as in Rodriguez-Verdugo et al. (2019) (20). Before running experiments, all strains were acclimated by inoculating 10 µl of frozen glycerol stocks in FAB minimal medium supplemented by either 0.6 mM benzoate or 0.6 mM benzyl alcohol and incubated for 24 hours at 26 °C with constant shaking (150 r.p.m.).

Evolution experiment

The evolution experiment had been previously described in Rodriguez-Verdugo and Ackermann (2021) (21). Briefly, the experiment consisted of evolving four replicates of *P. putida* in monoculture, four replicates of *A. johnsonii* in monoculture, and four replicates of *P. putida* with *A. johnsonii* (i.e., co-cultures) for 30 days, which equates to approximately 200 generations (~6.6 generations per day). All cultures were grown in batch culture with FAB minimal media and supplemented with 0.6 mM benzyl alcohol. The experimental lines were established from four ancestors of *P. putida* and *A. johnsonii*, isolated from single clones (Fig. S1). Thus, the ancestors diverged by a few generations when the evolution experiment was started. During the evolution experiment, the population densities for the monoculture and the co-culture from replicate #2 were estimated at the end of each day by plating cultures in LB agar

115 with streptomycin (64 μg/ml) and in LB agar with gentamycin (10 μg/ml) to track *A. johnsonii*
116 and *P. putida*, respectively (21).
117
118 **Growth curves and resource use traits**
119 To characterize the phenotypic diversification of *P. putida* in monoculture and co-culture,
120 we performed growth curve experiments on 30 single colonies isolated from each of the eight
121 experimental lines and from four ancestral populations. Briefly, bacteria were grown in FAB
122 medium supplemented with 0.6 mM benzoate for 24 hours and were then plated on Lysogeny
123 Broth (LB) agar and incubated for another 24 hours at 26 °C. We randomly selected 30 colonies
124 off the plate and resuspended them into wells filled with 200 μL 1% $MgSO_4$ in a 96-well plate.
125 We then transferred 2 μL of the diluted cells and transferred them to another 96-well plate
126 containing 198 μL of FAB supplemented with 0.6 mM benzoate. The plate was sealed with
127 vacuum grease to reduce evaporation. The plate was incubated in a photospectrometer plate
128 reader (Epoch2™, Agilent™) at 30 °C and continuous linear shaking. Optical density (OD)
129 measurements were taken every 10 minutes for 24 hours. From these growth curves, we
130 estimated four quantitative traits involved in resource use: the maximum growth rate in
131 exponential phase (μ_{max}), the yield (Y) defined as the amount of biomass produced per unit of
132 resource, the maximum uptake rate (V) defined as the maximum rate of resource consumption
133 per hour and the half-saturation constant (K) defined as the resource concentration supporting
134 half-maximum uptake rate. Parameters were estimated using the same method outlined in
135 Rodriguez-Verdugo et al. (2019) (20) using MATLAB (version R2017a, MathWorks). To
136 quantify the evolution of phenotypic traits, we compared the trait distributions from ancestral

137 populations (n = 30) to those from evolved populations (n = 30) using a two-tailed *t*-test (R,
138 version 4.3).
139
140 **Genome resequencing and variant calling**
141 We extracted DNA from the ancestors' clones, monoculture and co-culture populations,
142 and single clones from large and small morphotypes of all four experimental lines. Single clones
143 were revived from glycerol stocks in 3 mL of LB broth and grown overnight at 26 °C with
144 constant shaking (150 r.p.m.). The overnight cultures from monoculture and co-cultures whole
145 populations were grown FAB with 0.6 mM benzoate supplemented with gentamycin (10
146 µg/mL). We isolated and purified genomic DNA from 1 mL of the overnight cultures using the
147 Wizard DNA purification kit (Promega). Short-read sequencing (2 x 150bp paired-end reads)
148 was conducted on a NovaSeq6000 S4 flow cell (UCI Genomics High-Throughput Facility).
149 These sequences can be accessed at the NCBI Sequence Read Archive under Bioproject ID
150 number PRJNA623337.
151 All sequences were aligned to the reference genome of *P. putida* KT2440 (reference
152 NC_002947) using the computational pipeline *breseq* (version 0.36.1) and utility program
153 GDtools (22). We used consensus mode and polymorphism mode to call variants for clones and
154 whole populations, respectively. Each sample was sequenced to a depth of ~600X coverage.
155 Single-nucleotide polymorphisms in genic regions at or above 10% and passing the Fisher's
156 exact test for biased strand distribution were considered. *De novo* non-synonymous mutations
157 were defined as uniquely observed in the evolved sample and not the ancestral clone.
158
159 **Physiological tradeoffs**

160 *Biofilm formation* - We measured biofilm formation in the *P. putida* ancestors and the
161 evolved morphotypes using the method described in Martínez-García et al. (2014) (23). Samples
162 were inoculated into FAB media supplemented with 0.6 mM benzoate and incubated for 24
163 hours at 26 °C with constant shaking (150 r.p.m.). The tubes were then vortexed to homogenize
164 the culture and the OD_{600} was adjusted to 0.5. We added 2 µL of the adjusted acclimated culture
165 to a 96-well plate containing 198 µL of FAB and 0.6 mM benzoate and was incubated at 26 °C
166 with no shaking. Each sample was inoculated into 5 wells that serve as technical replicates. After
167 24 hours, the plate was removed from the incubator, and the OD_{600} was measured using the plate
168 reader. The media with planktonic cells was discarded by gently tapping the plate and was
169 washed 3 times with water. The cells attached to the plate were stained by adding 200 µL of
170 0.1% (w/v) crystal violet into each well and were incubated on the bench top for 30 minutes. We
171 then disposed of the crystal violet and gently washed the plate 2-3 times with deionized water.
172 The plate was left to completely dry. We added 200 µL of 33% (w/v) acetic acid to each well,
173 pipetting up and down until it was completely homogenized. The OD_{595} was measured, and an
174 index of biofilm formation was calculated, which was the crystal violet stained biofilm
175 (OD_{595}) divided by the initial culture with planktonic cells (OD_{600}). The higher the biofilm index
176 value, the greater the biofilm formation. These experiments were replicated three times. We
177 conducted a two-tailed *t*-test to determine if there were significant differences in biofilm
178 formation in the evolved morphotypes (n = 3) compared to the ancestor (n = 3).
179 *Motility* - We performed a swimming assay on the ancestors and evolved morphotypes of
180 *P. putida* using 0.3% (w/v) soft agar (23, 24). We inoculated 2 µL of an acclimated culture onto
181 FAB soft agar supplemented with 0.6 mM benzoate and incubated the plates at 30 °C. We
182 measured the halo around the initial inoculum after 24 hours to determine whether the evolved

183 morphotypes' swimming abilities differed from the ancestors' swimming abilities. A larger halo
184 indicates higher motility, while the absence of a halo suggests a lack of flagellar motility.
185
186 **Reciprocal invasion assays**
187 To test if frequency-dependent selection is a possible mechanism for maintenance of the
188 emergent diversity, we performed reciprocal invasion experiments. These experiments consisted
189 of culturing two morphotypes (large and small) at a frequency of 1 to 9 to see if they can invade
190 when rare in the presence and absence of *A. johnsonii.*
191 The two morphotypes of *P. putida* and *A. johnsonii* were revived from a freezer stock of
192 replicate 2 and were allowed to acclimate for 24 hours. The OD was then adjusted to 1.0 for
193 both morphotypes to standardize the starting ratio. For the condition without *A. johnsonii* (i.e.,
194 monoculture), 10 µL of the large morphotype and 90 µL of the small morphotype were
195 inoculated into 9.9 mL of fresh media supplemented with 0.6 mM benzyl alcohol and 0.06 mM
196 benzoate. The concentration of supplemented benzoate was selected based on the amount of
197 benzoate excreted to the external environment by *A. johnsonii* and measured by high-
198 performance liquid chromatography(20). The starting volumes were reversed when the small
199 morphotypes started from a rare frequency. For the condition with 100 µl of *A. johnsonii* (i.e. co-
200 culture), no external benzoate was added to the medium, and the large and small morphotypes
201 were inoculated at 1:9 and 9:1 ratios as previously described. Co-cultures and monocultures were
202 grown at 26°C with constant shaking (150 r.p.m.). After 24 hours, 1% of culture was transferred
203 into fresh media and these daily transfers were continued for three days. We counted colony-
204 forming units (CFU) at the start of the experiment (T0) and the end of each transfer cycle by

205 plating cultures onto LB agar plates supplemented with gentamicin (10 μg mL^{-1}). We included
206 two technical replicates per experiment, and each experiment was replicated three times.
207 The Malthusian parameters for the rare and common morphotypes were calculated from
208 cell densities at the initial and final time points, $m = \ln (N_f/N_0)$, with N_0 and N_f the initial and
209 final density after 24 hours, respectively. From the Malthusian parameters, we calculated the
210 relative growth rate of a morphotype against its competitor when starting from rare. For example,
211 $S_{i\text{-}rare} = m_{i\text{-}rare} - m_j$, for a pair of competitors *i* and *j* as in Zhao et al. (2016) (25). If the value of
212 S_{rare} for a given morphotype was significantly larger than zero, the morphotype could invade
213 when rare. If both the large and small morphotypes had a positive S_{rare} value, the pair was
214 considered to stably coexist through frequency-dependent selection.
215
216 **Results**
217 The evolution experiment was started with four experimental lines (replicates) of *P.*
218 *putida* in monoculture and four lines in co-culture with *A. johnsonii* grown in minimal growth
219 media supplemented with benzyl alcohol as the sole carbon source (Fig. S1). Following this
220 experiment, we conducted whole genome sequencing and identified *de novo* mutations in the
221 evolved lines. The effect of *de novo* mutations on yield, motility, and biofilm formation was then
222 evaluated by pairwise comparisons of the evolved lines to their ancestors. Finally, we tested if
223 frequency-dependent selection was a possible mechanism for maintaining diversity.
224
225 **Diversification evolved in co-culture but not in monoculture.**
226 After 200 generations of evolution, we observed diversification of *P. putida* in terms of
227 colony morphology relative to the ancestor (Fig. 1A). All four replicates were bimodal for colony

228 size and consisted of one cluster of strains that formed larger colonies on LB agar plates
229 compared to the ancestor (referred as 'large morphotype'), and one cluster that formed colonies
230 the size of the ancestor (referred as 'small morphotype'). This diversification in colony size was
231 only observed when *P. putida* was evolved in co-culture with *A. johnsonii*. In monocultures,
232 populations only formed larger colonies than the ancestor.

233 Next, we quantitively characterized this diversification in four traits involved in resource
234 use. In all monoculture and co-culture replicates, the phenotypic variance for each trait changed
235 compared to the variance of the ancestral population (Figs. S2 and S3). Changes in yield were
236 the most pronounced. We found that *P. putida* grown in co-culture with *A. johnsonii* had a
237 different distribution of yields compared to monoculture (Fig. S2). For example, co-cultures of
238 replicate #2 showed a bimodal distribution, with one subpopulation having a similar yield to the
239 ancestor and the other having a higher yield to the ancestor (Fig. 1B, right panel). On the other
240 hand, the monoculture had a distribution shifted towards higher yield (Fig. 1B, left panel). We
241 confirmed that the large morphotypes previously observed on LB agar plates had higher yields
242 compared to their ancestors, and the small morphotypes had similar yields to their ancestors. For
243 example, large morphotypes from replicate #2 had, on average, a 3.4-fold higher yield than the
244 ancestor (0.068 ± 0.001 and 0.020 ± 0.001 OD per mM for the large and ancestral morphotypes,
245 respectively; t-test, $p < 2.2*10^{-16}$). The small morphotypes achieved a yield of 0.023 ± 0.002 OD
246 per mM, which is comparable to that of the ancestor (t-test, $p = 0.122$).

247 Finally, leveraging the fact that large morphotypes with higher yield formed larger colonies
248 on LB agar plates that could be distinguished from the ancestral morphotype, we tracked the
249 evolution of phenotypic diversification for one of the replicates (replicate #2) during our
250 experiment. After approximately 40 and 70 generations of evolution, we observed the emergence

of a large morphotype in monoculture and co-culture, respectively (Fig. 2). In co-culture, the large and small morphotypes coexisted for the remaining 130 generations. Instead, in monoculture, the large morphotype rapidly outcompeted the ancestral morphotype, driving it to extinction after 140 generations. Thus, diversification only persisted in co-culture.

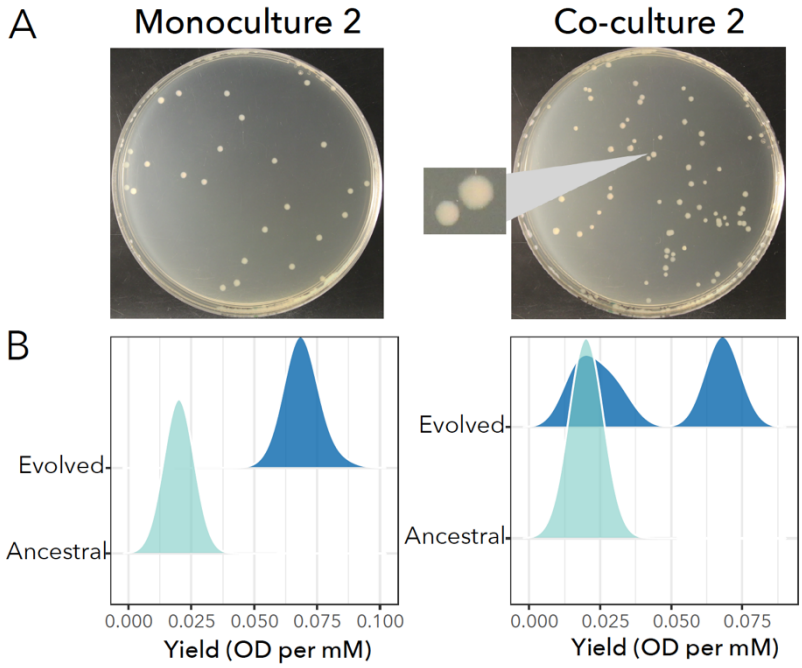

Fig. 1. *P. putida* population evolved in monoculture is monomorphic, while the population evolved in co-culture diversified into two sub-populations after 200 generations. A) Agar plates illustrating the difference in colony morphologies between the evolved population in monoculture (left panel) and the evolved population in co-culture (right panel) for replicate #2. B) In monoculture, *P. putida*'s population from replicate #2 evolved higher yield (t-test, $p < 2.2*10^{-16}$). In co-culture, *P. putida*'s population evolved bimodality (Hartigans' dip test for multimodality, $p < 2.2*10^{-16}$). The results from the other replicates and parameters are available in Fig. S2 and Fig. S3.

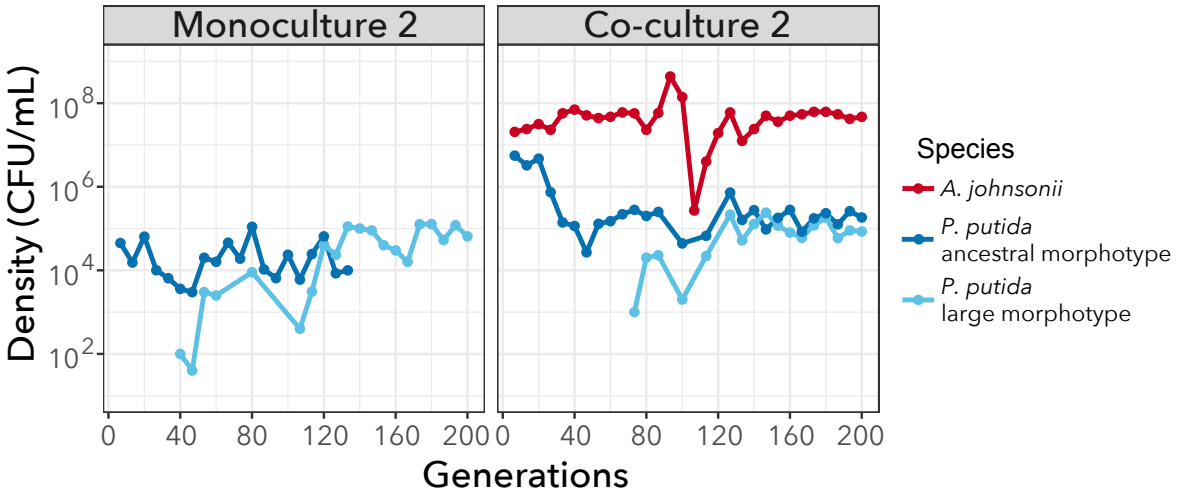

Fig. 2. Diversification was maintained in co-culture but not in monoculture. The large morphotype appears in the monoculture after approximately 50 generations and outcompetes the ancestral morphotype. In co-culture, both morphotypes coexist until the end of the experiment. Population density (CFU mL^{-1}) trajectories from replicate #2 are shown over 200 generations. *A. johnsonii*'s densities are from (21). Each circle represents the final density of each species and morphotypes after daily cycles of growth; lines connecting circles help visualization and do not have any biological meaning.

Morphological diversification in *P. putida* is based on genetic diversification

To determine if the observed phenotypic diversity (Fig. 1) reflected specific mutations, we performed whole population sequencing and identified *de novo* nonsynonymous mutations in the evolved lines (Table S1). We detected single substitution mutations in the *fleQ* gene at relatively high frequencies in evolved co-cultures from replicates #1 and #2 (Table 1). This gene codes for flagellar transcriptional regulator FleQ, which has been shown to affect motility and biofilm formation (26–28). Sequencing of single clones isolated from these populations

282 confirmed that the *fleQ* mutations were associated with a large morphotype. Instead, the small
283 morphotypes had mutations in genes other than flagellar genes in three out of four replicates
284 (Table S1), but most of these genes were unannotated. One exception was the small morphotype
285 from replicate #3, which had a 1 bp deletion in the *oplA* gene and was present at a 12.9%
286 frequency in the population (Table S1). The *oplA* gene codes for 5-oxoprolinase A, which has
287 been associated with the catabolism of amino acid derivatives (29, 30), but its functional
288 relevance in our experimental conditions is unclear.

289 The evolved monocultures also had mutations in flagellar genes. For example, replicate
290 #2 had a mutation in the *fleQ* gene at 100% frequency in the population (Table 1). This is
291 consistent with our observation that the large morphotype carrying the *fleQ* mutation was fixed
292 in the population (Fig. 2). Finally, we detected other flagellar mutations, including a mutation in
293 the *flgH* gene, coding for flagellar basal body L-ring protein FlgH in monoculture replicate #4
294 (Table 1). Thus, we infer that mutations in either *fleQ* or *flgH* lead to the enlarged morphotype.
295

296 **Table 1. Frequency of *de novo* flagellar mutations in the populations of *P. putida* in**
297 **monoculture and co-culture after 200 generations.**

Experimental populations	Gene	Mutation	Frequency
Co-culture 1	*fleQ*	A226V (GCC→GTC) *	23.1%
Co-culture 2	*fleQ*	L353Q (CTG→CAG) *	35.6%
Co-culture 3	-		-
Co-culture 4	-		-
Monoculture 1	-		-
Monoculture 2	*fleQ*	2 bp→ C †	100%

Monoculture 3	-		-
Monoculture 4	*flgH*	+C	100%

*Same as found in single clones of the large morphotype.
† Deletion of first base and substitution of second base with C.

Mutations in the large morphotype led to loss of motility and biofilm formation

We previously confirmed that the large morphotypes - commonly associated with *fleQ* mutations - had significantly higher yields than their ancestors as well as changes in other resource use traits (Figs. S2 and S3). To explore whether *fleQ* mutations affected other traits, we explored the mutants' abilities to swim and form biofilms. The motility assay showed a complete lack of motility in the *fleQ* mutants compared to their ancestors (Fig. 3). Other large morphotypes not carrying the *fleQ* mutations (e.g. from monoculture replicate #4) had a reduction in swimming, possibly due to a mutation in the flagellar component *flgH*. Replicate #4 also had a small morphotype colony that lacked motility, but the cause for the lack of motility remains unclear.

The *fleQ* mutation also resulted in a significant reduction in biofilm formation for the large morphotype (Fig. 4). The large morphotype, isolated from co-cultures #1 and 2, and monoculture #2, exhibited significant reductions in biofilm formation compared to their ancestors (t-test, p-values of 0.003, 0.023, and 0.008, respectively). The *flgH* mutations (large morphotype from monoculture #4) also had reduced biofilm formation but the difference was marginally significant (t-test, $p = 0.068$).

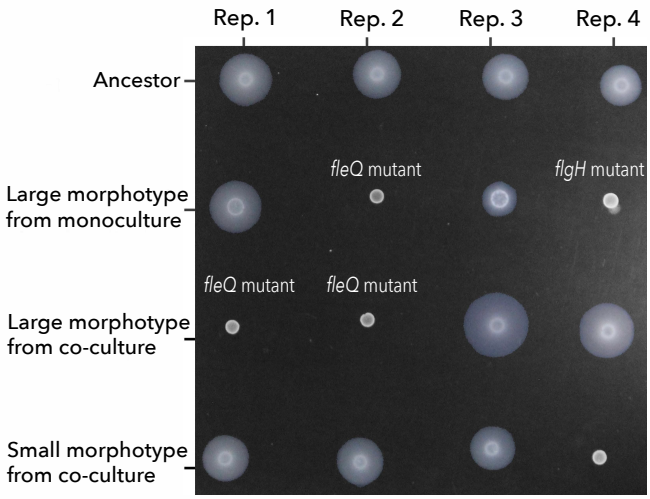

Fig. 3. Swimming assay shows the loss of flagellar motility in the large morphotypes with flagellar mutations. All samples were spotted on 0.3% (w/v) FAB soft agar. The absence of a halo around the initial inoculum indicates the loss of flagellum-driven motility.

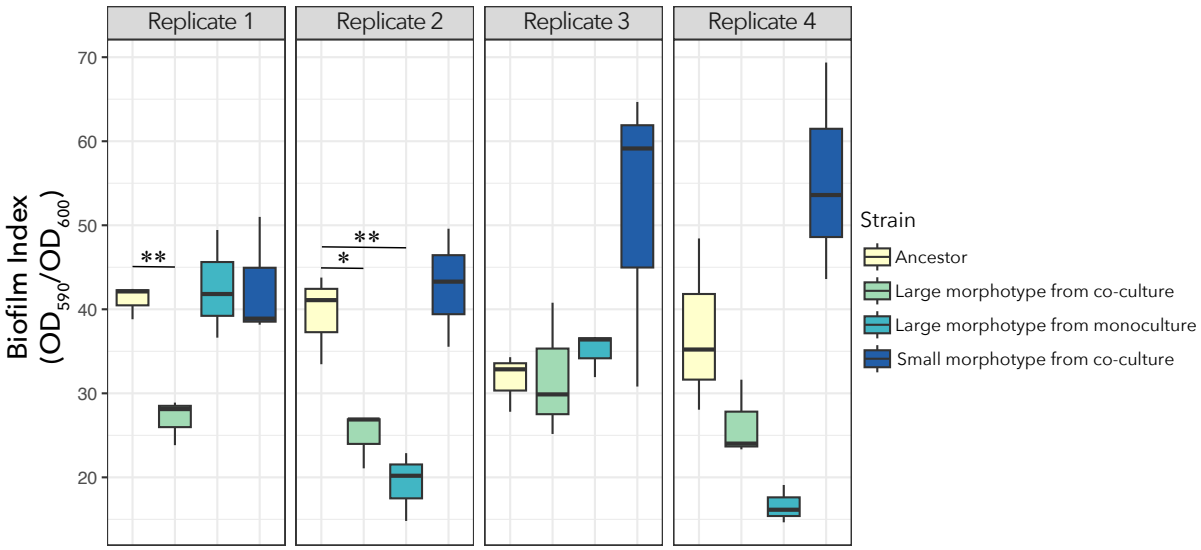

Fig. 4. Biofilm assay shows that *fleQ* mutants produce less biofilm than the ancestor. Biofilm index (CV/OD_{600}) after a 24-hour incubation period. All *fleQ* mutants (i.e. large morphotypes of co-cultures 1 and 2 and monoculture 2) had a significant reduction in the biofilm index compared to their respective ancestors. Note: * = $0.01 < p < 0.05$, ** = $p < 0.01$.

328

329 **Frequency-dependent selection does not explain the coexistence of morphotypes**

330 We previously observed that the large and small morphotypes stably coexisted for 150
331 generations in co-culture from replicate#2 (Fig. 2). To test if frequency-dependent selection is a
332 possible mechanism for the stable coexistence of these two morphotypes and if the
333 presence/absence of *A. johnsonii* influence this selection, we performed a reciprocal invasion
334 experiment. The reciprocal invasion assay did not show significant differences in the rare
335 morphotype's ability to invade depending on whether *A. johnsonii* was present (Table 2). One
336 exception was the large morphotype, which had a significantly positive relative growth rate when
337 rare (S_{rare}) in co-culture, meaning it could invade when rare (i.e., at 10% frequency). None of the
338 other morphotypes invaded from rare (Table 2). We confirmed that this was the case by tracking
339 the frequencies of the large and small morphotypes over two additional days (Fig. S4). Overall,
340 these results suggest that frequency-dependent selection is not the mechanism driving
341 coexistence.

342

343 **Table 2. The relative growth rate when rare was not affected by growth conditions**
344 **(monoculture or co-culture).** The values ± standard deviations were obtained from four to six
345 replicates.

Strain	Condition	Relative growth rate when rare (S_{rare})	p-value*
Small morphotype	Monoculture	0.65 ± 0.26	0.470
	Co-culture	0.19 ± 0.24	0.468
Large morphotype	Monoculture	0.58 ± 0.52	0.326
	Co-culture	0.94 ± 0.18	**0.019**

346 * One sample t-test testing whether S_{rare} is different than zero.
347

Discussion

The results from this study show that diversity can arise in commensal communities, even in a homogeneous environment with low resources. Diversity arose in both monoculture and co-culture conditions; however, it persisted only in co-culture conditions. In monoculture conditions, the ancestral morphotype was outcompeted by a new mutant. This suggests that the presence of *A. johnsonii* and, hence, positive interspecific interactions (cross-feeding) play a role in the maintenance of the emerging diversity.

We found that the phenotypic diversity of *P. putida* is based on genetic diversity. A mutation in *fleQ* gene was detected in two out of the four cocultures and one monoculture that corresponds to the large morphotype. The *fleQ* mutation led to an inability to swim and a significant decrease in biofilm formation. Mutants also had an increase in yield compared to their respective ancestors, which explains why they form larger colonies than the ancestor when plated in LB agar. This is unsurprising as the loss of flagellar function is a common response to shaking environments (31–35). The loss of flagellar function alleviates a large metabolic burden that can be invested in biomass production while still being able to passively move due to the continuous shaking of our culture conditions (23). The small morphotype also accumulated mutations that are at low frequency in the population (Table S1). However, these mutations are in genes that are not annotated, and their physiological effects on our culture conditions are unknown.

Even though our system lacked spatial structure initially, cross-feeding can lead to niche construction. The excretion or leakage of nutritional by-products leads to the construction of new niches, which can affect the generation and maintenance of biodiversity (36, 37). We hypothesize that *A. johnsonii* creates transient patches of resources, and this spatiotemporal heterogeneity maintains the diversity of newly formed lineages through a 'yield-foraging' tradeoff. That is, by

371 reducing the burden of flagellar motility, the *fleQ* mutants (large morphotypes) achieve higher
372 yields and have an advantage over the small morphotypes in monoculture (i.e., 'yield' strategy).
373 Instead, in co-culture, the small morphotypes retain flagellar motility and biofilm formation
374 abilities (Fig. 3 and 4), which may increase their abilities to cross-feed on the benzoate excreted
375 by *A. johnsonii* (i.e., 'foraging' strategy). A previous study showed that motility increases the
376 encounter rate between cross-feeding partners and cell-cell adhesion in a well-mixed
377 environment (38), supporting our hypothesis that *A. johnsonii* plays a key role in maintaining the
378 coexistence of *P. putida*'s lineages. Nevertheless, our findings did not support frequency-
379 dependent selection in the presence of *A. johnsonii* as the mechanism for maintaining lineages'
380 coexistence. It is possible that our representative clones did not capture the stable coexistence
381 dynamics. That is, maintenance of polymorphism through negative frequency-dependent
382 selection may only occur when considering the entire genetic diversity in *P. putida* populations.
383 Furthermore, other ecological and evolutionary processes could potentially maintain lineages
384 coexistence. One possibility is that small fitness differences between the two morphs could result
385 in neither of them able to invade, leading to neutral coexistence (39). Another possible
386 mechanism is multiple niche selection leading to the maintenance of stable polymorphisms in the
387 population (11, 40). An interesting avenue of research would be to explore further the
388 mechanisms by which *A. johnsonii* mediate the coexistence of newly formed lineages.
389 Our study has provided valuable insights into the emergence of diversity in commensal
390 microbial communities and suggests that interspecific interactions play a key role in the
391 diversification and maintenance of diversity. However, it is important to acknowledge certain
392 limitations in interpreting the results. First, while 200 generations was enough time to observe
393 diversification, we refrain from making a definitive conclusion about whether the diversity is

transient (short-lived) or if it can be sustained long-term. Another limitation in interpreting these results is the lack of control for the benzoate excreted by *A. johnsonii*. Although benzoate was not externally supplemented to the *P. putida* monocultures, they relied on residues of organic carbon present in the culture medium, i.e., assimilable organic carbon or AOC (21). Thus, an alternative hypothesis from the yield-foraging hypothesis is that diversification in the presence of *A. johnsonii* was maintained through resource partitioning. To discern between these two hypotheses, future evolution experiments should add an external source of benzoate to the monocultures. Such experiments would allow us to determine how important it is that benzoate is produced by *A. johnsonii* (i.e., a biogenic resource supply) versus being externally supplied to the medium.

Data Availability

The genome sequences have been deposited at the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under BioProject ID number PRJNA623337. All the other data generated or analyzed during this study are included in this published article and its supplemental materials.

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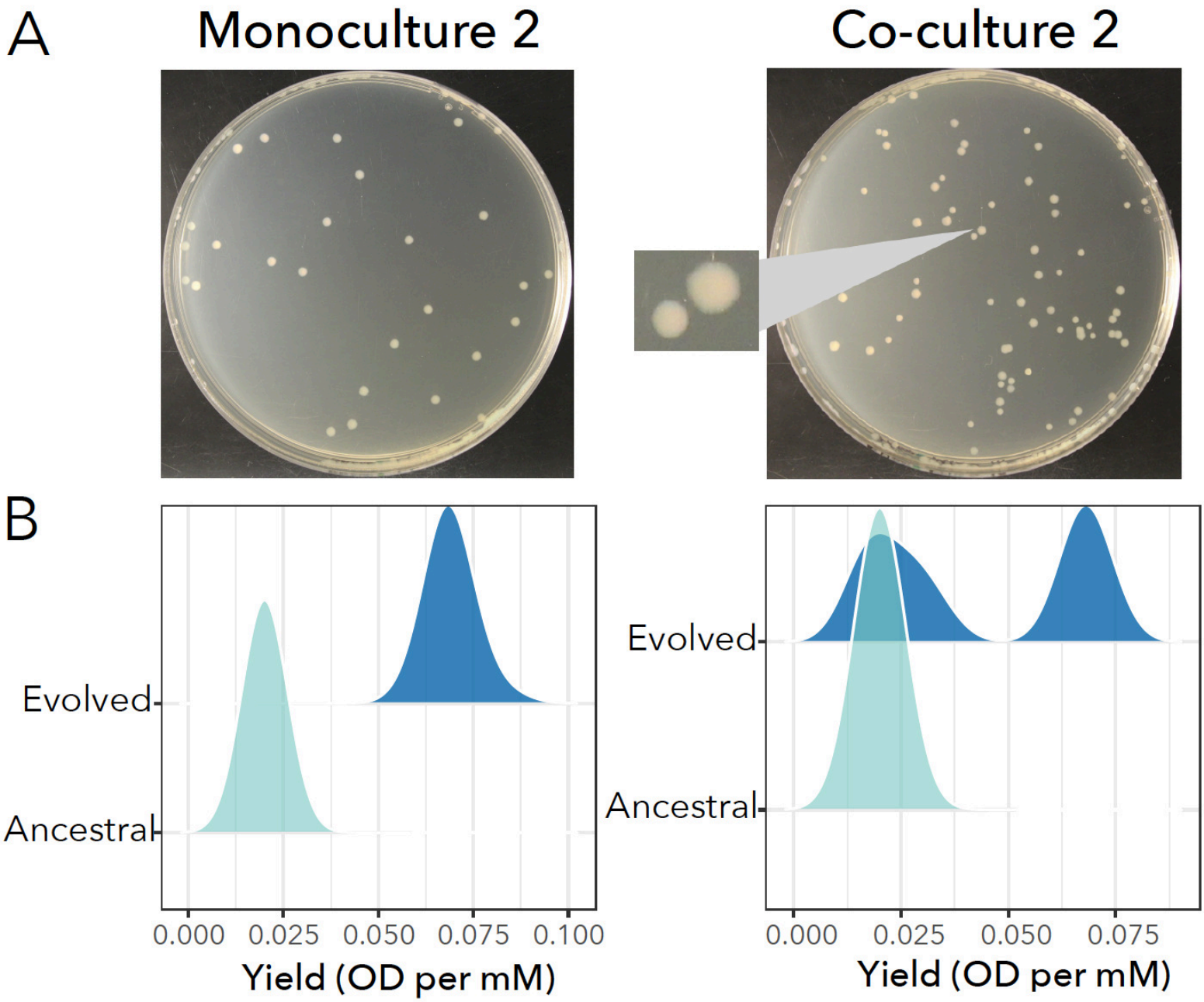
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Co-culture 2
B
Evolved
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0.025
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Yield (OD per mM)
Evolved
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0.000
0.025
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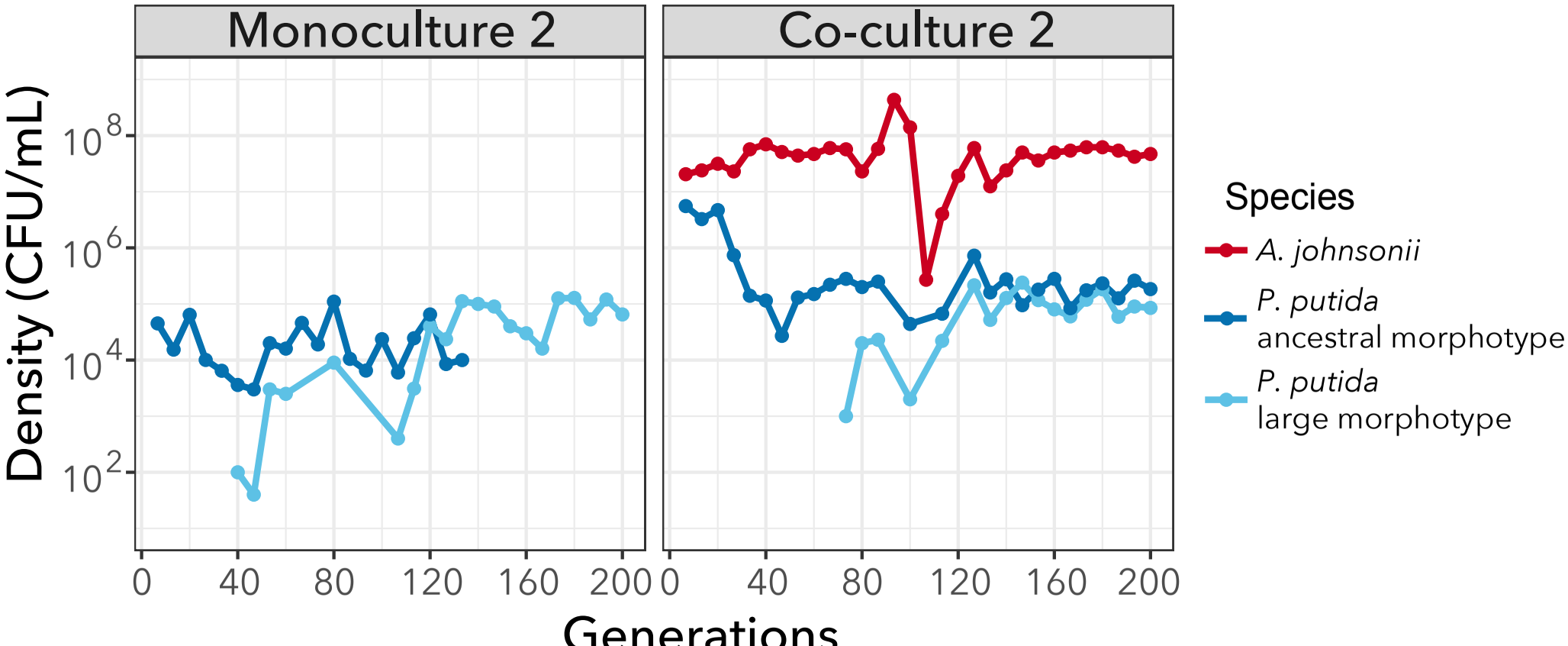
Yield (OD per mM)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Co-culture 2
Density (CFU/mL)
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Species
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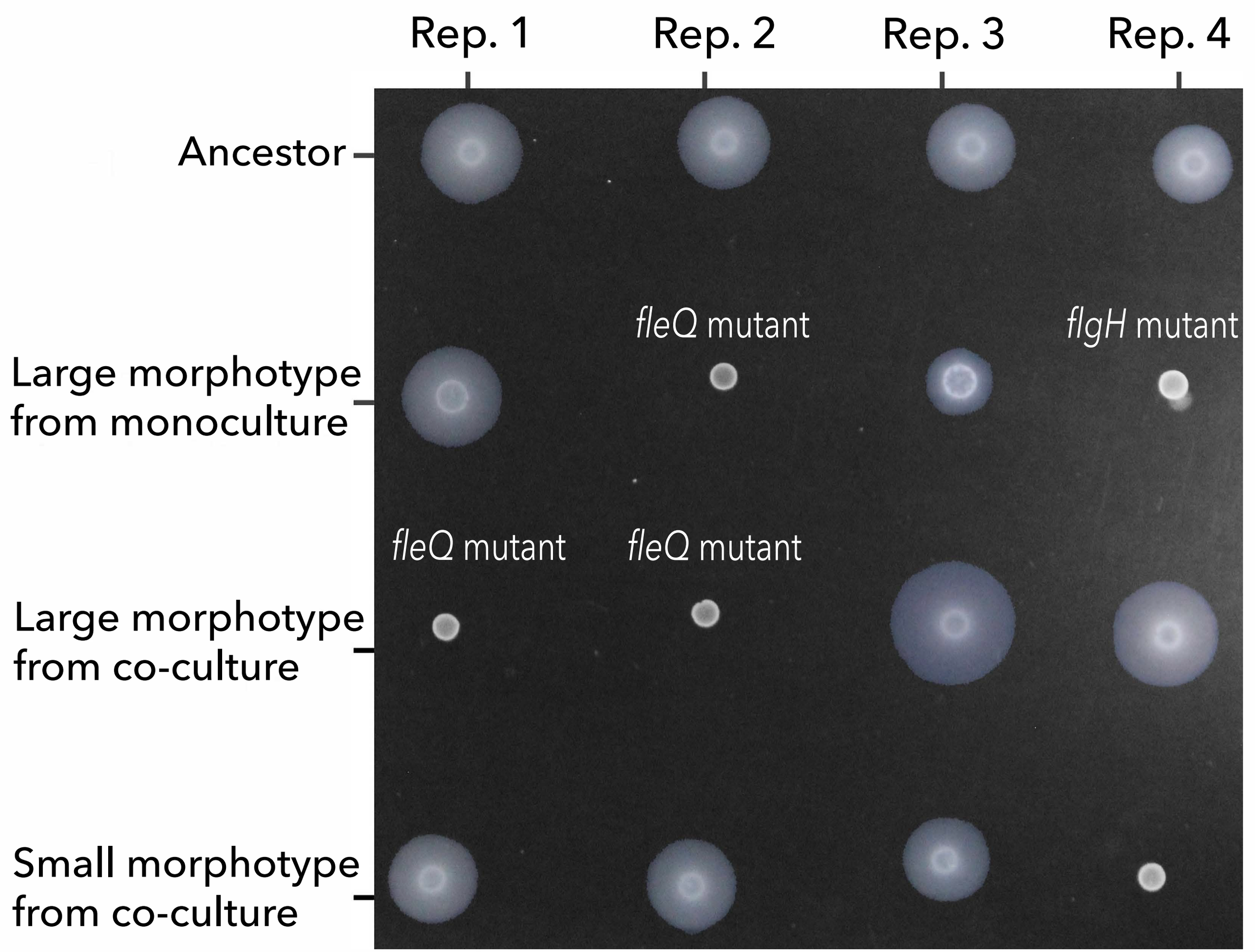
P. putida ancestral morphotype
P. putida large morphotype


Rep. 1
Rep. 2
Rep. 3
Rep. 4
Ancestor
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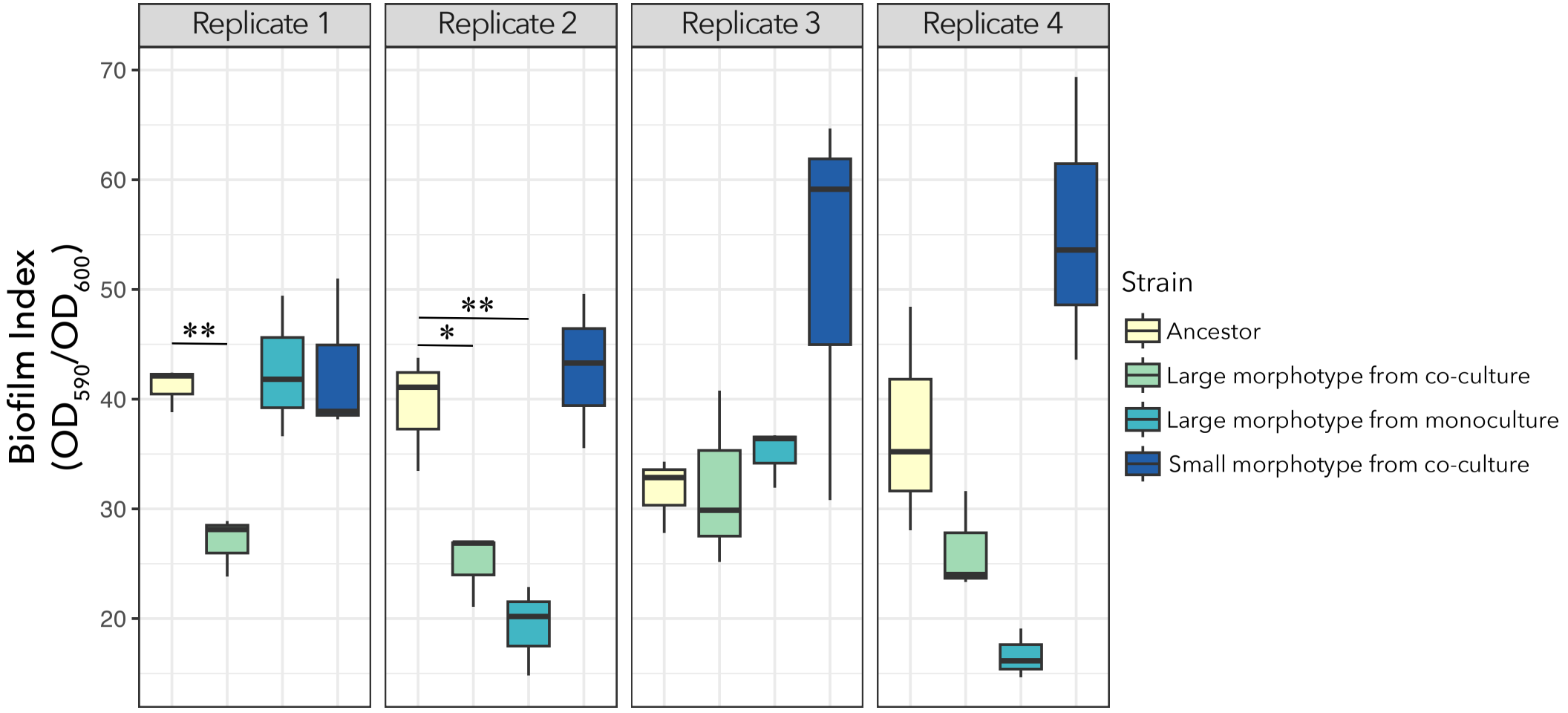
fleQ mutant


Replicate 1
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Strain
Ancestor
Large morphotype from co-culture
Large morphotype from monoculture
Small morphotype from co-culture