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Mumford, Rachel and Friman, Ville-Petri orcid.org/0000-0002-1592-157X (2016) Bacterial competition and quorum-sensing signalling shapes the eco-evolutionary outcomes of model in vitro phage therapy. Evolutionary applications. ISSN 1752-4571

https://doi.org/10.1111/eva.12435

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Bacterial competition and quorum-sensing signalling shapes the eco-evolutionary outcomes of model *in vitro* phage therapy

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Article Type: Original Research articles

Running head: Phage therapy in polymicrobial communities

ABSTRACT

The rapid rise of antibiotic resistance has renewed interest in phage therapy – the use of bacteria-specific viruses (phages) to treat bacterial infections. Even though phages are often pathogen-specific, little is known about the efficiency and eco-evolutionary outcomes of phage therapy in polymicrobial infections. We studied this experimentally by exposing both quorum sensing (QS) signalling PAO1 and QS-deficient *lasR Pseudomonas aeruginosa* genotypes (differing in their ability to signal intra-specifically) to lytic PT7 phage in the presence and absence of two bacterial competitors: *Staphylococcus aureus* and *Stenotrophomonas maltophilia* – two bacteria commonly associated with *P. aeruginosa* in polymicrobial cystic fibrosis lung infections. Both the *P. aeruginosa* genotype and the presence of competitors had profound effects on bacteria and phage densities and bacterial resistance evolution. In general, competition reduced the *P. aeruginosa* frequencies leading to a lower rate of resistance evolution. This effect was clearer with QS-signalling PAO1 strain due to lower bacteria and phage densities and relatively larger pleiotropic growth cost imposed by both phage and competitors. Unexpectedly, phage selection decreased the total bacterial densities in the QS-deficient *lasR* pathogen communities, while an increase was observed in the QS-signalling PAO1 pathogen communities. Together these results suggest that bacterial competition can shape the eco-evolutionary outcomes of phage therapy.

Keywords: Competition, coevolution, cost of resistance, host-parasite interactions, phage therapy, polymicrobial infections, quorum sensing signalling, resistance

1. INTRODUCTION

Growing concern for the evolution of antibiotic resistant bacteria and in particular for multi-resistant gram-negative bacteria (Levy and Marshall 2004), has led to renewed interest in alternative treatments including phage therapy (Rossolini et al. 2014). Phage therapy - the use of pathogen-specific parasitic viruses (bacteriophages) as a treatment for bacterial infections - is almost hundred years old and has been used for decades to treat bacterial infections in Eastern European countries such as Georgia and Poland (Alisky et al. 1998; Housby and Mann 2009; Abedon et al. 2011). While many studies have demonstrated the safety and benefits of phage therapy (Merabishvili et al. 2009; Abedon et al. 2011; Rose et al. 2014) phages have not yet been incorporated into western medicine partly due to lack of proper clinical trials and historically inconsistent treatment results (Kutateladze and Adamia 2008). While large-scale clinical trials are currently under way (e.g. Phagoburn; (Expert round table on and re-implementation of bacteriophage 2016)), the evolutionary outcomes of phage therapy are relatively unknown. Recent studies have shown that bacteria and phages can rapidly coevolve during model phage-therapy treatments (Betts et al. 2013; Friman et al. 2016) and that the diversity of phage communities can affect the bacterial resistance evolution (Hall et al. 2012; Betts et al. 2016). Besides rapid coevolution, further complications could arise from interspecific bacterial competition due to polymicrobial nature of bacterial infections: many human infections contain multiple different pathogenic bacterial and other microbial species (Peters et al. 2012). Considerable genotypic variation also exists between different strains of a pathogen and this variation is known to differ between different patients and to affect the pathogen susceptibility to phages (Debarbieux et al. 2010; Essoh et al. 2013; Friman et al. 2013). Understanding the relative importance and interactive effects of these potentially complicating factors is thus crucial for developing reliable and consistent phage therapy treatments. Here we focused explicitly on the ecological and evolutionary outcomes of phage therapy in polymicrobial communities and asked how focal bacterial genotype and the competition with other bacterial pathogens affect the total bacterial loads and focal pathogen resistance evolution during *in vitro* model phage therapy.

74 The bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen that commonly infects many
75 immunocompromised patients including cystic fibrosis (CF) and burn victim patients (Harrison
76 2007; Turner et al. 2014). *P. aeruginosa* is often characterised by multi-drug resistance to
77 conventional antibiotics (Strateva and Yordanov 2009), and hence, the development of novel phage
78 therapy treatments could potentially help a large number of patients (Harper and Enright 2011).
79 While *P. aeruginosa* can rapidly evolve resistance to various bacteriophages, which could decrease
80 the feasibility and long-term benefits of phage therapy (Hall et al. 2012; Betts et al. 2013; Friman et
81 al. 2013), it has also been shown that phages can counteract resistance evolution by coevolving to
82 be more infective (Betts et al. 2016; Friman et al. 2016). However, it is less clear how important
83 these coevolutionary dynamics are in more complex microbial communities. For example, lung and
84 wound infections are often very diverse and consist of multiple different non-pathogenic and
85 pathogenic bacterial species (Harrison 2007; Folkesson et al. 2012; Korgaonkar et al. 2013) that
86 could modify phage effects indirectly via competition.

87 Competition could affect the evolution of phage resistance via demographic and genetic
88 effects. Firstly, competition is likely to reduce focal pathogen population densities which could
89 weaken the selection for resistance due to less frequent phage-bacteria encounter rates and lowered
90 supply of resistance mutations (Levin and Bull 2004; Lopez-Pascua and Buckling 2008). These
91 demographic effects could be occurring indirectly via competition for shared resources in the site of
92 infection or directly via interference competition via bacteria-specific toxins such as bacteriocins
93 (Inglis et al. 2009; Ghoul et al. 2015). Furthermore, *P. aeruginosa* has been shown to display greater
94 virulence, antibiotic tolerance and growth when co-cultured with gram-positive *S. aureus* bacterium
95 (Korgaonkar et al. 2013; Michelsen et al. 2014), which suggests that the presence of other bacterial
96 species could also facilitate target pathogen coexistence in polymicrobial infections. Secondly, there
97 might be trade-offs between evolving phage resistance and retaining competitive ability or virulence
98 due to conflicting selection pressures (Friman and Buckling 2014). Such trade-offs are often
99 manifested as antagonistic pleiotropy where a mutation in the gene that confers benefit in the

presence of phage has a negative effect on some other function such as uptake of nutrients (Lenski and Levin 1985). The magnitude of such trade-offs is often dependent on environmental conditions, being larger in nutrient-poor environments (Yoshida, Hairston, and Ellner 2004) or in the presence of competitors (Kassen 2002). Lastly, it has been shown that the presence of a phage can change the competitive interactions between different bacterial species and that this effect depends on which competing bacterial species is affected by the phage (Harcombe and Bull 2005).

The effect of competitors on focal pathogen fitness, and pathogen potential to evolve resistance to phages, could further depend on the focal pathogen genotype. For example, *P. aeruginosa* CF lung infections are genetically diverse and this heterogeneity is driven by both temporal (Marvig et al. 2014) and spatial variation (Jorth et al. 2015). It has been recently shown that phages can have a different effect on *P. aeruginosa* density and resistance evolution depending on the strain and the genotype; specifically, the time bacteria spent adapting to the lung environment seem to make bacteria more susceptible to phages (Friman et al. 2013; Friman et al. 2016). One notable adaptation to the CF lung environment is the loss of quorum sensing related traits (Marvig et al. 2014; Michelsen et al. 2014; Andersen et al. 2015). Quorum sensing (QS) is a means by which bacteria communicate through the release of signalling molecules allowing cells to carry out density-dependent gene expression (Miller and Bassler 2001). In *P. aeruginosa,* the ability to quorum sense is critical for controlling behaviours such as the production of virulence factors (Folkesson et al. 2012) and it is known that strains from acute infections (early colonisations) are more virulent compared to strains from chronic infections (long-term colonisations) (Smith et al. 2006; Marvig et al. 2014). Interestingly, recent evidence suggests that QS-regulated genes can also affect bacterial resistance to phages. For example, with *E. coli,* QS genes regulate resistance to phage plastically via reduction of cell surface receptors (Hoyland-Kroghsbo, Maerkedahl, and Svenningsen 2013; Taj et al. 2014). Similarly, QS has been shown to be an important 'switch' for choosing between different anti-phage defence strategies in the bacterium *Vibrio anguillarum* (Tan,

125 Svenningsen, and Middelboe 2015). As a result, the decrease of phage resistance in *P. aeruginosa*
126 QS-mutants could be due to the loss of functional QS-genes.

127 Here we used *in vitro* experimental evolution approach to study the eco-evolutionary
128 outcomes of phage therapy with *P. aeruginosa* focal pathogen, that frequently co-infects the lungs
129 of CF patients (Harrison 2007). We manipulated both the presence of *Staphylococcus aureus* and
130 *Stenotrophomonas maltophilia* competitors (one or two competitors present - our definition of a
131 polymicrobial community from here on) and the PT7 phage, and used two *P. aeruginosa* pathogen
132 genotypes: QS-signalling PAO1 strain and QS-deficient *lasR* mutant strain, which does not produce
133 or respond to QS-signals (Diggle et al. 2007). These bacterial species were chosen because they
134 commonly coexist and infect humans patients suffering from burn wounds or cystic fibrosis (CF)
135 lung infections (Harrison 2007). We used fully factorial design where both *P. aeruginosa* genotypes
136 were evolved in all possible combinations and measured bacterial and phage densities and
137 coevolutionary changes between *P. aeruginosa* and PT7 phage at the end of the selection
138 experiment. We hypothesised that the rate of phage resistance evolution could be negatively
139 affected by competition via negative effects on population densities (lowered mutation supply rate
140 and phage-bacteria encounter rates) and that the effect of competition could further depend on the
141 focal pathogen genotype, the composition of competitor community, and the pleiotropic costs of
142 adaptation.
143

144 ## 2. MATERIALS AND METHODS

145 ***Bacterial and phage strains***

146 In addition to *Pseudomonas aeruginosa* (Diggle et al. 2007), we used *Staphylococcus aureus subsp.*
147 *aureus* (DSM-20231) and *Stenotrophomonas maltophilia* (DSM-50170) bacteria in our
148 experiments. We chose *P. aeruginosa* as our focal species as it is one of the most common causes of
149 morbidity for CF patients, while *S. aureus* and *S. maltophilia* often coexist with *P. aeruginosa*
150 among *Haemophilus influenza, Streptococcus pneumoniae, Burkholderia cenocepacia, Ralstonia*

and Achromobacter (Jelsbak et al. 2007; Folkesson et al. 2012). To compare the effect of pathogen genotype, two strains of *Pseudomonas aeruginosa* were used: QS-signalling PAO1 and QS-deficient PAO1 *lasR* mutant strains (Diggle et al. 2007). Apart from the mutation in QS-signalling pathway, the two isolates were otherwise isogenic (Fletcher et al. 2007). The *lasR* mutation is often associated with isolates from the later stages of long-term infections in CF patients (Marvig et al. 2014; Andersen et al. 2015) and its weakened virulence is due to inability to detect and produce quorum sensing signalling molecules that activate the expression of *P. aeruginosa* virulence factors (Smith et al. 2006). A lytic bacteriophage, PT7, which obligately kills *P. aeruginosa*, was used as a phage (Friman et al. 2016). Relatively little is known about PT7 phage. Even though its genome has not been sequenced, previous studies suggest that it is not closely related to PB1-like or phiKMV-like phages (Merabishvili et al. 2007). Similarly, it is unclear which receptors it uses to infect *P. aruginosa*. Prior the experiment, we confirmed that phage PT7 was not able to infect *S. aureus* or *S. maltophilia* (tested with streak assays), and that the presence of *S. aureus* or *S. maltophilia* had no effect on phage densities during short-term co-cultivation (24h). Moreover, both the PAO1 and *lasR* strains were susceptible to phage PT7 in the beginning of the experiment (streak assays) yielding similar phage population densities (phage efficiency of plating with plaque essay: $\sim 10^8$ phage particles mL^{-1} from the same ancestral phage stock).

Experimental design, growth conditions and selection experiment

We used a factorial design to independently manipulate bacterial community composition, the presence of phage and *P. aeruginosa* genotype. To this end, *P. aeruginosa* focal pathogen strains, PAO1 and *lasR*, were evolved in both the absence and presence of phage under four different competition treatments: alone, with *S. aureus,* with *S. maltophilia* and with both *S. aureus* and *S. maltophilia*. Each treatment (16 in total) was replicated five times.

The communities were grown in 1.5 mL of 10% nutrient broth (NB) media (containing 0.5g peptone and 0.3g beef extract per litre distilled water) in deep 96-well plates (Starlab; 2.2 mL of

177 total volume). All treatments were inoculated with approximately 3.8×10^5 bacterial cells per mL,
178 where two-competitor treatments were inoculated with 1:1 ratio of both bacteria and three-
179 competitor treatments with 1:1:1 ratio of every bacteria. Approximately 1.5×10^8 phage particles
180 were added to all phage treatments. All populations were incubated as static cultures at 37°C to
181 reflect human body temperature. The selection experiment was run for 16 days with transfers
182 carried out every fourth day. At each transfer, the cultures were first mixed and homogenised using
183 a pipette before an inoculum of 250 µL was transferred to new deep-well plates containing 1.5 mL
184 fresh media in each well, after 500 µL of each microbial community was cryopreserved in 20% of
185 glycerol at -80°C. Given nutrient broth concentration was chosen to allow prolonged growth during
186 4-day transfer intervals and to reduce the *P. aeruginosa* biofilm and exopolymer production.
187
188 ***Bacterial and phage density measurements***
189 Bacterial densities were measured only at the end of the experiment by serially diluting the samples
190 isolated from the last time point and plating out 10µl of each dilution onto NB agar plates (100%
191 NB media supplemented with 12g agar per litre). To determine *P. aeruginosa* densities in multi-
192 species communities, community treatment samples were also plated on *Pseudomonas* selective
193 agar plates (16g Peptic digest of animal tissue, 10g Casein enzymic hydrolysate, 10g K_2SO_4, 1.4g
194 $MgCl_2 \cdot 6H_2O$, 10ml glycerol and 11g Agar per litre with 200mg C-N selective supplement
195 dissolved in 4ml 1:1 ethanol:distilled water). Bacteria were incubated at 37°C for 48 hours before
196 counting the colonies and calculating the number of colony forming units (CFU) per ml. At every
197 transfer, phages were extracted by mixing with 10% chloroform to kill the bacteria. After vortexing
198 and centrifugation, chloroform-free phage supernatants were stored at 4°C. Phage densities were
199 estimated at every transfer with plaque assays where phage densities are defined as growth on a
200 lawn of ancestral PAO1 bacterial strain. PAO1 ancestral strain was grown at 37°C for 24hours and
201 200µl of this culture was then mixed with 20ml of 50°C soft agar and poured in an even overlay
202 over square NB agar plates. A 10µl of phage serial dilutions (10^{-4} – 10^{-7}) was then pipetted onto the

surface of the pseudomonas-agar overlay, plates were incubated at 37°C for 24 hours, and the number of phage plaques, i.e. phage particles, counted.

Phage resistance assays

A streak assay methodology was used to estimate the evolution of bacterial resistance and phage infectivity (Buckling and Rainey 2002). 12 randomly chosen colonies per each *P. aeruginosa* population were isolated at the end of the experiment and grown in 96-well microplates at 37°C in 150µl of NB media. After 24-hour growth, colonies were cryopreserved at -80°C as above for evolutionary analyses. Phage resistance was measured by pipetting 25µl of phage in a line across square NB plates. A sterilised 12-pin replicator (V&P Scientific) was then used to streak 12 bacterial colonies across the dried line of phage. Plates were incubated at 37°C for 24 hours (or until the bacterial streak became visible). Colonies with a clear reduction in growth over the phage line were scored as susceptible (0) and with normal growth over the phage as resistant (1). Phage resistance was determined at the population level in terms of a proportion of resistant colonies per population. All *P. aeruginosa* colonies were tested against the ancestral PT7 phage and evolved PT7 phages isolated from their own population (coevolved phage population isolated by the way of chloroforming as described above).

Measuring the pleiotropic cost of adaptation

The pleiotropic cost of adaptation was measured as the final bacterial density at 48h by using the same colonies that were used in the phage resistance assays. Colonies were inoculated in 96-well microplates containing 200µl NB media per well by using a sterilised 96-pin replicator (Boenik). The plates were then grown at 37°C and optical density (OD_{600}) measurements taken after 48 hours. The growth of the colonies, which had been subjected to competition and or phages in the selection experiment was compared to colonies that had evolved alone. A mean population density was calculated for all the colonies isolated from the same population. Even though this method results in

indirect fitness measures it was the only practical way to estimate the cost due to a high number of evolved clones (960 clones).

Statistical analysis

All models and test statistics are presented in the supplementary tables 1-5. For the bacterial density data, a linear model was fitted predicting square root transformed *P. aeruginosa* density as a function of phage treatment, competition and pathogen genotype. For the phage density data, a mixed model was used for log transformed phage density data as a function of competition and pathogen genotype with time set as a repeated factor. For the phage resistance data, a linear model was fitted predicting arsin transformed resistance data as a function of phage evolution (ancestral or coevolved), pathogen genotype, and competition. A similar model was used for data predicting the cost of adaptation with the exception that untransformed bacterial growth data was used for the analysis. Post hoc tukey honest significance difference tests were carried out to further investigate significant interactions between factor levels. All analyses were conducted in R, version 3.1.2. (Team. 2014).

3. RESULTS

Bacterial and phage densities during the selection experiment

Both phages ($F_{1, 64} = 8.67$, $p=0.005$) and competitors ($F_{3, 64} = 48.80$, $p<0.001$) significantly reduced *P. aeruginosa* densities in the end of the selection experiment (Fig. 1a-b, Supplementary Table 1). In the absence of phages, both PAO1 and *lasR* monocultures had higher *P. aeruginosa* densities compared to all polymicrobial communities, and PAO1 strain reached higher population densities compared to *lasR* strain when evolving in the absence of a phage and competitors ($p<0.001$ for all comparisons). However, the relative effect of competition was stronger for the PAO1 strain (genotype × competition: $F_{3, 64} = 5.02$, $p=0.003$). Moreover, phages reduced the densities of PAO1 strain more compared to a *lasR* strain (phage × competition: $F_{3, 64} = 7.70$, $p<0.001$). The phage effect depended also on the type of competitive community: in general, phage had a negative effect

on *P. aeruginosa* in the presence of *S. aureus* regardless of the pathogen genotype, while phages had mainly non-significant effects in the other polymicrobial communities (and even a positive effect in the presence of *S. maltophilia*, Fig. 1a-b). Unexpectedly, phage selection also affected the total bacterial biomasses in the polymicrobial communities (Fig. 1c, supplementary table 2) by increasing the total bacterial densities in the PAO1 communities, and decreasing the total bacterial densities in the *lasR* communities in general (genotype × phage: $F_{1, 56} = 8.04$, $p=0.006$; the effect varied depending on the community composition, supplementary figure 1).

Phage densities decreased during the selection experiment in general (Time: $F_{3, 30.35} = 17.34$, $p<0.001$, Fig. 2a-b, Supplementary table 3). While competition had no significant main effect on the phage densities, a significant interaction was found: even though competition had no effect in the weakly virulent pathogen communities, it reduced the phage densities in the PAO1 pathogen communities (genotype × competition: $F_{1, 32.1} = 2.96$, $p=0.047$, Fig. 2a-b). The number or type of competitors did not affect the phage densities with either PAO1 or *lasR* strain ($p>0.05$ in all comparisons). Together these results suggest that competitors had stronger negative effects on both the bacteria and phages in the PAO1 compared to *lasR* pathogen communities.

Bacteria-phage coevolution in different communities

Both initially susceptible PAO1 and *lasR* strains evolved increased levels of resistance to ancestral phage (Fig. 3a-b, supplementary table 4), while the *lasR* strain evolved higher levels of resistance compared to the PAO1 strain in general (genotype: $F_{1, 62} = 35.94$, $p<0.001$). While competitors had no effect on the *lasR* strain resistance evolution, they generally constrained PAO1 resistance evolution (phage origin × competition: $F_{1, 62} = 6.94$, $p<0.001$) with all competitive communities having similar effects ($p>0.05$ in all comparisons). We also found that phages coevolved to become more infective during the selection experiment (Fig. 3a-b): the resistance of evolved bacteria was lower when measured against evolved compared to ancestral phages (phage origin: $F_{1, 62} = 25.38$ $p<0.001$). Interestingly, PAO1 resistance was less affected by phage coevolutionary history

(ancestral vs. coevolved) compared to *lasR* strain (phage origin × genotype: $F_{1, 62} = 4.15$, p=0.046). Together these results suggest that competition altered the trajectory of bacteria–phage co-evolution.

Pleiotropic cost of adaptation

Coevolutionary history with the phage led to reduced bacterial growth in the absence of phages ($F_{1, 71} = 13.36$, p<0.001, Fig. 4a-b, Supplementary table 5). While the focal pathogen genotype ($F_{1, 71} = 2.34$, p=0.131) or the presence of competitors ($F_{1, 71} = 1.88$, p=0.175) had non-significant main effects on the pathogen growth, the growth cost imposed by phage selection was larger with the PAO1 strain (genotype × phage: $F_{1, 71}=6.27$, p=0.015). Moreover, already the presence of competitors led to reduced PAO1 strain growth in the absence of phage selection (genotype × competition: $F_{1, 71} = 7.08$, p=0.010; all competitive communities had similar effects: $F_{3, 63} = 2.38$, p=0.078). Consistent with the population density data, the evolved PAO1 strain reached higher population densities compared to *lasR* strain when bacteria had evolved in the absence of a phage and competitors (genotype × phage: $F_{1, 71}=6.27$, p=0.015). These results suggest that even though both pathogen genotypes suffered from a reduced growth due to phage selection in monocultures, only the PAO1 strain was affected by the presence of competitors and hence suffered relatively higher pleiotropic cost of adaptation in polymicrobial communities.

4. DISCUSSION

Here we studied the role of bacterial competition for the efficiency and eco-evolutionary outcomes of phage therapy in model polymicrobial infections *in vitro*. We found that both phages and competitors reduced the focal pathogen densities. However, this effect was strongly dependent on the focal pathogen genotype with both competitors and phage having a more severe effect on the QS-signalling PAO1 strain. The negative effects of competition observed at the population level correlated with reduced rate of resistance evolution. Interestingly, phage presence decreased the

total bacterial densities in *lasR* pathogen communities demonstrating an unexplored potential benefit of phage therapy: indirect, community-wide reduction in pathogenic bacterial loads in polymicrobial infections. However, a converse pattern was observed in PAO1 communities, which suggest that phages could also indirectly worsen the polymicrobial infections by increasing the density of other pathogenic bacteria. Together these results suggest that phage-mediated effects depend on bacterial competition and the focal pathogen genotype pinpointing the need to understand eco-evolutionary consequences of phage therapy in the community context.

Both competitors and phage had a negative effect on *P. aeruginosa* densities while the effect of competition was relatively larger compared to the effect of a phage. While the number or the composition of competitive communities had no clear effects on *P. aeruginosa* densities, the effect of competition depended on the *P. aeruginosa* genotype being more severe for the PAO1 compared to *lasR* strain in general. This suggests that QS may play an important role for *P. aeruginosa* competition against other bacterial species. For example, the proportion of *lasR* mutants typically increases during chronic polymicrobial CF-infections (Smith et al. 2006; Marvig et al. 2014; Ghoul et al. 2015) and this could be potentially partly explained with QS-mediated competitive interactions with other bacteria (Harrison et al. 2008). There are several mutually nonexclusive explanations for reduced *P. aeruginosa* growth in the presence of competitors. First, competition for limited resources was likely stronger in the presence of other bacterial species leading to lower *P. aeruginosa* densities in polymicrobial pathogen communities. Second, interference competition could have directly reduced *P. aeruginosa* growth directly. For example, *S. maltophilia* has been observed to influence *P. aeruginosa* biofilm architecture and protein synthesis (Ryan et al. 2008), while *P. aeruginosa* has been shown to have negative effects on *S. aureus* due to upregulation of antistaphylococcal substances such as pyocyanin and phenazine (Michelsen et al. 2014). Even though *S. aureus* has not been shown to have direct negative effects on *P. aeruginosa*, the *S. aureus* presence has been shown to favour the increase in the frequency of QS-deficient mutants (Harrison et al. 2008). In line with this study, it has been found that a QS-positive PAO1 strain interacts more

negatively with *S. aureus* compared to a QS-negative *lasR* strain (Michelsen et al. 2014). Recent evidence suggests that reduced antagonism between *S. aureus* on *P. aeruginosa lasR* mutants could be due to metabolic divergence (Frydenlund Michelsen et al. 2015). However, more detailed community level experiments are needed to understand these dynamics more profoundly.

The negative effect of phage was clearest in PAO1 monoculture and generally in the presence of *S. aureus* with both pathogen genotypes. The presence of *S. maltophilia* did not affect phage efficiency with the PAO1 strain and even increased the *lasR* densities in the presence of phage (Fig. 1a-b), while phage had no effects on *P. aeruginosa* densities in the presence of both *S. maltophilia* and *S. aureus*. Together these results suggest that phages can reduce *P. aeruginosa* densities additively in the presence of competitors but that this effect depends on the strength of competition and the composition of the competing bacterial community. Interestingly, phage presence decreased and increased the total bacterial densities of polymicrobial *lasR* and PAO1 communities, respectively. Reduction in PAO1 frequency by the phage could have led to a competitive release and increased the growth of *S. aureus, S. maltophilia* and total bacterial densities. Conversely, resource competition was likely more intense and more symmetric within *lasR* communities due to stronger levels of phage resistance evolution (and hence higher *P. aeruginosa* density). Lastly, it has been shown that phage selection can impose relatively higher competitive cost for the PAO1compared tothe *lasR* strain due to upregulation of siderophore production (Vasse, Torres-Barcelo, and Hochberg 2015). Such metabolic cost could also potentially explain relatively poorer PAO1 growth in the presence of competitors even in the non-social culture conditions used in this experiment. In addition to demographic explanations, the potential changes at the gene expression level warrant thus further investigation in the future.

In line with the bacterial density data, the phage abundances were also generally lower in the presence of competitors and this effect was clearer with the PAO1 strain that suffered more heavily from competition compared to the *lasR* strain. Competition-mediated reduction in bacterial and phage densities correlated with reduced levels of resistance evolution, and as a result,

PAO1 strain evolved lower levels of resistance compared to the *lasR* strain. Simple demographic effects that weaken the strength of selection via reduced bacteria and phage encounter rates and lowered mutation supply rate (Lopez-Pascua and Buckling 2008) could thus be important for the evolutionary outcomes of phage therapy in polymicrobial infections. We also found that phages coevolved to be more infective during the selection experiment as demonstrated by higher levels of resistance of evolved bacteria to the ancestral compared to evolved phage populations. In line with the population dynamics data, the coevolutionary signal was stronger in *lasR* pathogen communities where both bacterial and phage densities were also higher. Bacterial competition did not thus constrain only the bacterial resistance but also the phage infectivity evolution and the trajectory of phage-bacteria coevolution.

Also, some underlying genetic differences could have affected PAO1 and *lasR* strain response to phages. It has been shown that removing, altering and concealing cell surface receptors can prevent phage adsorption (Seed 2015) and that a functional QS system is important for regulating such phage defences (Hoyland-Kroghsbo, Maerkedahl, and Svenningsen 2013; Taj et al. 2014; Tan, Svenningsen, and Middelboe 2015). In contrast to these findings, we found that QS-defective strains were able to evolve higher levels of resistance to phages especially in the presence of bacterial competitors. A similar pattern has been found before, where the loss of QS impaired bacterial twitching motility leading to elevated resistance to pili-specific phages (Glessner et al. 1999). Even though the PT7 target receptor is unknown, both the PAO1 and *lasR* strains were equally susceptible to the phage in the beginning of the experiment. This suggests that initial differences in PAO1 and *lasR* strains' QS ability unlikely drove the long-term differences in the bacterial resistance and phage infectivity evolution. Phage receptors are also often important for other purposes including nutrient uptake (Lenski and Levin 1985), and hence, mutations in phage receptors often reduce bacterial competitive ability. In support for this, we found that both evolved PAO1 and *lasR* monoculture strains suffered reduced growth in the absence of phages and competitors if they had evolved in the presence of a phage during the selection experiment.

Interestingly, while competitors increased the magnitude of the growth cost with PAO1 strain, competitors had no effect or even a positive effect on *lasR* growth. One explanation for this is that less antagonistic interactions between the *lasR* and competitors allowed more rapid accumulation of compensatory mutations during the selection experiment due to relatively large population size and mutation supply rate compared to PAO1 strain. We also found that evolved PAO1 strain grew better in the growth media compared to *lasR* strain when the bacteria had evolved in the absence of a phage and competitors. This suggest that functional QS-system could help *P. aeruginosa* to adapt to abiotic environmental conditions potentially due to depressing of growth-limiting intracellular metabolism (Asfahl et al. 2015). In the community context our results suggest that even though both focal pathogen genotypes were able to evolve resistance to phage the PAO1 strain suffered more severe costs of adaptation due to both competition and phage.

Our results have important implications for the development of phage therapies in the context of polymicrobial infections. First, selection for phage resistance could be weaker in polymicrobial communities due to a competition-mediated reduction in the focal pathogen density and relatively higher pleiotropic costs of adaptation. Competition could thus enhance the phage efficacy when treating acute CF and burn infections that are commonly co-infected by QS-signalling *P. aeruginosa*, *S. aureus* and *S. maltophilia* (Harrison 2007; Turner et al. 2014). However, in contrary, *P. aeruginosa* resistance evolution to phages could be a more severe problem in chronic polymicrobial CF infections that are often dominated by *P. aeruginosa* mutants that have lost QS-signalling ability during the long-term adaptation (Smith et al. 2006; Marvig et al. 2014; Andersen et al. 2015). Interestingly, we found that higher levels of *lasR* strain resistance evolution were correlated with the higher rate of phage infectivity evolution, which could open up avenues for pre-adapting phages to be more infective before clinical phage therapy treatments (Betts et al. 2013; Friman et al. 2016). Moreover, it would be interesting to investigate if our results hold when multiple phage species are applied as a phage cocktail. We also want note that it is possible that both *S. aureus* and *S. maltophilia* strains evolved during the selection experiment. For example, it is

known that *P. aeruginosa* can promote the formation of small colony variants with *S. aureus* leading to changes in virulence and antibiotic resistance (Hoffman et al. 2006; Frydenlund Michelsen et al. 2015). It is thus important to expand the study the evolutionary effects of competition and phage selection across the whole polymicrobial community in the future and also link these phenotypic changes with the changes at the genotypic level.

In conclusion, here we show that the presence of competitors can modulate the phage-mediated effects on a focal pathogen. Crucially, phage selection imposed weaker selection for resistance evolution when the effect of competition with the focal pathogen was strong. Moreover, while the phage presence indirectly reduced the total bacterial loads in weakly virulent *lasR* pathogen communities, phages increased the total bacterial densities in highly virulent PAO1 pathogen communities. Bacterial competition is thus likely to be an important factor affecting both the ecological and evolutionary outcomes of phage therapy in polymicrobial infections. From a therapeutic perspective, the fact that overwhelming phage numbers were not able to eradicate *Pseudomonas* even in the presence of competitors reinforces the importance of studying phage-bacteria interactions in the polymicrobial context.

Acknowledgements

We thank Dr Stephen Diggle for providing the *P. aeruginosa* strains and Imperial College Junior Research Fellowship program, Wellcome Trust and British Ecological Society for the funding (VPF).

Data Archiving Statement

Data available from the Dryad Digital Repository: http://dx.doi..org/xxxxx

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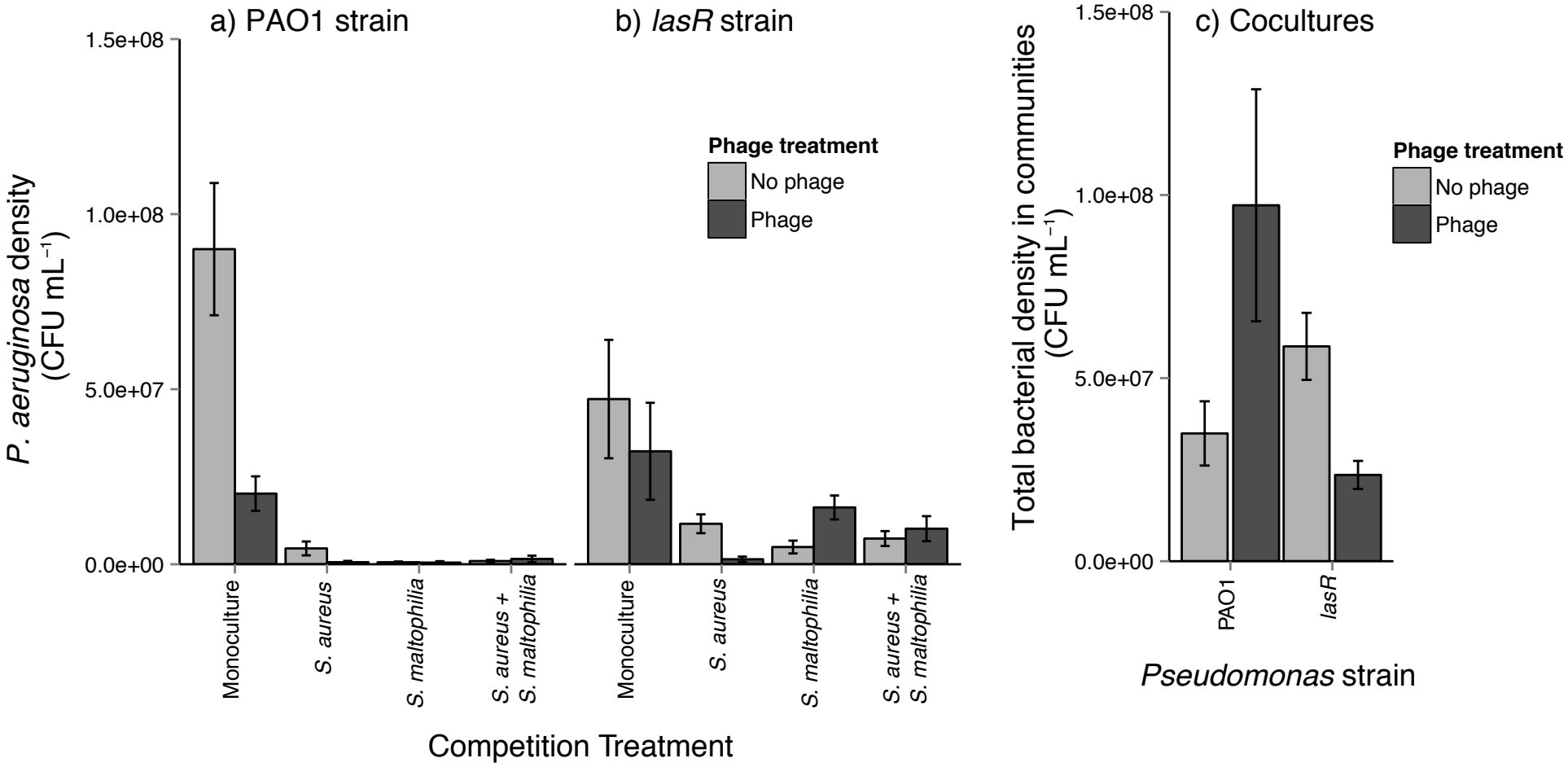
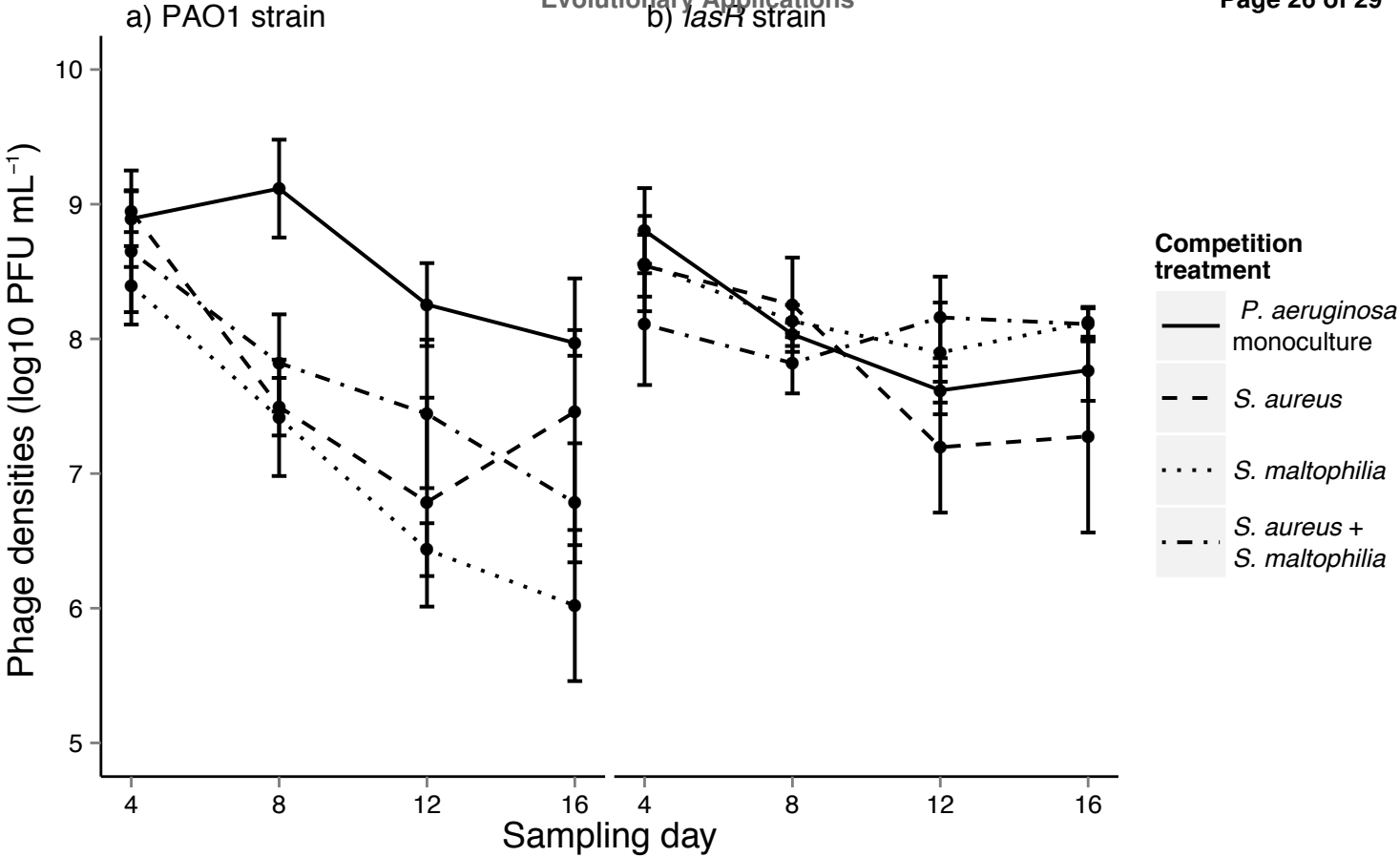
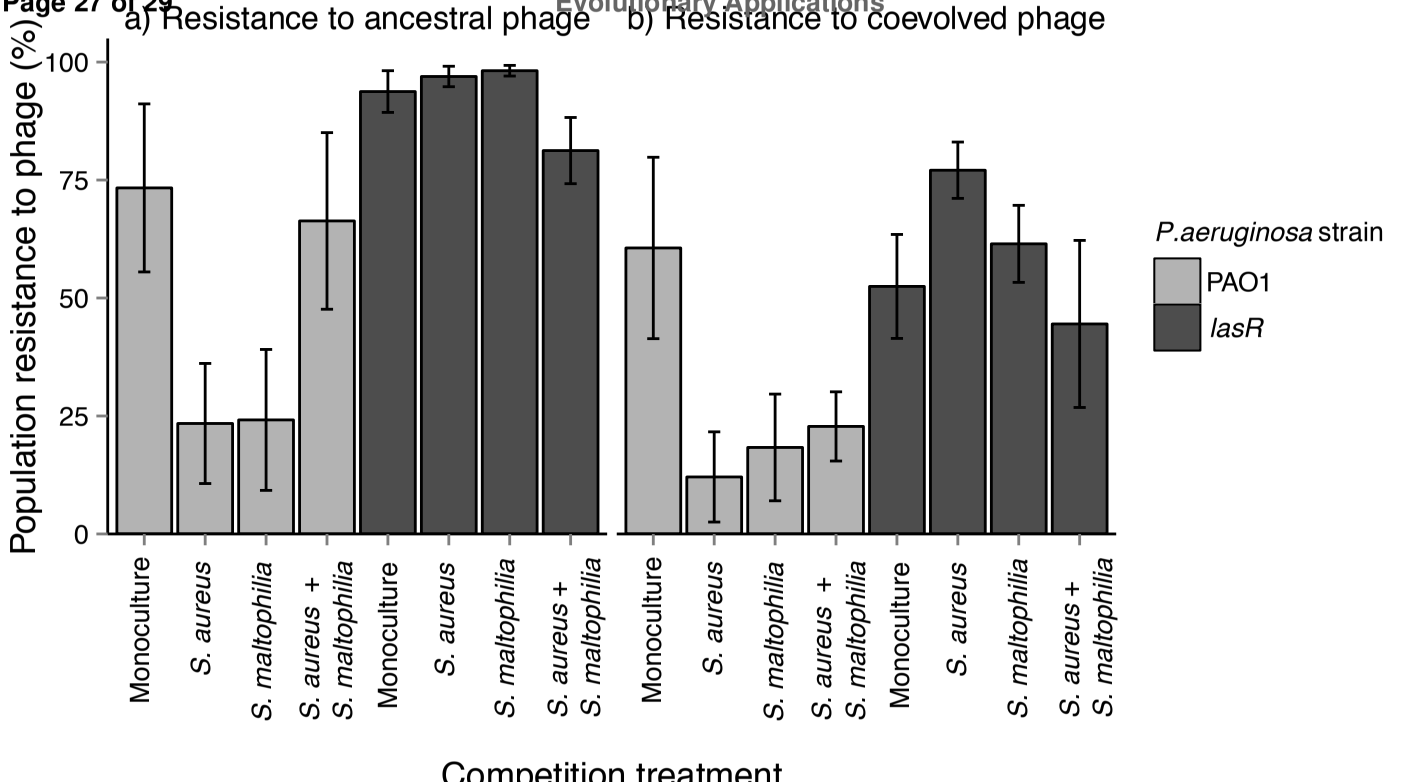
FIGURE LEGENDS

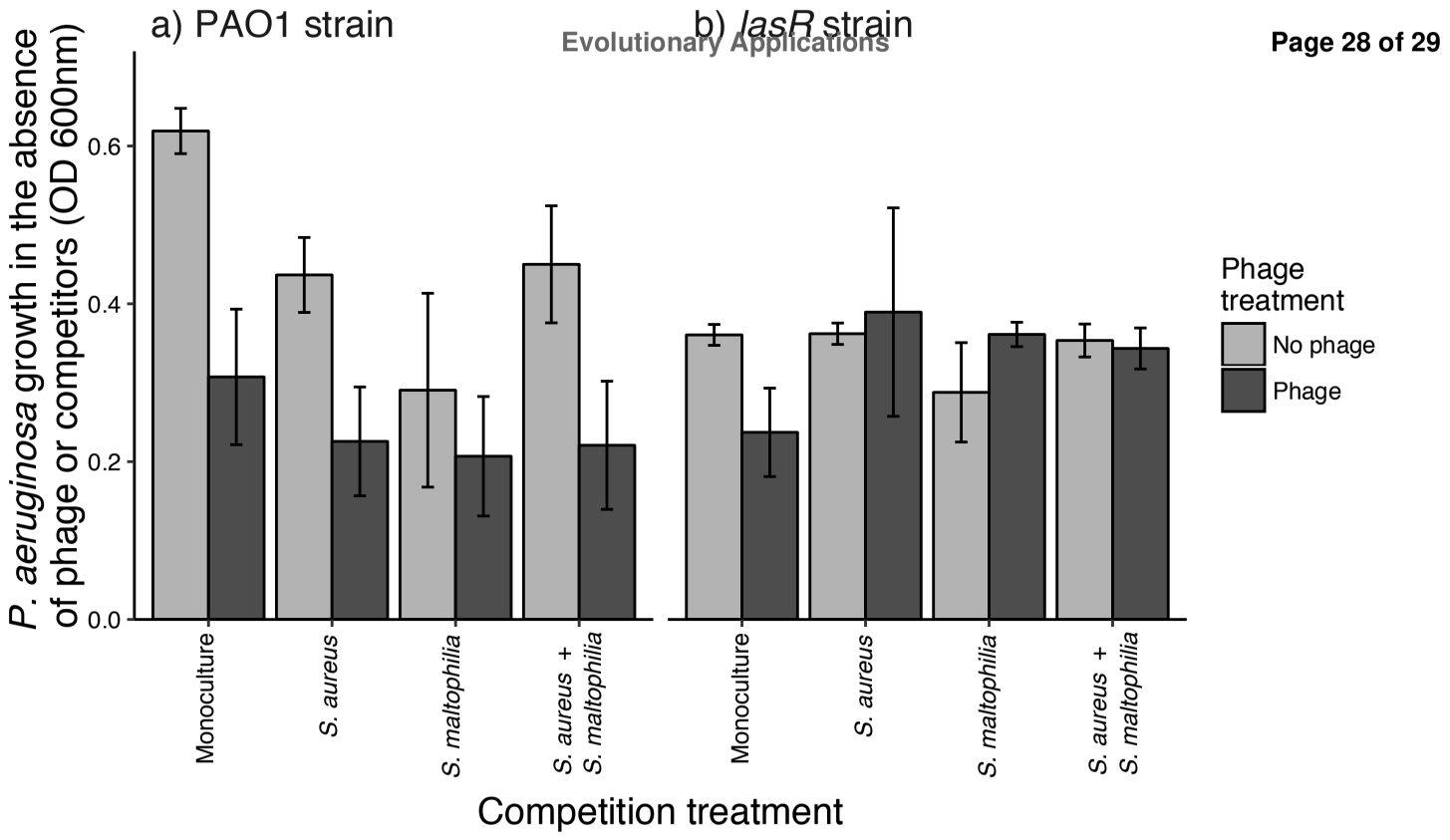
Figure 1. The comparison of *P. aeruginosa* (panels a and b) and total bacterial population densities (panel c) in the end of the selection experiment between different treatments (CFU denotes for

590 colony forming units per mL). Panel c shows the mean over all competition treatments for PAO1
591 and *lasR* strains, respectively. All bars show ±1 s.e.m.
592
593 **Figure 2.** Phage population densities in PAO1 (panel a) and *lasR* (panel b) focal pathogen
594 communities in the absence and presence of competitors (PFU denotes for plaque forming units,
595 i.e., phage particles per mL). All bars show ±1 s.e.m.
596
597 **Figure 3.** The resistance of evolved PAO1 (light grey) and *lasR* (dark grey) strains to ancestral and
598 coevolved phages measured at the end of the experiment. Competition treatment shows the absence
599 and presence of competitors during the selection experiment. Only populations that had evolved in
600 the presence of phage were used for the analysis; all *P. aeruginosa* populations that had evolved in
601 the absence of phage were susceptible to phages. All bars show ±1 s.e.m.
602
603 **Figure 4.** The cost of adaptation measured in terms of maximum population density after 48h of
604 growth. Panel (a) shows the growth of evolved PAO1 and panel (b) the growth of evolved *lasR*
605 strain in the absence of phage or competitors at the end of the selection experiment. Phage and
606 competition treatments denote the absence and presence of a phage and competitors during the
607 selection experiment. All bars show ±1 s.e.m.


a) PAO1 strain
b) lasR strain
c) Cocultures
P. aeruginosa density (CFU mL−1)
Total bacterial density in communities (CFU mL−1)
1.5e+08
1.0e+08
5.0e+07
0.0e+00
Phage treatment
No phage
Phage
Monoculture
S. aureus
S. maltophilia
S. aureus + S. maltophilia
Competition Treatment
PAO1
lasR
Pseudomonas strain


a) PAO1 strain
b) lasR strain
Phage densities (log10 PFU mL⁻¹)
10
9
8
7
6
5
4
8
12
16
Sampling day
Competition treatment
P. aeruginosa monoculture
S. aureus
S. maltophilia
S. aureus + S. maltophilia


a) Resistance to ancestral phage
b) Resistance to coevolved phage
Population resistance to phage (%)
0
25
50
75
100
Monoculture
S. aureus
S. maltophilia
S. aureus + S. maltophilia
Competition treatment
P.aeruginosa strain
PAO1
lasR


a) PAO1 strain
b) lasR strain
P. aeruginosa growth in the absence of phage or competitors (OD 600nm)
0.0
0.2
0.4
0.6
Monoculture
S. aureus
S. maltophilia
S. aureus + S. maltophilia
Competition treatment
Phage treatment
No phage
Phage

ID: EVA-2016-106-OA.R1
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Best,
Ville Friman and Rachel Mumford