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1 **Strain background, species frequency and environmental conditions are**
2 **important in determining population dynamics and species co-existence between**
3 ***Pseudomonas aeruginosa* and *Staphylococcus aureus***
4
5
6
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13
14 **Running title:** How ecology affects competition between two pathogens
15
16
17 **Keywords:** opportunistic human pathogens, interspecies interactions, polymicrobial
18 infections, ecological factors, community dynamics

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19 **Abstract**

20 Bacterial communities in the environment and in infections are typically diverse, yet we
21 know little about the factors that determine interspecies interactions. Here, we apply
22 concepts from ecological theory to understand how biotic and abiotic factors affect
23 interaction patterns between the two opportunistic human pathogens
24 *Pseudomonas aeruginosa* and *Staphyloccocus aureus*, which often co-occur in
25 polymicrobial infections. Specifically, we conducted a series of short- and long-term
26 competition experiments between *P. aeruginosa* PAO1 (as our reference strain) and
27 three different *S. aureus* strains (Cowan I, 6850, JE2) at three starting frequencies and
28 under three environmental (culturing) conditions. We found that the competitive ability of
29 *P. aeruginosa* strongly depended on the strain background of *S. aureus*, whereby
30 *P. aeruginosa* dominated against Cowan I and 6850, but not against JE2. In the latter
31 case, both species could end up as winners depending on conditions. Specifically, we
32 observed strong frequency-dependent fitness patterns, including positive frequency
33 dependence, where *P. aeruginosa* could dominate JE2 only when common, but not
34 when rare. Finally, changes in environmental (culturing) conditions fundamentally
35 altered the competitive balance between the two species, in a way that *P. aeruginosa*
36 dominance increased when moving from shaken to static environments. Altogether, our
37 results highlight that ecological details can have profound effects on the competitive
38 dynamics between co-infecting pathogens, and determine whether two species can co-
39 exist or invade each others' populations from rare. Moreover, our findings might parallel
40 certain dynamics observed in chronic polymicrobial infections.

41

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42 **Importance**

43 Bacterial infections are frequently caused by more than one species and such
44 polymicrobial infections are often considered more virulent and more difficult to treat
45 than the respective monospecies infections. *Pseudomonas aeruginosa* and
46 *Staphyloccocus aureus* are among the most important pathogens in polymicrobial
47 infections and their co-occurrence is linked to worse disease outcome. There is great
48 interest in understanding how these two species interact with each other and what the
49 consequences for the host are. While previous studies have mainly looked at molecular
50 mechanisms implicated in interactions between *P. aeruginosa* and *S. aureus*, here we
51 show that ecological factors such as strain background, species frequency and
52 environmental conditions are important elements determining population dynamics and
53 species co-existence patterns. We propose that the uncovered principles may also play
54 major roles in infections, and therefore proclaim that an integrative approach combining
55 molecular and ecological aspects is required to fully understand polymicrobial
56 infections.

57

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58 **Introduction**

59 Bacteria typically live in complex multi-species communities in the environment and
60 associated with host organisms (1–3). The same holds true in the case of disease, as it
61 is increasingly recognized that a majority of bacterial infections are polymicrobial,
62 meaning that they are caused by more than one bacterial species (4, 5). There is great
63 interest in understanding how bacteria interact and how interactions affect a community
64 and the associated hosts (6–8). At the mechanistic level, a multitude of ways have been
65 unraveled through which bacterial species can interact, with mechanisms including
66 cross-feeding, quorum sensing-based signaling, toxin-mediated interference and
67 physical interactions via contact-dependent systems (e.g. type VI secretion system) (9–
68 11). In the context of disease, a key question is how interactions affect species
69 successions in chronic infections and whether multispecies infections are more virulent
70 and more difficult to treat than the respective monospecies infections, as it is commonly
71 assumed (5, 12–14).
72
73 Studying interactions between the two opportunistic human pathogens
74 *Pseudomonas aeruginosa* (PA) and *Staphylococcus aureus* (SA) has emerged as a
75 popular and relevant model system (15–17). The reason for this is that the two species
76 often co-occur in infections, including cystic fibrosis (CF) lung and wound infections
77 (18–20). Results from laboratory experiments suggest that PA is the superior species,
78 suppressing growth of SA (21–23) and indeed, PA seems to be a well-equipped
79 competitor. For example, it has been shown that 4-hydroxy-2-heptylquinoline N-oxide
80 (HQNO) released by PA inhibits the electron transport chain of SA and induces the

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81 formation of small colony variants (SCVs) (22, 24). Furthermore, the PA endopeptidase
82 LasA is capable of lysing SA cells, a process that releases iron into the environment,
83 potentially providing a direct benefit to PA (21, 25). While it was observed that
84 experimental co-infections of PA and SA seem to be more virulent than the respective
85 monospecies infections (26, 27), evolutionary studies revealed that PA can adapt to the
86 presence of SA (28) and become more benign in the context of chronic co-infections
87 (29, 30). Moreover, of clinical relevance is the observation that PA and SA exhibited
88 increased antibiotic resistance or tolerance when co-cultured compared to being
89 cultured alone (31–33).
90
91 In this study, we follow a complementary approach to examine how biotic and abiotic
92 ecological factors influence interactions between PA and SA. Previous work has
93 primarily focused on the molecular mechanisms driving interactions between specific
94 strain pairs under defined laboratory conditions. Here we hypothesize that not only
95 molecular mechanisms, but also ecological factors will have a major impact on species
96 interactions, particularly on community composition and temporal dynamics between
97 species. To test our predictions, we used PA strain PAO1 as our focal strain and asked
98 how (competitive) interactions with SA vary when manipulating: (1) the genetic
99 background of SA; (2) the frequency of SA in competition with PA; and
100 (3) environmental (culturing) conditions.
101
102 To vary the genetic background of SA, we competed PA against the three different SA
103 strains Cowan I, 6850 and JE2. These strains fundamentally differ in several

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104 characteristics (Table 1). Cowan I is a methicillin-sensitive SA strain (MSSA), which is
105 highly invasive towards host cells, non-cytotoxic and defective in the accessory gene
106 regulator (agr) quorum-sensing system (34). 6850 is another MSSA strain, which is
107 highly invasive, cytotoxic and haemolytic (35–37). Finally, JE2 is a methicillin-resistant
108 (MRSA) USA300 strain, which is highly virulent, cytotoxic and hemolytic (38, 39). Given
109 the tremendous differences between these SA strains, we expect PA performance in
110 competition with SA to vary substantially.
111
112 To manipulate strain frequency, we competed PA against SA at three different starting
113 frequencies (1:9 ; 1:1 ; 9:1). Frequency-dependent fitness effects occur in many
114 microbiological systems (40–43). A common pattern is that species have a relative
115 fitness advantage when rare in a population, but not when common (so-called negative
116 frequency dependence). This phenomenon can lead to stable co-existence of
117 competitors. On the other hand, the fitness of a species can also be positive frequency
118 dependent, which means that a species is dominant when common in the community,
119 but not when rare. An important consequence of this pattern is that initially rare species
120 cannot invade an established population.
121
122 To manipulate environmental conditions, we changed simple parameters of our
123 culturing conditions. First, we compared the performance of PA against SA strains in
124 shaken liquid vs. viscous medium. Increased environmental viscosity has been shown
125 to increase spatial structure, thereby decreasing strain interaction rates (44–46).
126 Second, we compared the performance of PA against SA in shaken vs. static

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127 environments. While static conditions also reduce strain mixing, it further leads to a
128 more heterogeneous environment characterized by gradients from the aerated air-liquid
129 interface down to the microoxic bottom of a culture (47, 48).
130
131 In a first set of experiments, we assessed the growth performance of all strains in
132 monoculture under the three different environmental culturing conditions used. Basic
133 growth differences between strains could induce frequency shifts in co-cultures even in
134 the absence of direct interactions. We then performed high-throughput 24 hours batch
135 culture competition experiments between PA and SA using a full-factorial design. All
136 three species combinations were competed at all three starting frequencies under all
137 three environmental conditions (see Figure 1 for an illustration of the workflow). Finally,
138 we followed the temporal dynamics between PA and SA over five days to assess
139 whether results from 24 hours competitions are predictive for more long-term dynamics
140 between species and whether species coexistence is possible. (A copy of this
141 manuscript has been posted on bioRxiv doi: https://doi.org/10.1101/2020.04.21.052670)

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142 **Results**

143 **PA grows better than SA in monoculture**

144 We used tryptic soy broth (TSB) as the standard medium for all our assays. In this
145 medium, we found that the number of doublings varied significantly among strains
146 during a 24 hours growth cycle under all conditions tested (ANOVA, shaken: $F_{3,20}$ =
147 10.71, P = 0.0002; viscous: $F_{3,20}$ = 4.12, P = 0.0199; static: $F_{3,20}$ = 20.75, $P < 0.0001$,
148 Figure 2 and see Table S1 for the full statistical analysis). Under shaken conditions, PA
149 had the highest number of doublings (20.6 ± 0.63, mean ± SD), followed by SA strains
150 6850 (18.9 ± 1.16), Cowan I (18.1 ± 0.41) and JE2 (18.0 ± 1.18). While PA grew
151 significantly better than all SA strains, the number of doublings did not differ between
152 the three SA strains (TukeyHSD pairwise comparisons: Cowan I vs. 6850, P_{adj} = 0.3673;
153 6850 vs. JE2, P_{adj} = 0.3096; Cowan I vs. JE2, P_{adj} = 0.9994). Due to its moderate
154 growth advantage, PA is expected to slightly increase in frequency in competition with
155 SA strains under shaken conditions, even in the absence of any direct species
156 interactions.

157

158 Under viscous conditions, PA had the highest number of doublings as well (17.2 ±
159 1.02), followed by 6850 (17.0 ± 0.76), JE2 (16.3 ± 0.52) and Cowan I (15.6 ± 1.06).
160 However, differences in the number of doublings were only significant between PA and
161 Cowan I and between Cowan I and 6850 (TukeyHSD pairwise comparisons: PA vs.
162 Cowan I, P_{adj} = 0.0265; Cowan I vs. 6850, P_{adj} = 0.0496). Thus, based on growth rate
163 differences alone, one would expect PA to increase in frequency in competition with
164 Cowan I but not in competition with the other two SA strains.

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165

166 Under static conditions, PA again showed the highest number of doublings (18.6 ± 0.64)
167 followed by 6850 (16.5 ± 0.45), JE2 (15.9 ± 0.96) and Cowan I (15.1 ± 1.05). PA grew
168 significantly faster than all the three SA strains (TukeyHSD pairwise comparisons: PA
169 vs. Cowan I, $P_{adj} < 0.0001$; PA vs. 6850, $P_{adj} = 0.0009$; PA vs. JE2, $P_{adj} < 0.0001$), and
170 one would therefore expect PA to substantially increase in frequency against all three
171 SA strains under static conditions.

172

173 **Genetic background, strain frequency and environmental factors all influence**
174 **competition outcomes**

175 The full-factorial design allowed us to simultaneously analyze the impact of SA strain
176 genetic background, starting frequency, and culturing condition on the competitive
177 outcomes between PA and SA strains. Our linear statistical model yielded a significant
178 triple interaction between the three manipulated factors (strain genetic background,
179 starting frequency and culturing condition; ANCOVA: $F_{4,509} = 3.41$, $P = 0.0091$). While
180 this shows that all three manipulated factors influence the competitive outcomes
181 between PA and SA in complex ways, the triple interaction makes it difficult to tease
182 apart the various effects. The statistical procedure for such cases is to split the model
183 into sub-models. We followed this approach by first analyzing separate models for each
184 of the three environmental conditions (shaken, viscous, static), and then split models
185 according to SA strain background to test for differences between environmental
186 conditions.

187

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188 **The competitive ability of PA depends on the SA strain genetic background**

189 Under all three environmental conditions, we found that the relative fitness of PA
190 significantly depended on the SA strain background (ANCOVA, shaken: $F_{2,170} = 90.87$,
191 $P < 0.0001$; viscous: $F_{2,168} = 116.76$, $P < 0.0001$; static: $F_{2,170} = 56.52$, $P < 0.0001$;
192 Figure 3). Against Cowan I (Figure 3, column 1), we noted that PA consistently won the
193 competitions across all starting frequencies and culturing conditions. SA strain 6850
194 (Figure 3, column 2) turned out to be more competitive than Cowan I under shaken
195 conditions ($t_{176} = -6.74$, $P < 0.0001$), while it lost similarly against PA under viscous and
196 static conditions (viscous: $t_{174} = 0.78$, $P = 0.4350$; static: $t_{176} = -1.99$, $P = 0.0482$). In
197 contrast, JE2 was the most competitive SA strain in our panel (Figure 3, column 3),
198 performing significantly better than the other two SA strains under all conditions (see
199 Table S2 for the full statistical analysis), and outcompeted PA under shaken and
200 viscous conditions.

201

202 **The competitive ability of PA depends on its starting frequency in the population**

203 We found that the starting frequency of the two competitors had varying but always
204 significant effects on the competitive ability of PA (ANCOVA, shaken: $F_{1,170} = 52.81$,
205 $P < 0.0001$; viscous, interaction with strain background: $F_{2,168} = 10.05$, $P < 0.0001$;
206 static: $F_{1,170} = 162.32$, $P < 0.0001$). Under shaken conditions (Figure 3, row 1), we
207 observed that the relative fitness of PA increased when initially more common, thus
208 following a positive frequency-dependent pattern. Under viscous conditions (Figure 3,
209 row 2), the same positive frequency-dependent effect was only observed when PA
210 competed with JE2. In competition with Cowan I or 6850, we noted that the relative

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211 fitness of PA peaked at intermediate starting frequencies. Under static conditions
212 (Figure 3, row 3), we observed a pattern opposite to the one seen under shaken
213 conditions for all strain pair combinations. The relative fitness of PA decreased when
214 initially more common, thus following a negative frequency-dependent pattern (see
215 Table S2 for the full statistical analysis).
216
217 **The competitive ability of PA is highest under static conditions**
218 Next, we compared the competitive outcomes among the different culturing conditions
219 (shaken, viscous and static) for each strain combination separately. For all strain
220 combinations, the culturing condition significantly affected competition outcomes
221 (ANCOVA, Cowan I: $F_{2,168} = 461.73$, $P < 0.0001$; 6850: $F_{2,167} = 428.16$, $P < 0.0001$; JE2:
222 $F_{2,168} = 199.95$, $P < 0.0001$). In competition with all three SA strains, we found that the
223 relative fitness of PA was significantly higher under static compared to shaken
224 conditions (Cowan I: $t_{174} = 19.99$, $P < 0.0001$; 6850: $t_{174} = 17.99$, $P < 0.0001$; JE2: $t_{174} =$
225 15.39, $P < 0.0001$). In contrast, there were no significant differences in the relative
226 fitness of PA between shaken and viscous conditions for Cowan I ($t_{174} = 0.91$, $P =$
227 0.3644) and JE2 ($t_{174} = 0.82$, $P = 0.4117$), while against 6850, PA was more competitive
228 under viscous than shaken conditions ($t_{174} = 3.53$, $P = 0.0005$) (see Table S2 for the full
229 statistical analysis).
230
231 **Temporal dynamics between PA and SA**
232 In a next experiment, we competed PA and SA strains over five days under shaken
233 conditions using the same three starting frequencies and by transferring cultures to

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234 fresh medium every 24 hours. The aim of this experiment was to follow the more long-
235 term species dynamics and to assess whether stable coexistence between PA and SA
236 can arise.
237
238 In competition with Cowan I, we found PA to be the dominant species (Figure 4a). It
239 strongly increased in frequency already at day 1 under all starting frequencies and
240 almost completely outcompeted Cowan I by day 3 (i.e., Cowan I remained below
241 detection limit). Thus, we could not observe coexistence between PA and Cowan I. In
242 competition with 6850, we observed similar population dynamics (Figure 4b). PA
243 strongly increased in frequency from day 1 onwards at all starting frequencies and after
244 three days, the bacterial populations almost entirely consisted of PA. Only in 10 out of
245 30 populations, 6850 managed to persist at very low frequencies by day 5 (< 3% in nine
246 cases, and 13% in one case). In competition with JE2, we found community trajectories
247 that were strikingly different from the other two strain combinations (Figure 4c). First, we
248 observed that JE2 was a strong competitor, keeping PA at bay in many populations
249 during the first 24 hours of the experiment. Following day 1, community dynamics
250 followed positive frequency-dependent patterns. In all populations with intermediate or
251 high PA starting frequencies, PA became the dominant species, and SA was recovered
252 at low frequency in only a minority of populations by day 5 (3 out of 20 at < 10% of the
253 population). In stark contrast, in populations where PA was initially rare, it did not
254 increase in frequency, could not invade the SA populations and remained at a low
255 frequency (< 10%) throughout the 5 days.

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256 **Discussion**

257 *Pseudomonas aeruginosa* (PA) and *Staphylococcus aureus* (SA) frequently occur
258 together in polymicrobial infections, where they cause severe host damage and lead to
259 increased morbidity and mortality in patients (14, 49, 50). Consequently, there is high
260 interest in understanding how PA and SA interact and how their interactions may
261 influence disease outcome (12, 15). While most previous studies have focused on
262 molecular aspects (51, 52), we here examined how a set of ecological factors affect
263 competitive interactions between the two species. Our study, carried out in an *in vitro*
264 batch culture system, revealed that: (i) the competitive ability of PA varied extensively
265 as a function of the genetic background of SA; (ii) there were strong frequency-
266 dependent fitness patterns, including positive-frequency dependent relationships where
267 PA could only dominate a particular SA strain (JE2) when common, but not when rare;
268 and (iii) changes in environmental (culturing) conditions fundamentally affected the
269 competitive balance between the two species. The key conclusion from our results is
270 that ecology matters, and that variation in biotic and abiotic factors affect interactions
271 between pathogenic bacterial species. This is most likely not only the case in *in vitro*
272 systems, but also in the context of polymicrobial infections.

273

274 PA has often been described as the dominant pathogen possibly displacing SA in
275 infections (21, 53–55). Our results support this view, as PA dominated over SA under
276 many conditions in 24 hours and 5-day competition experiments (Figures 3 and 4).
277 However, PA did not always emerge as the winner and its success significantly varied in
278 response to the genetic background of SA. Specifically, JE2 was the strongest

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279 competitor, followed by 6850 and Cowan I. While differences in monoculture growth
280 performance can explain why PA dominates in many cases, they cannot explain the
281 variation in competitive abilities among SA strains, because the three SA strains grew
282 similarly under all conditions (Figure 2). PA vs. JE2 makes the strongest case for a
283 mismatch between growth performance in mono- vs. mixed cultures, as PA grew
284 significantly better than JE2 in monoculture under shaken conditions, but typically lost
285 the competition in mixed cultures in this environment (Figures 2 and 3). This suggests
286 that apart from resource competition via growth rate differences, other factors must
287 contribute to the competitive ability of SA strains towards PA. Such factors could include
288 interference mechanisms. For example, it is known that genetically different SA strains
289 widely differ in virulence among each other (56–58). Interestingly, we found that the
290 competitive ability of our three SA strains against PA correlated with their reported
291 virulence level in infections (34, 36, 39). This could indicate that factors important for SA
292 virulence (e.g. toxins or secreted enzymes) might also be involved in interactions with
293 competitor bacteria. For JE2 and related USA300 isolates, there are many genetic
294 determinants known to be important for their success as opportunistic human
295 pathogens (59). Among them are the cytotoxin Panton-Valentine leukocidin (PVL), the
296 arginine catabolic mobile element (ACME) and the phenol soluble modulins (PSMs)
297 (60). Derivatives of PSMs have previously been shown to exhibit inhibitory activity
298 against *Streptococcus pyogenes* (61). The authors of this work suggested that high
299 production of PSMs might not only benefit SA in host colonization, but also in
300 competition against co-infecting pathogens. Thus, it seems plausible that the USA300
301 derivative JE2 deploys a similar mechanism against PA in our competition experiments.

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302 Strain 6850 showed intermediate competitiveness against PA. As Cowan I, 6850 is a
303 MSSA strain, but it is known to be more virulent than Cowan I and therefore likely
304 produces certain substances that could also be important in competition with PA (34,
305 36). Conversely, Cowan I is known to have a nonfunctional accessory gene regulator
306 (agr) quorum sensing system (34). The agr controls most virulence determinants in SA
307 (62). If virulence determinants also played a role in interspecies competition, then this
308 could explain why Cowan I turned out to be the least competitive SA strain against PA.
309

310 Important to note is that we only manipulated the SA but not the PA strain background,
311 so that we cannot draw conclusions on genotype-by-genotype interactions. Such
312 interactions are likely to play a role as evidenced by previous studies showing that
313 genetically diverse PA clinical isolates widely differ in their ability to inhibit SA (29, 63,
314 64). More recently, it has also been shown that SA clinical isolates vary in their
315 interactions with PA, from being highly sensitive to completely tolerant against PA-
316 mediated effects (65). One aim of our future work is to follow up these proposed
317 mechanistic leads and test some of the outlined hypotheses above in order to explain
318 differences in the competitive abilities between SA strains towards PA.
319

320 Another insight from our experiments is that the competitive ability of PA often
321 depended on its starting frequency in the population, and that the type of frequency-
322 dependent interactions (positive or negative) varied across environmental conditions
323 (Figure 3). Our purpose was to compare three experimentally defined starting
324 frequencies to mimic what is happening when a species is either rare (1:9), at parity

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325 with its competitor (1:1) or dominant (9:1). Under natural conditions, including infections,
326 species frequencies could of course be more extreme, and invasion from rare could
327 start at species frequencies that would be below the detection limit of our methods.
328 In our experiments, we observed that under static conditions, the relative fitness of PA
329 declined when more common in the population, but PA still won at all frequencies. This
330 pattern is common for a highly dominant species that drives a competitor to extinction
331 (66). Its decline in relative fitness simply reflects the fact that the room for further
332 absolute frequency gains is reduced when a high frequency is already reached. In stark
333 contrast, under shaken conditions, we found that the relative fitness of PA increased
334 when it was more common in the population. Against Cowan I and 6850, this positive
335 frequency-dependent fitness pattern did not affect the long-term community dynamics
336 and PA won at all frequencies (Figure 4a+b). Against JE2, however, the 24 hours
337 competition data suggest that, in most cases, PA cannot invade populations when
338 initially rare and this is exactly what we observed in the long-term experiments: when its
339 initial frequency was below 10%, PA did not increase in frequency, while it always fixed
340 in the population or reached very high frequencies when initially occurring above 10%.
341 There were two additional interesting observations with regards to PA-JE2 long-term
342 dynamics. First, there were no major changes in PA frequency relative to JE2 during the
343 first 24 hours (compatible with the competition assay data in Figure 3), and clear
344 positive-frequency dependent patterns only emerged from 48 hours onwards. One
345 possible explanation for this pattern is that PA is initially naïve, but then senses and
346 mounts a more competitive response over time (67). Similarly, SA might also respond,
347 for example through increased formation of small colony variants (SCVs), which are

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348 known to be induced by inhibitory exoproducts released by PA (22, 24). Second, one
349 replicate (starting frequency 1:1) did not follow the above rules: PA continuously
350 dropped in frequency until day 4 (11%) and then sharply increased to 93% on day 5.
351 This frequency "zigzag" pattern is an indicator of antagonistic co-evolution (68), where
352 the spread of a beneficial mutation in one species (SA) is followed by a counter-
353 adaptation in the competing species (PA). It therefore seems that such evolutionary
354 dynamics can already occur within relatively short periods of time. This finding supports
355 evidence from studies on clinical PA isolates, which showed patterns of adaptations
356 towards SA favoring co-existence between the two species over time (29, 30).
357
358 Our results further show that the competitive ability of PA is profoundly influenced by
359 environmental (culturing) conditions (Figure 3). The largest differences arose between
360 shaken and static culturing conditions with PA being most competitive in the latter
361 environment. PA is known to be metabolically versatile, it is motile and grows well under
362 microoxic conditions (69, 70). Static conditions introduce strong oxygen and nutrient
363 gradients, and our results from monoculture growth show that PA grows better under
364 these conditions than SA (Figure 2). This is certainly part of the reason why PA ends up
365 as the competition winner against all SA strains under static conditions. With regard to
366 medium viscosity, we initially hypothesized that increased spatial structure could temper
367 competitive interactions and favor species co-existence, as competitors are spatially
368 more segregated from each other (44, 71, 72). However, we found no support for this
369 hypothesis as the competitive ability of PA did not much differ between shaken and
370 viscous environments. While the spatial structure, introduced through the addition of

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371 agar to the liquid growth medium, had significant effects on within-species social
372 interactions in other study systems (66, 73), it did not appreciably affect the between-
373 species interactions in our setup. One reason might be that the degree of spatial
374 structure introduced (0.2% agar in TSB) was simply not high enough to see an effect.
375 This could especially be true if toxins were involved in mediating interactions – small
376 molecules that can freely diffuse and target competitors that are not physically close-by.
377

378 We argue that our results, even though they stem from an *in vitro* system, could have at
379 least three important implications for our understanding of polymicrobial infections. First,
380 we show that the biological details of the strain background matter and determine who
381 is dominant in a co-infection and whether co-existence between species is possible.
382 Thus, we need to be careful not to overinterpret interaction data from a single PA-SA
383 strain pair and conclude that the specific details found apply to PA-SA interactions in
384 general. Second, there might be strong order effects, such that the species that infects
385 a host first cannot be invaded by a later arriving species. This scenario applied to the
386 interactions between PA and SA strain JE2, which were both unable to invade
387 populations of the other species from rare. Finally, local physiological conditions at the
388 infection site, like the degree of spatial structure or oxygen supply, can shift the
389 competitive balance between species. This suggests that infections at certain sites
390 might be more prone than others to polymicrobial infections, or to experience ecological
391 shifts from one pathogen to another. To sum up, we wish to reiterate our take home
392 message that the ecology of interactions between pathogens should receive more
393 attention and may explain so far unresolved aspects of polymicrobial infections.

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394 **Materials and Methods**

395 **Bacterial strains, media and growth conditions**

396 We used the *Pseudomonas aeruginosa* (PA) strain PAO1 (74) as our PA reference
397 strain and the *Staphylococcus aureus* (SA) strains Cowan I, 6850 and JE2 for all
398 experiments (Table 1). To distinguish PA from SA strains, we used a variant of our PA
399 strain PAO1, which constitutively expresses the green fluorescent protein, from a single-
400 copy gene (attTn7::ptac-gfp), stably integrated in the chromosome (75, 76). We chose
401 the rich laboratory medium tryptic soy broth (TSB, Becton Dickinson) for all our
402 experiments, because it supports growth of all the strains used. Bacterial stocks were
403 prepared by mixing 50% of culture with 50% of a 85% glycerol solution and were stored
404 at -80 °C. For all experiments, overnight cultures were grown in 10 ml TSB in 50 ml
405 falcon tubes for ± 16 hours at 37 °C and 220 rpm with aeration. After centrifugation and
406 removal of the supernatant, we washed bacterial cells using 10 ml 0.8% NaCl solution
407 and adjusted the OD_{600} (optical density at 600 nm) to obtain similar cell numbers per ml
408 for each strain. All media, buffer and washing solutions were sterilized by autoclaving at
409 121°C for 20 minutes and subsequently stored at room temperature in the dark. For all
410 experiments, blanks were used to ensure the sterility of the media during
411 experimentation.

412

413 **Calculating number of doublings for each strain in monoculture**

414 To assess the number of doublings of each strain in monoculture, we grew our strains in
415 TSB (or TSB + 0.2% agar, respectively) under the same conditions and using the same
416 starting OD_{600} as for the competition experiments (see below). We serially diluted cells

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417 at the start (t_0) and after 24 hours (t_{24}), and plated aliquots on TSB + 1.2% agar. The
418 plates were incubated overnight at 37 °C and colony- forming units (CFUs) counted for
419 both timepoints on the following day. We estimated the number of doublings (D) for
420 each strain as $D = (\ln(x_{24}/x_0))/\ln(2)$, where x_0 and x_{24} are the initial and the final CFU/ml,
421 respectively (23). We performed this experiment two times with three replicates per
422 strain per experiment.
423

424 **Competition experiments**

425 To initiate competitions, we mixed PA and SA strain pairs at three different starting
426 frequencies (1:9, 1:1, 9:1) from washed and OD_{600}-adjusted overnight cultures (see
427 above). Competitions occurred in 24-well plates filled with 1.5 ml TSB per well. The
428 starting OD_{600} of both mixed and monocultures was 10^{-5}. Monocultures of each strain
429 served as controls in each experiment. We incubated plates for 24 hours at 37 °C under
430 three different culturing conditions: shaken (170 rpm), viscous (170 rpm with 0.2% agar
431 in TSB) and static. Prior and after the 24 hours competition period, we estimated the
432 actual strain frequencies for each replicate using flow cytometry. We performed four
433 independent experiments each featuring five replicates for each strain/starting
434 frequency/condition combination. A graphical representation of the competition workflow
435 is provided in Figure 1.
436

437 To follow community dynamics over time, we set up competitions in the same way as
438 described above. After the first 24 hours of competition, we diluted cultures 1:10,000
439 into fresh TSB medium. This process was repeated for five consecutive days. Strain

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440 frequencies were assessed using flow cytometry prior and after each 24 hours
441 competition cycle. We carried out two independent experiments for each strain pair and
442 starting frequency combination with 5 replicates per strain pair and frequency.
443

444 **Flow cytometry to estimate relative species frequency**

445 We assessed the relative strain frequencies at the beginning and at the end of each
446 competition using a BD LSR II Fortessa flow cytometer (flow cytometry facility,
447 University of Zürich) and the FlowJo™ software (BD, Bioscience) for data analysis. As
448 our PA strain expresses a constitutive gfp tag, PA cells could unambiguously be
449 distinguished from the gfp-negative SA cells with a blue laser line (excitation at 488 nm)
450 and the FITC channel (emission: mirror 505 longpass, filter 530/30) (see supplementary
451 Figure 1). Cytometer Setup and Tracking settings of the instrument were used for each
452 experiment and the threshold of particle detection was set to 200 V (lowest possible
453 value). We diluted cultures appropriately in sterile-filtered 1x phosphate buffered saline
454 (PBS, Gibco, Thermo Fisher) and recorded 100,000 events with a low flow rate. The
455 following controls were used for data acquisition in every experiment: 1) PBS blank
456 samples (to estimate number of background counts of the flow cytometer), 2) untagged
457 monocultures (negative fluorescence control, used to set a fluorescence threshold in
458 FlowJo™) and 3) constitutive gfp-expressing monocultures (positive fluorescence
459 control, set to 100% gfp-positive cells). Using our fluorescence threshold, we extracted
460 the percentage of gfp-positive cells for each sample and scaled these values to the
461 positive fluorescence control. The resulting percentage corresponds to the frequency of
462 PA present in the respective replicate. Initial and final strain frequencies were used to

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463 calculate the relative fitness (v) of the focal strain PA as $v = [a_t \times (1-a_0)]/ [a_0 \times (1-a_t)]$,
464 where a_0 and a_t are the initial and final frequencies of PA, respectively (43). We ln-
465 transformed all relative fitness values to obtain normally distributed residuals. Values of
466 $\ln(v) > 0$ or $\ln(v) < 0$ indicate whether the frequency of the focal strain PA increased (i.e.
467 PA won the competition) or decreased (i.e. PA lost competition) relative to its SA
468 competitor.
469
470 We know from previous experiments in our laboratory that due to the gfp tag, our PA
471 strain does have a slight fitness defect in competition with its untagged parental strain
472 ($\ln(v) = -0.358 \pm 0.13$, mean ± 95% CI, see (66)). As we consistently used the same
473 gfp-tagged PA strain for all experiments in this study, results are fully comparable
474 among treatments.
475
476 To test whether flow cytometry counts (measuring all viable and non-viable cells)
477 correlate with CFUs (measuring only viable cells), we serially diluted and plated initial
478 and final strain frequencies from competitions performed under shaken conditions for all
479 three strain combinations on TSB + 1.2% agar. We compared the obtained CFUs with
480 the flow cytometry counts from the same samples and found strong positive correlations
481 for the strain frequency estimates between the two methods (see supplementary
482 figure 2). This means that flow cytometry adequately measures strain frequencies and
483 that the two methods (flow cytometry and CFU counts) yield similar results.
484

485 **Statistical analysis**

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486 All statistical analyses were performed with R Studio version 3.6.1. We used analysis of
487 variance (ANOVA) and Tukey's HSD to compare number of doublings in monocultures
488 of PA and SA. To test whether the relative fitness of PA varies in response to the SA
489 strain genetic background, starting frequency and culturing conditions, we first built a
490 factorial analysis of co-variance (ANCOVA), with SA strain genetic background and
491 culturing conditions as factors and the starting frequency as covariate. We further
492 included 'experimental block' as an additional factor to account for variation between
493 experiments. This full model yielded a significant triple interaction between SA strain
494 genetic background, starting frequency and culturing condition. We therefore split the
495 full model into a set of ANCOVA sub-models, separated either by culturing condition
496 (shaken, viscous, static) or by SA strain genetic background (Cowan I, 6850, JE2). For
497 post-hoc pairwise comparisons between culturing conditions or SA strains in the sub-
498 models, we removed 'experimental block' as additional factor from the model. To test
499 whether PA relative fitness is significantly different from zero under a given
500 strain/starting frequency/condition combination, we performed one sample t-tests and
501 used the false discovery rate method to correct p-values for multiple comparisons (77).
502 To compare strain frequencies obtained by flow cytometry with those obtained by
503 plating (CFUs), we used Pearson correlation analysis. For all data sets, we consulted
504 Q-Q plots and results from the Shapiro-Wilk test to ensure that our residuals were
505 normally distributed. Summary tables for linear models and t-tests used to analyze
506 Figures 2 and 3 can be found in the supplemental material (Tables 1-3).
507
508

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509 **Data availability**

510 All raw data sets have been deposited in the figshare repository (DOI will be provided

511 upon the acceptance of the manuscript).

512

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513 **Conflict of Interest**

514 The authors declare no conflict of interest.

515

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523

524 **Author contributions**

525 S.N. and R.K. designed research, S.N. performed research, S.N. and R.K. analysed
526 data and wrote the paper.

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765 **Table and figures**

766 **Table 1.** PA and SA strains used for this study.

Strain name	Origin	Description	Reference
***Pseudomonas aeruginosa* (PA)**			
PAO1::gfp	Wound	Constitutive GFP expression from the chromosome (attTn7::Ptac-GFP).	Our laboratory
***Staphylococcus aureus* (SA)**			
Cowan I	Septic arthritis	MSSA isolate. Highly invasive, but not cytotoxic. Agr-defective.	ATCC 12598
6850	Osteomyelitis	MSSA isolate. Highly invasive, cytotoxic and hemolytic.	ATCC 53657
JE2	Skin and soft tissue infection	USA300 CA-MRSA isolate. Highly virulent, cytotoxic and hemolytic.	NARSA

767

768 CA-MRSA: Community-acquired methicillin-resistant *S. aureus*

769 MSSA: Methicillin-sensitive *S. aureus*

770 Agr: Accessory gene regulator

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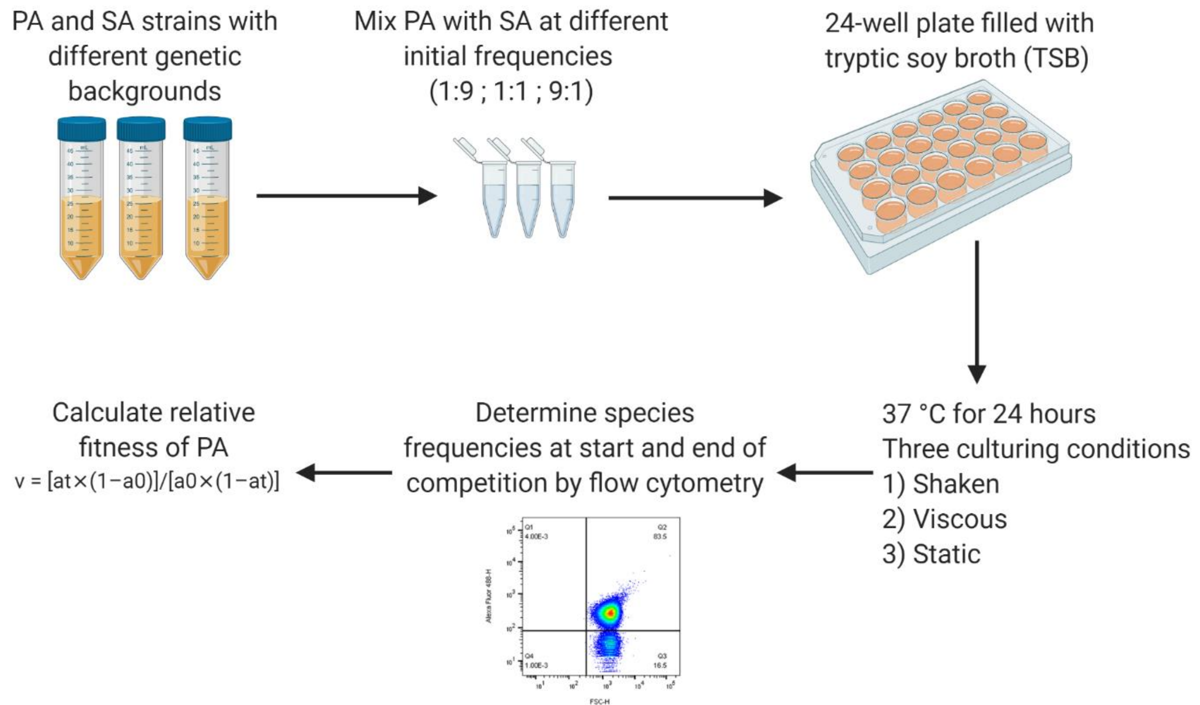

772

773 **Figure 1.** Workflow for the competition experiments. Bacterial overnight cultures were
774 grown in 10 ml TSB in 50 ml falcon tubes for ~ 16 hours at 37 °C and 220 rpm with
775 aeration. After washing and adjustment of OD_{600} to obtain similar cell numbers for all
776 strains, strain pairs (PA-Cowan I ; PA-6850 ; PA-JE2) were mixed at three different
777 volumetric starting frequencies (1:9 ; 1:1 ; 9:1). Flow cytometry was used to measure
778 the actual starting frequencies. Competitions were started with diluted cultures (OD_{600} =
779 10^{-5}) in 24-well plates filled with 1.5 ml TSB per well. Plates were incubated for 24 hours
780 at 37 °C under three different culturing conditions: shaken (170 rpm), viscous (170 rpm
781 + 0.2% agar in TSB) and static. After the 24 hours competition period, final strain
782 frequencies were measured for each replicate by flow cytometry. Using the initial and
783 final strain frequencies, the relative fitness (v) of the focal strain PA was calculated as
784 $v = [a_t \times (1-a_0)]/ [a_0 \times (1-a_t)]$, where a_0 and a_t are the initial and final frequencies of PA,
785 respectively.

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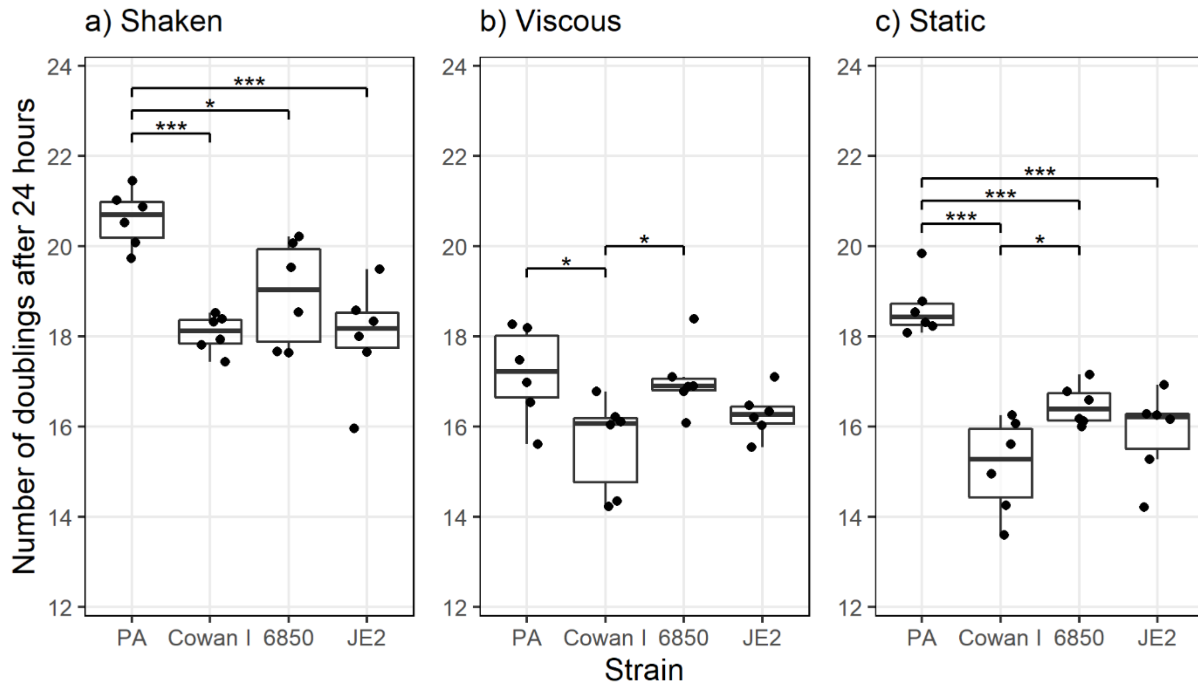

786 **Figure 2.** The number of doublings in monoculture is higher for *P. aeruginosa* PAO1
787 (PA) than for the three *S. aureus* strains (Cowan I, 6850, JE2) under most conditions.
788 Strains were grown as monocultures in TSB for 24 hours at 37 °C under the same
789 conditions and using the same starting OD_{600} as for the competition experiments. The
790 box plots show the median (bold line) with the first and the third quartiles. The whiskers
791 cover the 1.5* inter-quartile range (IQR) or extend from the lowest to the highest value if
792 they fall within the 1.5* IQR. Data are shown from two independent experiments with
793 three replicates each. * $p < 0.05$, *** $p < 0.001$, pairwise comparisons without bars are
794 all not significant.

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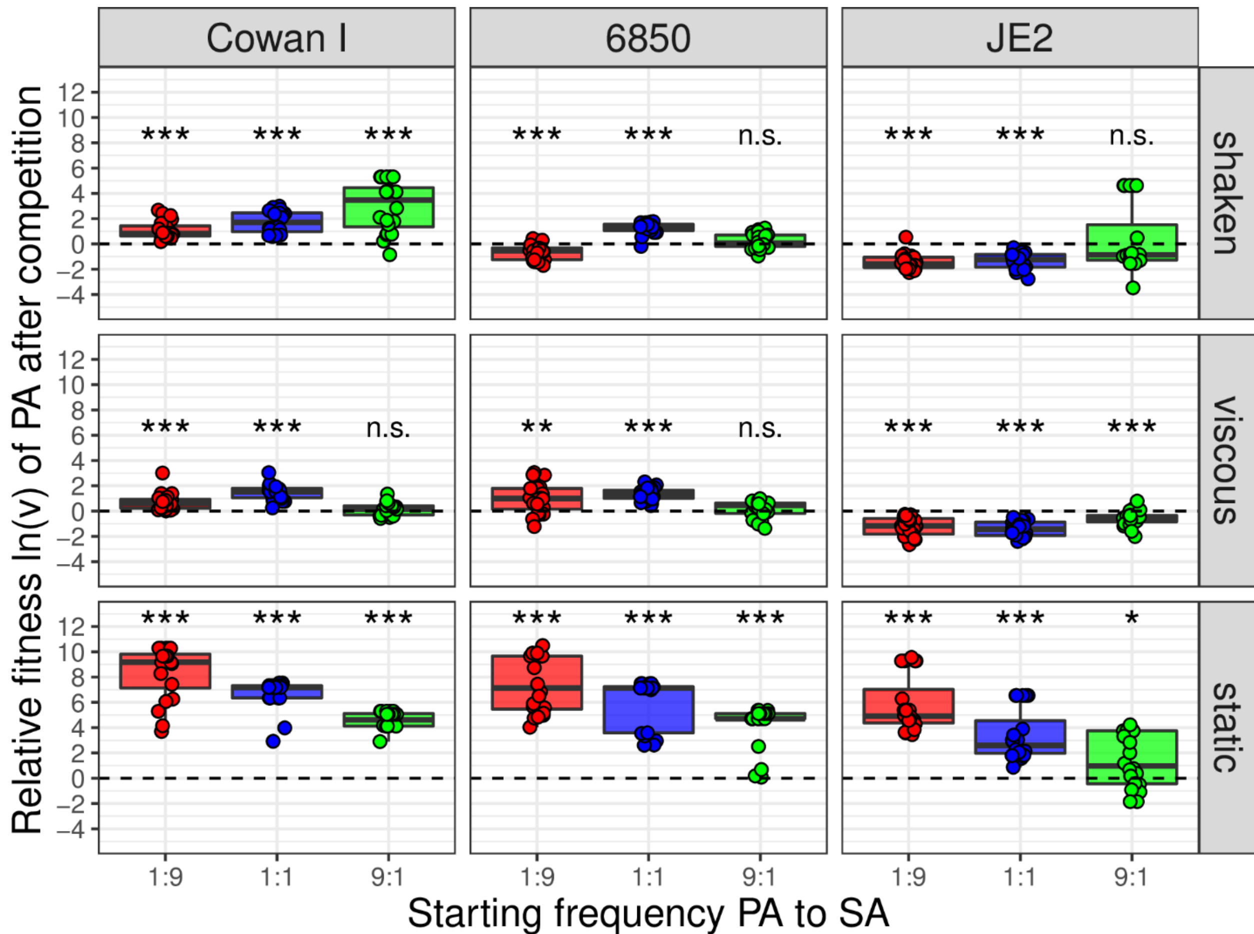

795

796 **Figure 3.** Relative fitness ln(v) of *P. aeruginosa* PAO1 (PA) after 24-hours competitions
797 against three different *S. aureus* (SA) strains (Cowan I, 6850, JE2) at three different
798 starting frequencies (1:9 ; 1:1 ; 9:1) and across three different environmental conditions
799 (shaken, viscous, static). Values of ln(v) < 0, ln(v) > 0, or ln(v) = 0 (dotted line), indicate
800 whether PA lost, won, or performed equally well in competition against the respective
801 SA strain. The box plots show the median (bold line) with the first and third quartiles.
802 The whiskers cover the 1.5* inter-quartile range (IQR) or extend from the lowest to the
803 highest value if they fall within the 1.5* IQR. Each strain pair/culturing condition/starting
804 frequency combination was repeated 20 times (four experiments featuring five
805 replicates each). Asterisks indicate whether the relative fitness of PA is significantly

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806 different from zero in a specific treatment (one-sample t-tests with p-values corrected for
807 multiple comparisons using the false discovery rate method: n.s. = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Detailed information on all statistical comparisons are
809 provided in Table S3.

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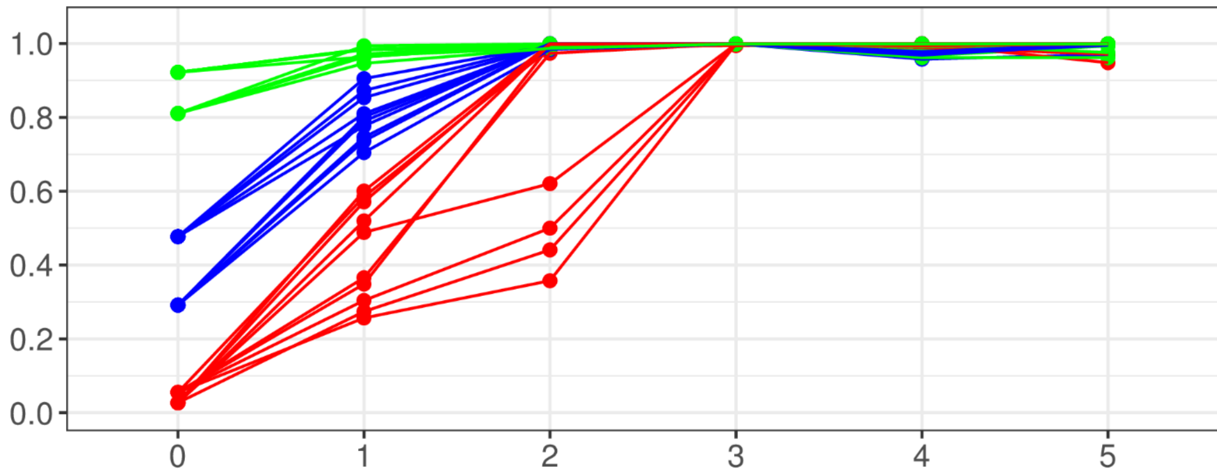

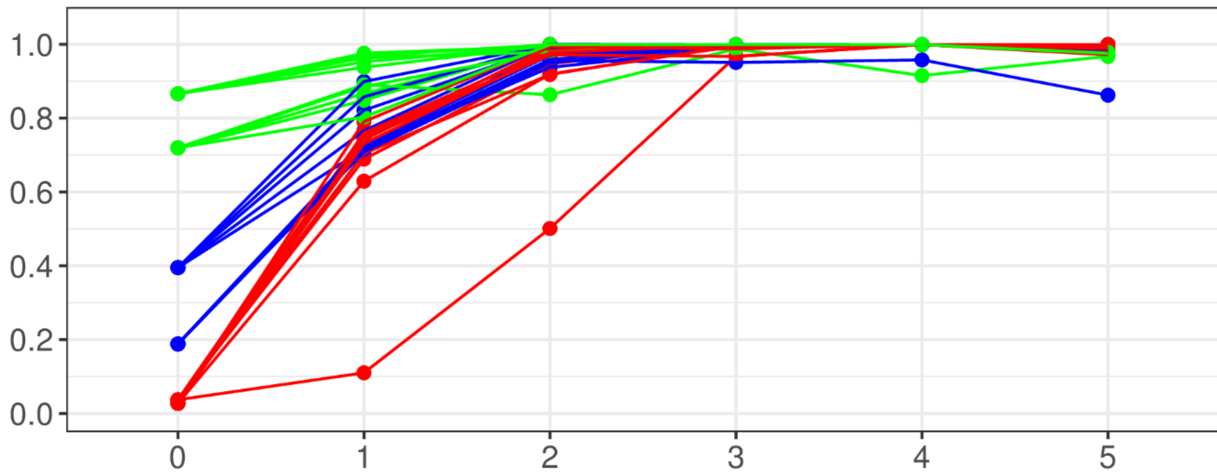

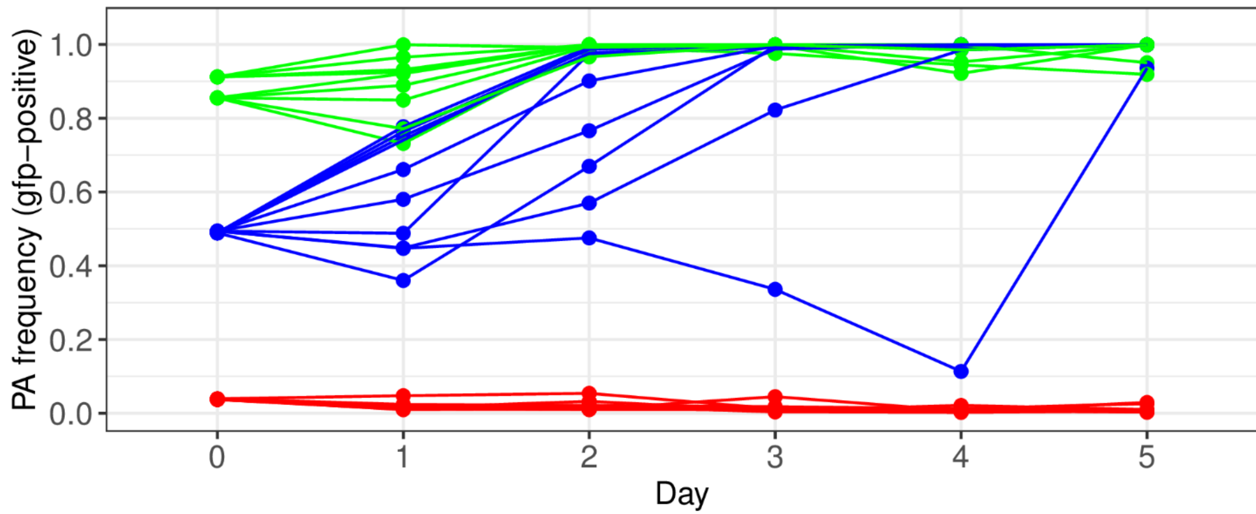

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811 **Figure 4.** Multi-day competitive dynamics between *P. aeruginosa* PAO1 (PA) and the
812 three *S. aureus* strains (a) Cowan I, (b) 6850 and (c) JE2 under shaken conditions.
813 Competitions started at three volumetric starting frequencies of PA:SA (red 1:9, blue
814 1:1, green 9:1). Community composition was followed over five days with daily transfer
815 of diluted cultures to fresh TSB medium. Strain frequencies were assessed using flow
816 cytometry. The experiment was carried out two times with five replicates per treatment
817 combination and experiment.