

1 **Division of labor, bet hedging, and the evolution of mixed biofilm investment strategies**

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3 Nick Vallespir Lowery^{1*}, Luke McNally^{1,2}, William C. Ratcliff³ & Sam P. Brown^{1,2,3}

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5 1. Institute of Evolutionary Biology, School of Biological Sciences, University of

6 Edinburgh, Edinburgh EH9 3FL, UK

7 2. Centre for Immunity, Infection and Evolution, School of Biological Sciences,

8 University of Edinburgh, Edinburgh EH9 3FL, UK

9 3. School of Biology, Georgia Institute of Technology, Atlanta GA 30332, USA

10 *Current address: META Center for Systems Biology, University of Oregon, Eugene OR

11 97403, USA

12

13 **Correspondence to: Nick Lowery (nlowery@uoregon.edu) or Sam Brown**

14 **(sam.brown@biology.gatech.edu)**

15 **ABSTRACT**

16 Bacterial cells, like many other organisms, face a tradeoff between longevity and fecundity. Planktonic
17 cells are fast growing and fragile, while biofilm cells are often slower growing but stress resistant. Here
18 we ask: why do bacterial lineages invest simultaneously in both fast and slow growing types? We
19 develop a population dynamical model of lineage expansion across a patchy environment, and find that
20 mixed investment is favored across a broad range of environmental conditions, even when transmission
21 is entirely via biofilm cells. This mixed strategy is favored because of a division of labor, where
22 exponentially dividing planktonic cells can act as an engine for the production of future biofilm cells,
23 which grow more slowly. We use experimental evolution to test our predictions, and show that
24 phenotypic heterogeneity is persistent even under selection for purely planktonic or purely biofilm
25 transmission. Furthermore, simulations suggest that maintenance of a biofilm subpopulation serves as a
26 cost-effective hedge against environmental uncertainty, which is also consistent with our experimental
27 findings.

28 **INTRODUCTION**

29 After billions of years of evolution, many organisms retain an impressive capacity for innovation and
30 adaptation to their environment. However, core traits such as durability and reproductive rate are often
31 optimized such that improvements in one will often come at the cost of another - indeed, understanding
32 how adaptation occurs when key fitness parameters are constrained by tradeoffs lies at the core of life
33 history theory (1). While most life history theory has been developed with large multicellular organisms
34 in mind, microbes also exhibit classical trade-offs in fecundity and longevity, with faster growing lineages
35 tending to be more fragile (2, 3). Understanding how microbes manage such trade-offs remains a major
36 goal in microbiology, both from mechanistic (4) and ecological (5) perspectives.

37 Multiple mechanisms of enhancing durability and longevity are available to microbes, but typically come
38 at the cost of reduced metabolic proficiency. Spore formation is perhaps the clearest example of a high
39 survival, low fecundity phenotype: by encasing the genome and some essential metabolic machinery in
40 a thick and extremely resistant cell wall, dormant spores can survive for extraordinarily long durations
41 (6, 7). Alternatively, cells may form metabolically dormant persister cells capable of surviving diverse
42 environmental insults (8, 9). Finally, many microbial species form biofilms, where dense cell packing and
43 production of a protective extracellular matrix provides broad resistance to stressors such as
44 desiccation, predation, or chemical insult (10), but also limits space and nutrient diffusion, thereby
45 reducing growth rates.

46 Clonally reproducing microbes present an interesting and experimentally tractable system to examine
47 mixed-behavioral strategies. Across many species of microbe, single genotypes can produce coexisting
48 subpopulations of rapidly dividing planktonic cells and slow-growing or dormant stress-tolerant
49 phenotypes, but focus is often given to a specific phenotype of interest rather than the balance between
50 alternate phenotypes. In this study, we examine how the trade-off between survival and growth of
51 individual cells drives the evolution of mixed biofilm / planktonic investments on a lineage scale under
52 diverse environmental conditions. Specifically, we build population dynamical models of bacteria in
53 patchy environments, where cells can switch between biofilm and planktonic states within ephemeral
54 patches (via planktonic colonization of the biofilm, and dispersal from the biofilm to the planktonic
55 state), and can also transmit among patches either as biofilm or planktonic cells. We then ask, under
56 what conditions is investment into biofilm favored, given that biofilms grow more slowly? If only one
57 phenotype (i.e. biofilm or plankton) is favored for transmission to a new patch, does it ever pay to
58 diversify into the cell type that is, from a transmission perspective, a 'dead end'? Our model predicts
59 that phenotypic diversification can pay across a range of environmental conditions, as rapidly growing
60 planktonic cells can function as a 'growth engine' providing higher levels of future planktonic and biofilm

61 cells for transmission. We then test our model predictions using stochastic simulations and experimental
62 evolution of biofilm allocation in the environmental microbe *Pseudomonas aeruginosa*.

63 **RESULTS**

64 **Biofilm Growth Dynamics**

65 While it is well known that planktonic cells accumulate exponentially in nutrient-rich environments, it is
66 less clear whether close-packed biofilm cells would follow the same functional form (note that here we
67 are considering biofilm growth in the absence of any coupling with the planktonic compartment, i.e. no
68 cells dispersing from the biofilm or colonizing from the bulk). We hypothesize that sparse colonization
69 allows for lineages to grow exponentially, as there is little steric inhibition or nutrient depletion to slow
70 growth. However, once confluence across the surface is reached, further growth is restricted to a fixed
71 depth within the outermost layer in biofilms due to space and diffusion limitations (11, 12). We explore
72 this conjecture using the individual-based simulation platform iDynoMiCS (13) to simulate a simple two-
73 dimensional biofilm, and find that after an initial period of exponential growth, cell accumulation decays
74 to a linear function in time (Figure 1). More generally, we find that the rate of biofilm growth depends
75 on the geometry of the system being considered (Supplemental Text 1), with the growth rate following a
76 polynomial of order equal to the dimensionality of the system (i.e. for a three dimensional sphere,
77 biofilm cells accumulate as a cubic in time). However, it should be noted that for finite volumes there is
78 a constant downward pressure through time on the order of the growth polynomial as the biofilm
79 reaches confluence across each dimension (e.g., initially cubic expansion in three dimensions will decay
80 to quadratic expansion in two dimensions once the limit in the z-direction is reached). These findings
81 highlight that while biofilm cells do not face the extreme growth penalty of resistant spores or persister
82 cells, they face a significant and compounding growth deficit in comparison with the exponential growth
83 of planktonic populations.

84 **Coupled Biofilm-Plankton Dynamics**

85 We next model a growing bacterial microcosm within which cells grow in one of two compartments, the
86 planktonic (P) phase within the bulk fluid, and the biofilm (B) phase attached to a surface and in contact
87 with the bulk. The compartments are coupled, such that biofilm cells can disperse to the planktonic
88 phase, and planktonic cells can colonize the biofilm. Cells in each compartment also divide, with
89 planktonic cells growing exponentially, and biofilm cells growing linearly (i.e. we assume a finite 2-D
90 surface available for biofilm colonization, and ignore the initial super-linear growth period).

91 With the biofilm limited to linear expansion, we reasoned that the effects of growth within and dispersal
92 from the biofilm would be negligible when coupled to exponential growth in the planktonic phase. This
93 simplification was shown to be reasonable by comparing numerical simulations of the full model and a
94 simplified model with no biofilm growth or dispersal (Figure S1), yielding the model system outlined in
95 Figure 2A, and Equations 1.1, 1.2, 2.1 and 2.2 for within-patch growth. By setting the growth of the
96 biofilm to zero, this simplified framework renders biofilm cells functionally equivalent to spores or
97 persisters as described above, i.e. a subpopulation of non-dividing cells supported by the growth of
98 vegetative cells, which presumably must provide some other benefit (e.g. environmental resistance) to
99 the overall population to counteract this loss in fitness or else be lost from the population.

100 Our simplified model framework results in the following coupled differential equations:

101 1.1)
$$\frac{dP}{dt} = (r - c)P$$

102 1.2)
$$\frac{dB}{dt} = cP$$

103 where r is the exponential growth rate, c is the rate of colonization of the biofilm (with $0 \leq c < r$). Note
104 that in general c need not be bounded by r , and arbitrarily high values for c would result in a decline in P

105 as switching to the biofilm phase outpaces planktonic growth, giving a sharp trade-off between the two
106 compartments; we discuss this case in the context in which it arises below. Solving equations 1.1 and
107 1.2 as a function of time yields our within-patch population model,

108 2.1) $P(t) = P_0 e^{(r-c)t}$

109 2.2) $B(t) = \frac{c}{r-c} (P(t) - P_0)$

110 where P_0 is the planktonic inoculum.

111 The within-patch model reveals a temporal trade-off in biofilm accumulation with increasing
112 colonization (Figure 2). As expected, planktonic cells decline monotonically with increasing colonization
113 rate c , as more cells are siphoned from the planktonic to the biofilm compartment (Figure 2, A and C).

114 The biofilm, however, shows more interesting dynamics with changing rates of colonization (Figure 2, B
115 and D). At $c = 0$, no biofilm cells accumulate, and as c approaches r , all new planktonic cells colonize the
116 biofilm, resulting in a static planktonic population and linear accumulation of biofilm cells (Figure 2 A
117 and B, yellow lines). However, when the planktonic fraction is allowed to expand exponentially (with $0 <$
118 $c < r$), the biofilm also accumulates cells roughly exponentially (once $P(t) \gg P_0$) at a constant fraction $\frac{c}{r-c}$
119 of the planktonic population. High colonization rates thus provide more biofilm cells at short time
120 scales, while lower colonization rates maximize biofilm over longer periods of growth (Figure 2B).

121 In Figure 2D, we find that the colonization rate maximizing biofilm cells declines with increasing
122 planktonic growth rates, giving a humped shape in biofilm as a function of colonization rate. We can
123 find an analytical condition for this relationship by examining the slope of B as a function of c as c
124 approaches r (Equation 2.3, see Supplemental Text 2 for derivation).

125 2.3) $\lim_{c \rightarrow r} \left(\frac{dB}{dc} \right) = -\frac{1}{2} P_0 t (rt - 2)$

126 If the slope is negative, this would imply an interior maximum in B at some $c < r$ (as biofilm cells
127 necessarily increase as c increases from zero). We find that this limit is negative for $rt > 2$, i.e. the
128 presence of a humped relationship in B requires patch quality (the product rt) to exceed a minimal
129 threshold value.

130 These results suggest that the colonization rate maximizing biofilm will depend on opportunities for
131 growth in the planktonic state (governed by growth rate r and growth duration t), which we explore
132 further in Figure 2E and F. In Figure 2E, plotting biofilm cells against planktonic cells reveals that while
133 limited growth (blue lines) leads to an allocation trade-off (i.e. increasing B necessarily comes at the cost
134 of decreasing P), increasing growth rate decouples this trade-off, with B maximized at diminishing
135 colonization rates c , depicted explicitly in Figure 2F. However, as colonization decreases beyond this
136 point, biofilm declines sharply as colonization tends to zero.

137 Under high growth regimes, the planktonic fraction may therefore be viewed as a ‘growth engine’ to
138 maximize biofilm: when within-patch conditions are sufficiently favorable to planktonic division
139 (Equation 2.3), reducing the biofilm colonization rate c below the maximum increases the net flux of
140 cells into the biofilm by expanding the pool of dividing planktonic cells, P . This growth engine effect is
141 sufficient to drive colonization rates maximizing biofilm down to a fraction of the growth rate (Figure
142 2F).

143 **Evolutionary Model**

144 While intermediate colonization rates may maximize biofilm, we note that any allocation towards the
145 biofilm comes at the cost total population size in Equation 2.4:

$$146 \quad 2.4) \quad \frac{d(P(t) + B(t))}{dc} = -\frac{rP_0(1 + ((r - c)t - 1)e^{(r-c)t})}{(r - c)^2}$$

147 which is strictly negative for $t > 0$ and equal to 0 at $t = 0$. Given this trade-off between the biofilm and
148 total population size, what conditions would favor biofilm investment ($c > 0$)? We can examine the
149 evolutionary consequences of allocation in the within-patch population model by constructing a life
150 cycle in which a population colonizes successive patches through space and/or time (i.e. migration
151 between patches, or remaining in a single patch that experiences periodic disturbances). We define a
152 fitness function (Equations 3.1 and 3.2) by assigning transmission probabilities k_p and k_b that a given cell
153 from the respective planktonic or biofilm compartments will go on to found a new patch:

154 3.1) $W = k_p P(t) + k_b B(t)$,

155 or, explicitly, per-founding cell (analogous to the reproductive number 'R0' framework common to
156 parasite epidemiology and evolution, e.g. in (Frank, 1996))

157 3.2) $W(c, r, t) = k_p e^{(r-c)t} + k_b (e^{(r-c)t} - 1) \frac{c}{r - c}$

158 Equation 3.2 allows us to interrogate the fitness consequences of biofilm investment strategies
159 (colonization rate c) across a wide array of ecological parameters. k_p and k_b capture the reproductive
160 value of each cell type, and can be interpreted equivalently as a per-cell transmission probability or as
161 the fraction of each subpopulation able to transmit successfully; they will dictate how well a given cell
162 type (biofilm or planktonic) can survive the inter-patch transition, and be influenced by the nature of the
163 environment. Growth time t describes the disturbance regime, i.e. how long a population can stay in a
164 single patch, and r measures the nutrient quality of individual patches, or how rapidly planktonic cells
165 can divide within the patch. We define c^* as the optimal colonization rate maximizing fitness W under a
166 given ecological condition, and display the behavior of c^* as a function of transmission parameters k_p
167 and k_b in Figure 3.

168 There are three general strategies microbes may adopt in maximizing fitness: devoting all resources to
169 the biofilm fraction ($c^* = r$, below black dotted lines in Figure 3), splitting resources between the two
170 fractions ($0 < c^* < r$, between dotted lines in Figure 3), or devoting all resources to the plankton ($c^* = 0$,
171 above white dotted lines in Figure 3). In Supplemental Text 3, we investigate the conditions governing
172 the two transitions defining these regimes by examining the behavior of Equation 3.2 in more detail.

173 *Trade-offs in allocation*

174 When growth opportunities are limited (Figure 3, upper left panel) we see evidence of a sharp tradeoff
175 between B and P investment, with very little parameter space allowing intermediate investments ($0 < c^*$
176 $< r$). In the limit of zero within-patch growth, the allocation decision becomes a ‘zero-sum’ game, with
177 no allowance for intermediate investment:

$$178 \quad 3.6) \quad \left. \frac{dW}{dc} \right|_{r=0} = te^{-ct}(k_b - k_p)$$

179 In this no growth scenario ($r = 0$), if biofilm cells have greater transmission value ($k_b > k_p$), then total
180 biofilm investment is favored; if not, then total planktonic cell investment is favored, giving a strict
181 trade-off defined by whichever fraction is preferentially transmitted between patches.

182 This simple zero-sum logic is intuitive, but fails significantly under more permissive growth conditions
183 (i.e. increasing r and/or t ; Figure 3 and Figure S2), where we see an intermediate level of colonization is
184 favored over a relatively large portion of the parameter space. Despite large transmission advantages to
185 biofilm cells, the intermediate colonization regime extends to the boundary of $k_p = 0$ (black dashed line
186 undefined for $r > 0.04$, Figure 3), such that even when planktonic cells have zero probability of founding
187 a new patch, the vast majority of the population is still allocated to that fraction. This result follows
188 from the dynamics of the within-patch model (Equations 2.1, 2.2 and Figure 2): when $k_p = 0$, fitness is

189 determined entirely by the size of the biofilm population, which is maximized at low colonization rates
190 under conditions favoring growth due to the driving force of the planktonic growth engine.

191 **Biofilm as a bet hedge against environmental instability**

192 In our evolutionary model (Figure 3), we assume that lineages can adapt their allocation decision making
193 (c^*) in the context of constant transmission weightings k_p and k_b . However, the relative success of
194 biofilm vs. planktonic cell propagules is likely to vary extensively in time as a function of unpredictable
195 biotic and abiotic stresses (i.e. changing k_p relative to k_b). Despite reduced colonization rates leading to
196 larger planktonic populations, sharply diminishing returns from further decreases in c (Figure 2E) suggest
197 that the biofilm compartment has the potential to function as a cost-effective hedge against
198 unpredictable selective events. At all growth rates, populations can exchange a small reduction in the
199 size of the planktonic population for a massive increase in the biofilm population by raising the rate of
200 colonization from a minimal value: returning to Equations 1.1 and 1.2, taking the ratio of the biofilm to
201 planktonic growth rate yields the fraction $\frac{c}{r-c}$, which approaches infinity as c approaches r .

202 Maintaining a small biofilm presence is therefore relatively low-cost, even in the absence of
203 environmental stresses that favor biofilm cells, suggesting that selection against low rates of biofilm
204 production will be weak. To test this hypothesis, we performed a selection experiment, transferring 12
205 replicate populations of *P. aeruginosa* for 20 transfers (approximately 100 generations), allowing only
206 planktonic or only biofilm cells to survive (Figure 4). While relative biofilm production rapidly decreased
207 in the plankton passaged lines ($k_b = 0$, $k_p = 1$, red points in Figure 4A), declining from ~40% to ~13% of
208 cells in biofilms after seven transfers (paired t-test, mean difference = 0.265, $t = 18.7$, $df = 11$, $p = 1.1 \times 10^{-9}$),
209 biofilm production did not evolve to be any lower over the remaining 13 transfers of the experiment
210 (Figure 4A, fraction biofilm from passages 7-20 best fit by an intercept model [AIC = -580] vs. a linear

211 model [AIC = -424]; also note in Figure 4C the biofilm OD holds roughly constant over the course of the
212 experiment).

213 Interestingly, in the biofilm-only selection line ($k_b = 1$, $k_p = 0$; Figure 4A, blue points), the proportion of
214 cells in biofilms did not increase over the course of the experiment, despite the total number of cells in
215 the biofilm increasing by more than 100% (Figure 4B). Particularly, we see that the planktonic cell
216 density increases steadily, while the biofilm fraction increases initially then stagnates after roughly 10
217 passages (Figure 4B, Table S1). This result is consistent with our models above, with planktonic growth
218 driving biofilm accumulation, and experimentally demonstrates the utility of a mixed strategy under
219 strict selection regimes.

220 The low cost of maintaining biofilm makes it well suited as a potential bet-hedging strategy, increasing
221 the long-term geometric mean fitness in unpredictable environments by minimizing the variance in
222 fitness through time. To test this prediction, we used our model framework to construct a simulated
223 passaging regime in which growth and transmission parameters were subject to differing levels of
224 variance. We assigned inoculum populations of planktonic cells a fixed colonization rate c (applied
225 relative to r), which were then subjected to alternating periods of growth and transmission. Parameters
226 r , t , k_p and k_b were drawn from normal distributions with fixed means μ , and differing variances σ^2
227 between treatments. The values for r , t and c were used to solve Equations 2.1 and 2.2; proportions k_p
228 and k_b of these new cells were then passaged to the next growth phase, as in Equation 3.1. The fitness
229 function defined in equation 3.2 (effectively a reproductive number R_0 (14)) was used as our fitness
230 metric for these simulations, calculated as the ratio of founding cells in a given passage relative to the
231 number of founding cells in the previous passage. Simulation results are displayed in Figure 5.

232 For all treatments, the variance in R_0 declines with increasing c , consistent with biofilm colonization
233 functioning as a bet-hedging strategy for bacteria facing unpredictable selection (Figure 6A). For low

234 and medium degrees of unpredictability, the average R_0 declines monotonically with increasing c ,
235 reflecting the penalty imposed by environmental variance on fitness (Figure 6B). Interestingly, under
236 the high variance treatment, a pronounced hump shape in average R_0 appears (Table S2), indicating that
237 under extremely unpredictable environments the trade-off between mean fitness and reduced variance
238 in fitness breaks down and biofilm formation is generally beneficial (Figure 6B). The combination of
239 direct fitness benefits and bet hedging effects lead to maxima in geometric mean R_0 at intermediate
240 colonization rates across all variance treatments (Figure 6C).

241 **DISCUSSION**

242 In this work, we constructed a simple deterministic model of allocation between coupled biofilm and
243 planktonic compartments within a growing bacterial population. Within the biofilm, cell division limited
244 by geometry and nutrient diffusion rendered its effects inconsequential to the dynamics of the
245 population as a whole, relative to the exponentially expanding pool of planktonic cells. Under
246 inhospitable conditions, in which the population experienced restricted growth and/or frequent
247 disruption, trade-offs between the biofilm and the planktonic compartment forced lineages to specialize
248 in whichever fraction was favored to transmit between patches. When conditions become more
249 permissive, the lineage is able to leverage exponential planktonic growth to maintain robust populations
250 in both compartments, and at a fraction of the cellular cost of direct biofilm allocation (i.e. at reduced
251 colonization rates c); in general, such cost saving measures are likely to be favored in any cooperative
252 trait during periods of growth (15). Because maintenance of a biofilm comes at little cost under
253 conditions favoring planktonic growth, biofilm is able to function as a robust and cost-effective hedge
254 against unpredictable environmental change. Conversely, the planktonic fraction is a useful amplifier of
255 biofilm cells even when biofilm is the transmissible propagule – this growth/transmission division of
256 labor is more obvious for strictly non-growing phenotypes (e.g. spores), but the same logic holds for
257 slow growth in biofilms as well.

258 We focus primarily on biofilm-planktonic cell populations as a model of survival-fecundity alternate
259 states, and therefore use the language of colonization (of biofilms by planktonic cells) and dispersal
260 (from the biofilm) to represent the switching processes between the two phenotypes. However, the
261 model logic applies to other classes of resistant cells as well, such as persisters and spores. Indeed,
262 there are instances where biofilm formation functions as a prerequisite or amplifying step in the
263 formation of other types of resistant cells: persister cells are often enriched in biofilms (8, 16–19), and
264 biofilm formation is a prerequisite step in fruiting body formation (the preferential site of sporulation) in
265 *Bacillus subtilis* (20, 21); it would be interesting to investigate how investment would be optimized with
266 multiple survival phenotypes available in both simultaneous and sequential contexts.

267 Given the costs inherent to cellular investment into a growth-limited state, one may expect lineages to
268 evolve the ability to efficiently switch between biofilm and planktonic phenotypes, thereby optimizing
269 fitness by reducing lag times and minimizing unnecessary mortality in the event of environmental
270 disturbance, and indeed such systems appear to be abundant in nature (22–27). However, we note that
271 environmental sensing in this case would not supplant the need to maintain some level of biofilm as a
272 hedge (Figure 5, Figure 6), but rather to enhance the efficiency of such maintenance; in environments
273 where catastrophic disturbances occur even at very low frequency, lineages that maintain biofilm
274 regardless will still have better chances of survival. One would therefore predict biofilm to be
275 completely lost in only the most constant environments (Figure 5).

276 Phenotypic regulation to further optimize allocation between biofilm and planktonic lifestyles would
277 also help expedite the evolution of rudimentary life cycles at the population level, alternating between a
278 growing ‘soma’ and dispersive ‘propagules’ with distinct demographics associated with each phase.
279 Under the formalism presented here, either the biofilm or planktonic compartments alone, or some
280 combination thereof, may serve as dispersing propagules. The historical archetype has generally held
281 that the biofilm functions as the soma, with motile planktonic cells as dispersive propagules (28–30).

282 More recently Hammerschmidt et al. (31) found that alternating selection on dispersive and biofilm
283 phenotypes in *Pseudomonas fluorescens* leads to the evolution of a lifestyle in which cooperative biofilm
284 cells producing shared adhesive molecules form a pellicle that functions as the growing soma, and
285 planktonic non-adhesive cheats are co-opted as dispersive propagules, thereby dividing labor between
286 the two cellular fractions and increasing the overall fitness of the lineage. Our results indicate that the
287 opposite cycle (biofilm cells as propagules, planktonic cells as soma) could also be viable, as the
288 population can still reap the benefits of dividing labor between specialized cellular fractions. Indeed,
289 where individual patches are permissive to growth, but transmission between patches is exceedingly
290 harsh (e.g. wind- or animal-dispersal), dispersal via biofilm aggregates and growth within a planktonic
291 ‘soma’ would offer the greatest advantage, as the ‘soma’ would accumulate biomass rapidly, and
292 dispersal propagules would enjoy increased survival at little reproductive cost given the hostile
293 transmission conditions. For example, biofilm formation and other survival phenotypes are likely
294 important to successful transmission via fomites, upon which bacteria can remain viable for months
295 (32). Dispersal in physically linked groups (i.e. budding dispersal, (33)) may also help maintain
296 cooperative traits during dispersal, thereby potentially accelerating colonization when a new patch is
297 reached. The biofilm ‘streamers’ observed by Drescher et al. (34) may be another example of this mode
298 of transmission, where flow rates are such that the biofilm forms physical bridges to allow colonization
299 of vacant surfaces in a topographically complex environment, as full detachment would prevent
300 recolonization of adjacent surfaces due to extreme shear forces.

301 Taken together, our results highlight the evolutionary significance of within-population phenotypic
302 heterogeneity and its consequences for survival and fecundity in mixed transmission environments. By
303 optimizing the switching rate between robust and fecund specialists (here, the colonization rate from
304 the planktonic to biofilm fractions, though we note that other mechanisms could lead to equivalent
305 outcomes, such as the steady-state frequencies of genotypes arising from within-population

306 diversification, as in (35, 36)), lineages were able to maximize fitness and transmission across a wide
307 range of environments, as well as enhance survival in the face of catastrophic changes within the
308 environment. The rate of phenotypic switching is therefore an essential parameter upon which
309 selection may act when multiple phenotypes can persist within lineages.

310 **METHODS**

311 *Passaging experiment*

312 A mid-exponential phase culture of *P. aeruginosa* PA01 was used to inoculate 200 uL LB and one 3 mm
313 sterile glass bead in each of 24 wells in a 96 well plate at an OD of 0.05. Plates were sealed with
314 Aeraseal tape and grown statically at 24 °C in a humidified chamber. Every 12 hours (growth conditions
315 were chosen to prevent entry into stationary phase, where multiple regulatory systems that modulate
316 biofilm and growth behaviors are engaged), biofilm allocation was measured by removing and
317 measuring the OD of the liquid phase, then washing, resuspending and measuring the density of the
318 attached biofilm as above in 200 uL fresh LB. 12 lines had only the planktonic cells passaged, while the
319 other 12 had only biofilm cells passaged; in each case, cells were diluted to an inoculum OD of 0.05, and
320 20 passages were performed.

321 *Statistics and mathematical analysis*

322 Agent based simulations were performed using iDynoMiCs (13), and analytical analyses were performed
323 using Mathematica. Numerical modelling (37) and statistics were performed in R (38), unless otherwise
324 noted.

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410

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419 **FIGURE LEGENDS**

420 **Figure 1: Biofilms grow sub-exponentially.** **A**, accumulated cells and **B**, lineage growth rate through
421 time for agent-based simulations of a 2-D biofilm growing on a 1-D surface. Varying inocula (see legend)
422 were allowed to grow for a fixed time period under nutrient-rich conditions. Simulations were
423 implemented using the agent-based simulation platform iDynoMiCS (13). **C**, Schematic depiction of
424 nutrient depletion leading to growth arrest in the biofilm interior. Diffusion and active consumption of
425 nutrients in the outermost layers of the biofilm (yellow cells) result in starvation conditions for cells in
426 the interior regions (red cells).

427 **Figure 2: Maximal biofilm can be driven by planktonic growth.** **A**, time series of planktonic and **B**,
428 biofilm cells, for colonization rates c between $0.1r$ (purple) and $0.99r$ (yellow), with $r = 0.08$, $P_0 =$
429 $5,000,000$. **C**, planktonic, and **D**, biofilm cells as a function of colonization rate relative to growth rate
430 for $t = 40$, $0.02 \leq r \leq 0.12$ (blue-red color scale), $P_0 = 5,000,000$. **E**, Biofilm cells plotted against planktonic
431 cells under the same conditions as C and D. The colonization rate varies over each curve, with the end
432 points indicated by labels (relative colonization c/r approaches 1 at the left, and zero at the right).
433 Diamonds indicate the maximum in biofilm cells. **F**, Relative colonization rate c/r at which biofilm is

434 maximized. Note in panels D and E the limit of $c = 0$ is omitted, as this prevents any formation of biofilm
435 cells.

436 **Figure 3: Optimal colonization rate c^* as a function of reproductive value for planktonic and biofilm**
437 **cells.** Contour plots showing the relative colonization rate (c^*/r , yellow-purple color scale) optimizing
438 fitness (Equation 3.2). Each panel displays c^*/r as a function of k_p and k_b . Across panels, the growth rate
439 r increases from $r = 0.02$ to 0.12 , with $t = 40$ in all cases; similar plots varying t are displayed in Figure S2.
440 White dotted line indicates the threshold at which $c^* > 0$, and black dotted line indicates the threshold
441 at which $c^* < 1$ (see main text and Supplemental Text 3).

442 **Figure 4: Selection for planktonic transmission fails to purge biofilm from experimental populations.**
443 *P. aeruginosa* (PAO1) was grown in 96-well plates containing a glass bead at room temperature, and
444 every 12 hours populations were fractionated, measured, and passaged to a new well. **A**, Proportion
445 biofilm (defined as the ratio of the biofilm optical density (OD) to the sum of the ODs of the two
446 fractions), **B**, fractionated ODs of B-selected ($k_b = 1$, $k_p = 0$), and **C**, fractionated ODs of P-selected ($k_b = 0$,
447 $k_p = 1$) lines over the course of the passaging experiment. Points represent 12 independently evolving
448 lineages per treatment, and curves from best-fit regression (adjusted $R^2 = 0.92$, Table S1). All ODs are
449 reported corrected for the OD of blank medium.

450 **Figure 5: Biofilm production can act as a bet-hedging strategy.** Log-scale plots of **A**, variance in R_0 , **B**,
451 average R_0 , and **C**, geometric mean of R_0 , are plotted as a function of relative colonization rate (ratio of
452 c to the expected value for r , see below) for lineages subject to passaging simulations with different
453 levels of environmental instability. 100 replicate inocula of 5000 planktonic cells with the same fixed
454 colonization rates $c \in [0, 0.2]$ were subjected to 10 passages. For all cases, $\mu_r = 0.06$, $\mu_t = 40$, $\mu_{kp} = 0.1$
455 and $\mu_{kb} = 0.6$. Under fixed conditions these parameters would favor $c^* = 0.49$. Variance treatments
456 modified σ^2 of the distributions from which r , t , k_p and k_b were drawn, with $\sigma^2_{low} = \mu/100$, $\sigma^2_{mid} = \mu/9$ and

457 $\sigma^2_{\text{high}} = \mu/2.75$. Parameters were restricted to logical values, with $0 \leq k_b, k_p \leq 1$, $r > 0$, and $t \geq 1$; this
458 thresholding did not change the overall mean of any parameter by more than $\pm 3\%$. R_0 was calculated as
459 the ratio of founding cells at a given passage relative to the founding cells of the previous passage.
460 Points and error bars (offset to reduce overlap) represent means with 95% confidence intervals, and
461 solid lines display best-fit regressions (Table S2).









