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Burns, N, James, C and Harrison, E

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1 **Polylysogeny magnifies competitiveness of a bacterial pathogen *in vivo***

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3 Nicola Burns¹, Chloe E James² & Ellie Harrison^{1#}

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5 1. Department of Biology, University of York, UK, ncb508@york.ac.uk

6 2. Biomedical Science Research center, University of Salford, UK,

7 c.james@salford.ac.uk

8 3. Department of Biology, University of York, UK, ellie.harrison@york.ac.uk

9

10 # corresponding author

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15 Running head: Polylysogeny aids bacterial competitiveness

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27

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30 Abstract

31 The rise of next generation sequencing is revealing a hidden diversity of temperate phages within the
32 microbial community. While a handful of these phages have been well characterized, for the vast
33 majority, the role of phage carriage, and especially multiple phage carriage, is poorly understood. The
34 Liverpool Epidemic Strain of *Pseudomonas aeruginosa* is an aggressive pathogen in Cystic Fibrosis lung
35 infections that has recently been found to contain several unique prophages within its genome. Here
36 we experimentally investigate the role of two of these phages *in vivo*, using an insect model of
37 infection. We find that while no benefit is conferred by phage carriage in single bacterial infections,
38 phages confer a large fitness advantage during mixed infections by mediating bacteria-bacteria
39 competition. Differences between the two phages appeared to be associated with the rate at which
40 the competitor acquired the phage, and therefore resistance. However the advantage was greatest in
41 the polylysogen, carrying both phages. These finding suggest that the LES phages may play an
42 important role in host invasions and more generally show that the carriage of multiple phages may
43 itself be beneficial by hindering the spread of resistance in rival bacterial populations.

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60 *Introduction*

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62 Temperate bacteriophages (phages) are widespread and important viruses of bacteria. Unlike purely
63 lytic phages, temperate phages can be transmitted both horizontally through the lytic cycle, and
64 vertically through lysogeny, whereby the phage genome is inserted into the bacterial chromosome.
65 This dual lifecycle confers several potential benefits to the host bacteria. Firstly, many phages carry
66 useful bacterial accessory traits which often, although not exclusively (Mann et al 2003), encode gene
67 products associated with pathogenicity, such as toxins and antigenic molecules (Brussow et al 2004).
68 Secondly, phages may act as an anti-competitor mechanism: phages integrated into the chromosome
69 (prophages) can undergo spontaneous induction and enter the lytic cycle, producing lytic phages able
70 to infect and kill susceptible bacteria in the local area. While this results in the death of the individual
71 host cell, it can provide a large competitive advantage for the remaining phage-carrying population,
72 which are resistant to infection (Brown et al 2006). Thirdly, the integration of phages into host
73 genomes may itself act as a driver of genome innovation through increased mutagenesis and the
74 supply of novel DNA (Goerke and Wolz 2010). Temperate phages therefore have the potential to play
75 a major role in the ecology and evolution of bacterial populations.

76 Genome sequencing has revealed the presence of prophages in a large proportion of bacterial
77 genomes, many of which carry multiple phages (polylysogens) (Figuroa-Bossi et al 2001; Schuch and
78 Fischetti 2009; Winstanley et al 2009; Wang et al 2010). While a small number of prophages have
79 been identified as carrying specific, useful bacterial traits, the role of majority of these prophages, and
80 in particular the benefits of polylysogeny, remains unclear. The Liverpool Epidemic Strain (LES) of
81 *Pseudomonas aeruginosa*, a major pathogen in lung infections of Cystic Fibrosis (CF) sufferers, has
82 been shown to carry multiple novel prophages of unknown function. Originally isolated in Liverpool,
83 LES has been found to be extremely transmissible, infecting non-CF sufferers (McCallum et al 2002)
84 and spreading throughout hospitals in the UK and worldwide (Fothergill et al 2012). It is also
85 associated with increased patient morbidity (McCallum et al 2002). Recent work has shown that
86 disruption of these phages, through signature-tagged mutagenesis, leads to a significant reduction
87 bacterial fitness in a rat model of chronic lung infection (Winstanley et al 2009). Infective phage

88 particles from four prophages have been detected in the sputa of CF sufferers (Fothergill et al 2010;
89 James et al 2014), suggesting that these phages are actively entering the lytic cycle during infection.
90 These results imply a role for these phages in LES infections, however what benefit they confer is
91 poorly understood.

92
93 In this study we investigate the ecological consequences of single and multiple phage carriage using
94 two temperate phages isolated from the LES strain in experimental infections using an insect model
95 system; larvae of the greater wax moth (*Galleria mellonella*). *Galleria* larvae have been successfully
96 used as an *in vivo* model of both bacterial (Ingis et al 2009; Yasmin et al 2010; Evans and Rozen 2012;
97 Hall et al 2012) and fungal (Scully and Bidochka 2005) infection as they provide a tractable model host
98 environment. Using this system we find that, in single genotype infections, these phages appear to
99 have no impact on the growth or virulence of their bacterial host. However in mixed infections with
100 phage-free bacteria, phage carriage, and especially multiple phage carriage, is highly beneficial. These
101 results suggest that polylysogeny can in itself be beneficial to the bacterial host by mediating bacteria-
102 bacteria competition.

103

104 *Methods*

105

106 **Strain construction and culture conditions**

107 To allow us to make direct comparisons between isogenic strains experiments were conducted using
108 a phage-free lab strain of *P. aeruginosa*, PAO-1, as the bacterial host. Two marked PAO-1 strains were
109 produced following the methods of Koch et al (2001); a focal strain PAO-1-*gm*, which carries a
110 gentamycin resistance marker and a 'reference' strain PAO-1-*sm*, which carries a streptomycin
111 resistance marker and was used as a competitor strain during competitive fitness assays. Phages
112 LES ϕ 2 and LES ϕ 4 have been previously isolated from the LES strain LES-B58 (James et al 2012). Single
113 and double lysogens of the PAO-1-*gm* strain were produced following the methods of James et al
114 (2012), resulting in 3 lysogenic genotypes; PAO-1-*gm* -LES ϕ 2 (from now on referred to as P2), PAO-1-

115 *gm*-LES ϕ 4 (P4) and PAO-1-*gm*-LES ϕ 2 LES ϕ 4 (P2&4). The non-lysogenic PAO-*gm* strain was used as
116 the phage free control strain (PF).

117

118 Wax moth larvae were obtained from wigglywigglers.com. Larvae were selected at random for
119 inoculation and discarded if they showed any sign of melanisation, which may indicate disease or the
120 onset of pupation. Larvae were chilled briefly on ice prior to injection to reduce movement and the
121 hind section dipped in ethanol to minimize contamination. To initiate infections, overnight bacterial
122 cultures were diluted 100x in phosphate buffered saline (PBS) and 5 μ l (approx. 10^6 bacteria) injected
123 into the larvae using a disposable micro-syringe. Larvae were incubated individually in separate sterile
124 petri dishes at 37 °C.

125

126 **Growth rate and time-to-death assays**

127 Four replicate overnight cultures were established for each of the four treatments, PF, P2, P4 and
128 P2&4 making a total of 16 cultures. Each culture was used to inoculate seven larvae, in addition to
129 which four larvae were injected with PBS only. Replicate larvae were then randomly split into seven
130 groups with one group to be homogenized after 2, 4, 6, 8, 10, 12 and 24 hours respectively. At each
131 time point, the homogenate was serially diluted and plated onto Lysogeny Broth (LB) agar containing
132 gentamicin to determine bacterial density. In addition, the homogenate was filter sterilized to isolate
133 free-phage, serially diluted in PBS and plated onto PAO-1 lawns. Throughout the experiment, the
134 24hr group was monitored for survival at hourly intervals. Larvae were scored as dead when there
135 was no observable response when prodded lightly.

136

137 **Competitive fitness**

138 Six replicate competitions were established for each treatment from a 1:1 mix of PAO1-*sm* and either
139 the PF, P2, P4 or P2&4 strain depending on the treatment. Therefore 24 competitive cultures were
140 established in total. These cultures were serially diluted and plated onto LB agar selecting for either
141 streptomycin or gentamicin resistance to determine the bacterial density of the PAO1-*sm* and
142 treatment specific test strain respectively at 0 hours.

143

144 In order to sample fitness at multiple time points each of the 24 cultures was diluted 100 fold in PBS
145 and three larvae were inoculated per replicate. After 6, 12 and 24 hours one of the three larvae,
146 chosen at random, was homogenized, serially diluted and the bacteria plated on to streptomycin and
147 gentamicin selective media. Competitive fitness was estimated for each time point from the
148 Malthusian parameters (Lenski et al 1991).

149

150 In addition we quantified the proportion of lysogens in the originally phage-free PA01-*sm* population
151 by PCR screening. Ten streptomycin resistant clones from each competition were restreaked to
152 remove contaminating free-phage particles and colonies were screened using primers targeting
153 LES ϕ 2 (LES1nestF: TTTGGTGATGATCGGCTTAGC, LES1nestR: TGTGGAAGCGATCAGTCT) and LES ϕ 4
154 (4tot1F: GCTCATGAGTGGCTGACAAC, 4tot1R: TCTTGGGCAGAGAACCATTC) (14).

155

156 **Statistical analysis**

157 All analyses were conducted in R statistical package (R Foundation for Statistical Computing). All data
158 sets were analyzed in a fully factorial linear model and further investigated using tukeys post hoc
159 comparisons.

160

161

162 *Results*

163

164 **Effects of lysogeny in single infections.** The impact of prophage carriage was first examined in single
165 infections of each of the four strains. Average doubling time of the *in vivo* infections was 38.2
166 minutes (± 1.3 SE), with no difference between the strains (Fig. 1a: $F_{3,12}=1.02$, $p=0.42$). In all
167 competitions containing lysogens, infectious free-phage particles were present at an average
168 frequency of 0.12 phage per bacteria (± 0.02 SE), with no difference between strains ($F_{2,92}=1.01$,
169 $p=0.34$), or through time ($F_{1,92}=1.02$, $p=0.89$).

170

171 The virulence of each strain in the wax moth larvae host was estimated by time-to-death assays by
172 monitoring infected larvae hourly for 16 hours. Control larvae that were inoculated with PBS

173 remained alive after 24hrs with no signs of illness. All larvae inoculated with bacteria became highly
174 melanised and died within between 9 - 14 hours, with no significant difference between strains (Fig.
175 1c: $F_{3,12}=0.383$, $p=0.77$).

176

177 **Effects of lysogeny in mixed-infection.** The relative fitness of the four strains was examined in
178 competition with a labeled, phage-free isogenic competitor. As the competitor strain is susceptible
179 to infection and can therefore become lysogenized during the competition, fitness was estimated
180 after 6, 12 and 24 hours to examine the effect of time on this relationship. Competitive fitness of the
181 non-lysogenic PF strain relative to the test strain (which is isogenic with the exception of the antibiotic
182 resistance markers) maintained a fitness not significantly different from 1 (Fig. 2: $F_{1,16}=-0.15$, $p=0.7$)
183 with no variation through time ($t_{11}=-0.41$, $p=0.69$) demonstrating that neither marker alters fitness
184 relative to the other.

185

186 In contrast, the three lysogenic strains showed different fitness profiles both through time and
187 relative to each other (STRAIN X TIME: $F_{2,48}=4.14$, $p=0.022$). At 6 hours, fitness estimates for each of the
188 lysogenic strains was less than 1 (on average $0.85\pm 2.06\%$) indicating a fitness cost relative to the
189 plasmid free strain. No significant difference between the three lysogenic strains was observed at
190 this time point (for each pairwise post hoc comparison $p>0.851$), but both the fitness of the P2 and
191 P2&4 strains was significantly reduced relative to the non-lysogenic PF strain ($t_{20}=-2.48$, $p=0.022$ and
192 $t_{20}=-2.16$, $p=0.043$ respectively). After 12 hours however, this fitness interaction was reversed and all
193 three strains had an average fitness greater than 1. By 24 hours, the three lysogenic strains all
194 exhibited a large significant fitness advantage relative to the test strain (P2: $t= 2.645$, $p= 0.0155$; P4: $t=$
195 7.243 , $p<0.0001$ and P2&4: $t= 10.363$, $p<0.0001$). This fitness advantage varied widely between
196 strains. Among the two single lysogens P4 had a significantly larger fitness advantage than P2
197 ($1.74\pm 0.057\%$ compared to $1.25\pm 0.030\%$, $t=4.599$, $P<0.001$). However the double lysogen, P2&4, had
198 a fitness advantage of $2.07\pm 0.135\%$ indicating a 100% fitness advantage over the phage free strain,
199 and significantly larger than both single lysogens (compared to P2: $t=7.719$, $p<0.001$ and P4: $t=-3.120$,
200 $p=0.025$).

201

202 **Lysogenic conversion during competition.** The proportion of the lysogens among the initially
203 susceptible competitor population was estimated by PCR for 10 clones per competition at each time
204 point (Fig. 3). After 6 hours, no lysogens were identified from any of the competitions. After 12
205 hours however, lysogens were identified in all populations of each treatment. When comparing the
206 total proportion of lysogens (i.e. the proportion of clones carrying at least one prophage), both the
207 main effects of time and strain were significant (TIME $F_{2,50}=4.22$, $p=0.0202$ and STRAIN $F_{1,50}=86.71$,
208 $p<0.0001$). After 24 hours, between 90 – 100% of competitor clones from both the P2 and the P2&4
209 treatments carried at least one prophage. In comparison, prophages were identified in an average of
210 80.0% ($\pm 8.6\%$ SE) of clones from competitions with the P4 strain ($t_{6,11}= -2.98$, $p=0.024$). In the double
211 lysogen however not all lysogenic competitor clones carried both prophages. Between the six
212 replicate competitions run for 24 hours between 20% (2/10) and 50% (5/10) of clones screened
213 carried both phages.

214

215

216 *Discussion*

217

218 Many bacterial strains carry multiple ‘cryptic’ prophages whose function remains largely unknown.
219 Using an insect model of infection we have investigated the role of two such phages, LES ϕ 2 and
220 LES ϕ 4, in the success of the aggressive LES strain of *P. aeruginosa in vivo*. We find that both phages
221 confer a large fitness benefit to their bacterial hosts during competition with susceptible bacteria.
222 However this benefit does not appear to be due to any intrinsic advantage of phage carriage; no
223 differences in growth rates or virulence were identified between lysogens and their phage-free
224 counterpart under the conditions of this study. While we are unable to exclude the possibility that,
225 within the native lung environment these phages may provide additional benefits not expressed in
226 the wax moth larvae, these results clearly demonstrate a large competitive advantage due to
227 ‘apparent competition’, mediated by phages in their infectious state, which are able to infect and lyse
228 susceptible non-lysogens. This is also supported by the finding that in the initial stages of infection
229 (i.e. after 6 hours of competition) lysogens were not beneficial and in fact were associated with a
230 $\sim 10\%$ fitness cost relative to the non-lysogen. This can be explained by the fact that the benefits of

231 producing phages in their lytic form (i.e. lysis of competitors) will be density dependent and therefore
232 greater in the latter stages of infection where the encounter rate between infectious phage particles
233 and susceptible bacteria is high. The cost of phage carriage, which may include the biosynthetic
234 burden of carrying additional DNA (Glick 1995; Rozkov et al 2004) and cell death through spontaneous
235 induction, is paid regardless of the presence of susceptible hosts, therefore potentially explaining the
236 cost observed over shorter time scales.

237

238 Our results also revealed a marked difference in the scale of the fitness advantage conferred by the
239 different phages and between single and multiple phage infections. Lysogens carrying LES ϕ 2 had
240 significantly lower fitness than those carrying the LES ϕ 4 phage, however no difference was observed
241 in the growth rate, virulence or the proportion of infectious phage particles released during growth.
242 This difference instead appears to be associated with the ability of these phages to lysogenize the
243 competitor strain, which has previously been shown to be a major determinant of the benefit of
244 phage carriage (Gama et al 2013). Among single lysogens, the proportion of lysogenized competitor
245 clones was higher in the P2 competitions compared to those in competition with P4 lysogens. Rapid
246 lysogenisation of the competitor effectively 'levels the playing field' between the strains earlier in the
247 competition, reducing the benefit of phage carriage to original host strain.

248

249 The largest fitness benefit (>100%) was observed in the double lysogen. During competition with this
250 strain nearly 100% of the competitor strain became lysogenized, however, on average less than 50%
251 carried both prophages and were therefore completely resistant to lysis. Previous work has shown
252 that single lysogens of the LES ϕ 4 phage are fully susceptible to infection by LES ϕ 2, while LES ϕ 2
253 carrying strains are partially, though not completely resistant to LES ϕ 4 (James et al 2012), therefore
254 making a large proportion of the population susceptible to lysis. These results suggest that
255 polylysogeny, which is observed commonly among pathogenic bacteria (Figuroa-Bossi et al 2001;
256 Schuch and Fischetti 2009; Winstanley et al 2009; Wang et al 2010), may in itself be adaptive for the
257 bacterial host as it hinders the process of immunization of competitor strains and prolongs the
258 effectiveness of a lysogen's 'armoury' of viruses.

259

260 This relationship between polylysogeny and host fitness also has interesting consequences for the
261 phages themselves. As integrated elements prophages gain a benefit from polylysogeny through
262 increased fitness of their host, therefore it may be imagined that between-host selection may favour
263 phages that accommodate superinfection. However, polylysogeny may also have fitness costs for the
264 phages themselves. Most significantly, prophages which cohabit hosts containing more virulent
265 phages (i.e. those that are more likely to initiate lysis and/or are able to replicate faster) are at a
266 significant numeric disadvantage (Refardt 2011). Therefore selection occurring within-hosts (i.e.
267 between phages) may in fact favour more aggressive phages or resistance to superinfection. The LES
268 phages themselves display a hierarchy (James et al 2012) of resistance which may be indicative of this
269 conflict.

270

271 These findings therefore suggest that the LES phages are effectively acting as weapons in bacterial
272 warfare. Recent work has shown that lytic LES particles are consistently released throughout long
273 term lung infections, implying that these phages are playing a significant role in ecology of this chronic
274 infection (James et al 2014). Similar results have been identified in several other bacterial pathogens,
275 namely *Escherichia coli* (Brown et al 2006; Gama et al 2013), *Bordetella* (Joo et al 2006) and
276 *Salmonella* (Bossi et al 2003) and *Enterococcus faecalis* (Duerkop et al 2012) suggesting that phage
277 mediated competition may be a common strategy employed by pathogenic bacteria. More broadly
278 temperate phages form part of a wider group of horizontally transmitted elements that influence host
279 fitness through apparent competition including plasmids and integrative elements which encode
280 genes for bacteriocins (Riley & Wertz 2002). The benefits of carrying such elements however are likely
281 to be short lived as susceptible bacteria can become infected, negating the competitive advantage to
282 the original hosts, suggesting that these elements are particularly important in the initial stages of
283 invasion of new environments already occupied by resident bacteria or in repelling potential invading
284 bacteria. The carriage of multiple phages may be one strategy employed to get around this short-
285 coming, as multiple steps are required for immunization to occur and thus the benefit of the phage
286 'armoury' is prolonged. This finding suggests that 'cryptic' phages may in fact be playing a major role
287 in the ecology and evolution of bacterial pathogens.

288

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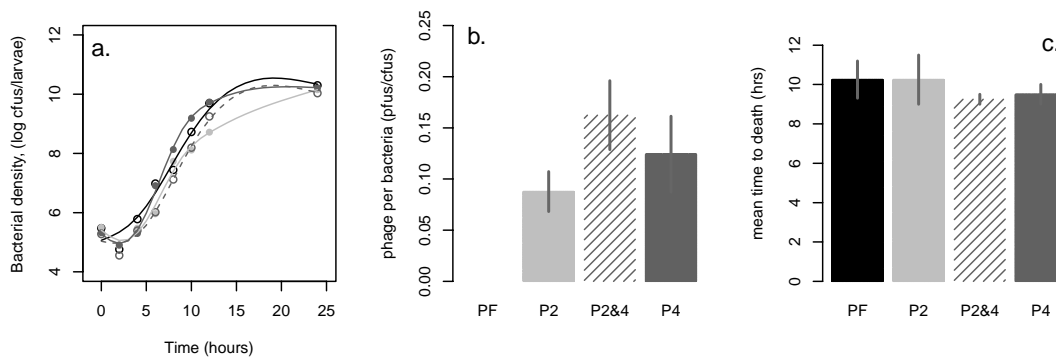
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378 **Figure 1. Life history of single infections of lysogenic strains.** a. Bacterial density thorough time

379 during infection. Points show average bacterial density (n=4) for the phage-free strain (black, empty),

380 P2 (light grey, filled), P4 (dark grey, filled), P2&4 (dark grey, empty). Lines show splines predicted by a

381 general additive model. b. The average number of phage particles per bacteria during infection. As

382 phage:bacteria ratio did not vary over time bars show means of replicates (n=4) averaged through

383 time. Lines show standard error. c. Average virulence of the four strains shown as mean time-to-

384 death of larval hosts observed over a 24 hour period (n=4). Lines show standard error. Controls,

385 inoculated with PBS only are not shown as no deaths were observed for the duration of the

386 experiment.

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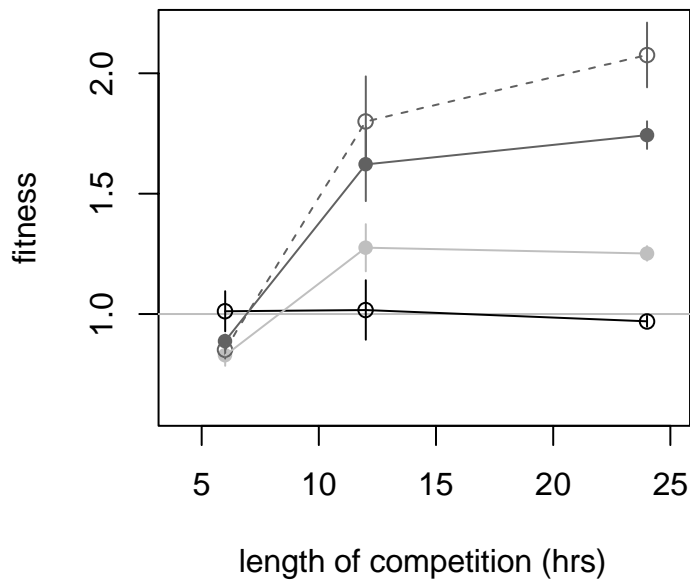
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400 **Fig. 2 Relative fitness of lysogens in competition with an isogenic phage-free strain.** Points show

401 mean competitive fitness estimated after 6, 12 and 24 hours of competition for the four different

402 strains with a non-lysogenic competitor. A fitness of 1 indicates no difference in fitness from the

403 competitor strain. Points show mean fitness of 6 replicates for treatments PF (black, empty), P2

404 (light grey, filled), P4 (dark grey, filled) and P2&4 (dark grey, empty). Error bars denote standard

405 error.

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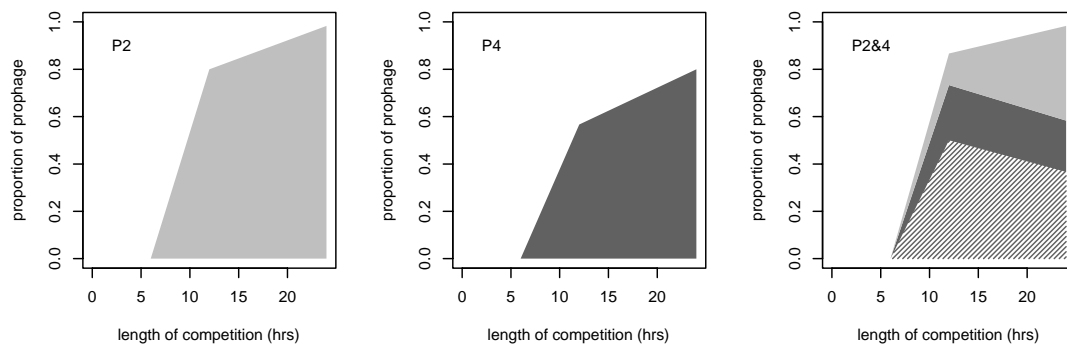
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418 **Fig. 3. Rate of lysogeny during competition.** The average proportion of lysogens in the initially non-

419 lysogenic PAO-1-*sm* population during competition with lysogens P2, P4 and P2&4 (left to right).

420 Colours denote the proportion of lysogens carrying LES ϕ 2 (light grey), LES ϕ 4 (dark grey) and both

421 LES ϕ 2 and LES ϕ 4 prophages (shaded). Values shown are the means of 6 populations. For each

422 population the proportion of lysogens was estimated by screening 10 clones for the presence or

423 absence of phages.