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Using experimental evolution to explore natural patterns between bacterial motility and resistance to bacteriophages

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Abstract

Resistance of bacteria to phages may be gained by alteration of surface proteins to which phages bind, a mechanism that is likely to be costly as these molecules typically have critical functions such as movement or nutrient uptake. To address this potential trade-off we combine a systematic study of natural bacteria and phage populations with an experimental evolution approach. We compare motility, growth rate, and susceptibility to local phages for 80 bacteria isolated from horse chestnut leaves and, contrary to expectation, find no negative association between resistance to phages and bacterial motility or growth rate. However, because correlational patterns (and their absence) are open to numerous interpretations, we test for any causal association between resistance to phages and bacterial motility using experimental evolution of a subset of bacteria in both the presence and absence of naturally associated phages. Again, we find no clear link between the acquisition of resistance and bacterial motility, suggesting that for these natural bacterial populations, phage-mediated selection is unlikely to shape bacterial motility; a key fitness trait for many bacteria in the phyllosphere. The agreement between the observed natural pattern and the experimental evolution results presented here demonstrates the power of this combined approach for testing evolutionary trade-offs.

Keywords: Coevolution/ Dispersal/ Host-parasite/ Trade-off/ Cost of Resistance

Introduction

Resistance to parasites is typically believed to be associated with fitness costs,

25 such as reduced growth rate or competitive ability. Such costs are thought to play a

key role in maintaining polymorphism in host resistance, and hence parasite

persistence within populations (Antonovics & Thrall 1994; Burdon & Thrall 2003;

Boots & Bowers 2004; Morgan *et al.* 2005; Morgan *et al.* 2009), and may also impact

on interactions with other species both within and across trophic levels (Clancy &

30 Price 1986; Omacini *et al.* 2001; Lennon & Martiny 2008; Hall *et al.* 2009). For

example, resistance of bacteria to bacteriophages has been associated with

substantial fitness costs (Lenski 1988a; Bohannan *et al.* 1999), including an

increased cost of deleterious mutations (Buckling *et al.* 2006), and decreased

competitive ability (Brockhurst et al. 2005; Lennon *et al.* 2007; Quance & Travisano

35 2009). Given the ubiquity of bacteria-phage interactions, and their key role in all

ecosystems, these costs are likely to have important ecological consequences

(Bohannan & Lenski 2000a; Fuhrman & Schwalbach 2003).

Here, we investigate fitness costs associated with resistance to lytic phages in

the bacterial pathogen, *Pseudomonas syringae* using two complementary

40 approaches. First, we carry out a correlational study between resistance to phages

and other bacterial fitness traits (growth rate and motility) in natural plant-associated

isolates. This approach allows for the direct characterization of phenotypic diversity in

natural bacterial and phage populations, but can be difficult to interpret because

isolates will inevitably have numerous genetic differences between them in addition

45 to resistance to phages. Next, we test for a causal link between resistance to phages

and other fitness traits using a subset of these isolates by experimentally evolving

bacteria in the presence and absence of phages. This experimental evolution

approach is known to be a powerful tool for studying evolutionary trade-offs among fitness traits (Ebert 1998; Kassen 2002; Buckling *et al.* 2009), but may be less helpful

50   in elucidating the importance of these trade-offs in explaining natural diversity. Combining these two approaches allows for a more robust assessment for the role of trade-offs in maintaining phenotypic diversity in natural populations.

Lytic phages have the potential to impose strong selection on host populations, as they are obligate killers. They replicate by injecting their viral DNA

55   into a host bacterium, hijacking the host replication machinery to propagate, and then bursting the host cell in order to release their viral progeny (Lenski 1988b). Infection begins with the binding of molecules on the phage tail fiber to a bacterial cell surface receptor (Lindberg 1973) and resistance to phages can be gained by loss or change of these receptors, which is likely to impact on other bacterial functions (Whitchurch &

60   Mattick 1994; Brockhurst et al. 2005). For example, phages commonly exploit bacterial surface motility appendages (flagella and pili); flagellatropic phages are known to reversibly bind to helical grooves on the bacterial flagellum and use the rotation of the flagellum to spiral towards the cell surface (Samuel *et al.* 1999), and similarly, pilus-specific phages will attach to pili and fuse their membrane with that of

65   the bacterial cell during pili retraction (Romantschuk & Bamford 1985; Mattick 2002). A first step towards bacterial resistance may therefore be the loss or alteration of these structures. For example, phage-resistant mutants often show defective flagella that are unable to rotate (Icho & Iino 1978) and abnormal unpiliated or hyperpiliated bacteria may arise to prevent phage attachment (Bradley 1980; Mattick 2002;

70   Brockhurst et al. 2005).

Reduced motility function is likely to have important implications for bacterial fitness in both pathogenic and non-pathogenic bacteria (Drake & Montie 1988;

Korber et al. 1994; O'Toole & Kolter 1998). Immotile mutants of the opportunistic animal pathogen *Pseudomonas aeruginosa* show reduced infectivity on human hosts

75　and impaired biofilm formation (Drake & Montie 1988; O'Toole & Kolter 1998). Similarly, epiphytic, non-motile strains of the plant pathogen *Pseudomonas syringae* were found to have reduced fitness and competitive ability compared to more motile strains, especially under conditions of environmental stress (Haefele & Lindow 1987). Indeed, motility is a key component of fitness for bacteria in the plant phyllosphere

80　and is necessary for successful pathogenicity, as bacteria colonizing leaf surfaces are better able to invade the leaf interior through the stomata if they are motile (Panopoulos & Schroth 1974; Beattie & Lindow 1999; Melotto *et al.* 2006).

　　　　Despite the predicted link between phage resistance and motility, and some correlative work suggesting a trade-off between the two (Joys 1965; Whitchurch &

85　Mattick 1994), the association has never been systematically investigated. Here, we combine an examination of natural bacteria and phage isolates (from the leaves of horse chestnut trees) with experimental evolution to address this relationship. Contrary to our expectations, while natural populations of Pseudomonads show a positive relationship between bacterial motility and resistance to phages, subsequent

90　experimental work suggests that this relationship is not clearly causal.

Materials and methods

Relationship between resistance and motility in natural populations

　　　　To investigate the natural variation in resistance to phage and motility, we examined 80 natural isolates, from either the surface or the interior of horse chestnut

95　leaves collected around Oxfordshire, United Kingdom, that were part of a larger sampling design from a previous experiment (Koskella et al. 2011). The study included a reciprocal cross-inoculation of culturable bacteria and communities of

phages isolated from each of 32 leaves. Bacteria were isolated from either the leaf surface, using buffer from leaf washes, or leaf interior, using homogenates from

100   surface-sterilized leaves. Washes/homogenates were plated on 1.2% King's medium B (KB) broth (10 g/litre glycerol, 20 g/L proteose peptone #3 (Becton Dickinson UK Ltd), 12 g/L agar, 1.5 g/L $K_2HPO_4\bullet3H_2O$, 1.5 g/L $MgSO_4\bullet7H_2O$) and, after 48 hours of growth, colonies were picked at random based on proximity to a randomly chosen spot on the plate. Phages from the interior and surface of each leaf were separated

105   from bacteria by chloroform treatment of the buffer solutions. This allowed us to generate an inoculum that was representative of the natural phage community as it did not require passaging through a bacterial host. Using a cross-inoculation design, 7 μl of each phage inoculum was spotted in a grid formation onto a lawn, grown in soft KB agar (0.6% wt/vol), of each bacterial isolate. Phage plaque formation within

110   the spot was compared with bacterial growth across the lawn, allowing us to define each bacterial isolate as either susceptible or resistant to the local, sympatric phage population (*i.e.,* to quantify susceptibility to phages from the same leaf). We then randomly chose 40 bacterial isolates from each category (susceptible or resistant) to further characterize and measure motility and growth rate. Of the 80 isolates

115   examined, only 6 pairs showed strong phenotypic and genotypic similarity, and each of these pairs was combined in our statistical analyses.

    Bacteria typically exhibit three types of motility: swimming, swarming, which are primarily flagella-dependent, and twitching, which is dependent on type IV pili (Mattick 2002; Harshey 2003). To examine these motility mechanisms, we measured

120   dispersal capability of each of the bacterial isolates under different agar environments. For each motility assay, bacterial isolates were first grown overnight from freezer stocks in KB broth at 28 ˚C. A small amount of each culture was then

used to inoculate the center of 90 cm^2 petri dishes containing 25 mL of KB medium

with the appropriate concentration of agar, dried briefly before use. Twitching motility

125 was assessed on KB medium solidified with 1.2% (wt/vol) agar and estimated using

the bacterial movement between the interface of the petri dish and agar surface,

while swarming and swimming motility assays were performed on KB medium

containing 0.6% and 0.3% (wt/vol) agar, respectively (Rashid & Kornberg 2000), and

estimated by area dispersed through the agar. The area of dispersal was measured

130 after either 24 hours (for swimming) or 48 hours (for swarming and twitching) of

incubation at 24 °C by demarcating the area covered, photographing the plate with a

measurement standard, and analyzing the area digitally using ImageJ 1.41o

(Abramoff *et al.* 2004). All area data were square root transformed and three replicate

assays were run for each bacterial isolate.

135 In vitro *growth rate and density assays*

We measured the growth rate and final bacterial density for each of the 80

natural bacterial isolates. For growth rate assays, KB cultures were grown overnight

at 28°C and diluted by a factor of 1:100. Then, 10 µl of each dilution was added to a

96-well microplate containing 90 µL of KB per well and optical density at 600 nm was

140 measured every 45 minutes at an incubation temperature of 24 ^{o}C with 5 second

shaking prior to read for 24 hours using a microplate spectrophotometer (BioTek

Powerwave XS, Northstar Scientific Ltd., Bedfordshire, UK). The period of

exponential growth occurred between 4 and 12 hours, during which time V_{max}

(measured as milli-optical density units per minute (mOD/ min)), the maximal rate of

145 change in optical density during log growth, was calculated. For density assays,

cultures incubated for 24 hours were diluted by a factor of 1:1 (in order to bring them

within a range of measurements for which the readings were most accurate) and measured for optical density at 600 nm.

Characterization of isolates

150   The identity of each bacterial isolate, to the genus level, was determined by sequencing an 800 bp region of the 16S rRNA subunit using the forward primer 27F (Lane 1991) and reverse primer 907R (Muyzer *et al.* 1998). The reaction contained 1 U *Taq* DNA polymerase (Invitrogen, Paisley, UK), 1X *Taq* Buffer, 3 mM MgCl$_2$, 0.2 µM dNTP's, 0.2 pm of each primer, and 0.5 µL of a 1:10 dilution of an overnight KB

155   culture. PCR amplification was performed at 95 °C for 4 minutes, 29 cycles of 95 °C for 45 seconds, 52 °C for 1 minute, and 72 °C for 2 minutes, with a final elongation at 72 °C for 10 minutes. The product was then sequenced by Geneservice (Oxford, UK) using the reverse primer. These sequence data have been submitted to the GenBank database under accession Numbers HQ529384-HQ529465. Each bacterial isolate

160   was assigned to the genus level, using the NCBI database, based on highest sequence similarity; all but five of the isolates had over a 97% similarity to a previously characterized isolate, with the other five being between 93 and 95% similar and all isolates had an e value of 0. Isolates were not assigned to the species level due to the highly conserved nature of the sequenced 16S rRNA region.

165   *Selection Experiment*

To specifically examine how the acquisition of phage resistance might alter motility function we performed an experimental evolution study using a random subset of the natural isolates. Because of both the observed positive correlation between motility and resistance to phages and the epidemiological significance of the

170   species (Hirano & Upper 2000; Webber *et al.* 2008; Green *et al.* 2009), we chose to

focus exclusively on 10 bacterial isolates from the leaf interior that had >99%

sequence similarity to known isolates of *Pseudomonas syringae.* Importantly, these

isolates were all sampled from separate leaves to decrease the probability of pseudo-

replication. To experimentally examine the relationship between swarming motility

175 and phages in the environment, we performed 10 serial transfers (approximately 10-

16 bacterial generations per transfer) of each bacterial isolate into fresh soft agar

(0.6% wt/vol) that contained either high concentrations, low concentrations or no

phages (Figure 1). To initiate the experiment, overnight cultures from a single

bacterial colony were grown in KB broth, which was then pipetted directly onto the

180 centre of a 144 cm^2 square petri-dish (Fisherbrand, Leicestershire, UK) containing 40

ml soft KB agar. Plates were left in a humid incubator at 24 ^{o}C for 24 hours, after

which samples were taken for the next transfer under one of two selection regimes:

positive (termed 'dispersal' treatment) or neutral (termed 'random' treatment)

selection for dispersal (Figure 1). For the dispersal selection lines, we took six

185 samples, equally spaced apart, from the outer edge of the colony range. For the

random selection bacterial lines we took six samples from throughout the colony, as

chosen by a random number grid. Each sample was taken by stabbing a sterile 1 ml

pipette (Finnpippette, Northumberland, UK) through the agar to the bottom of the

plate and then transferring the agar stab to 1 ml of M9 solution (1 mM thiamine

190 hydrochloride, 0.4% glycerol, 0.2% casamino acids, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$).

The pipette tip was washed thoroughly in the solution and, after vortexing, 2.5 μl of

the solution was used to inoculate fresh agar plates, as described above. This was

repeated every 24 hours for ten transfers. Plates were poured with fresh media at

each transfer so as not to confound age of agar plate with dispersal ability.

195   The phage inoculum was generated by isolating 16 individual phage types (*i.e.,* independent plaques) from 16 different horse chestnut leaves used in the previous cross-inoculation (Koskella et al. 2011). Each phage isolate was passaged once through one of two previously characterized strains of *P. syringae pv. aesculi* (*P.s. pv aesculi* 6617 and 6623; Green *et al.* 2010), in order to amplify numbers of

200   phage particles, and separated from the bacteria using chloroform. The 16 phage isolates were mixed to produce a stock inoculum, representing a subset of the naturally occurring phage community in the leaf environment, and stored at 4 °C. This design allowed us to hold the phage environment relatively constant while the bacteria evolved in response. To create a homogeneous selective environment,

205   phage inoculum was vortexed into the soft agar prior to solidifying, when the agar reached about 40 °C. After ten transfers, each line was assayed for growth rate and motility in a phage-free common garden (*i.e.,* all treatments were grown under the same laboratory environment), and under three agar concentrations. Bacterial densities (OD $_{600}$) of overnight cultures of each evolved and ancestral strain were

210   also measured in both the presence and absence of phages.

Statistical analyses comparing bacterial dispersal and susceptibility to phages

        Analyses and figures were produced on PASW Statistics 18 (SPSS; part of IBM UK ltd, Middlesex, UK). We first used a two-way analysis of variance to compare the area dispersed (square-root transformed) across bacterial isolates of different

215   genera and susceptibility to phages. In addition, independent samples t-tests were run to compare susceptibility to phages and dispersal within the *Pseudomonas* and *Erwinia* isolates from both the leaf surface and leaf interior. For the experimental evolution results, we examined the initially susceptible and initially resistant isolates separately, due to the dramatic differences in means and variance between them,

220   and we included bacterial strain as a random factor in each model. At the end of the

experiment, we examined the area dispersed within a common garden (in the

absence of phages) at the end of the experiment using a separate two-way analysis

of variance for (1) the 30 susceptible lines and (2) the 30 resistant lines and

compared population growth parameters to dispersal using Pearson correlation

225   coefficients. In all cases, the area dispersed was square root transformed.

Results

Relationship between resistance, growth and motility in natural populations

Sequencing of the chosen bacterial isolates revealed that the surface of the leaf

was primarily dominated by *Erwinia*-like species (>85%), while the leaf interior

230   community was comprised of both *Erwinia*-like (50%) and *Pseudomonas*-like species

(47%). We excluded bacterial isolates from other genera, including *Rhanella* and

Pantoea, from subsequent analyses due to low replication. Overall, 44%

(N/N_{TOT}=24/54) of the *Erwinia*-like isolates and 61% (N/N_{TOT}=14/23) of

Pseudomonas-like isolates were susceptible to sympatric phages, i.e. those collected

235   directly from the same leaf as the bacteria being tested. We chose to focus

specifically on susceptibility to sympatric phages, using the leaf homogenate as an

inoculum, because this measure more accurately reflects local selection pressures

and did not require amplification through a bacterial host.

The relationship between swarming motility and resistance to sympatric

240   phages significantly differed across bacterial genera (Figure 2a; interaction effect for

genus x phage susceptibility: $F_{1,38}$= 5.99, P = 0.020). Specifically, there was no

difference in dispersal capability between resistant and susceptible strains of the

Erwinia-like isolates from the leaf surface or interior (Figure 2a). However, for the

Pseudomonas-like isolates from the leaf interior (surface isolates were excluded due

245   to low sample size) resistant isolates showed a higher swarming motility range than

the susceptible isolates (Figure 2a; t_{17}= 2.241, P= 0.039) but did not show a

difference for swimming or twitching motilities (swimming: t_{17}= 1.143, P= 0.271;

twitching: t_{17}= 1.219, P= 0.099).

There was no difference in growth rate, measured as V_{max} (mOD/ min)

250   between resistant and susceptible *Erwinia*-like isolates from the surface or the interior

($P > 0.05$), but for *Pseudomonas*-like isolates from the leaf interior, resistant isolates

had a higher growth rate than susceptible isolates (Figure 2b; t_{17}= 3.234, P= 0.005).

Importantly, the dispersal area during swimming and swarming motility was

significantly correlated with growth rate for both the *Pseudomonas*-like isolates

255   (Pearson correlation, swimming: r= 0.517, P= 0.012; swarming: r= 0.669, $P<$ 0.001)

and the *Erwinia*-like isolates (swimming; r= 0.317, P= 0.019; swarming: r= 0.359, P=

0.008). However, twitching dispersal was not correlated with growth rate for either

Pseudomonas-like isolates (r= 0.310, P= 0.150) or *Erwinia*-like isolates (r= -0.100, P=

0.473).

260   *Selection for bacterial resistance to phages and/or motility*

Of the ten Pseudomonad isolates chosen for experimental evolution, five were

initially susceptible and five were initially resistant to the phage inoculum. Of the

initially susceptible strains, all were susceptible to at least half of the 16 phage

isolates used in the inoculum (mean susceptibility of 70.0% ±19.7 SD). Of the five

265   initially resistant bacterial isolates, resistance was complete across all 16 phage

isolates (susceptibility of 0%). At the start of the experiment the presence of phage

had a significant negative effect on motility for the initially susceptible strains ($F_{1,24}$=

7.223, P= 0.013), but had no effect on the initially resistant strains ($F_{1,24}$= 1.624, P=

0.215). After ten transfers of experimental evolution in the presence of phages (at

270 both high and low concentration and across random and dispersal selection

regimes), each of the five bacterial isolates that were initially susceptible to phages

had evolved resistance to most or all of the 16 phage isolates used in the inoculum

(Figure 3). For these isolates there was no longer an effect of phages in the

environment on motility ($F_{1,24}$= 0.400, P= 0.533). These strains did not evolve

275 increased resistance in the absence of phages (GLM for mean proportion of infective

phages, effect of Time: $F_{1,18}$= 16.56, P= 0.002; Phage treatment: $F_{2,18}$= 4.173, P=

0.032; Time x Phage treatment interaction: $F_{2,18}$= 4.173, P= 0.032). The bacterial

isolates that were initially resistant remained resistant throughout the duration of the

experiment. Although we did not directly allow for coevolution between bacteria and

280 phages (Bohannan & Lenski 2000a; Buckling et al. 2009), some degree of

coevolution may have occurred between phages that were passively collected along

with bacteria at each transfer; creating an additional benefit to dispersing away from

the inoculation site, as coevolved phages are likely to have increased infectivity to the

evolving bacteria (Bohannan & Lenski 2000a; Brockhurst et al. 2005). However, all

285 resistance assays were run using ancestral phages, as these represented the

selection pressure throughout the experimental environment.

To confirm our measures of phage resistance, we also measured growth rates

of ancestral and evolved lines in the presence of phages. For those strains that were

initially resistant, we found no difference in population density in either the presence

290 or absence of phages (GLM with arcsinsqrt transformed density, interaction effect of

Time x Phage presence: $F_{1, 42}$= 0.206, P= 0.652). However, for those strains that

were initially susceptible and evolved resistance to phages over the course of the

experiment, the ancestral bacterial lines had a significantly lower density than

evolved bacterial lines in the presence of phages, but not in the absence of phages

295   (Time x Phage presence: $F_{1, 42}$= 5.977, P= 0.019), indicating that phages were

initially reducing population size of susceptible bacteria but that population size was

not affected by phages once resistance had evolved.

We measured area dispersed and growth rate of all isolates at the start of the

experiment in the absence of phages and found that the initially resistant bacterial

300   isolates had a higher mean swarming dispersal (mean area dispersed 56.45 cm^2 ±

7.05 SD in 0.6% agar) than the susceptible isolates (3.14 cm^2 ± 3.36 SD) and that

initially resistant isolates had a higher growth rate (mean Vmax 3.49 ± 0.36 SD) than

susceptible isolates (1.55 ± 0.79 SD). These results were consistent with the findings

from the full sample of natural bacterial isolates.

305   After the ten serial transfers of experimental evolution we again assayed

motility in a phage-free environment to examine the effect of both phage and

dispersal selection regime on the evolution of dispersal, without the confounding

ecological effects of phages (Figure 4). The initially susceptible strains did not show a

response to selection for increased dispersal ($F_{1,20}$= 0.333, P= 0.571) and there was

310   no evidence for a direct effect of phage treatment on dispersal ($F_{2,20}$= 0.337, P=

0.718) nor for any interaction between phage and selection ($F_{2,20}$= 0.080, P= 0.923),

suggesting a lack of a causal link between phage resistance and motility (Figure 4a).

However, the initially resistant strains were able to respond to selection for increased

dispersal ($F_{1,20}$= 10.505, P= 0.004), regardless of phage treatment (main effect of

315   Phage: $F_{2,20}$= 1.995, P= 0.162, interaction between Phage and Selection: $F_{2,20}$ =

0.514, P= 0.606, Figure 4b). Finally, there were few correlated changes in swimming

or twitching motility resulting from either the dispersal or phage selection regimes: the

only significant effect, after controlling for multiple tests, was increased swimming

motility under positive selection for dispersal (compared with random) in the initially

320   resistant lines (Figure 4b; main effect of selection: $F_{1,20}= 8.268$, $P= 0.009$).

We also investigated how the selection regimes affected growth rate of each

population, with the specific focus on whether there were costs associated with the

acquisition of resistance. We found no evidence that dispersal selection regime or

phage-imposed selection affected population growth rate for either initially resistant

325   or initially susceptible bacteria ($P> 0.10$ for all treatments). Importantly, although we

found a correlation between growth rate and dispersal for the initially resistant strains

(Pearson $r= 0.390$, $P= 0.033$) there was no correlation between growth rate and

dispersal for the initially susceptible lines ($r= 0.000$, $P= 0.999$), demonstrating that

dispersal estimates were not simply a function of population growth. This result was

330   qualitatively the same when comparing population density and dispersal.

Discussion

We combined an examination of natural phenotypic variation with

experimental evolution to investigate the potential link between resistance to phages

335   and bacterial fitness (growth rate and motility). We found that, contrary to

expectation, natural bacterial isolates that were resistant to local phages had neither

reduced motility nor reduced growth rates relative to those that were susceptible.

Generally, there was no relationship between bacterial motility or growth rate and

resistance to phages from the local environment (as present in the leaf homogenate)

340   for *Erwinia*-like isolates and a positive correlation for *Pseudomonas*-like isolates

under favorable, laboratory conditions (Figure 2). This result is in line with previous

work from marine Cyanobacteria showing that costs of resistance to phage are not

ubiquitous and instead, are highly dependent on the virus and bacteria strain being

examined and on whether strains are in direct competition (Lennon *et al.* 2007).

345   Importantly, we could not rule out the possibility that more motile and resistant strains

represented different species or pathovars than less motile and susceptible strains.

Therefore to investigate any causal links between resistance to phages and bacterial

motility, we experimentally evolved 10 *Pseudomonas*-like bacterial lines in either the

presence or absence of phages, and under either positive or random selection for

350   dispersal.

After ten serial transfers of experimental evolution we found that bacteria that

were initially susceptible to phages had evolved resistance in the presence, but not

the absence, of phages (Figure 3). Moreover, at the start of the experiment we saw

decreased motility in the presence of phages for those strains that were susceptible

355   to infection, but not for those that were initially resistant. This result adds to a growing

body of evidence that parasites can have a direct impact on host demography

(Fellous et al. 2010; Bradley et al. 2005; Cameron et al. 1993). In addition, we found

that bacterial motility decreased over the course of the experiment for most bacterial

isolates (regardless of phage treatment and even under selection for increased

360   dispersal), suggesting a cost to motility such that flagella and/or pili function is

reduced under favorable laboratory conditions. Importantly, the fact that the initially

resistant bacteria showed reduced motility but no loss of resistance to phages in the

control (no-phage) treatments, suggests that there is no negative correlation between

resistance and either growth rate or motility in the laboratory. This overall reduction in

365   motility is initially surprising given that half the lines were selected for increased

dispersal and suggests that the imposed selection regime was relatively weak, as

only the initially resistant, and not the initially susceptible, lines showed increased

motility under selection for high dispersal (Figure 4). This result warrants further study

as we cannot provide a clear mechanistic explanation, and understanding whether

370   phages directly hinder the response of susceptible bacteria to selection for increased

motility is of key interest. In addition, under the no-phage, common garden conditions

at the end of the experiment, we found no evidence that phage-imposed selection for

resistance in the initially susceptible lines was associated with a change in either the

motility trait under selection (swarming), or twitching and swimming motility (Figure

375   4). Finally, there was no evidence of growth costs associated with the acquisition of

resistance (although the strains were never put in direct competition). These data

suggest no causal relationship, either positive or negative, between the acquisition of

phage resistance and dispersal ability.

        A number of other studies, like ours, have found no clear cost in terms of

380   population growth associated with resistance to phages (Lenski 1988a; Lythgoe &

Chao 2003; Meyer et al. 2010). However, the lack of an observed pattern is

somewhat surprising given the predicted association of many phages with flagella or

pili number and function (Icho & Iino 1978; Bradley 1980; Mattick 2002). There are a

number of possible explanations. First, phages that use motility organelles (*i.e.*, pili

385   and flagella) as attachment sites may be relatively uncommon in the natural leaf

environment and hence do not impose very strong selection against bacterial motility.

This interpretation may have been influenced by our phage isolation method;

chloroform treatment is known to destroy primarily lipid-based phages (Leers 1969),

and if a correlation exists between phage-targeted bacterial receptors and phage

390   coat composition we could have missed an effect of phage-mediated selection.

Second, observed dispersal behaviors may be influenced by traits other than motility

organelles, such as cell size, chemotaxis or quorum sensing (Harshey 2003), which

are not altered by the acquisition of phage resistance; a possibility that would indicate

bacterial motility can evolve independently of phage-mediated selection. Third, it is

395   plausible that motility-associated costs may only be visible under stressful

environmental conditions. For example, previous experimental work has shown that

costs of resistance to phages in both *Escherichia coli* B (Bohannan & Lenski 2000b)

and *Pseudomonas fluorescens* (Lopez-Pascua & Buckling 2008) are increased in

nutrient-poor environments. Fourth, it is possible that any costs were rapidly

400   compensated by second site mutations, as is commonly observed with antibiotic

resistance (MacLean *et al.* 2010). However, such rapid compensation would suggest

that costs of phage resistance are relatively unimportant in natural populations.

Overall, our results do not support a causal link between the evolution of

resistance to phages and bacterial motility in natural populations, although we did

405   find a surprising positive association between phage resistance and bacterial motility

for Pseudomonads in the phyllosphere. This of course does not rule out an important

role of phage-imposed selection on the evolution of motility, as costs associated with

resistance are likely to be contingent upon genetic background, local environment

and the precise measures of fitness, but does suggest that the effect of phage-

410   imposed selection is unpredictable and is likely to depend on the natural phage

community and bacterial environment. This is an important consideration for phage

therapy of pathogenic bacteria, where virulence is often associated with motility traits

and any association between phage resistance and bacterial motility could have

important consequences over coevolutionary time (Josenhans & Suerbaum 2002).

415   Understanding these potential costs will be key as phage therapy becomes a more

common method for controlling pathogenic bacterial populations (Goodridge 2004;

Levin & Bull 2004). More generally, these results add to a growing body of work

across a range of taxa that points to the wide range of host fitness traits correlated

with pathogen resistance (Boots & Begon 1993; Ferdig et al. 1993; Fellowes et al.

420   1998; Langand et al. 1998; Zhong et al. 2005; Buckling et al. 2006; Morgan et al.

2009; Williams et al. 1999; Yourth et al. 2002; Lythgoe & Chao 2003; Sanders et al.

2005). This method of combining natural observations with laboratory selection

experiments shows promise in furthering our understanding of the importance of

phages, and other selective pressures, as drivers of bacterial evolution and diversity

425   in natural environments.

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Figure 1. Ten natural isolates were experimentally evolved over ten transfers in one of three phage environments: high phage concentration (40 µl phage inoculum /40 ml

595   0.6 % agar), low phage concentration (4 µl phage inoculum /40 ml 0.6 % agar) or no phage treatment (where no phage was added to 0.6 % agar). In addition, each line was evolved under one of two selection regimes: positive (termed 'dispersal' treatment) or neutral (termed 'random' treatment) selection for dispersal. In total, we had 60 selection lines evolved over approximately 140 generations of selection.

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Figure 2. Relationship between dispersal (a) and growth rate (b) for the 40 *Pseudomonas*-like and *Erwinia*-like isolates collected from the leaf interior. Resistance (dark grey) or susceptibility (light grey) to local phage is compared to determine whether there exists a cost to resistance with regard to bacterial motility or

605   growth. Dispersal was measured as area covered (cm^2) over 48 hours on soft agar (0.6%). Values were square root transformed to correct for non-normality. Error bars represent ± 1 SEM.

Figure 3. Evolution of resistance for the five initially susceptible isolates over ten

610   serial transfers in either the presence (dashed lines) or absence (solid line) of phage. Proportion of infective phage represents susceptibility to each of the 16 phage clones used in the experimental evolution inoculum. Error bars represent ± 1 SEM.

Figure 4. Results from common garden experiment run at the end of the selection

615   experiment (*i.e.,* transfer 10) for initially resistant (a) and initially susceptible (b) isolates. Measured on 0.3 % agar (swimming; left panel), 0.6 % agar (swarming; middle panel); and 1.2 % agar (twitching; right panel). These experiments were run in

the absence of phage in the environment to examine differences between the lines that are not resulting from interactions with or ecological feedback from phage.

620 "Treatment" therefore represents the phage environment of each line during the course of experimental evolution and not within the common garden experiment. Area dispersed is square root transformed and error bars represent ± 1 SEM.
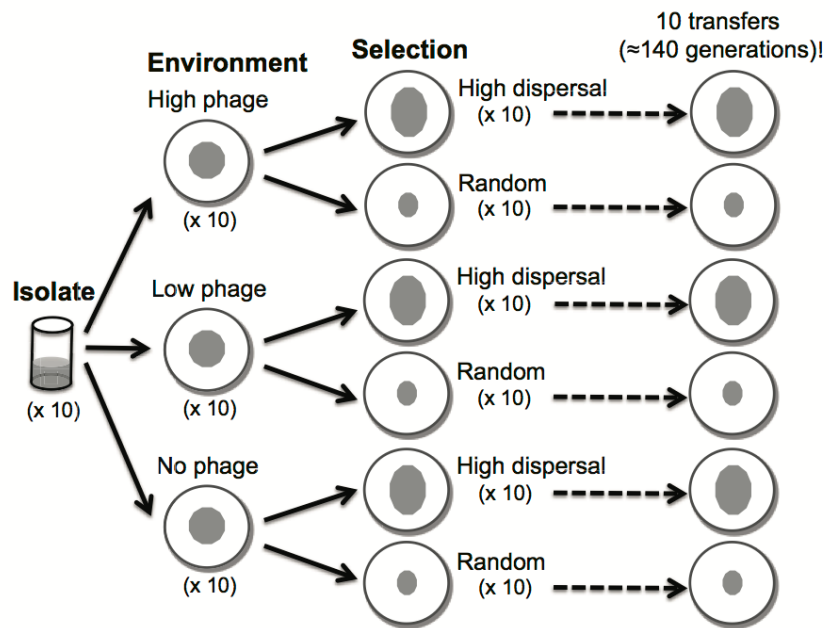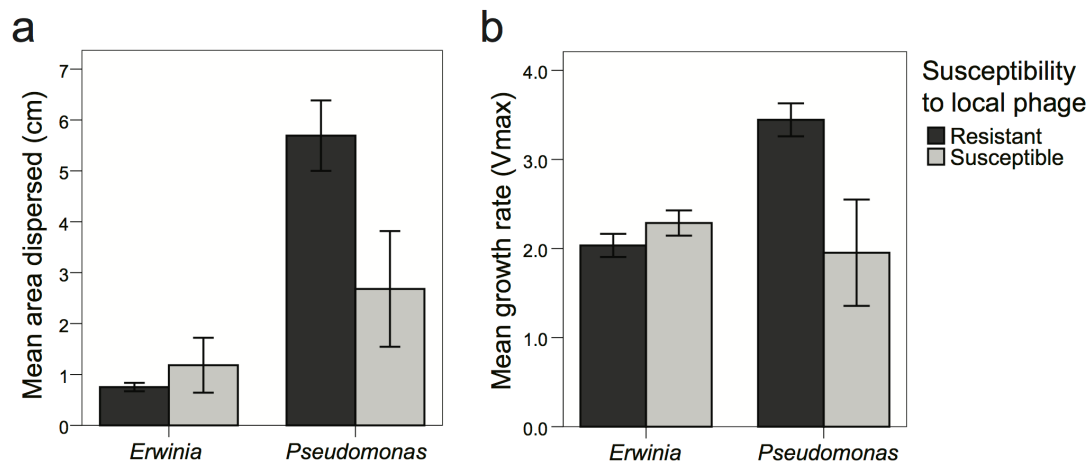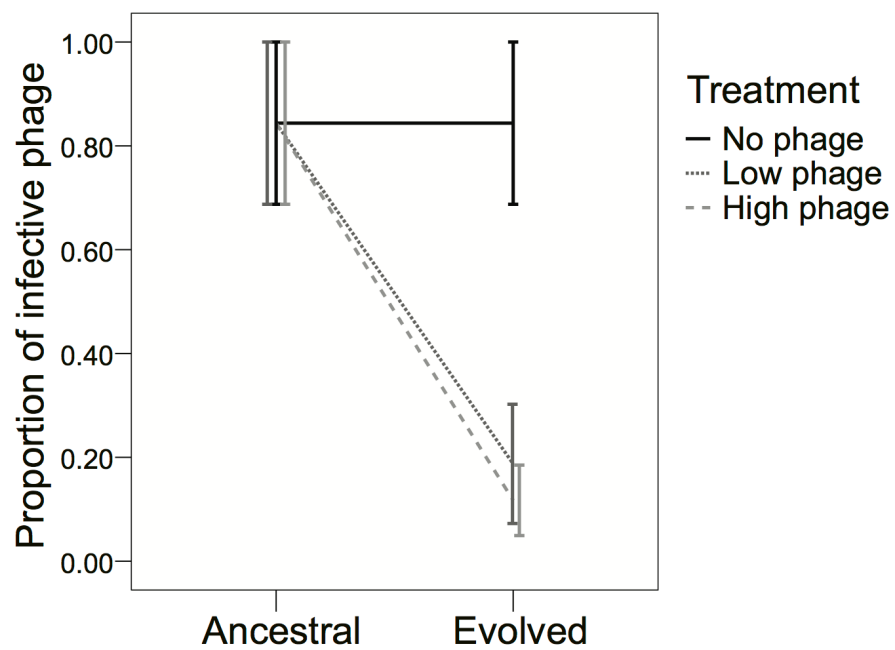

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Figure 1.

Figure 2.

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Figure 3.

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