Microbial community structure is affected by phage-resistance associated increases in host density

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Data availability: All data and R code used in the analysis are available on GitHub (https://github.com/mcastledine96/Variovorax_phage_resistance_increases_density_2024). Assemblies and sequencing data are available from ENA (Project Accession PRJEB74093).

Abstract

Lytic bacteriophages (‘phages’) can limit bacterial densities and shape community structure, either directly through lysis or indirectly through costs to resistance. However, phages have also been reported to have no, and in some cases even positive, effects on host densities. Here, we investigate the mechanisms behind an increase in host density in *Variovorax* sp. populations following a fixation of resistance that was maintained after phage extinction. Our results demonstrate that the density increase was a genetic trait coinciding with resistance emergence. Growth curves showed that phage resistance shifted population growth curves such that density was higher in the death-phase. This density-increasing effect of resistance had important implications for community structure with phage resistant *Variovorax* decreasing the density of a conspecific. That resistance to lytic phage can increase host densities has implications for wider ecology and phage therapy where lytic phages are presumed to have negative effects on their hosts.

Introduction

Bacteriophages are hypothesised to have major impacts on structuring microbial communities, and in regulation of wider nutrient cycles (Winter *et al.* 2010; Breitbart 2012; Breitbart *et al.* 2018). Part of this assumption comes from the ubiquity of bacteriophages (Clokie *et al.* 2011). By lysing their hosts, phages can suppress fast-growing bacteria densities and give a relative advantage to slow-growing or rarer microbial taxa (Winter *et al.* 2010). Phages are shown to lyse algal blooms, resulting in crashes in algae abundances and nutrient release which benefits the surrounding community (Breitbart *et al.* 2018; Flynn *et al.* 2022; Liao *et al.* 2023). Furthermore, a common assumption of many models is that phage resistance is costly (Winter *et al.* 2010), which leads to suppression of dominant or previously-fast growing bacteria populations (Gandon *et al.* 2008; Winter *et al.* 2010).

However, the effects of phage resistance on host phenotypes, and the associated cost, is highly system specific. If evolving phage resistance typically involves modification of a receptor essential to host growth, resistance is likely to be associated with a growth rate cost (van Houte, Buckling and Westra 2016). For example, to become resistant to OMKO1, *Pseudomonas aeruginosa* mutates an efflux pump required for drug resistance (Chan *et al.* 2016). As a result, while antibiotics are present, resistance to OMKO1 imposes a cost to *P. aeruginosa* (Chan *et al.* 2016). Similarly, phage resistance involving mutations to flagella or pili are further shown to slow bacteria growth rates (Buckling *et al.* 2006; Li *et al.* 2022). However, there are numerous examples of phage resistance being non costly to growth including in *Pseudomonas syringae* (Koskella *et al.* 2012), *Synechococcus* (Lennon *et al.* 2007), and *P. aeruginosa* (Castledine *et al.* 2022).

Phage resistance may also indirectly increase host fitness (beyond the direct effect of resisting phage). For instance, increased biofilm production is a phage resistance mechanism which physically blocks phage access to bacteria cells (Salathé and Soyer 2008; Harper *et al.* 2014; Abedon 2023). Biofilm production has consequential fitness benefits in resisting wider environmental stress such as antibiotics (Crabbé *et al.* 2019), and can be advantageous in competition (Oliveira *et al.* 2015). Phages can also accelerate the molecular evolution of their hosts and select for hypermutator strains, therefore giving increased adaptive potential to secondary stressors (Pal *et al.* 2007; Jayaraman 2011). *P. fluorescens* phage has been shown to increase host densities in soil, although only when grown in monoculture (in the absence of a soil community) (Gómez and Buckling 2011). Here, phages are hypothesised to have increased bacterial densities by reducing intraspecific competition through slowing of growth rates or facilitating diversification into separate ecotypes (Gómez and Buckling 2011), although the exact mechanism is unknown.

Understanding how phages can increase bacteria densities is important for phage therapy where increasing bacteria densities would be undesirable (Abedon 2023). Furthermore, investigating this effect has important implications for our understanding of how phages shape microbial communities where, in nature, phages are presumed to have predominantly negative effects their host densities (1, 3). In a previous study, we noted that densities of *Variovorax* sp. increased following phage exposure and fixation of phage resistance (Castledine *et al.* 2024a) even when phage had been driven extinct. Here, we investigate the mechanism underpinning phage-mediated increases in *Variovorax* densities, and the consequence this has for the composition

of a three species synthetic bacteria community. We show that phage resistance is associated with density increases resulting from a shift in growth curves that led to higher densities in the death-phase. Phage resistance had ramifications for community structure with decreases in the proportion of another community member which is parasitised by *Variovorax*.

Materials and Methods

Experimental evolution

In a previous study, species *Ochrobactrum* sp., *Pseudomonas* sp., and *Variovorax* sp. were grown as monocultures and as a three-species polyculture with and without their respective phage (*Ochrobactrum* phage ORM_20, *Pseudomonas* phage CH7FMC, *Variovorax* phage VAC_51) (Castledine *et al.* 2024a). Experimental treatments included: monoculture, monoculture with phage (each bacterial species individually with only its species-specific bacteriophage), polyculture (all three bacteria present) and polyculture with phage (all three bacteria with all three phages present) (Figure 1). Each treatment was replicated six times. Experimental set-up is described in (Castledine *et al.* 2024a). Briefly, cultures were established using isogenic strains of each bacteria and phage, with phage added at an MOI of 0.01 (to phage present treatments). Cultures were grown in 1/64 TSB (tryptic soy broth diluted in demineralised H_2O), at 28 °C and transferred weekly to fresh medium. Bacteria and phage densities were estimated every two weeks. For *Variovorax* cultures, we estimated phage resistance in week two and eight monocultures (phage present and absent). Phage resistance was analysed using spot assays with ancestral phage.

Clonal density measures

We first wanted to assess whether higher densities conferred to phage-exposed populations was a heritable trait observable in single clones picked and regrown in the absence of phage. From week two and four cultures (the weeks preceding and following higher densities), twelve colonies were isolated from each replicate from monoculture treatments. Colonies were individually grown in 1/64 TSB and density normalised as before. To fresh 6 mL 1/64 TSB, ~10^6 CFUs of each clone were added

129 to individual vials. Cultures were grown for 1 week, frozen, and plated from frozen onto
130 KB agar as previous.
131
132 **Clonal and population sequencing and bioinformatics**
133 To gain insight into the mechanism of resistance and whether this could be linked to
134 changes in bacterial density and growth rate, isolates were sent for whole genome
135 sequencing (WGS). Three phage resistant and three phage susceptible clones from
136 three independent week two polyculture lines were selected for WGS. Polyculture
137 lines were selected as these were the only treatment which had phage susceptible
138 and resistant clones coexisting (100% resistance was found in monoculture lines).
139 This allowed for direct comparison between different clones that have evolved from
140 the same starting population but only differed in their resistance profile. DNA
141 extractions were done on each isolate by MicrobesNG using in-house protocols
142 (MicrobesNG Sequencing Service Methods, 2021). WGS was performed by
143 MicrobesNG using an Illumina Novaseq 6000 to create 250 base pair (bp) paired-end
144 reads. In-house processing by MicrobesNG trimmed the adapters and removed reads
145 with a quality <15.
146
147 To further understand genetic changes that resulted in increased growth rates of
148 phage resistant *Variovorax*, cultures from week 7 monoculture lines of evolution were
149 sent for population sequencing. Overnight cultures (120 µL frozen stock in 1/64 TSB
150 grown shaking at 28 ºC. 1.8 mL was spun at 16000 x g for 5 mins (Progen GenFuge
151 24D centrifuge). The supernatant was removed and another 1.8 mL of fresh sample
152 was added to each tube and centrifuged for a further 5 mins and supernatant removed.
153 The Qiagen DNeasy Ultra Clean DNA Extraction Kit reagents and protocol were used

154 to extract the gDNA from the cultures. The concentration of gDNA in each sample was
155 determined using the QuBit dsDNA HS Assay kit regents and protocol. Sequencing
156 was done by the University of Liverpool's Centre for Genomic Research (CGR) using
157 an Illumina Novaseq to create 150bp paired end reads. The CGR trimmed for the
158 presence of Illumina adapter sequences using Cutadapt (v1.2.1) and removed reads
159 with a minimum quality score of 20 and which were shorter 15bp in length.
160
161 For both clonal and population sequencing, we further filtered the trimmed reads to
162 remove Illumina adapter sequences and for reads with a quality score below 10 using
163 TrimGalore (v0.6.4_dev). We used the breseq pipeline (v0.36) – a computational tool
164 for analysing short-read DNA data - to map the trimmed reads to a high quality
165 reference genome of *Variovorax* sp. (GenBank accession: GCA_037482385.1) and
166 predict genetic variants (Deatherage and Barrick 2014). As our reference genome has
167 two contigs, we added the parameters "-c" and "—contig-reference" so that all the
168 sequences are assumed to have the same average coverage and coverage
169 distribution. In the clonal sequencing, 97.6% of reads mapped to the reference
170 genome (minimum of 95.4%), giving on average ~876,989 reads per replicate
171 (minimum 444,292) with an average coverage of 62.21 (minimum of 31.84). We only
172 investigated genetic variants that were at a frequency of 1 (we do not expect
173 polymorphisms in any of the predicted genetic variants). For the population
174 sequencing, we ran breseq in polymorphism mode (-p). Variants with <5% frequency
175 in the population samples were removed.
176
177 **Phage resistance and competition assay**

178 We determined if phage resistance conferred a growth rate benefit to *Variovorax*,
179 thereby leading to higher bacterial densities. Twelve clones per replicate from week
180 two monoculture-lines were isolated and grown in 96-well plates with 150 µL TSB.
181 Phage resistance was determined by streaking isolated colonies against the ancestral
182 phage (streak assay). Week two isolates were chosen as this time-point preceded
183 changes in bacterial density from phage exposure and phage extinction. We elected
184 for phage resistant clones generated during experimental evolution (instead of
185 selecting spontaneous mutants) in the hope that resistance mutations/mechanisms
186 (and therefore phenotypic effects) were consistent with those from the evolution
187 experiments. One phage resistant clone from the phage resistance assays was
188 chosen for each replicate (n = 6), and one clone from each no-phage treatment
189 replicate was isolated as a phage susceptible control (n = 6). Each colony was grown
190 individually in 1/64 TSB individually as described previously. In addition, marked
191 (ancestor-cp; chloramphenicol resistance (Sünderhauf *et al.* 2023)) and unmarked
192 ancestral *Variovorax* sp. were grown. Cultures were diluted to normalise densities as
193 described previously. The unmarked ancestral strain and each phage resistant and
194 susceptible colony was competed against the marked ancestral strain at equal starting
195 densities (~3 x 10^6 CFUs of each competitor inoculated (30 µL diluted culture)). Diluted
196 cultures used in inoculation were frozen and plated on KB agar as above for starting
197 density estimation. Cultures were grown for one week as previous and plated from
198 frozen onto KB and KB-chloramphenicol (25 µg/mL) agar. Relative fitness was
199 calculated from the ratio of the estimated Malthusian parameters, $m_{resistant}$, $m_{susceptible}$
200 or $m_{ancestor}$:$m_{ancestor\text{-}cp}$, which were calculated as $m = \ln(N_1/N_0)$, where N_1 is the final
201 density and N_0 is the starting density (Lenski *et al.* 1991).
202

203 **Growth curves**

204 Next, we were interested if phage resistance had shifted the growth curve of
205 *Variovorax* populations thereby leading to higher densities at transfer. 120 µL from
206 each monoculture line from week seven were revived in 6 mL growth medium and
207 grown shaking for two days at 28 ºC. Densities were normalised as previous and 10
208 µL was inoculated into fresh growth medium. Every 24 hrs for one week, cultures were
209 vortexed and optical density (OD_{600}; Multiskan Sky) was measured from a 200 µL
210 sample.

211

212 To characterise the log/exponential growth phase of phage resistant and susceptible
213 cultures at a higher resolution, week seven cultures were revived as previous and 20
214 µL of diluted culture (~10^6 CFUs) were inoculated into 180 µL growth medium. Optical
215 density reads were measured every 45 mins for 24 hrs.

216

217 **Supernatant assay**

218 We hypothesised that higher densities and shifts in growth curve may be caused by
219 production of less-toxic metabolites. This hypothesis was tested using a supernatant
220 assay. Week seven cultures were revived and normalised as previous using five
221 replicates of resistant and susceptible populations (one replicate each of susceptible
222 and resistant cultures became contaminated and were not used in the experiment).
223 Two replicates of each week seven culture (two technical replicates for each biological
224 replicate) were established to ensure enough supernatant was available for the
225 reciprocal transplant assay. Cultures were grown for one week. On day five, fresh
226 week seven cultures were revived and grown for two days. Week-old cultures were
227 sterilised by passing medium through a 0.22 µm filter syringe. Replicate supernatants

228 from phage resistant and susceptible populations were pooled into separate
229 supernatant stocks. To separate vials, 3 mL of phage resistant and susceptible
230 supernatant was added to 25 mL vials. Two-day (fresh) culture densities were
231 normalised and each culture was inoculated into both supernatant types. This
232 established two controls in which cultures were grown in the same supernatant (e.g.
233 phage resistant in phage resistant supernatant) and treatments in which cultures were
234 transplanted into the opposite supernatant (e.g. phage resistant in phage susceptible
235 supernatant and vice versa). Cultures were grown for two days and plated as previous.
236

237 **Phage resistance and community structure**

238 Next we assayed whether resistance to phage gives *Variovorax* an advantage over
239 *Ochrobactrum* and *Pseudomonas* when phages are absent. Six independent phage
240 resistant clones and six independent phage susceptible clones were isolated from
241 phage and no phage week two monoculture evolution lines respectively. 'No phage'
242 lines were selected for isolation of phage susceptible clones as phage resistance was
243 too high in 'phage' lines for enough individual susceptible clones to be isolated.
244 Comparison between 'no phage' and 'phage' lines accounts for any change in general
245 laboratory adaptation. Each clone and ancestral *Variovorax* were individually grown
246 for two days at 28 °C, shaking (180 r.p.m.) in 6 mL 1/64 TSB. Ancestral *Pseudomonas*
247 and *Ochrobactrum* isolates were also grown in the same conditions. Isolate densities
248 were normalised to 10^5 CFUs/mL and 10 µL added to relevant vials. Each *Variovorax*
249 clone (ancestral, resistant, susceptible) was grown as a polyculture with
250 *Pseudomonas* and *Ochrobactrum*, with six replicates per treatment. Cultures were
251 grown static for 1 week at 28 °C, then frozen as previous and plated from frozen.
252

253 **Statistical analyses**

254 All data were analysed using R (v.4.2.1) in RStudio (Team 2013) and all plots were
255 made using the package '*ggplot2*' (Wickham 2016). Model simplification was
256 conducted using likelihood ratio tests and Tukey's post hoc multiple comparison tests
257 were done using the R package '*emmeans*' (Lenth 2018).

258

259 *Variovorax* densities were analysed in a linear mixed effects model with density (log10
260 CFU/mL) tested against interacting fixed effects of phage exposure, culture type
261 (polyculture or monoculture) and time (weeks two, four, six, eight) with a random effect
262 of treatment replicate. Clonal densities (log10 CFU/mL) from clonal growth
263 experiments were analysed against interacting fixed effects of phage exposure and
264 time (week two or four) with random effects of treatment replicate and block
265 (experiment conducted in two blocks for feasibility). In competition assays, relative
266 fitness was analysed in a linear model with a fixed effect of competitor (ancestor,
267 phage resistant or susceptible).

268

269 One week growth curves were analysed in a mixed effects model with optical density
270 tested against a fixed term of day and interacting fixed effects of treatment (phage
271 resistant / susceptible) and a quadratic term of day to account for the non-linear
272 relationship. A random effect of treatment replicate was included. 24-hour assays of
273 bacterial growth were used to estimate exponential growth rate. One replicate was
274 removed from the no-phage treatment for failing to grow. Per replicate, exponential
275 growth was estimated using rolling regression, taking the steepest slope of the linear
276 regression between OD_{600} and time in hours in a shifting window of every nine time
277 points (~6.75 hr). A linear model tested growth rate against treatment.

278

279 The supernatant assay was analysed in a linear mixed effects model with density
280 (log10 CFU/mL) analysed against interacting fixed effects of supernatant type and
281 original treatment with a random effect of treatment replicate.

282

283 To test whether consistent genetic differences occur within treatments, we performed
284 nonmetric multidimensional scaling on the Euclidean distance matrix of SNPs/indels
285 and their proportions in each population using '*metaMDS*' in the R package '*vegan*'
286 (Oksanen *et al.* 2019). Nonmetric multidimensional scaling aims to collapse
287 information from multiple dimensions (i.e., from different populations and multiple
288 SNPs/indels per population) into just a few, allowing differences between samples to
289 be visualized and interpreted. Permutational ANOVA tests were run using
290 '*vegan::adonis*', with Euclidean distance as the response term and treatment as the
291 predictor variable. Additionally, we analysed whether evolution with phage affected
292 the genetic difference of each population (phage, no phage) from the ancestral
293 population. The genetic distance from the ancestral population was calculated as the
294 sum of the difference of the proportion of each SNP/indel in each population from the
295 ancestral proportion. Genetic distance was then analysed in a linear model analysing
296 distance against phage presence/absence.

297

298 To assess whether *Variovorax* phage resistance influences community structure in the
299 absence of phage, we analysed the relative proportion of each species in a
300 generalised linear mixed effects model. Here, each species proportion was analysed
301 against interacting fixed effects of *Variovorax* phage resistance and species identity,
302 with a random effect of treatment replicate and a binomial error structure.

Results

Phage resistant clones have higher densities than phage susceptible clones

In a previous study (Castledine *et al.* 2024a), we evolved *Variovorax* and its lytic phage in the presence and absence of two other bacteria-phage pairs (Figure 1). We observed an interesting interaction with *Variovorax* densities increasing after two weeks of phage exposure (phage present: $\bar{x}$ = $10^{7.79}$ CFU/mL, 95%CI = $10^{7.74}$ - $10^{7.85}$; phage absent: $\bar{x}$ = $10^{7.69}$ CFU/mL, 95%CI = $10^{7.63}$ - $10^{7.74}$; ANOVA comparing models with and without phage: χ_1^2= 7.21, p = 0.007; Tukey HSD: p = 0.013). This coincided with a fixation of phage resistance in polyculture (90.3%; SE ±6.6) and monoculture (100%), and extinction of phage after two weeks. As such, we investigated the hypothesis that phage resistance has led to increased *Variovorax* densities.

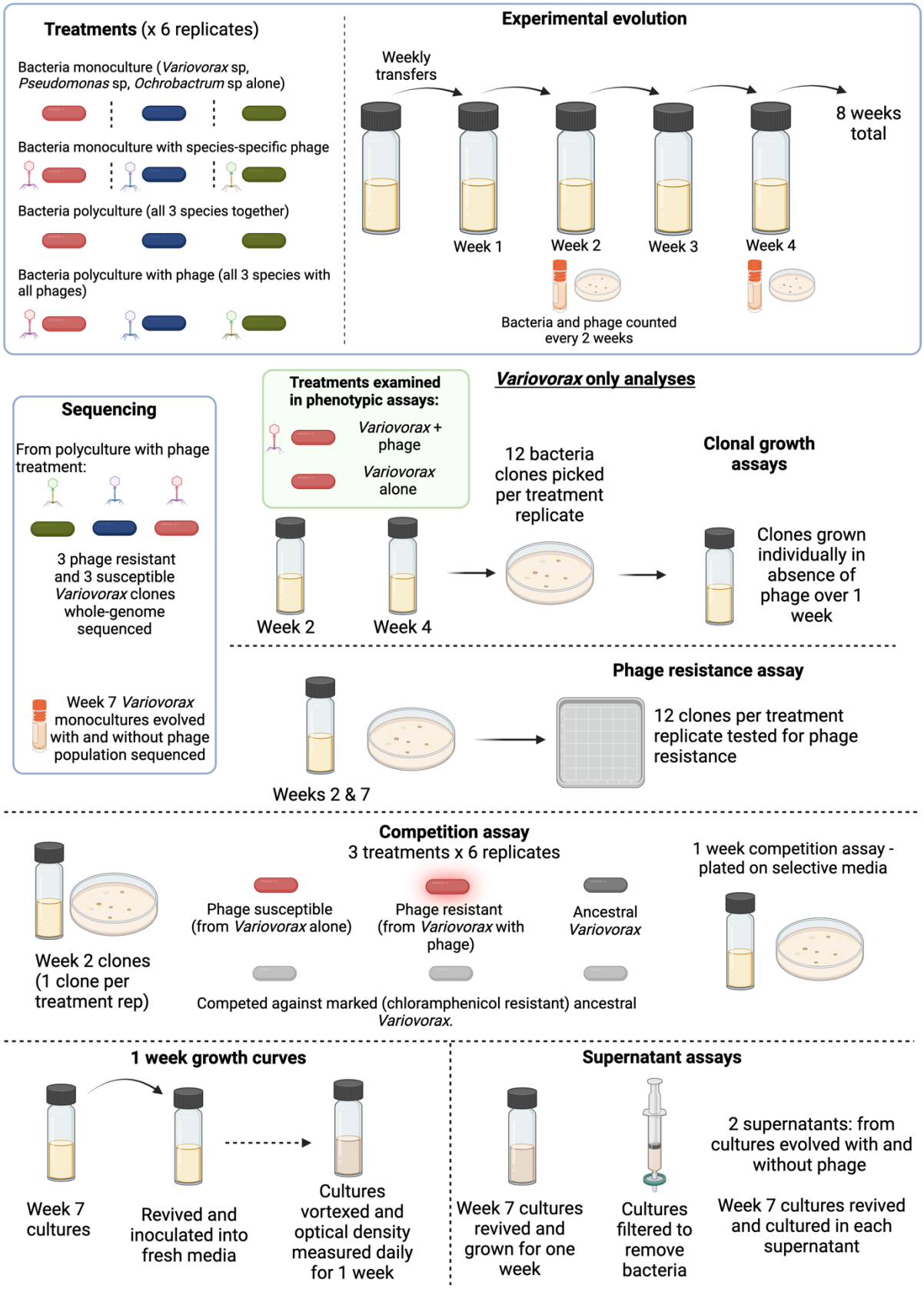
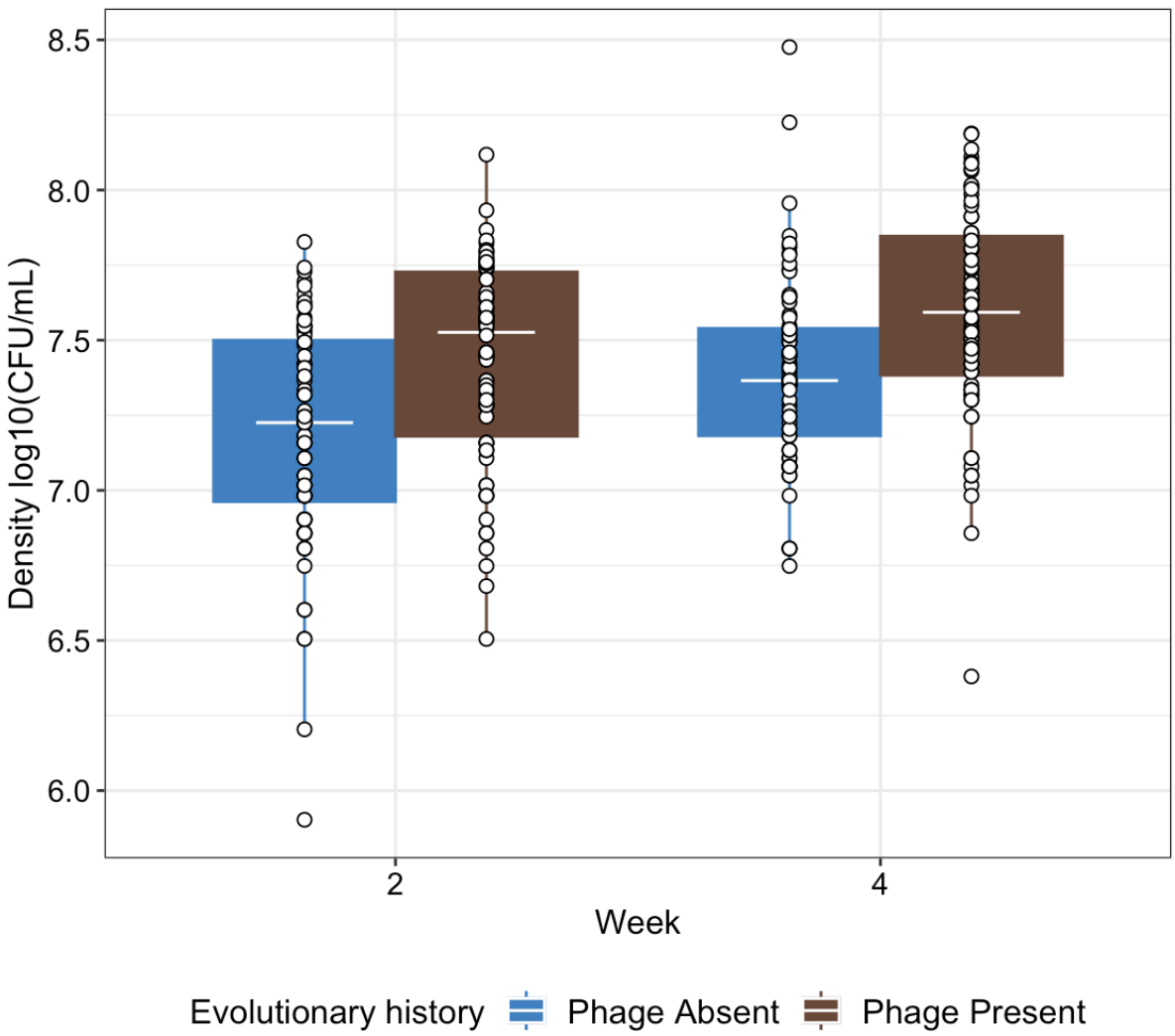


Figure 1. Overview of experimental design in which *Variovorax* and its phage evolved in monoculture and polyculture, and assays performed to understand

318 **the mechanism of this density increase.** Sequencing analyses and phenotypic
319 assays aimed to determine the mechanism by which phages led to increased
320 *Variovorax* densities across experimental evolution. Created in BioRender.
321 Castledine, M. (2024)
322 BioRender.com/c73o264.
323
324 To assess whether higher densities in phage-exposed populations was likely a result
325 of a genetic change (as opposed to epigenetic or physiological), we measured the
326 densities of individual clones (isolated from monoculture treatment on weeks two (pre
327 phage loss) and four (post phage loss)) grown in isolation (no phage or other clones
328 present so any density effects are purely heritable). All clones isolated from phage
329 treatments were phage resistant as phage resistance reached fixation in monoculture,
330 while all clones from the no-phage treatment were susceptible. Consistent with the
331 population dynamics during experimental evolution, clones isolated from phage-
332 exposed replicates had 66% higher densities ($\bar{x}$ = $10^{7.51}$ CFU/mL, 95%CI = $10^{5.60}$ -
333 $10^{9.41}$) than phage-unexposed clones ($\bar{x}$ = $10^{7.29}$ CFU/mL, 95%CI = $10^{5.38}$ - $10^{9.19}$;
334 ANOVA comparing models with and without phage: χ^2_1= 6.60, p = 0.0102; Tukey HSD:
335 p = 0.018; Figure 2). Additionally, there was a significant independent effect of time
336 with clones from week four ($\bar{x}$ = $10^{7.49}$ CFU/mL, 95%CI = $10^{5.30}$ - $10^{9.68}$) reaching higher
337 densities than clones from week two ($\bar{x}$ = $10^{7.31}$ CFU/mL, 95%CI = $10^{5.12}$ - $10^{9.49}$; Tukey
338 HSD: p < 0.001; Figure 2). Overall, these results suggest effects imposed by phage
339 were hereditary as phage did not need to be actively present for effects to be
340 observed.
341



342

343 **Figure 2. The density of individual clones (points) which have been grown after**
344 **being isolated from evolution lines where phage have been absent or present.**
345 Tops and bottoms of the bars represent the 75th and 25th percentiles of the data, the
346 middle lines are the medians, and the whiskers extend from their respective hinge to
347 the smallest or largest value no further than 1.5* interquartile range. Points represent
348 individual clones.

349

350 **Phage resistant populations have distinct mutations**

351 Given the consistent higher growth rate that phage exposed populations and phage
352 resistant clones had over phage absent populations and phage susceptible clones,

353 we looked at whether there were underlying genetic changes that could explain this.
354 We sequenced 3 resistant and 3 sensitive clones coexisting within 3 polyculture
355 treatment replicates, allowing for a direct comparison between different clones that
356 evolved from the same starting population but differed in their resistance profile. Of
357 the phage resistant clones, 2/3 clones had a missense mutation at the same locus
358 (gene ID: 01575) and 1 had a substitute mutation at a nearby locus (gene ID: 01584;
359 10.4k base pairs downstream; this was also the only mutation in this clone) that were
360 not found in phage susceptible isolates. No other mutations were found at the same
361 loci across clones. Single point mutations are consistent with surface modification
362 forms of resistance (Beckett and Williams 2013; van Houte, Buckling and Westra
363 2016). Generally, few mutations were found, with one resistant clone having four
364 mutations while the other two had only one mutation each. Similarly, one phage
365 susceptible clone had two mutations while the other two had one mutation. 01584
366 sequence (mutation found in 1/3 resistant clones) had no comparable genes in
367 BLAST, however its proximity to the other genes could imply a similar function
368 (Demerec and Hartman 1959; Ballouz *et al.* 2010).
369
370 We conducted pool sequencing analysis of seven weeks of evolution in monoculture
371 (*Pseudomonas* and *Ochrobactrum* absent), with the aim of identifying further genetic
372 changes linked to phage resistance and increased density. 5/6 phage resistant
373 populations had at least one genetic variant at high frequency (>50%) that was not
374 present in the phage susceptible populations, compared to only 2/6 of the phage
375 susceptible populations, suggesting specific mutations were selected by phage
376 (Figure S1). However, a lack of convergence suggests phage resistance may be
377 maintained by several different mechanisms that have the same phenotypic effects

(resistance and higher population density) (see Supplementary Results for full analysis).

Phage resistant clones do not have a fitness advantage

We next determined whether phage resistance conferred a fitness advantage in the absence of phage. In competition assays with a marked ancestral strain, relative fitness was not significantly affected by phage resistance ($F_{2,15} = 0.460$, $p = 0.640$; Figure 3). The greater density associated with resistance did not therefore result in a competitive fitness advantage.

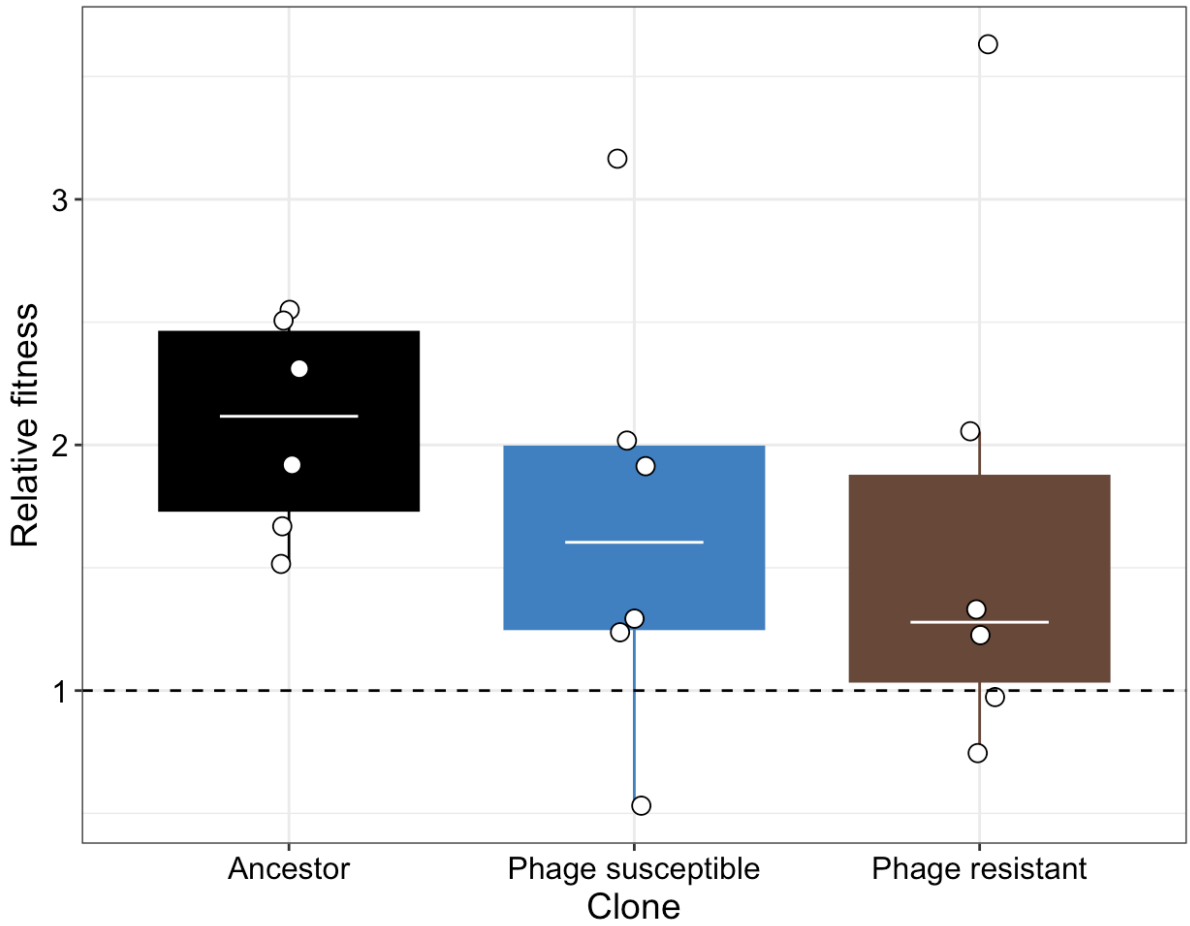


Figure 3. Relative fitness of ancestral, phage susceptible and resistant isolates against a marked ancestral strain of *Variovorax*. Points represent independent

replicates. Tops and bottoms of the bars represent the 75th and 25th percentiles of the data, the middle lines are the medians, and the whiskers extend from their respective hinge to the smallest or largest value no further than 1.5* interquartile range.

Phage resistant populations have greater persistence during the death-phase

We next investigated if density benefits resulting from resistance arose from changes in growth dynamics. This may have arisen if, for example, logistic growth rates of resistant isolates were slowed, resulting in resources becoming less exhausted when cultures were transferred to fresh media on day seven. As such, we measured the growth curves of *Variovorax* populations isolated from week seven of experimental evolution over one week. Here, there was a significant interaction between treatment and day of measurement (ANOVA comparing models with and without the interaction: χ_1^2 = 7.25, p = 0.007), suggesting phage resistance had altered population growth dynamics (Figure 4). Consistent with our previous observation based on CFU counts (Figure 2), optical density after one week was higher in phage resistant ($\bar{x}$ = 0.067, 95%CI = 0.062 - 0.072) than susceptible ($\bar{x}$ = 0.057, 95%CI = 0.053 - 0.062) populations (Figure 4). Consistent with an absence of fitness differences between susceptible and resistant isolates (Figure 3), there was no significant difference in exponential growth rate measured in the 24 hr growth rate assay ($F_{1,9}$ = 0.622, p = 0.451) or carrying capacity in the one week growth rate assay (phage resistant: $\bar{x}$ = 0.08, 95%CI = 0.075 – 0.085; phage susceptible: $\bar{x}$ = 0.076, 95%CI = 0.072 – 0.081; Figure 5). Phage resistant isolates show similar growth dynamics to susceptible isolates, except they maintain higher densities during the death phase at day 7.

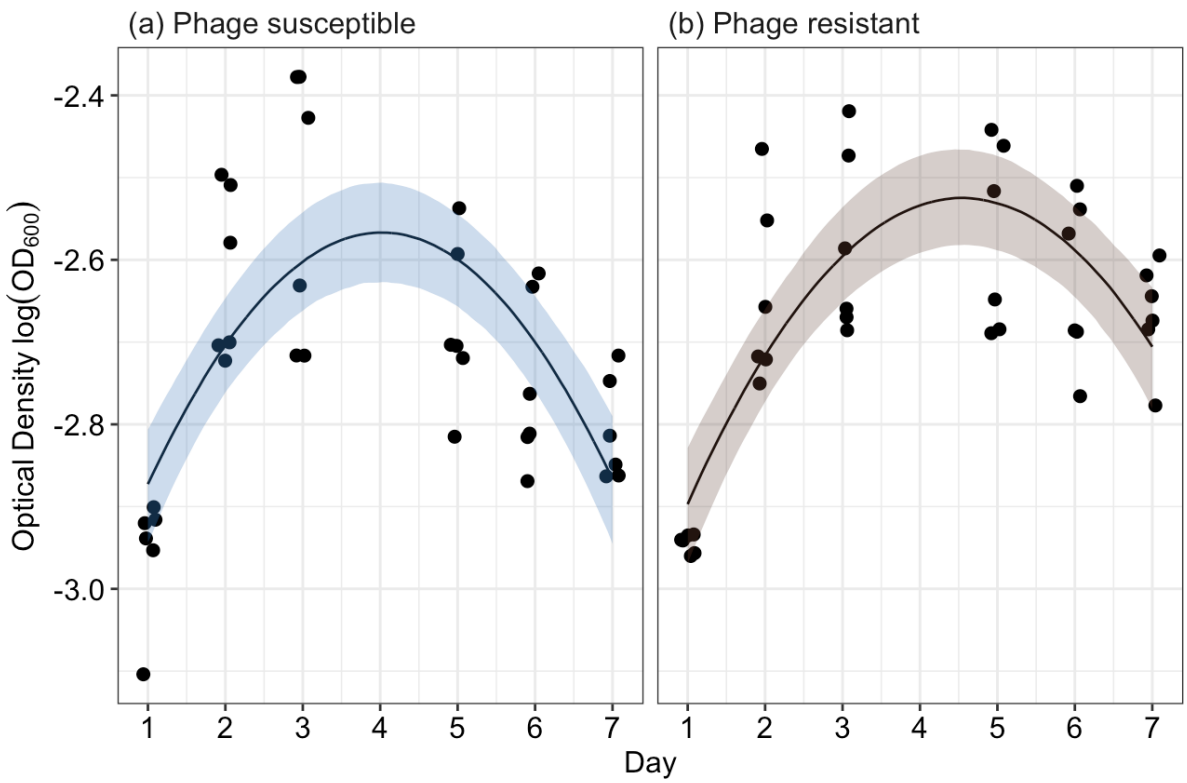


Figure 4. The seven-day growth curves of (a) phage susceptible and (b) phage resistant *Variovorax* populations that had evolved for seven weeks. Points represent individual replicate populations measured repeatedly through time. Solid lines represent the mean predictions, and shaded bands represent the 95% confidence intervals of the best fitted model.

The supernatant of phage resistant populations is not less toxic

If bacterial populations can maintain a higher density during the stationary/death phase, this suggests that bacteria are potentially producing fewer toxic metabolites and/or are less susceptible to them. We investigated these possibilities by growing week seven populations in spent supernatants from either their own treatment or the opposite treatment. However, population density was non-significantly different between populations irrespective of prior phage exposure (χ_1^2 = 1.48, p = 0.224) or

supernatant type (χ_1^2 = 2.12, p = 0.145; interaction between effects: χ_1^2 = 0.148, p = 0.7004; Figure 5).

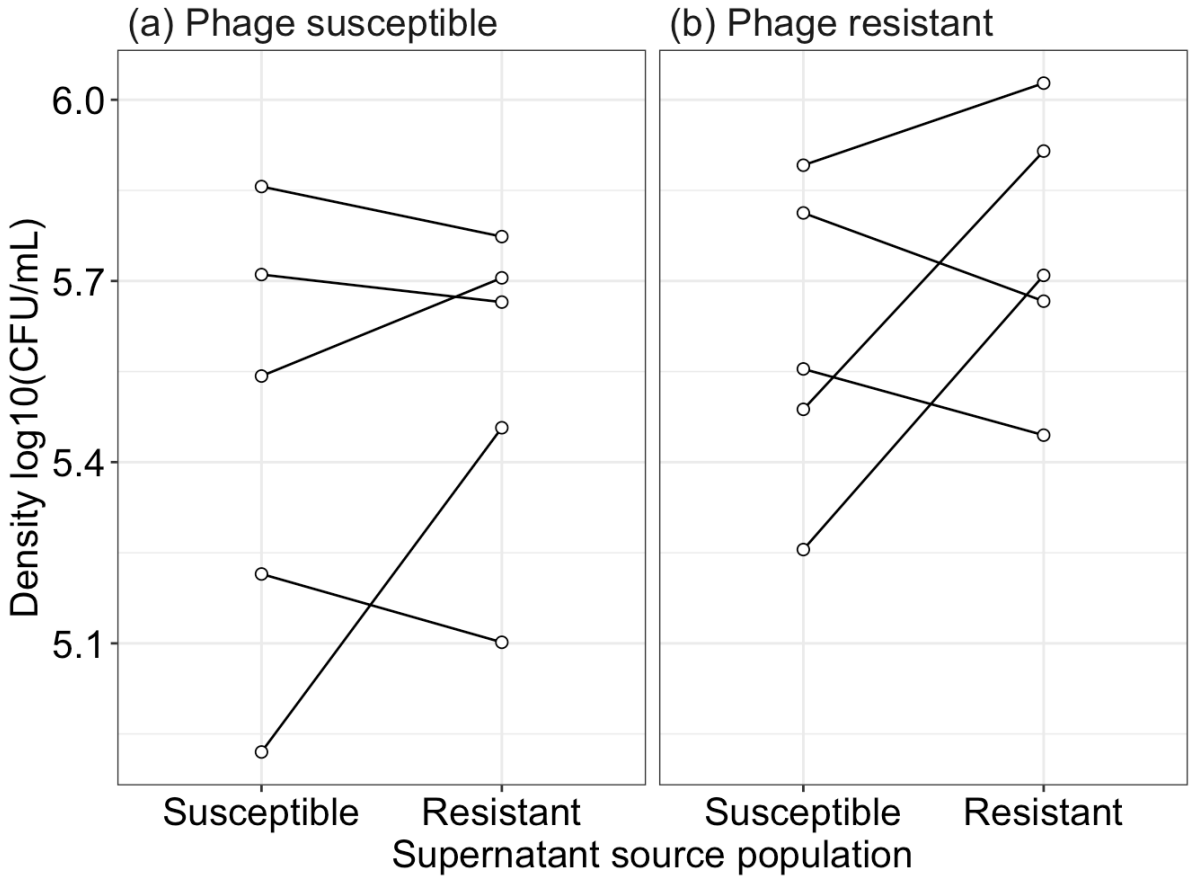


Figure 5. Density changes when populations which are (a) phage susceptible and (b) phage resistant, are grown in supernatant isolated from either population. E.g. phage susceptible growing in supernatant from phage susceptible populations (same evolutionary history) or phage susceptible growing in supernatant from phage resistant populations (different evolutionary history). Points represent densities of individual replicates while lines indicate the change when that same population is grown in a different supernatant.

Indirect benefit of phage resistance impacts community structure

442 Considering that phage resistance increased *Variovorax* densities, we considered
443 whether this had implications for the structure of a simple microbial community. We
444 had previously cultured *Variovorax* with two other species, *Ochrobactrum* and
445 *Pseudomonas*, from which it derives a fitness benefit to the other species' detriment
446 (Castledine *et al.* 2024b). As such, we predicted that phage resistance increasing
447 *Variovorax*'s density may have negative implications for the other two species. To this
448 end, we re-assembled communities with different *Variovorax* isolates with ancestral
449 *Ochrobactrum* and *Pseudomonas*. Consistent with this prediction, the relative
450 proportion of *Pseudomonas* in the community was significantly lower with phage
451 resistant *Variovorax* ($\bar{x}$ = 0.092, 95%CI = 0.08 – 0.11) compared to ancestral ($\bar{x}$ =
452 0.143, 95%CI = 0.128 – 0.158; Tukey HSD comparing *Pseudomonas* proportion with
453 ancestral vs resistant *Variovorax*: estimate = 0.495, z-ratio = 5.02, $p < 0.001$) or phage
454 susceptible *Variovorax* ($\bar{x}$ = 0.128, 95%CI = 0.114 – 0.143; ANOVA comparing models
455 with and without interaction between *Variovorax* phage resistance and species
456 identity: χ^2_4 = 34.1, $p < 0.001$; Tukey HSD comparing *Pseudomonas* proportion with
457 resistant vs susceptible *Variovorax*: estimate = -0.367, z-ratio = -3.64, $p < 0.001$;
458 Figure 6). *Pseudomonas*'s relative proportion was non-significantly different when
459 cultured with ancestral or phage susceptible *Variovorax* (Tukey HSD: estimate =
460 0.129, z-ratio = 1.403, p = 0.339). Neither *Ochrobactrum*'s or *Variovorax*'s relative
461 proportion was significantly affected by phage resistance in *Variovorax* (Tukey HSDs
462 > 0.05, Table S1; Figure 6).

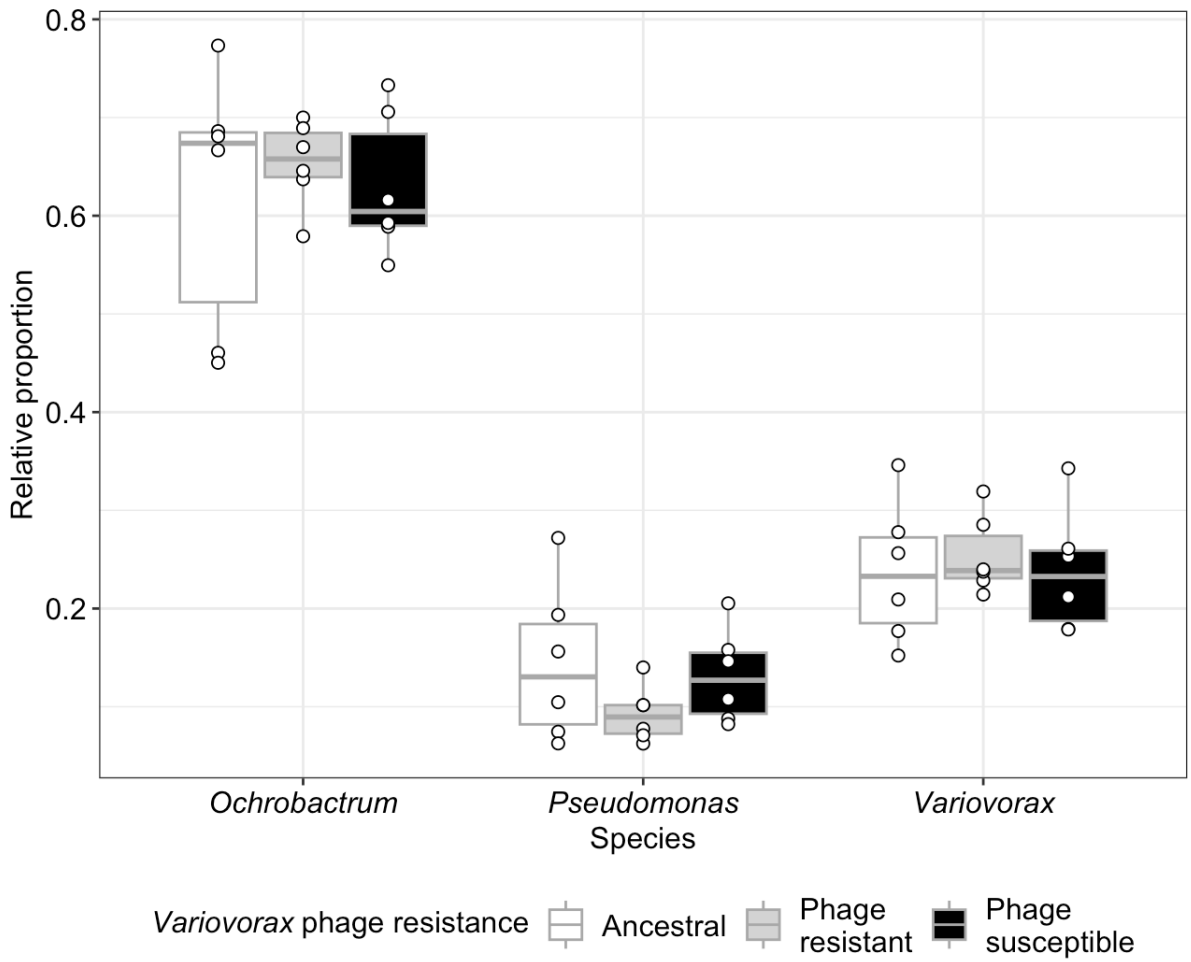


Figure 6. The relative proportion of each species in replicate communities when cultured with ancestral, phage resistant or phage susceptible *Variovorax* isolates. Points represent individual treatment replicates. Tops and bottoms of the bars represent the 75th and 25th percentiles of the data, white lines indicate the medians, and whiskers extend from their respective hinge to the smallest or largest value no further than 1.5× the interquartile range.

Discussion

Bacteriophages have been previously shown to positively influence bacteria densities, but only when propagated in monoculture and the mechanism was not determined (Gómez and Buckling 2011). Here, we showed that evolved resistance to phage

475 changed the growth curves of *Variovorax* populations resulting in higher densities
476 during the death phase. High levels of resistance, and the associated positive effect
477 on density, was observed for several weeks past phage extinction within monocultures
478 (no other bacterial species present) and in a community context. That this effect was
479 consistent in both the presence and absence of other species is particularly interesting
480 as the growth of this species is enhanced by the presence of the other two species
481 (Castledine, Padfield and Buckling 2020). As such, irrespective of the metabolic state
482 the bacteria are in (feeding on the media or on supernatant), the phage had a positive
483 effect on *Variovorax* density. This effect had consequences for community structure
484 with phage resistance in *Variovorax* suppressing *Pseudomonas* in communities.
485 These results demonstrate that phage resistance can give some species a relative
486 advantage against others in community contexts, contrary to the prevailing wisdom of
487 phage community ecology (Winter *et al.* 2010).
488
489 Phage resistance did not have a significant effect on exponential growth rate but did
490 affect the death phase, with phage resistant populations maintaining a higher density.
491 Although we did not find evidence that this was linked to a change in metabolites
492 produced after one week, it may reflect a broader ability of the bacteria being able to
493 cope in stressful conditions. *Variovorax* is a metabolically plastic species and is
494 capable of being lithoautotrophic or chemoorganotrophic, including the metabolism of
495 toxic or complex compounds (Satola, Wübbeler and Steinbüchel 2013). A lack of
496 convergent evolution meant we could not link genotype to phenotype in this study.
497 Clones had few genetic changes, but fitness benefits from phage-exposure were not
498 linked to a single phage resistance mutation, although 2/3 clones had a missense
499 mutation in the same protein. If this mutation and protein was the main mechanistic

500 way phage-resistance increased fitness, we would expect it to also be present in the
501 population sequencing, but no genetic changes in that locus were found. Instead,
502 population sequencing revealed multiple mutations emerging and several reached
503 high frequencies (>50%) in 5/6 phage treatment populations, although with a lack of
504 convergent evolution. All high frequency genetic variants were in hypothetical proteins,
505 making mechanistic inferences impossible within this project. Interestingly, only two
506 no-phage culture had a unique mutation that reached high frequency (>50%)
507 compared to 5/6 phage cultures, suggesting phage presence increased the likelihood
508 of variants reaching a high frequency. Different mutations can confer the same
509 phenotypic outcome, including phage resistance (Wright *et al.* 2018, 2019; Debray,
510 De Luna and Koskella 2022), which is likely to be the case in this study.
511
512 While resistance was clearly beneficial in the presence and absence of phage,
513 reaching and being maintained at very high frequencies, we did not find any phage
514 resistance emerge in phage-free controls across eight weeks. This was presumably
515 because there was no selective benefit of phage resistant mutants competing against
516 phage sensitive clones in the absence of phage, and hence resistant mutations would
517 not have increased in frequency. Nevertheless, we may expect this resistance-linked
518 growth phenotype to be favoured in highly structured metapopulations, where limited
519 dispersal can result in different patches seeded by single clones with the most
520 productive patches (i.e. those seeded with resistant clones) producing more
521 individuals to colonise new patches (Griffin, West and Buckling 2004). The resistance
522 mutations may also allow populations to subsequently explore alternative evolutionary
523 trajectories in the longer term (Salathé and Soyer 2008).
524

525 Importantly, phage resistance increasing *Variovorax* densities shifted community
526 structure, resulting in lower proportions of *Pseudomonas*. *Variovorax* has been shown
527 in previous work to benefit from the presence of *Pseudomonas* to *Pseudomonas*'
528 detriment (Castledine *et al.* 2024b). Increasing densities of phage resistant *Variovorax*
529 therefore likely resulted in increased costs experienced by *Pseudomonas* in coculture.
530 Phage lysis and phage resistance are typically assumed to be most costly to dominant
531 community members (Winter *et al.* 2010). However, many examples exist of non-
532 costly phage resistance and species dominating communities with high levels of
533 phage resistance (Zhao *et al.* 2013; Castledine and Buckling 2024). Consequently,
534 our results in-part support the king-of-the-mountain' hypothesis in which dominant
535 community members are more able to evolve resistance owing to greater population
536 sizes (Zhao *et al.* 2013; Giovannoni 2017) – albeit in this case likely by mutation than
537 horizontal-gene-transfer (starting population isogenic). Synonymously, the 'king-of-
538 the-mountain' hypothesis also relies on a low trade-off in defence and competition
539 (Thingstad *et al.* 2014), evident in *Variovorax*.
540
541 Few examples exist of phages improving bacteria densities (Gómez and Buckling
542 2011), suggesting this observation may not be ubiquitous. However, much research
543 has heavily focussed on model strains of bacteria and phage which are unlikely to be
544 generalisable to all bacteria and phages (Castledine and Buckling 2024; Castledine *et*
545 *al.* 2024a). Furthermore, different mechanisms of phage resistance have the potential
546 to improve bacteria densities in specific contexts. For example, phages can select for
547 increased biofilm production (Scanlan and Buckling 2012; Castledine *et al.* 2022) and
548 persister populations (Fernández-García *et al.* 2023) which can further confer
549 resistance to stressors such as antibiotics and immune cells (Crabbé *et al.* 2019; Niu,

550 Gu and Zhang 2024). If phages can improve the density of soil bacteria, such as by
551 driving host diversification (Brockhurst, Rainey and Buckling 2004), this may have
552 positive impacts on carbon cycling and crop yield (Dy, Rigano and Fineran 2018).
553 *Variovorax* species, for instance, are symbionts to plants and are found in the
554 rhizosphere where they can improve plant growth (Satola, Wübbeler and Steinbüchel
555 2013). Alternatively, if phages are used therapeutically (Oechslin 2018; Abedon 2019)
556 then density enhancement of target bacterial pathogens is clearly detrimental. Work
557 considering wider bacteria and phage pairs, beyond model systems, will allow greater
558 generalisations to be made and understanding of how phages can affect their hosts in
559 the short and long-term.
560
561 The influence of phages on bacterial populations are highly diverse, making
562 predictions and generalisations challenging from model systems to phages in nature.
563 Our work highlights the surprising outcomes that can arise from bacteria-phage
564 interactions, and that the simple assumption that lytic phages negatively affect host
565 densities may not always be true. Wider research analysing the effects of different
566 phages on host bacteria, both during and after phage extinction would give important
567 insight into how phage therapy may affect pathogens during infection and how phages
568 operate in nature.
569

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679

680

681 **Supplementary Information**

682 **Supplementary results: pool sequencing**

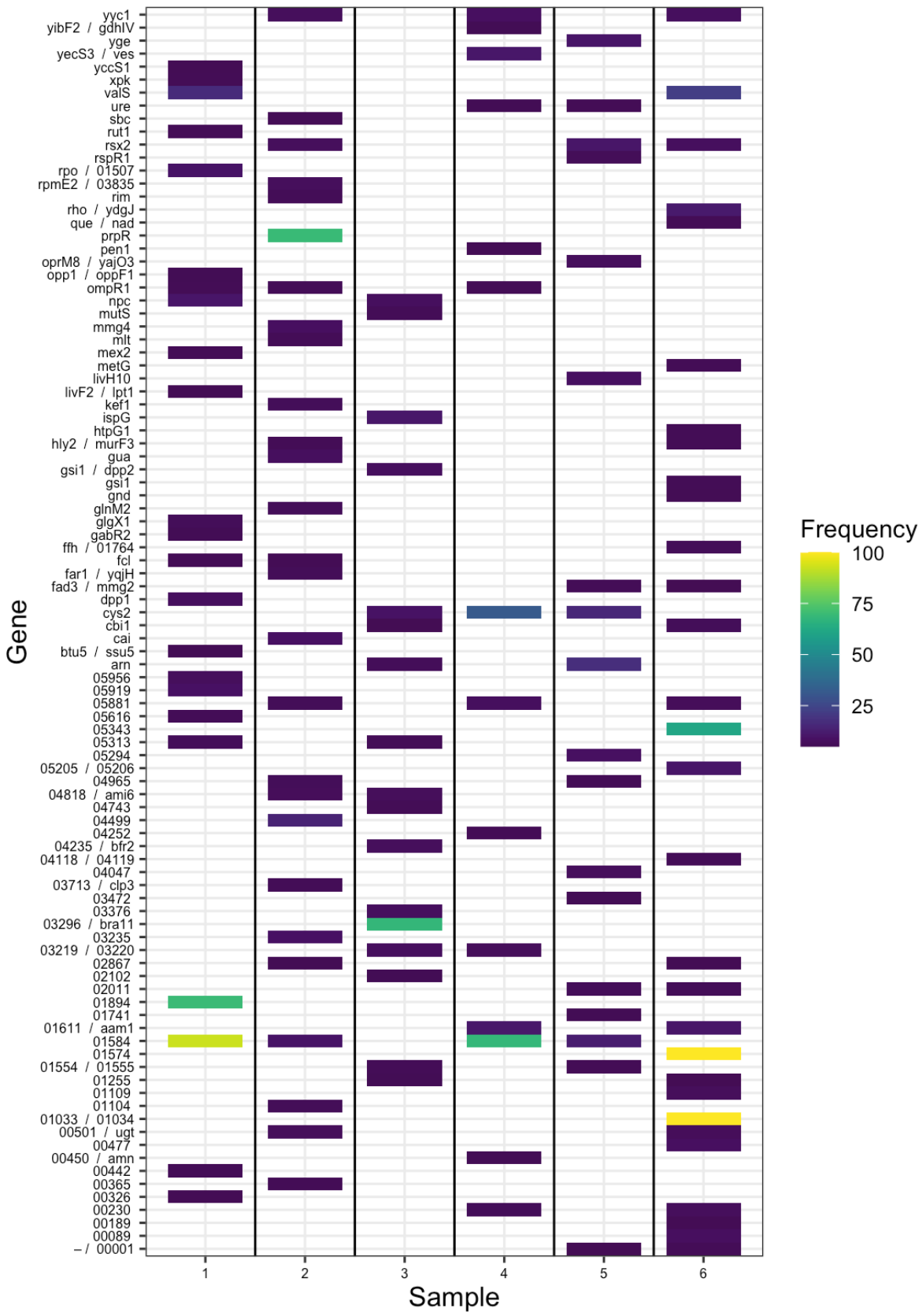
683 In total, we found 470 genetic variants, with 239 shared between resistant and
684 susceptible populations. Phage resistant populations had 117 unique variants (within
685 60 genes) while phage susceptible populations had 114 unique variants (within 64
686 genes). Most variants were found at low frequency (96.8% of variants at <50%
687 frequency) with resistant and susceptible populations having on average 147 variants.
688 5/6 phage resistant populations had at least one unique genetic variant at high
689 frequency (>50%) compared to only 2/6 of the phage susceptible populations,
690 suggesting specific mutations were selected by phage.

691

692 While we did not observe mutations in 01575 (mutation found in 2/3 resistant clones),
693 mutations were found in 01584 (mutation found in 1/3 resistant clones) in 4/6 phage
694 resistant populations (92.7%, 68.2%, 12.3%, 9.6%). Mutations selected to high
695 frequency were also found in *prpR* (69.3% in 1 population; transcription regulator
696 gene) and in five other hypothetical proteins or intergenic regions (Figure S1). The
697 lack of convergence suggests phage resistance may be maintained by several
698 different mechanisms that have the same phenotypic effects (resistance and higher
699 population density). As a result, we did not find phage presence significantly differed
700 in genetic composition (PERMANOVA: $F_{1,10} = 0.889$, $p = 0.81$, Figure S2) or distance
701 from the ancestral genome (ANOVA: $F_{1,10} = 0.547$, $p = 0.477$).

702

703

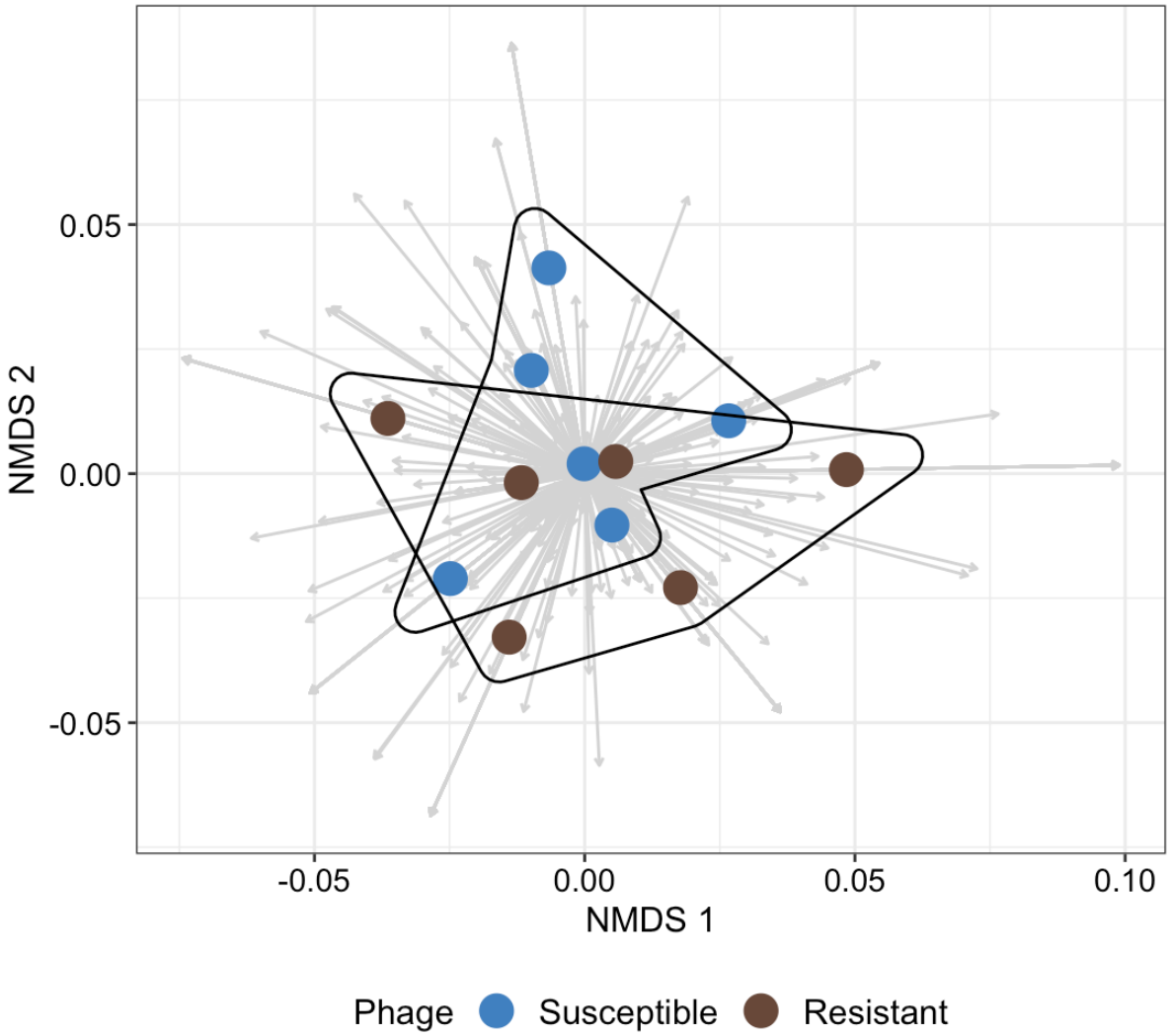


704

705 Figure S1. The frequency of genetic variants found in phage resistant but not phage

706 susceptible populations. Where multiple variants are found in the same gene, the

707 average has been presented. Each replicate population is identified on the x-axis.
708 Genes of known function are named on the y-axis while hypothetical proteins are given
709 a numerical denomination. Gene names separated by a forward slash indicate
710 mutations in intergenic regions. Samples indicate each of the six replicate populations
711 that evolved in the presence of phage.
712



713
714 Figure S2. The divergence of each treatment group as indicated by Euclidean
715 distances of each gene (arrows) from the ancestral genome. Points represent
716 individual replicates sequenced from phage resistant or susceptible treatment groups.
717

718 **Supplementary Table**

719 Table S1. Tukey HSD comparisons comparing the relative proportion of

720 *Ochrobactrum* (O), *Pseudomonas* (P) and *Variovorax* (V) when the *Variovorax* strain

721 is the ancestral type, phage resistant or susceptible. P-values are adjusted by the

722 tukey method for comparing a family of three estimates.

Species	Contrast	Estimate	z-ratio	p-value
O	Ancestral - Resistant	-0.146	-2.252	0.063
O	Ancestral - Susceptible	-0.049	-0.763	0.726
O	Resistant - Susceptible	0.096	1.483	0.299
P	Ancestral - Resistant	0.495	5.018	<0.001
P	Ancestral - Susceptible	0.129	1.403	0.339
P	Resistant - Susceptible	-0.367	-3.637	0.001
V	Ancestral - Resistant	-0.091	-1.257	0.42
V	Ancestral - Susceptible	-0.019	-0.259	0.964
V	Resistant - Susceptible	0.072	0.995	0.58

723
724