HIGHER VIRAL VIRULENCE SELECTS FOR FASTER EVOLUTION OF HOST RESISTANCE

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39 ABSTRACT

40 Infectious organisms can vary tremendously in their virulence. While the evolution
 41 of virulence and different levels thereof has received much attention over the past
 42 decades, the evolution of host resistance in response to different levels of virulence is
 43 far less understood and as of now mainly based on theoretical predictions. Increasing
 44 virulence can drive the evolution of host resistance if the costs of resistance are
 45 outweighed by the benefits of avoiding infection. Accordingly, we hypothesised that
 46 higher virulence will therefore select for faster resistance evolution, and ultimately
 47 shorter epidemics if parasites fail to overcome evolved host resistance. To test this
 48 hypothesis, we performed a bacteria-phage co-evolution experiment using two
 49 filamentous phages that differ in their virulence. Overall, we found two alternative
 50 defence strategies against filamentous phage infections: (1) immunity through super
 51 infection exclusion whereby the phage becomes part of the bacterial genome and
 52 protects its host against subsequent phage infections, and (2) resistance: surface-
 53 receptor modifications which prevent phages from entering the host cell. While
 54 immunity emerged rapidly and at a similar rate against both viruses, it did not persist
 55 long against the higher virulence virus, against which resistance evolved significantly
 56 faster compared to the lower virulence virus. The faster replacement of immunity by
 57 resistance in populations with higher virulence infections was driven by higher costs of
 58 being infected outweighing the fitness costs of resistance mutations. This resulted in a
 59 faster extinction of higher virulence viruses and implies that more virulent infections
 60 should cause shorter epidemics when viruses are unable to overcome evolved host
 61 resistance. Given that closely related viruses often vary substantially in their virulence,
 62 these findings suggest that resistance evolution will play a greater role in the
 63 epidemiological dynamics of higher virulence infections.

64

65 **Keywords:** virus, virulence, filamentous phages, experimental evolution, resistance

66 evolution

67 INTRODUCTION

68 Infectious organisms vary strikingly in their level of virulence and the resulting
69 selection they impose on their host. While some human viruses such as Ebola or rabies
70 can cause a deadly disease, others (including many cold viruses) often remain
71 asymptomatic. Even closely related viruses, such as different strains of myxoma
72 (Fenner and Marshall 1957) or corona viruses (Weiss and Leibowitz 2011), can differ
73 greatly in virulence. The evolution of different levels of virulence has been suggested to
74 result from a trade-off between parasite reproduction and transmission (Anderson and
75 May 1982, May and Anderson 1983). Higher reproduction results in higher virulence
76 but also in higher host morbidity and mortality and thus limited chances for
77 transmission. Accordingly, virulence may be expected to peak at intermediate levels,
78 maximising onward transmission. However, this pattern is not universal and selection
79 for virulence is expected to vary according to a wide variety of epidemiological
80 parameters (Alizon, Hurford et al. 2009).

81 The evolution of virulence has been studied extensively during the last two
82 decades, both using selection experiments (Bull, Molineux et al. 1991, Turner, Cooper
83 et al. 1998, Messenger, Molineux et al. 1999) and observations of parasites evolved in
84 nature (Herre 1993, Ebert 1994). What remains open, however, is the question of how
85 virulence will impact evolutionary trajectories of resistance in a host population, and
86 how these trajectories change with different levels of virulence. Severe disease
87 symptoms resulting from highly virulent infections will significantly reduce host fitness
88 imposing strong selection for hosts to quickly acquire resistance, for instance through
89 evolutionary changes. Thus, we expect benefits of host resistance relative to the costs of
90 disease symptoms to be higher against highly virulent compared to low virulent
91 infections. If this holds true, high virulence will lead to a stronger selection for and thus

92 faster evolution of host resistance. Ultimately this should lead to shorter epidemics if
93 parasites fail to overcome these evolved host resistances.

94 To explore how variation in virulence between closely related viruses influences
95 the dynamics of host resistance evolution, we designed a co-evolution experiment using
96 the model bacterium *Vibrio alginolyticus* K01M1 as a host and two closely related
97 versions of the filamentous *Vibrio* phage VALGΦ8, that differ in their replication rate
98 and hence in their virulence (Table 1)(Chibani, Hertel et al. 2020). Filamentous phages
99 (family *Inoviridae*), i.e., long, thin proteinaceous filaments which contain a circular
100 single-stranded DNA genome have been shown to be ideal model systems to study
101 virulence evolution (Bull, Molineux et al. 1991, Messenger, Molineux et al. 1999).
102 These phages can establish chronic infections whereby virions are continuously released
103 without lysis. Although filamentous phages do not kill their host, infections cause harm
104 by reducing host growth rates. This is because the host cell pays the metabolic costs
105 resulting from phage replication and through phage-encoded proteins inserted into the
106 bacterial membrane (Mai-Prochnow, Hui et al. 2015). Thus, the virulence of
107 filamentous phages, which can vary greatly across phage types (Rakonjac 2012), can be
108 directly quantified by measuring the reduction in bacterial growth rate caused by phage
109 infection.

110 By combining experimental evolution and whole genome sequencing, we show that
111 bacteria can defend themselves against these infections via two alternative strategies:
112 (1) immunity (i.e., infected bacteria became resistant to further infection) and (2)
113 resistance (surface receptor modifications). We show that immunity is faster than
114 resistance evolution and occurs at a similar rate in both treatments. In contrast, selection
115 for resistance evolution is stronger (because immunity is more costly), and this form of
116 resistance evolves more rapidly against high virulence compared to low virulence

117 phages. This resulted in faster extinction and ultimately shorter epidemics for high

118 virulence phage.

119

120 MATERIAL AND METHODS

121 (a) Strains and culture conditions

122 Experiments were conducted using the *Vibrio alginolyticus* strain K01M1 (Chibani,
123 Roth et al. 2020). K01M1 contains one integrated filamentous *Vibrio* phage VALGΦ6
124 (later called: resident K01M1Φ-phage throughout the manuscript), which replicates at a
125 very low frequency (Chibani, Hertel et al. 2020). Compared to other, closely related *V.*
126 *alginolyticus* strains, K01M1 is highly susceptible to infections by filamentous phages
127 (Wendling, Piecyk et al. 2017). For the selection experiment we used two different
128 versions of the filamentous *Vibrio* phage VALGΦ8 (Table 1), one integrative (isolated
129 from the host strain K04M5) and one episomal (isolated from the host strain K04M1).
130 While both phages have been shown to significantly reduce the growth of K01M1
131 (Wendling, Piecyk et al. 2017, Wendling, Goehlich et al. 2018), infections with the high
132 virulent phage impose a significantly stronger reduction in bacterial growth than
133 infections with the low virulent phage. All experiments were carried out in liquid
134 medium (Medium101: 0.5% (w/v) peptone, 0.3% (w/v) meat extract, 3.0% (w/v) NaCl
135 in MilliQ water) at 25° C in 30-ml microcosms containing 6 ml of medium with
136 constant shaking at 180 rpm.

137

138 **Table 1** Bacteria and phages (including NCBI accession numbers) used in the present study.

Isolate	Accession Number(s)	Phages	Role in evolution experiment
<i>V. alginolyticus</i> K01M1	CP017889.1 CP017890.1	<i>Vibrio</i> phage VALGΦ6	Host strain during evolution experiment
<i>V. alginolyticus</i> K04M1	CP017891.1 CP017892.1	<i>Vibrio</i> phage VALGΦ6 <i>Vibrio</i> phage VALGΦ8 (episomal)	Donor of the episomal, low virulent phage
<i>V. alginolyticus</i> K04M5	CP017899.1 CP017900.1	<i>Vibrio</i> phage VALGΦ6 <i>Vibrio</i> phage VALGΦ8 (integrative on CR2)	Donor of the integrative, high virulent phage
<i>Vibrio</i> phage VALGΦ6	MN690600		Resident phage in ancestral K01M1
<i>Vibrio</i> phage VALGΦ8	MN719123		Co-evolving phage in evolution experiment

139

140 (b) Selection experiment

141 Six replicate populations were founded for each of three treatments from
 142 independent clones of K01M1. Treatments comprised (a) a high virulent, integrative
 143 version of the filamentous *Vibrio* phage VALGΦ8, later called VALGΦ8_{K04M1}, (b) a
 144 low virulent, episomal version of the filamentous *Vibrio* phage VALGΦ8, later called
 145 VALGΦ8_{K04M5}, and (c) no phage as control. Each population was established from 60 µl
 146 of an independent overnight culture (5×10^8 CFU/ml). At the beginning of the
 147 experiment, we inoculated phage-containing treatments with 300 µl of a 5×10^{10} PFU/ml
 148 stock solution. Populations were propagated by transferring 1% to fresh medium every
 149 24 hours for a total of 30 transfers. On transfer T0, T1, T2 followed by every other
 150 transfer, phage and bacterial densities were determined, as described below and whole
 151 population samples were frozen at -80° C at a final concentration of 33% glycerol. In
 152 addition, on transfer T0, T1, T2, T6, followed by every sixth transfer 24 single colonies
 153 were isolated at random from each population and stored at -80° C. Two populations
 154 from the control treatment tested positive for virus infection, indicating contamination,
 155 were excluded from later assays.

156

157 (c) Bacterial and phage densities

158 *Bacterial densities*: bacterial densities were determined by plating out 100 µl of a
 159 dilution series ranging from 10^{-5} to 10^{-7} on *Vibrio* selective Thiosulfate Citrate Bile
 160 Sucrose Agar (TCBS) plates (Fluka Analytica). Plates were incubated over night at 25°
 161 C and the total amount of colonies was counted the following day.

162 *Phage densities*: Quantification of filamentous phages by standard spot assays is
 163 often not possible (Rakonjac 2011). Instead of typical lytic plaques we mostly observed
 164 opaque zonas of reduced growth. Thus, we used spectrometry to quantify phage

165 prevalence ([http://www.abdesignlabs.com/technical-resources/bacteriophage-](http://www.abdesignlabs.com/technical-resources/bacteriophage-spectrophotometry)
166 [spectrophotometry](http://www.abdesignlabs.com/technical-resources/bacteriophage-spectrophotometry)), which uses the constant relationship between the length of viral
167 DNA and the amount of the major coat protein VIII of filamentous phages, which,
168 together, are the main contributors of the absorption spectrum in the UV range. The
169 amount of phage particles per ml can be calculated according to the following formula:

$$170 \quad phages / ml = \frac{(OD_{269} - OD_{320}) * 6e16}{bp},$$

171 where OD₂₆₉ and OD₃₂₀ stand for optical density at 269 and 320 nm and bp stands for
172 number of base pairs per phage.

173 This method is based on small-scale precipitation of phages by single PEG-
174 precipitation. After centrifuging 1500 µl of the phage containing overnight culture at
175 13,000 ×g for 2 min, 1200 µl of the supernatant was mixed with 300 µl PEG/NaCl 5×
176 and incubated on ice for 30 min. Afterwards phage particles were pelleted by two
177 rounds of centrifugation at 13,000 ×g for 2 min, resuspended in 120 µl TBS 1× and
178 incubated on ice. After one hour the suspension was cleaned by centrifugation at 13,000
179 ×g for 1 min and absorbance was measured at 269 and 320 nm.

180 Quantification of filamentous phages using spectrometry is likely to be erroneous if
181 viral load is low. Therefore, we additionally quantified phage prevalence/ phage
182 extinction in each of the populations on every second transfer day by standard spot
183 assays with a serial dilution (10⁻¹ to 10⁻⁶) on the ancestral host (for details see
184 (Wendling, Piecyk et al. 2017)) and measured until which dilution the typical opaque
185 zones of reduced bacterial growth were visible.

186

187 (d) Measuring phage-resistance

188 We measured the rate of phage resistance evolution among bacteria against the

ancestral phage by determining the reduction in bacterial growth rate (RBG) imposed by the phage, adapted from (Poullain, Gandon et al. 2008) with some modifications according to (Goehlich, Roth et al. 2019). Twenty-four random colonies from each population from transfer T0, T1, T2, T6, T12, T18, T24, and T30 were introduced into 96-well microtiter plates containing Medium101 at a concentration of 5×10^6 cells/ml and inoculated with $\sim 2.5 \times 10^6$ PFU/ml of each of the two ancestral phages used for the selection experiment or without phage (control). Absorbance at 600 nm was measured using an automated plate reader (TECAN infinite M200) at T0 and again after 20 hours of static incubation at 25°C. The reduction in bacterial absorbance ‘RBG’ was calculated according to the following formula:

$$RBG = \frac{OD600(t=20) - OD600(t=0)[Phage]}{OD600(t=20) - OD600(t=0)[Control]},$$

where OD stands for optical density at 600nm.

(e) Frequency of prophage carriage

On transfer T0, T1, T2, T6 followed by every sixth transfer we measured the frequency of phage carriage of 24 random clones per population using standard PCR. Primers (VALGΦ8_Forward TGGAAAGTGCCAAGGTTTGGT, VALGΦ8_Revers GAAGACCAGGTGGCGGTAAA) that specifically target the co-evolving *Vibrio* phage VALGΦ8 have been designed using NCBI Primer-BLAST webpage (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Note, while this primer-pair detects the presence/ absence of *Vibrio* phage VALGΦ8, it does not confirm chromosomal integration of the respective phage. Glycerol stocks were inoculated overnight (25°C, 180 rpm) in Medium 101 and subsequently diluted (1:10) in HPLC purified H₂O and frozen at -80° C. One µl of this suspension was used as DNA template in the PCR assay. Reaction comprised 1 µl Dream Tag Buffer, 0.1 µl Dream Tag DNA polymerase

(Thermo Scientific, USA), 4.9 µl H₂O, 1 µl dNTPs [5 mM] and 1 µl of each primer [50 µM]. The amplification program used consisted of: (i) 3 min at 95° C, (ii) 35 cycles of 45 sec at 95° C, 30 sec at 63° C, 45 sec at 72° C, (iii) 7 min at 72° C. Afterwards, 5 µl of each reaction was mixed with 2 µl loading dye (10×) and loaded onto a 1.2% agarose gel dissolved in 1×TAE gel buffer. GeneRuler Express DNA-ladder was used as size marker. Agarose gels were run 15 min at 70 V in 0.5× TAE running buffer and subsequently stained with ethidium bromide for 10 min. DNA was visualized using UV light and documentation took place using Intas Gel iX20 Imager. Phage presence was recorded positive if a PCR product of 1400 bp was visible.

For all subsequent assays, we randomly picked one phage-resistant clone with a positive PCR product (later called: Φ-carrier) and one phage-resistant clone with a negative PCR product (later called: mutant) from each phage-evolved population as well as two randomly selected non-resistant clones from the control populations.

(f) Competition experiments

To determine differences in fitness between both resistance forms, we measured the competitive fitness of Φ-carrier relative to mutants. Each competition culture was done in triplicates as described in (Harrison, Guymer et al. 2015). In brief, overnight cultures of both competing strains (of which one was labelled with a GFP-marker) were mixed 1:1 and 60 µl of this mixture was inoculated to 6 ml Medium 101 to initiate each competitive culture. After 24 hours, fitness was estimated by means of flow cytometry (FACS-Calibur Becton & Dickinson, Heidelberg, GER), where absolute fluorescent cells and non-fluorescent cells were calculated. Competitive fitness was estimated as the ratio in Malthusian parameters (Lenski, Rose et al. 1991):

$$W = \ln(abundance_{t=24}/abundance_{t=0})_{competitor1} / \ln(abundance_{t=24}/abundance_{t=0})_{competitor2}$$

239 (g) Bacterial growth rate and phage production

240 To determine fitness parameters that could explain observed differences in
241 competitive fitness we additionally quantified bacterial growth rate (μ) by means of 24-
242 hour growth curves and phage production using PEG precipitation (as described in (c))
243 of the same clones used for the competition assays (i.e., one Φ -carrier and one mutant
244 from each phage-treated population and two random phage-susceptible clones from the
245 control populations plus the ancestor).

246

247 (h) Whole genome sequencing

248 We used a combination of long- and short read sequencing to obtain complete
249 genomes of the same clones from the assays above, i.e., one Φ -carrier and one mutant
250 from each phage-treated population and one random phage-susceptible clone from each
251 control population, which corresponds to six independently evolved clones per
252 treatment and resistance form. High molecular weight DNA was extracted from cell
253 pellets of overnight cultures following the protocol for gram negative bacteria from the
254 DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). For long-read sequencing the
255 library was prepared using Pacific Bioscience protocol for SMRTbell™ Libraries using
256 PacBio® Barcoded Adapters for Multiplex SMRT® Sequencing. To do so, DNA was
257 fragmented into 10kb fragments using g-tubes (Covaris). Samples were pooled during
258 library preparation aiming for equimolar pooling and library size was selected using
259 Ampure beads. The library was sequenced on a PacBio Sequel instrument using Sequel
260 Polymerase v3.9, SMRT cells v3 LR and Sequencing chemistry v3.0. Loading was
261 performed by diffusion. Two SMRT cells were sequenced (movie time: 600min, pre-
262 extension time: 240 min). Reads were demultiplexed using Barcoding pipeline on
263 SMRT Link (v6.0.0.47841, SMRT Link Analysis Services and GUI v6.0.0.47836) with

264 40 as a minimum barcode score.

265 Short-read sequencing was done on an Illumina 2500 platform and resulted in a
266 minimum average coverage of 88× per strain (coverage range was from 88× to 157×).
267 The reads were quality controlled using the program FastQC Version 0.11.5. High
268 quality reads were used for hybrid assemblies as well as for single nucleotide variation
269 analysis.

270 Genome assemblies were performed in two different ways: In a first approach
271 assemblies were performed using the Unicycler pipeline (v0.4.7) and the programs
272 within. Assemblies were performed as hybrid assemblies using short-read and long read
273 data in a short-read first approach. In brief: An initial assembly was performed with
274 short-read only using spades (v3.13.0) as provided within Unicycler. The resulting
275 contigs were co-assembled with long-read data using miniasm (v0.2-r168) and curated
276 using the racoon software. This step resulted in complete closed replicons. All long
277 reads were mapped and integrated into the contigs. All replicons were polished using
278 the Pilon software (1.22) to clear any small-scale assembly errors. Finally, all replicons
279 were rearranged according to the origin of replication. The assembly for the ancestral
280 K01M1 strain, as has been described in (Wendling, Piecyk et al. 2017) was performed
281 following the Hierarchical Genome Assembly Process (HGAP3) protocol, developed
282 for Pacific Biosciences Single Molecule Real-Time (SMRT) sequencing data (Chin,
283 Alexander et al. 2013). HGAP is available for use within PacBio's Secondary Analysis
284 Software SMRTPortal. Methodically, the longest subreads of a single SMRT Cell
285 (usually 25x genome coverage, e.g., 25 x 5 Mbp = 125 Mbp) are being chosen to be
286 error-corrected with "shorter" long reads in a process named preassembly. Hereby, a
287 length cut-off is computed automatically separating the "longer" reads (for genome
288 assembly) and the "shorter" reads (for error-correction). The level of error-correction is

289 being estimated with a per-read accuracy of 99%. Finally, error-corrected long read data
290 is being assembled with Celera Assembler 7.0.

291

292 (i) SNV analysis and reconstruction of infecting phages

293 All short-read sequences were mapped on a high quality closed reference genome
294 of *Vibrio alginolyticus* Strain K01M1 (Wendling et al., 2017) using Bowtie2
295 (Langmead & Salzberg, 2012). Single nucleotide variation (SNV) analysis was done
296 using the Breseq pipeline as described in Deatherage & Barrick, (Deatherage & Barrick,
297 2014). Assembly of reads were done using Spades (Bankevich et al., 2012). Whole
298 genome alignments have been calculated using the MAUVE aligner (Darling, Mau, &
299 Perna, 2010). Presence of infecting phage genomes were determined by assembling
300 NGS-reads that did not map on the K01M1 genome in a bowtie2 mapping using Spades.
301 The resulting contigs were annotated based on the review of Mai-Prochnow on
302 filamentous phages (Mai-Prochnow et al., 2015). The genomes of the evolved phages
303 were compared to the infecting phage genomes *Vibrio* phage VALGΦ8 as well as to the
304 genome of the resident prophage *Vibrio* phage VALGΦ6 from the challenged strain
305 K01M1 using BLAST and Easyfig 2.1 (Sullivan, Petty, & Beatson, 2011).

306 Coverage analysis of phage derived short reads were mapped against the complete
307 ancestral genome of *V. alginolyticus* K01M1 (NCBI accession numbers CP017889.1,
308 CP017890.1) as well as the phage genomes VALGΦ6 and VALGΦ8 using Bowtie2.
309 The resulting coverage data was visualized using Artemis (Version) and compared for
310 differences in coverage. Coverage analysis of Sequel long-reads was performed within
311 the “Base Modification Analysis” within SMRTlink 8 using the same reference as stated
312 above or additionally including the infecting phage. Coverage data was visualized
313 within SMRTlink.

314

315 (j) Statistical analyses

316 All statistics were performed in the R 3.1.2 statistical environment (Team 2011).

317 For all analysis aimed to compare the two different phage treatments to one another,
318 control populations (i.e., those that evolved without phages) were excluded. When
319 comparing temporal dynamics between phage-treatments, we excluded the starting
320 time-point T0, because these measurements were taken before phages were added to the
321 populations.

322

323 *Bacteria and phage dynamics*

324 Bacterial and phage densities were analysed over time using a generalized least
325 squares model to control for autocorrelation of residuals over time using the gls function
326 (package nlme) with phage treatment, transfer as categorical variable as well as their
327 interaction as fixed effect.

328 We considered phages to be prevalent in the population if opaque zones of reduced
329 growth were visible during standard spot assays. Phage prevalence was subsequently
330 quantified by a serial dilution, which were assigned with invers values (i.e., if reduced
331 growth zones were visible up to dilution of 10^{-6} we assigned to it a value of 7, whereas
332 if they were only visible on an undiluted spot, we assigned to it a value of 1, if no zone
333 of reduced growth was visible it was scored as 0). Phage extinction events across phage-
334 treatments were analysed using a log-rank test.

335

336 *Measuring bacterial resistance*

337 We observed a bimodal histogram on all RBG values with a local minimum at RBG
338 = 0.82 (Figure S1). Thus, we considered an infection as positive if $RBG < 0.82$. The

339 proportion of resistant clones per population as well as the proportion of clones that
 340 tested positive for PCR (targeting the co-evolving phage) were analysed using a
 341 generalized linear model with a binomial error distribution using the glm function
 342 (package lme4) with phage treatment, transfer and their interaction as fixed effect.

343

344 *Fitness effects*

345 We determined differences in relative fitness between MSHA-mutants and phage-
 346 carrier using a linear model with resistance mechanisms and GFP-label and the
 347 interaction thereof as fixed effects. To determine differences in the amount of free
 348 phages and in growth rates produced between ancestral strains and evolved strains and
 349 between both resistance forms, we used Welch's pairwise *t*-tests with sequential
 350 Bonferroni correction. We further performed a Pearson's correlation analysis to
 351 determine whether phage production impacted bacterial growth rates.

352

353 RESULTS

354 *Bacterial densities*

355 We propagated six replicate populations of *Vibrio alginolyticus* K01M1 with or
 356 without either of two closely related filamentous phages that differed in their virulence
 357 — VALGΦ8_{K04M5} (high virulence), VALGΦ8_{K04M1} (low virulence) — for 30 serial
 358 transfers (~240 bacterial generations). Phages reduced bacterial densities in both phage-
 359 containing treatments by several orders of magnitude compared to no phage control
 360 populations (Figure 1a). However, the immediate reduction (measured 24 hours post
 361 infection [hpi]) in bacterial density was stronger in populations co-evolving with the
 362 high virulence phage than with the low virulence phage (Figure 1a), confirming that
 363 VALGΦ8_{K04M5} is more virulent than VALGΦ8_{K04M1}. Over time, however, the densities
 364 of bacterial populations co-evolving with the high virulence phage recovered three times
 365 faster than populations co-evolving with the low virulence phage (significant
 366 phage:transfer interaction in gls-model: $F_{15,186}=6.58$, $p<0.001$, Figure 1a).

367

368 *Phage densities*

369 In both phage containing treatments, phages, that had initially been added at a titre
 370 of $\sim 5 \times 10^{10}$ PFU/ml, amplified massively during the first couple of bacterial generations
 371 and reached levels of 3.01×10^{12} PFU/ml (VALGΦ8_{K04M5}) 24 hpi and 2.83×10^{12} PFU/ml
 372 (VALGΦ8_{K04M1}) 48 hpi (Figure 1b), before densities decreased to levels comparable to
 373 phage-free populations, which stem from the resident phage VALGΦ6. These data
 374 suggest that the strong reduction in bacterial densities at the beginning of the
 375 experiment (Figure 1a) directly resulted from the production of viral particles (Figure
 376 1b).

We further observed that phage extinction events differed significantly between treatments (log-rank test: $\text{Chisq}_1=4.9$, $p=0.03$). High virulent phages went extinct in five out of six populations after 12 transfers, whereas low virulent phages survived in four out of six populations until transfer 28, before they finally went extinct on transfer 30 (Figure S2).

Figure 1 Population dynamics over 30 transfers of bacteria-phage co-evolution. (a) Bacteria in CFU/ml, (b) Phages in PFU/ml, the grey dashed line represents the detection limit for quantifying filamentous phages using spectrophotometry, (c) Fraction of phage-resistant clones ($n=24$), and (d) Fraction of Φ -carrier within phage-resistant clones. Fractions are based on 24 random clones per replicate population. In all panels, data are represented as mean of six replicate populations per treatments, error bars represent standard errors. Colours correspond to one of three phage-treatments, low virulent VALG Φ _{8K04M1} (red), high virulent VALG Φ _{8K04M5} (yellow), no phage (grey).

390

391

392 *Modes of phage resistance selected by high and low virulence phage*

393 To understand the evolutionary drivers behind these different selection dynamics
394 against both phages, we next isolated 24 random clones per population from seven
395 timepoints and quantified the fraction of phage-resistant clones per population. The
396 proportion of phage-resistant clones per population increased rapidly within the first 24
397 hours (Figure 1c). This was true for both phage treatments in which we observed almost
398 100% phage-resistant clones after 24 hours, and subsequently resistance levels remained
399 high in both treatments for the duration of the experiment.

400 Different mechanisms can confer resistance to filamentous vibriophages, such as
401 superinfection exclusion, (i.e. when the same phage is already present in the host cell
402 (Wendling, Piecyk et al. 2017)) or surface-receptor modifications, in particular at the
403 mannose-sensitive hemagglutinin (MSHA) type IV pilus, that prevents filamentous
404 vibriophages from entering the host (Jouravleva, McDonald et al. 1998). To determine
405 the underlying genetic basis of the resistance mechanism in the present study, we first
406 performed a PCR-based analysis to determine presence of newly acquired phages in
407 bacterial genomes, which confers resistance through superinfection exclusion. After 24
408 hours, Φ -carriers, were the dominating form among the resistant clones (Figure 1d),
409 suggesting that superinfection exclusion is a fast way to acquire phage resistance.
410 However, 48 hours post infection (i.e., on transfer 2) the proportion of Φ -carriers
411 declined, and did so significantly faster in populations co-evolving with the high
412 virulence, compared to the low virulence phage (Figure 1d, significant phage:transfer
413 interaction: $F_{6,60}=10.18$, $p<0.001$).

414 To test if the decline of Φ -carriers was driven by the invasion of surface receptor
 415 modification resistance we used whole genome sequencing (WGS) of two randomly
 416 chosen clones from each population (one PCR-positive and one PCR-negative clone) to
 417 identify mutations. We observed no loci with mutations on chromosome 2 or the
 418 plasmid pI9064, but on chromosome 1 we identified 12 loci with mutations that were
 419 not present in clones from the control treatment, suggesting an association with phage-
 420 mediated selection. Of these 12 loci, three were randomly distributed across PCR-
 421 positive and PCR-negative clones while the remaining nine loci were exclusive to PCR-
 422 negative clones suggesting a potential role in evolved phage resistance. Of these nine
 423 loci, eight had substitutions, duplications, insertions or deletions in four different
 424 proteins belonging to the MSHA type IV pilus operon (*mshL*, *mshE*, *mshG*,
 425 *K01M1_28150*; Figure 2a/ Table S1). Of these, five caused severe frameshift mutations
 426 that presumably have a high impact on the function of this protein. Overall mutated
 427 MSHA-loci occurred in 8/12 PCR-negative clones, which suggests strongly parallel
 428 evolution of phage resistance. The MSHA operon is highly conserved across *Vibrio*
 429 clades (Figure 2b), suggesting that, similar to other vibrios (Jouravleva, McDonald et al.
 430 1998), the MSHA type IV pilus plays an important role in resistance against the
 431 filamentous *Vibrio* phage VALGΦ8. Note, a search of all assembled genomes for
 432 CRISPR associated genes as well as for CRISPR array like repetitive sequence patterns
 433 did not yield any results. All PCR-negative phage resistant clones are from here
 434 onwards referred to as MSHA-mutants.

435 The genomic data also confirmed that clones with a positive PCR result (i.e., Φ -
 436 carrier) did indeed contain the respective co-evolving phage. Interestingly, in all
 437 sequenced PCR-positive clones, the co-evolving phage existed exclusively as an
 438 episome, despite the K01M1 genome containing at least two known integration sites

459 Φ -carriers went to extinction in all six populations co-evolving with the high
 460 virulence phage 12 days post infection but were able to persist until the end of the
 461 experiment (i.e., transfer 30), albeit at very low frequencies, in five out of six
 462 populations co-evolving with the low virulence phage. We hypothesised therefore that
 463 the fitness benefit of MSHA-mutants relative to Φ -carriers would be higher for the high
 464 virulence phage compared to the low virulence phage, because MSHA-mutants do not
 465 suffer virulence from phage infection. This was confirmed in a separate pairwise
 466 competition experiment, in which we quantified fitness advantages of MSHA-mutants
 467 relative to each Φ -carrier (Figure S4). The fitness benefit of MSHA-mutants was higher
 468 against bacteria carrying the high virulence phage compared to bacteria carrying the low
 469 virulence phage (significant treatment term in linear model with treatment, GFP-label
 470 and the interaction thereof as fixed factors: $F_{1,8}=18.63$, $p=0.003$, Table S2). These
 471 results support the dynamics observed in the selection experiment, (i.e., VALG $\Phi 8_{K04M5}$ -
 472 carriers went extinct significantly faster than VALG $\Phi 8_{K04M1}$ -carriers) and suggests
 473 stronger selection for MSHA-mutants when coevolving with high virulence phage.

474 Bacterial population densities during the selection experiment were negatively
 475 correlated with the number of Φ -carriers per population (Pearson's correlation without
 476 zero inflation Φ -K04M1: $r=0.69$, $t_{21}=-4.38$, $p<0.001$, Φ -K04M5: $r=0.92$, $t_7=-6.29$,
 477 $p<0.001$; Figure S5). This implies that, even though the majority of the clones in the
 478 populations were resistant to infection, bacterial populations were not able to recover as
 479 long as the dominating mechanism of phage-resistance was superinfection exclusion,
 480 presumably due to virulence. To further quantify the parameters that influence the
 481 fitness of MSHA-mutants relative to Φ -carriers, we measured the amount of free phages
 482 and the absolute growth rate of selected clones. Specifically, we quantified differences
 483 in phage production and tested if phage production impairs bacterial growth. We

484 observed an increase in phage production relative to the ancestor in Φ -carriers isolated
 485 from populations coevolved with the high virulence phage (paired t -test: $t_5=-4.31$,
 486 $p=0.008$) but not those coevolved with the low virulence phage (non-significant paired
 487 t -test: $t_{4.2}=-1.18$, $p=0.3$, Figure S6a). Also, VALG Φ _{8K04M5}-carriers produced
 488 significantly more free phages than VALG Φ _{8K04M1}-carriers (paired t -test: $t_{5.61}=-3.36$,
 489 $p=0.017$).

490 Direct comparisons of growth rates among evolved clones revealed a significant
 491 difference between both resistance forms with Φ -carriers growing on average slower
 492 than MSHA-mutants (VALG Φ _{8K04M5}: paired t -test: $t_{6.61}=-3.39$, $p=0.006$;
 493 VALG Φ _{8K04M1}: paired t -test: $t_{7.5}=-3.32$, $p=0.01$, Figure S6b). We further observed that
 494 phage production significantly impaired bacterial growth (significant negative
 495 correlation between the amount of produced phages and bacterial growth rate Figure 3).

496 Taken together these data suggest that superinfection exclusion buys time, offering
 497 resistance to subsequent infection, but at the cost of suffering the virulence of being
 498 infected; this resistance mechanism is replaced by surface receptor modification in
 499 populations coevolving with both the high and the low virulence phage, but that
 500 selection for resistance mutations is stronger with higher virulence.

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Figure 3 Correlation between bacterial growth rate [μ] and production of free phages measured as PFU/ml per clone (left VALGΦ_{K04M1}, right: VALGΦ_{K04M5}).

506 **DISCUSSION**

507 We hypothesised that selection for resistance evolution in viral infections will be
 508 stronger for high virulent compared to low virulent viral infections. Resistance against
 509 filamentous phages can emerge via two ways: (1) super-infection exclusion mediated by
 510 the presence of the infecting phage in the bacterial host cell, or (2) molecular resistance
 511 evolution, for instance surface-receptor modifications, which prevent phages from
 512 entering host cells. Here, we show that upon phage infection, the first mechanism of
 513 resistance acquisition was by super-infection exclusion, which occurred at a similar rate
 514 in both treatments. However, over time, these phage-carrying clones were rapidly
 515 replaced by phage resistant mutants. This replacement happened significantly faster in
 516 high virulent compared to low virulent infections, suggesting that selection for
 517 molecular resistance evolution is stronger in high virulent infection. Ultimately this
 518 faster resistance evolution caused a faster extinction of phages, which were not able to
 519 co-evolve in order to overcome evolved host-resistance, and a shorter epidemic.

520 At least two mutually non-exclusive scenarios can explain why mutants became the
 521 dominant form in our experiments: (1) cost of resistance, and (2) stability of resistance.
 522 While resistance acquisition is faster via super-infection exclusion than by molecular
 523 changes, our data suggest that this process is less stable. That is because in episomal,
 524 non-integrative phage infections some bacterial daughter cells will not inherit the
 525 infecting phage due to spontaneous segregation-loss, rendering them once again
 526 susceptible to subsequent infections. In contrast, molecular resistance evolution is more
 527 stable and due to their higher fitness, those resistant clones can rapidly sweep through
 528 the population and reach fixation.

529 The rapid extinction of phages and phage-carriers in our experiment raises the
 530 questions, why filamentous phages are omnipresent in nature (Roux, Krupovic et al.

2019), including in environmental *Vibrio* strains closely related to our model strain K01M1 (Chibani, Hertel et al. 2020). In other words, what selects for the presence of filamentous phage-carriers in nature? In some cases filamentous phages can contribute positively to the host's evolutionary fitness (Hay and Lithgow 2019) through lysogenic conversion, i.e., by providing the bacterial host with new phenotypes induced by phage-encoded proteins (Waldor and Mekalanos 1996). In certain environments this may be beneficial enough to outweigh the costs, resulting in a successful chronic infection. Among those, the most prominent example is CTX ϕ , which carries the genes for the cholera toxin (Waldor and Mekalanos 1996). For the phage from our study, however, we can only speculate, that its presence provides a fitness advantage in the natural environment, which we did not capture in our laboratory experiments. These might include for instance advantages during colonization (Davis and Waldor 2003), or increased stress tolerance (Yu, Chen et al. 2015). The annotation and comparative genomic analyses of VALG Φ 8 based on the available information in today's databases, does however not reveal any known-accessory traits that would support this hypothesis (Chibani, Hertel et al. 2020). Answers might come from co-evolution experiments in more natural environments, for instance inside the gut of marine animals. Such results could then be used to predict the chances of establishing a persistent chronic infection which we assume is likely to depend on ecological and evolutionary factors that determine the net-benefit of the phage on its host (Bull, Molineux et al. 1991, Shapiro, Williams et al. 2016, Shapiro and Turner 2018).

Ecological benefits of filamentous phages can arise during species interactions, e.g., by protecting hosts against infection by other phages (superinfection exclusion), by acting as decoys for mammalian immunity (Sweere, Van Bellegheem et al. 2019), or from interactions with the abiotic environment e.g., through stress tolerance (Yu, Chen

et al. 2015). Evolutionary benefits include increased mutation supply, and lysogenic conversion (Waldor and Mekalanos 1996). However, filamentous phages can also be costly, for instance through disruption of functional host genes following chromosomal insertion or a fitness loss due to increased transcriptional load, in particular for phages that reproduce at high frequencies. We predict that the net-benefit of a filamentous phage on its host determines the co-evolutionary outcome of both species. If costs associated with phage carriage exceed the benefits, we expect that phages will be quickly lost from the population as we have seen in the present experiment. By contrast, if phages increase their host's fitness we expect successful chronic infections.

Conclusion

Consistent with our hypothesis we could show that selection for resistance evolution is stronger against high virulence compared to low virulence viral infections. Accordingly, high virulence infections may be cleared faster leading to shorter epidemics which may additionally limit the transmission success of highly virulent viruses. However, since the virus in our study failed to co-evolve, this system should not be used as a general model to study resistance evolution. Other outcomes in terms of resistance evolution are to be expected, if viruses co-evolve with their hosts. It might be possible that co-evolution of high virulent viruses is constrained as they have much less time to acquire the necessary mutations that would allow them to overcome host resistance. If this holds true, natural selection might favour low virulent variants, that are able to persist longer inside an organism which not only increases their transmission potential but also their time to co-evolve. Future work will focus on the impact of virulence on the evolutionary potential during host-virus co-evolution.

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References

588

589 Alizon, S., A. Hurford, N. Mideo and M. Van Baalen (2009). "Virulence evolution and
590 the trade-off hypothesis: history, current state of affairs and the future." J Evol Biol
591 **22**(2): 245-259.

592 Anderson, R. M. and R. M. May (1982). "Coevolution of hosts and parasites."
593 Parasitology **85** (Pt 2): 411-426.

594 Bull, J. J., I. J. Molineux and W. R. Rice (1991). "Selection of Benevolence in a Host-
595 Parasite System." Evolution **45**(4): 875-882.

596 Chibani, C. M., R. Hertel, M. Hoppert, H. Liesegang and C. C. Wendling (2020).
597 "Closely Related *Vibrio alginolyticus* Strains Encode an Identical Repertoire of
598 Caudovirales-Like Regions and Filamentous Phages." Viruses **12**(12).

599 Chibani, C. M., O. Roth, H. Liesegang and C. C. Wendling (2020). "Genomic
600 variation among closely related *Vibrio alginolyticus* strains is located on mobile
601 genetic elements." BMC Genomics **21**(1): 354.

602 Chin, C. S., D. H. Alexander, P. Marks, A. A. Klammer, J. Drake, C. Heiner, A. Clum,
603 A. Copeland, J. Huddleston, E. E. Eichler, S. W. Turner and J. Korlach (2013).
604 "Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing
605 data." Nat Methods **10**(6): 563-569.

606 Davis, B. M. and M. K. Waldor (2003). "Filamentous phages linked to virulence of
607 *Vibrio cholerae*." Curr Opin Microbiol **6**(1): 35-42.

608 Ebert, D. (1994). "Virulence and Local Adaptation of a Horizontally Transmitted
609 Parasite." Science **265**(5175): 1084-1086.

610 Fenner, F. and I. D. Marshall (1957). "A comparison of the virulence for European
611 rabbits (*Oryctolagus cuniculus*) of strains of myxoma virus recovered in the field in
612 Australia, Europe and America." J Hyg (Lond) **55**(2): 149-191.

613 Goehlich, H., O. Roth and C. C. Wendling (2019). "Filamentous phages reduce
614 bacterial growth in low salinities." R Soc Open Sci **6**(12): 191669.

615 Harrison, E., D. Guymer, A. J. Spiers, S. Paterson and M. A. Brockhurst (2015).
616 "Parallel Compensatory Evolution Stabilizes Plasmids across the Parasitism-
617 Mutualism Continuum." Current Biology **25**(15): 2034-2039.

618 Herre, E. A. (1993). "Population structure and the evolution of virulence in nematode
619 parasites of fig wasps." Science **259**(5100): 1442-1445.

- 620 Jouravleva, E. A., G. A. McDonald, J. W. Marsh, R. K. Taylor, M. Boesman-
621 Finkelstein and R. A. Finkelstein (1998). "The *Vibrio cholerae* mannose-sensitive
622 hemagglutinin is the receptor for a filamentous bacteriophage from *V. cholerae* O139."
623 Infect Immun **66**(6): 2535-2539.
- 624 Lenski, R. E., M. R. Rose, S. C. Simpson and S. C. Tadler (1991). "Long-Term
625 Experimental Evolution in *Escherichia-Coli* .1. Adaptation and Divergence during
626 2,000 Generations." American Naturalist **138**(6): 1315-1341.
- 627 Mai-Prochnow, A., J. G. Hui, S. Kjelleberg, J. Rakonjac, D. McDougald and S. A.
628 Rice (2015). "'Big things in small packages: the genetics of filamentous phage and
629 effects on fitness of their host'." FEMS Microbiol Rev.
- 630 May, R. M. and R. M. Anderson (1983). "Epidemiology and genetics in the
631 coevolution of parasites and hosts." Proc R Soc Lond B Biol Sci **219**(1216): 281-313.
- 632 Messenger, S. L., I. J. Molineux and J. J. Bull (1999). "Virulence evolution in a virus
633 obeys a trade-off." Proc Biol Sci **266**(1417): 397-404.
- 634 Poullain, V., S. Gandon, M. A. Brockhurst, A. Buckling and M. E. Hochberg (2008).
635 "The evolution of specificity in evolving and coevolving antagonistic interactions
636 between a bacteria and its phage." Evolution **62**(1): 1-11.
- 637 Rakonjac, J. (2012). Filamentous Bacteriophages: Biology and Applications eLS. Ltd:
638 Chichester, John Wiley & Sons.
- 639 Roux, S., M. Krupovic, R. A. Daly, A. L. Borges, S. Nayfach, F. Schulz, A. Sharrar, P.
640 B. Matheus Carnevali, J. F. Cheng, N. N. Ivanova, J. Bondy-Denomy, K. C. Wrighton,
641 T. Woyke, A. Visel, N. C. Kyrpides and E. A. Elie-Fadrosh (2019). "Cryptic
642 inoviruses revealed as pervasive in bacteria and archaea across Earth's biomes." Nat
643 Microbiol **4**(11): 1895-1906.
- 644 Shapiro, J. W. and P. E. Turner (2018). "Evolution of mutualism from parasitism in
645 experimental virus populations." Evolution **72**(3): 707-712.
- 646 Shapiro, J. W., E. S. C. P. Williams and P. E. Turner (2016). "Evolution of parasitism
647 and mutualism between filamentous phage M13 and *Escherichia coli*." PeerJ **4**.
- 648 Sweere, J. M., J. D. Van Belleghem, H. Ishak, M. S. Bach, M. Popescu, V. Sunkari, G.
649 Kaber, R. Manasherob, G. A. Suh, X. Cao, C. R. de Vries, D. N. Lam, P. L. Marshall,
650 M. Birukova, E. Katznelson, D. V. Lazzareschi, S. Balaji, S. G. Keswani, T. R. Hawn,
651 P. R. Secor and P. L. Bollyky (2019). "Bacteriophage trigger antiviral immunity and
652 prevent clearance of bacterial infection." Science **363**(6434): 1416+.
- 653 Team, R. D. C. (2011). " R: A language and environment for statistical computing. R
654 Foundation for Statistical Computing." Vienna, Austria. ISBN 3-900051-07-0, URL
655 <http://www.R-project.org/>.

656 Turner, P. E., V. S. Cooper and R. E. Lenski (1998). "Tradeoff between Horizontal
657 and Vertical Modes of Transmission in Bacterial Plasmids." Evolution **52**(2): 315-329.

658 Waldor, M. K. and J. J. Mekalanos (1996). "Lysogenic conversion by a filamentous
659 phage encoding cholera toxin." Science **272**(5270): 1910-1914.

660 Weiss, S. R. and J. L. Leibowitz (2011). "Chapter 4 - Coronavirus Pathogenesis."
661 Advances in Virus Research **81**: 85-164.

662 Wendling, C. C., H. Goehlich and O. Roth (2018). "The structure of temperate phage-
663 bacteria infection networks changes with the phylogenetic distance of the host
664 bacteria." Biol Lett **14**(11).

665 Wendling, C. C., A. Piecyk, D. Refardt, C. Chibani, R. Hertel, H. Liesegang, B. Bunk,
666 J. Overmann and O. Roth (2017). "Tripartite species interaction: eukaryotic hosts
667 suffer more from phage susceptible than from phage resistant bacteria." BMC Evol
668 Biol **17**(98).

669 Yu, Z. C., X. L. Chen, Q. T. Shen, D. L. Zhao, B. L. Tang, H. N. Su, Z. Y. Wu, Q. L.
670 Qin, B. B. Xie, X. Y. Zhang, Y. Yu, B. C. Zhou, B. Chen and Y. Z. Zhang (2015).
671 "Filamentous phages prevalent in *Pseudoalteromonas* spp. confer properties
672 advantageous to host survival in Arctic sea ice." ISME J **9**(4): 871-881.
673