

1 Genetic determinism of phage-bacteria coevolution in natural populations

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38 **ABSTRACT**

39

40 Coevolution between bacteriophage (or phage) and their bacterial host is thought to be key for
41 the coexistence of these antagonists. Recent studies have revealed the major role of mobile
42 genetic elements in the emergence of phage resistant hosts but how phage escape these
43 defenses in the wild remained to be explored. Here we show a striking parallel in phage
44 evolving counter defenses to host defenses in natural population. We established a large
45 collection of phages and their bacterial hosts and we explored the genetic structure of their
46 interaction. We find that clearly delineated genomic clusters of phage are specific for distinct
47 clades within a bacterial species, *Vibrio crassostreae*, yet while all phages can adsorb, only a
48 subset of hosts are killed due to intracellular defense mechanisms. Host genomes contain
49 multiple mobile defense genes and susceptibility to phage is negatively correlated with
50 genome size. Phages also display extensive gene content variation, but their genome size
51 remains conserved. We show that this gene content variation in hosts and phage is due to
52 rapid turnover of genes involved in defense and escape, and that by exchanging anti-defense
53 genes, phages irreversibly switch host. This could be indicative of co-evolution following the
54 matching-allele-model of specificity and the spatial and temporal variability of phage
55 infectivity further suggests that negative-frequency dependent selection drives phage-vibrio
56 coevolutionary dynamics. We propose a “pan-escape system” that can be shared among
57 phages by homologous recombination within a population that infects a bacterial host.

58

59 **MAIN TEXT**

60

61 The ongoing battle between marine bacteriophages (phages) and their bacterial hosts is
62 probably billions of years old and involves an arsenal of defense and counter-defense
63 systems^{1,2}. This arms race is fueled by the underlying coevolutionary dynamics going on at
64 different steps of the infection process³⁻⁵. Viral infections requires the phage to adsorb to
65 specific receptors at the bacteria cell surface and then bypass intracellular host defenses⁶⁻⁸.
66 Recent work on marine *Vibrionaceae* (herein named vibrios) highlighted that phage defense
67 genes turn over exceedingly fast, differentiating clonal isolates, while phage receptors can be
68 highly monomorphic across diverse members of a species, likely due to recent positive
69 selection effecting gene specific sweeps⁹. As a result, phage can enter much more diverse
70 hosts than they can kill. Intracellular anti-phage defense systems are largely encoded by
71 complex, chromosomally inserted mobile genetic elements (MGEs), and single bacterial
72 strain can encode numerous anti-phage systems, suggesting that a wide variety of phages
73 select for multiple resistance systems and/or more than one system is necessary to efficiently
74 prevent infection^{10,11}. Bernheim and Sorek recently proposed the ‘pan-immune system’ model
75 which suggests that, although a single bacterial strain cannot carry all possible defense
76 systems because of fitness costs, horizontal gene transfer can allow access to immune defense
77 mechanisms encoded by closely related strains¹². As phages are thought to rapidly evolve
78 counter-defenses to thrive in the environment, it is expected that the diversity of escape
79 mechanisms within closely related phages mirror the host pan-immune system¹². However,
80 the counter-defense by phages has been insufficiently studied. While bacteria can
81 considerably expand their genome size, in phage, the size of the capsid also constrains the
82 genome size, likely limiting the number of escape mechanisms a phage can encode. An open
83 question is, therefore, how phage populations counter the numerous defense systems in their
84 hosts and how this influences phage specificity in the wild.

85

86 Here we explore the dynamics of phage-bacteria coevolution by focusing on the genetic
87 determinisms of phage-host interactions in natural populations. We combined cultivation,

88 genome sequencing and molecular genetics to analyze a large collection of sympatric and
89 allopatric environmental vibrios and their phages as a model system. We show that genome
90 size, MGE and phage defense element correlate with host resistance to phages. In contrast,
91 phage genome size is conserved among closely related phages, but variable gene content is
92 nonetheless extensive suggesting a role in escaping the host defenses. We demonstrate this in
93 several phages and show that by exchanging anti-defense genes, phages irreversibly switch
94 host.

95
96 **Oysters and their vibrio pathogens as a model system.** Oysters affected by the Pacific
97 oyster mortality syndrome are infected by diverse virulent strains of *Vibrio crassostreae* that
98 rise to similar abundances in diseased animals^{13,14,15,16}. To study the dynamics of *V.*
99 *crassostreae* at fine temporal resolution, we sampled vibrios from a pool of five juvenile
100 oysters deployed in an oyster farm (Bay of Brest, France) and from the surrounding seawater
101 on 57 dates over five months (each Monday, Wednesday and Friday from May 3 to
102 September 11, 2017) (see Methods). Roughly 48 colonies were picked from *Vibrio* selective
103 plates and screened by PCR targeting the *r5.2* gene, which was previously identified as a *V.*
104 *crassostreae*-specific marker¹⁴. Sequencing of the *gyrB* gene confirmed that 195 isolates were
105 *V. crassostreae* (Table S1). Seawater temperature reached 16°C on the 22nd of May, a
106 previously observed threshold for oyster mortalities¹⁷, and mortalities began on the 29th of
107 May and persisted until the 25th of August (Fig.S1a). *V. crassostreae* occurred only during the
108 disease outbreak with frequencies varying from 0–16% for seawater and 0–58% for oysters
109 (Fig. S1a), consistent with the previously determined increased prevalence of this species in
110 diseased oysters¹³. The quantification of *V. crassostreae* DNA by qPCR further revealed an
111 equal distribution between seawater size fractions (Fig. S1b).

112
113 In order to establish a large collection of phages infecting *V. crassostreae*, we combined a
114 sympatric and allopatric sampling strategy. First, we used the 195 *V. crassostreae* strains as
115 “bait” to isolate phages from 20mL-seawater equivalents of viral concentrate (1,000X) or
116 oyster tissues (0.1mg) collected on the same day (Fig. S2). Phage infection was assessed by
117 plaque formation in soft agar overlays of host lawns mixed with a viral source. This approach
118 yielded 45 phages from 18 of 195 tested hosts (9.2%). Second, 90 *V. crassostreae* isolated
119 from 12 June–28 July were screened for phages with: (1) ten pooled seawater viral
120 concentrates from five consecutive dates, and (2) twenty time-shift combinations of single
121 seawater viral concentrates. Each approach resulted in the isolation of 21 and 177 additional
122 phages from 5/90 (5%) and 38/90 (42%) plaque-positive hosts, respectively, in total resulting
123 in a collection of 243 phages from Brest (Table S2). Finally, to better understand the spatial
124 variability of phage infectivity, this collection was complemented with 51 bacteria and 31
125 phages previously isolated in Sylt (Germany, 2016), where the oyster beds have not yet
126 suffered from *V. crassostreae*-related disease outbreaks¹⁵.

127
128 **Phage-bacteria infection network revealed sparse but modular interaction.** To investigate
129 phage host-range, phages isolated in Brest (n=243) and Sylt (n=31) were tested against *V.*
130 *crassostreae* isolates from the time series (n=117), previously sampled in Brest (n=34) or in
131 Sylt (n=51) and representative members of other *Vibrio* species (n=97), summing to 299
132 potential hosts. Interactions were assessed by drop-spotting viral lysates onto host lawns to
133 test for plaque formation in an all-by-all assay. To prevent “lysis from without” sometimes
134 observed with high phage concentrations¹⁸, all phages were normalized to 10³ PFU/drop using
135 the original host of isolation. Clearing on plates was assessed after 48 hours. Of the 81,926
136 tested interactions, only 1,861 were positive (2.2%, Fig. S3). Most phages were specific to *V.*
137 *crassostreae* with only 14 phages infecting member(s) of other *Vibrio* species. Among these,

138 phage 6E35.1 showed the broadest host range with 26 sensitive *V. crassostreae* isolates from
139 Brest or Sylt and eight strains from other species. Focusing on the *V. crassostreae* species, the
140 matrix suggested that killing tends to occur between subsets of hosts and phages (Fig. S3).
141 However, due to our experimental design, the host-range analysis may be confounded by
142 clonal isolates from the same samples. We considered as potential clones, vibrio isolates with
143 100% *gyrB* sequence identities and identical patterns of susceptibility in the cross-infection
144 matrix assays. Removal of potential clones resulted in 157 strains, 90 from the time series,
145 and 34 and 33 previously sampled in Brest and Sylt, respectively (Table S3). Phages showing
146 identical patterns of infectivity were also considered clonal, with 76 phages selected as
147 representative of each clone. We found that a minimum set of 24 out of 76 phages was
148 sufficient to kill a maximum of 107 out of 157 (68%) *V. crassostreae* isolates, with a mean
149 infectivity of six hosts per phage.

150
151 We sought to explore how the phylogenetic diversity shapes the structure of *V. crassostreae*-
152 phage interaction. We assembled the genome sequences of the 157 isolates, with the number
153 of contigs ranging from 16–824 (Table S3). We observed that the *V. crassostreae* core
154 genome phylogeny formed eight tight clades (V1 to V8) but with different phylogenetic depth
155 (Fig. 1a). Within the less diverse clades, the median single nucleotide polymorphism (SNP)
156 were 900 (clade V1), 1650 (clade V2), 2183 (clade V3), 104 (clade V4) and 2335 (clade V5).
157 The most closely related genomes were separated by 16 single nucleotide polymorphisms
158 (SNP) and considerable gene content variation (Fig. S4), confirming the non-clonality of the
159 isolates. We next characterized the diversity of the *V. crassostreae* infecting phages. Electron
160 microscopy revealed that all phages belong to the *Caudovirales*, with 32, 15, and 29 of 76
161 being podoviruses, myoviruses, and siphoviruses, respectively (Fig. S5-7). Genome
162 sequencing of these double-stranded DNA viruses revealed that many phages share common
163 genes, and that the genomes overall form distinct genomic clusters using the prefuse force
164 directed layout implemented in Cytoscape¹⁹ (Fig 1b, Fig. S8).

165
166 We thus considered the cross-test matrix in light of vibrio core genome phylogeny and phage
167 clustering (Fig. 1a). This revealed that each cluster of phages specifically kills a single clade
168 of *V. crassostreae*. The only exception was the phages from cluster P6a that infect, in addition
169 to strains from clade V6, a single strain from clade V7. This strain was otherwise resistant to
170 phages belonging to cluster P7. Vibrios from a specific clade could be infected by more than
171 one cluster, e.g., vibrios from V1 were killed by phages from cluster P1a, P1b and P1c. We
172 further asked whether the observed specificity of a phage cluster for a vibrio clade results
173 from adsorption variation or intracellular defenses. We show that a representative phage of
174 each of the 16 clusters was only able to adsorb to bacteria from its specific clade of vibrios,
175 with the exception of a phage from cluster P5b that adsorbs to vibrios from clade V5 and V6
176 (Fig. 2; Fig S9). However, within the same vibrio clade, phages adsorb similarly to all tested
177 vibrios regardless of the production of progeny and cell lysis. Hence clade-specific receptor(s)
178 and cluster-specific receptor binding protein(s) appear to constitute a first level of specificity
179 while within each clade, intracellular mechanisms likely result in a narrower range of vibrio
180 strains that are killed.

181
182 **Flexible genomes of both hosts and phages can be extensive.** We observed that *V.*
183 *crassostreae* genome sequences showed large size variation (Table S3). Strains from clade
184 V1, V6 and V7 had the smallest genomes (medium size 5-5.2 Mbp) and strains from clades
185 V2, V3, V4, V5 and V8 the largest genomes (5.4-5.8 Mbp) (Fig. 3a). The larger genomes
186 contain a higher number of genes encoding for integrases and partitions systems, indicative of
187 a higher number of MGEs. This might allow vibrios to acquire defense elements, as illustrated

188 by a higher frequency of restriction-modification (RM) systems and other known phage
189 defense systems in larger genomes. Accordingly, we observed a negative correlation between
190 genome size and the number of phages able to kill the host (Spearman's rank correlation of
191 phylogenetic independent contrasts, $\rho = -0.232$, $p\text{-value} = 0.004$). Hence, our results are
192 consistent with a major role of MGEs in the emergence of phage resistance among closely-
193 related strains as well as with the observation that the majority of the pan genome among
194 closely related strains consists of MGEs harboring phage defense genes^{9,12}.

195
196 A considerable diversity in genome length (from 21–253 kbp) and gene content (23–407
197 predicted coding sequences) was also observed for phages (Table S4). Notably the genomes
198 of the podoviruses were found to be significantly smaller than those of the myoviruses and
199 siphoviruses (21–58 kbp, Tukey's HSD, all p values <0.017) (Fig. S8b) while the broader host
200 range myovirus 6E35.1 has a comparatively larger capsid (Fig. S6) and genome size (253 kb)
201 (Table S4). Intra-cluster genomic comparisons revealed a high conservation of the genome
202 size, a high ANI value of the core genes ($>99\%$ in most clusters) but an extensive variation in
203 gene content among phages in some clusters (Fig 3b). Notably a total of 65 putative
204 recombinases (UvsX, Erf, Sak, Sak4, RedB and Gp2.5) were identified in 50 out of 76 phage
205 genomes (Table S4). Altogether our results indicate a recent phage diversification by
206 recombination possibly involving escape mechanisms to host defense, a hypothesis we test
207 further below.

208
209 **Within host clades, diverse intracellular mechanisms control phage production.** Our
210 genome analysis revealed that vibrios with smaller genomes carry fewer genes with phage
211 defense annotation and tend to be infected by more phages (Fig.3a). We therefore
212 hypothesized that the identification of immunity mechanism should be facilitated in smaller
213 genome hosts that are resistant to phages. In clade V1 (medium genome size 5Mbp), only one
214 strain (7F1_18) out of 12, appeared to be resistant to all siphoviruses from cluster P1a and all
215 podoviruses from cluster P1b. Because cross-infection tests were done at a constant, relatively
216 low phage concentration (10^3 PFU), we sought to refine estimates of host susceptibility of
217 vibrios from clade V1. First, pairwise interactions were assessed by drop-spotting serial
218 dilution of the phage lysates on host lawns. Second the production of phages was assessed by
219 efficiency of plating (EOP). Combined methods allowed us to classify the strains as
220 “sensitive” or “partially sensitive” if a clearing and viable phage production was obtained at a
221 low or high titer, respectively. The strains were classified as “resistant but impaired” if we
222 observed a turbid clearing zone but no production of viable phages when using high titers.
223 This phenotype may either arise from “lysis from without” (lysis is effected by viral
224 adsorption or extracellular compounds) or abortive infection¹⁸. Our exploration of host
225 susceptibility at a finer resolution led us to classify the strain 7F1_18 as resistant but impaired
226 to all nine siphoviruses from cluster P1a and a subset of three podoviruses from cluster P1b
227 (named P1b_{blue}) (Fig. 4a, b). However, the strain 7F1_18 was partially sensitive to a second
228 subset of P1b phages (named P1b_{red}), highlighting diversity among podoviruses from P1b.

229
230 We next explored the genetic determinants of resistance in strain 7F1_18. Genome
231 comparison identified only two genomic regions (1 α and 1 β) specific to this strain (Fig. 4c).
232 These regions encode for known anti-phage systems. In region 1 α , the RADAR defense
233 system consists of an adenosine triphosphatase and a divergent adenosine deaminase that
234 might cause editing-dependent abortive infection in the presence of the phage¹¹. The genes
235 *yeeA* and *yeeB*, encode a DNA methylase and a helicase respectively. A type III restriction
236 modification (RM) system²⁰ was identified in the region 1 β . Genetic knock out of regions
237 1 α and 1 β further demonstrated their role in 7F1_18 immunity. The deletion of region 1 α was

238 sufficient to restore full sensitivity to all P1a and P1b_{red} phages (Fig. 4a, b and Fig. S10, S11).
239 Single gene inactivation further showed that only Radar was involved in defense to phages
240 P1a while only *yeeAB* mediated resistance to P1b_{red} (Fig. 4a, b and Fig. S10, S11). A
241 subsequent deletion of region 1 β or the inactivation of the RM III system was necessary to
242 confer full sensitivity to all P1b_{blue} phages (Fig. 4, Fig. S11). This suggested that P1b_{red} but
243 not P1b_{blue} evolved a RM III escape mechanism. Genome comparison identified two genes
244 encoding for unknown function that are present in all P1b_{red} and absent in all P1b_{blue} phages
245 (Fig. S12). Altogether, our results demonstrate that testing diverse phages will often be
246 necessary to define the role of defense genes and some of them can act additively. Our data
247 also suggested that phages P1b_{red} diversified from P1b_{blue} by acquiring a protection toward a
248 restriction system.

249
250 **Bacterial defense and phage anti-defense interplay led to host shift.** The examination of
251 the interactions between vibrios from clade V5 and phages from cluster P5a revealed an
252 additional level of modularity (Fig. 5a). Subsets of phages, designated P5a_{red} and P5a_{blue}
253 exclusively killed a subset of vibrios designated V5_{red} and V5_{blue}, respectively. We showed
254 above that representatives of P5a_{red} and P5a_{blue} were able to adsorb to all tested V5 strains
255 (Fig. 2). We hypothesized that the specificity of killing depends on the interplay between
256 bacterial defense and phage anti-defense with consequences for phage specificity.

257
258 Comparative genomics revealed that six genomic regions (5 α , 5 β , 5 χ , 5 δ , 5 ϵ , 5 ϕ) are found
259 only in all V5_{red} vibrio strains (Fig. S13). This is consistent with the observation that vibrios
260 from clade V5 have larger genomes (medium size 5.7 Mbp) and a higher number of known
261 phage defense elements (Fig. 1). Simultaneous deletions within regions 5 β and 5 ϵ resulted in
262 V5_{red} (strain 29_O_45) sensitivity to P5a_{blue} phages (Fig. 5c and Fig. S14). In region 5 β (Fig.
263 5b), the deleted genes (*dndFGH*) are part of the Dnd system, an innate defense system with
264 functional similarity to methylation-based R-M systems²¹⁻²³. DndA-E proteins catalyse
265 phosphorothionate modifications (replacement of oxygen by sulfur in the DNA sugar-
266 phosphate backbone) and the DndFGH proteins use the absence of this modification to
267 identify foreign DNA and cause double-stranded breaks. In region 5 ϵ (Fig. 5b), the two
268 deleted genes encode a reverse transcriptase and a trans-membrane domain protein, homologs
269 of a two-gene phage resistance system, the retron family Ec48, which confers resistance to
270 phage via abortive infection²⁴.

271
272 EOP allowed higher accuracy in assessment of phage infectivity. P5a_{blue} phages were pro-
273 duced at high levels (10¹⁰ PFU/ml) in a V5_{blue} host (strain 28_O_24) whereas two orders of
274 magnitude fewer phages were produced in a V5_{red} derivative lacking both Dnd and Ec48
275 retron defense (Δ Dnd Δ retron) (Fig. 6b). A third deletion in regions 5 α , χ , δ or ϕ did not modi-
276 fy this phenotype, suggesting that additional unknown defense mechanism(s) control the full
277 production of phage progeny in V5_{red}, strain 29_O_45. No P5a_{blue} phage progeny was pro-
278 duced in the V5_{red} wild type host or a derivative carrying the Ec48 retron and lacking Dnd
279 (Δ Dnd). However, P5a_{blue} phages were produced, but at lower titers (10⁴ PFU/ml), in a V5_{red}
280 derivative carrying Dnd and lacking Ec48 (Δ retron). In summary, among six genomic regions
281 specific to V5_{red} vibrio, we identified two anti-phage systems that are cumulative, the Ec48
282 retron being more effective in preventing P5a_{blue} phage production than the Dnd defense sys-
283 tem.

284
285 To understand how P5a_{red} phages evolved to counter vibrio V5_{red} defense systems, Dnd and
286 Ec48 retron, we compared the genomes of podoviruses from cluster P5a. We found only two
287 genes that are specific to P5a_{red} phages (Fig. 6a and S15). Gene p0019 in 44E38.1 encodes a

288 protein of unknown function and gene p0018 encodes a 479 amino acid (aa) protein
289 consisting of two domains: an N-terminal (aa 4–175) phosphoadenosine phosphosulphate
290 reductase (PAPS²⁵) domain and a C-terminal (aa 299–470) DNA N-6-adenine-
291 methyltransferase (Dam²⁶) domain. A PAPS domain (aa 46–228) is also present in the
292 sulphotransferase encoded by *dndC* in V5_{red} vibrios Dnd defense system. The P5a_{red} Dam
293 domain shares 96% identity with a 178 aa methylase gene (p0019) encoded by P5a_{blue} phage
294 66E30.1. This suggests a chimeric origin for the p0018 encoded protein, as described for an
295 endonuclease that provides ICP1 phage immunity in *V. cholerae*²⁷. Genome comparison also
296 revealed a 5.7kb sequence that diverges between the P5a_{blue} and P5a_{red} phages (Fig. 6a). The
297 region encodes an exonuclease with an RNaseT/DNA polymerase III domain, a single-strand
298 DNA binding protein, two proteins of unknown function and a putative low fidelity single-
299 strand annealing protein (SSAP)-based recombinase system, consisting of two genes similar
300 to λ red²⁸.

301
302 We hypothesized that the incorporation of a PAPS domain by the phage P5a_{red}- conferred
303 resistance to the vibrio V5_{red} defense system Dnd. A P5a_{blue} phage was engineered using
304 homologous recombination with a plasmid carrying regions identical to the P5a_{red} and P5a_{blue}
305 phages genome and flanking the two P5a_{red} specific genes (see Methods). This plasmid was
306 transferred by conjugation into a V5_{blue} strain or the V5_{red} derivative Δ retron strain.
307 Conjugants were infected by a P5a_{blue} phage (66E30.1) and recombinants were enriched using
308 Δ retron as host, because Δ retron (i) is partially sensitive to phage P5a_{blue} and therefore allows
309 the production of progeny, (ii) carries the Dnd defense system that might select Dnd-resistant
310 recombinants, and (iii) recombinants might remain sensitive to the retron. We obtained
311 recombinant phages at a high frequency (30%) using Δ retron as host for both recombination
312 and selection. All isolated recombinants (designated P5a_{blue}-PAPS) were able to infect the V5_{red}
313 derivative Δ retron (10¹¹ PFU/ml) (Fig. 6b and S16). Thus, the P5a_{red} specific genes encode an
314 anti-Dnd system that is related at least in part to the acquisition of a PAPS domain fused to a
315 methylase.

316
317 P5a_{blue}-PAPS phage lost infectivity for V5_{blue} (Fig. 6b, c and S16), demonstrating that the
318 P5a_{blue}-specific genes are necessary to infect V5_{blue}. Two of the genes encode for methylases
319 (Fig. 6a) in 66E30.1: p0019, annotated as a Dam methylase, and p0020 is a N-4 cytosine-
320 specific and N-6 adenine-specific DNA methylase²⁹. These genes likely counteract
321 degradation by V5_{blue} restriction enzyme(s) yet to be identified. Thus by exchanging anti-
322 defense genes, phages irreversibly switch host. This could be indicative of co-evolution
323 following the matching-allele-model of specificity³⁰ where an exact genetic match is required
324 for infection.

325
326 In an attempt to identify phages that can escape the retron system, we noticed that infection by
327 P5a_{blue}-PAPS resulted in the production of plaques that escape retron immunity (Fig. 6a).
328 Compared to the Δ retron vibrio strain, EOPs were, respectively, 10⁻⁵ and 10⁻³ using V5_{red} and
329 Δ Dnd as host. When isolated and further propagated on V5_{red} these escapers showed the same
330 infectivity as P5_{red} (Fig. S16), and are thus likely spontaneous mutants (P5a_{blue}-PAPS-retronR). We
331 hypothesized that the 5.7kb sequence that diverges between the P5a_{blue} and P5a_{red} phages
332 isolated in nature is involved in P5a_{red} resistance to retron. Sequencing this region of three
333 laboratory generated retron-escaper phages revealed non-synonymous mutations that
334 distinguished the mutants from the ancestor (Fig. S17), all localized in the exonuclease
335 (p0028 in 66E30.1). Mutations in the exonuclease gene (single mutations, deletions, or
336 integrations) were also observed in 8/10 additional mutants. Spontaneous coliphage mutants
337 have previously been isolated that overcome the defense conferred by Ec48 retron¹². All

338 mutations abolished the function of RecBCD phage-encoded inhibitors (Gam in λ , gp5.9 in
339 T7), a host complex involved in DNA repair and anti-phage activity. It was proposed that
340 Ec48 “guard” RecBCD and that Ec48 activity is triggered by phage-mediated RecBCD
341 inhibition¹². None of these inhibitors were identified in the P5a genomes, suggesting that: (i)
342 P5a phages encode non-orthologous protein(s) with similar inhibitory effects on RecBCD; or
343 (ii) retron activity or phage retron escape depends on a mechanism distinct from RecBCD
344 guarding.

345
346 **Negative-frequency dependent selection might drive phage-vibrio coevolution in this**
347 **natural system.** Our analyses characterize the genetic basis of antagonistic coevolution
348 between *V. crassostreae* and its phages. While our present sampling density does not allow
349 for an in-depth analysis of coevolutionary dynamics, the spatial and temporal variability of
350 phage infectivity and results from cross-inoculation experiments are consistent with the
351 hypothesis that negative-frequency dependent selection drives phage-vibrio coevolution at the
352 level of phage clusters and vibrio clades. First, across space (Brest *versus* Sylt), phage
353 infectivity is higher on sympatric than on allopatric phage-host combinations (Fig. S18a).
354 This spatial pattern results from the non-overlapping distribution of *Vibrio* clades across
355 locations (Fig. S18b) implying that phages sampled from a given location have a lower
356 chance of finding compatible hosts to infect in allopatry. Second, over time, the mean
357 infectivity peaked for contemporary combinations and declined as phages were inoculated on
358 bacterial strains sampled from more distant sampling dates (“past and future”) ³¹⁻³³ (Fig. S19).
359 The presence of phages of a given clade was significantly associated with the presence of the
360 corresponding *V. crassostreae* clade at that time. This pattern is characteristic of fluctuating
361 selection dynamics, whereby phage populations are maximally adapted to their contemporary
362 bacterial populations³⁴. Evidence of modular patterns of specificity at the between-clade level
363 (Fig. 1a) and the matching-allele model of specificity within clade V5 (Fig. 6c) further support
364 this hypothesis.

365 366 **CONCLUSION**

367
368 We dissected the genetic mechanisms driving the specificity of the interaction between *V.*
369 *crassostreae* and their viral predators at different stages of the infection. Phage adsorption
370 matched bacterial clades within the *V. crassostreae* species, suggesting clade-specific
371 receptor(s) and cluster-specific receptor binding protein(s). In the future, the identification of
372 the receptor(s) of each phage cluster should allow us exploring their presence and diversity
373 among the *V. crassostreae* clades and interpretation of how selection acts on these. We can
374 expect that receptor evolution might be constrained due to a role of these surface structures
375 for the fitness of a bacterial clade in the natural environment³⁵. Our results are consistent with
376 the previously described major role of MGE in the emergence of phage resistance among
377 closely-related strains and these MGEs constituting the majority of the flexible genome ^{9,12}
378 which is supported here by the variation in vibrio genome size which correlates with
379 resistance, the number of phage defense elements and the identification of diverse anti-phage
380 mechanisms localized in defense genomic regions.

381
382 We identify a striking parallel in phage evolving counter defenses to these highly mobile host
383 defenses. First, within a cluster of closely related phages, gene variation can be extensive but
384 the total number of genes per genome is known to be constrained by the capsid size.
385 Accordingly, we identified in podoviruses a mechanism of adaptation by gene exchange
386 rather than gene addition. Exploring phages with larger genomes (such as the myovirus) will
387 decipher whether these phages are more permissive to gene acquisition at multiple loci and/or

388 prone to faster coevolutionary dynamics. Second, homologous recombination system such as
389 the SSAP-like recombinases identified in phage P5a, have been identified in numerous
390 vibriophage genomes and have been suggested to play a role in overcoming bacterial anti-
391 phage defenses by allowing survival of recombinant progeny (Kauffman [*co-submitted*] in
392 revision). This leads us to speculate that gene variation in phage mirror the turnover of MGEs
393 encoding for resistance in the hosts and by analogy to the Bernheim and Sorek model¹², we
394 propose a “pan-escape system” that can be shared among phages by homologous
395 recombination within a population that infect a bacterial host.

396

397 MATERIAL AND METHODS

398

399 Sampling.

400 Samples were collected from an oyster farm located at the Bay of Brest (Pointe du Château,
401 48° 20' 06.19" N, 4° 19' 06.37" W), every Monday, Wednesday and Friday from the 3rd of
402 May to the 11th of September 2017. Specific Pathogen Free (SPF) juvenile oysters^{17,36} were
403 deployed in the field in batches of 100 animals. When the first mortalities were observed in
404 the first batch, another batch of SPF animals was placed in the field, leading to the
405 consecutive deployment of 7 batches from the 26th of April to the 11th of September. Oyster
406 mortalities were recorded on each sampling day. Oysters were always collected after a
407 minimum of 7 days of incubation in the field.

408 On each sampling date, five living oysters were collected from a batch showing <50%
409 mortalities. The animals were cleaned, shucked, weighed and 2216 Marine Broth (MB) was
410 added (10mg/ml) for homogenization using an ultra-turrax. A volume of 100 µL homogenate
411 was used for vibrio isolation, the remaining volume was centrifuged (10 min, 10,000 rpm),
412 the supernatant filtered through a 0.2 µm filter and stored at 4°C until the phage isolation
413 stage. Two liters of seawater were collected and size fractionated as previously described¹³.
414 Bacterial cells from 0.2 µm filters were suspended in 2 mL MB and 100 µL of this suspension
415 was used for vibrio isolation. The iron chloride flocculation method³⁷ was used to generate
416 1000-fold concentrated viral samples from 2 liters passaged through a 0.2µm filter, following
417 the previously described protocol³⁸. Virus-flocculates were suspended in 2mL 0.1M EDTA,
418 0.2M MgCl₂, 0.2M oxalate buffer at pH6 and stored at 4 °C until the phage isolation stage.

419

420 *Vibrio crassostreae* isolation, identification and genome analysis.

421 *Isolation and identification.* Vibrios from seawater or oyster tissues were selected on
422 Thiosulfate-citrate-bile salts-sucrose agar (TCBS). Roughly 48 colonies were randomly
423 picked from each plate and re-isolated once on TCBS, then on 2216 Marine agar (MA). *V.*
424 *crassostreae* isolates were first identified by PCR using a primer set targeting the *r5.2* gene
425 (previously identified as population specific marker¹⁴ (Table S5) and colonies as template.
426 PCR positive isolates were grown in MB and stored at -80°C in 10% DMSO. Their taxonomic
427 assignment was further refined by *gyrB* gene sequencing¹⁴. Bacteria were grown overnight in
428 MB and DNA extracted using an extraction kit (Wizard, Promega) according to the
429 manufacturer's instructions. The partial *gyrB* gene was amplified using degenerate primers
430 (Table S5), Sanger sequenced (Macrogen) were manually corrected with the chromatogram.
431 Sequences were aligned with Muscle and phylogenetic reconstruction was done with RAxML
432 version 8 GTR model of evolution, a gamma model and default parameters³⁹.

433 *Quantification of V. crassostreae from seawater fractions.* Quantification of *V. crassostreae*
434 from seawater size fractions (>60µM, 60-1 µM, 5-1 µM and <1 µM) was performed using
435 quantitative PCR (qPCR). DNA was extracted from filters or 1mg of oyster tissues using the
436 Wizard Genomic DNA extraction kit (Promega). All amplification reactions were analysed
437 using a Roche LightCycler 480 Real-Time thermocycler (Genomic platform SBR). The total

438 qPCR reaction volume was 25 μl and consisted of 4 μl DNA (2,5 $\text{ng } \mu\text{l}^{-1}$) and 12,5 μl
439 LightCycler 480 SYBR Green I Master mix (Roche) containing 0.2 μM PCR primer (Table
440 S5) (Eurofins SA) with the following program: enzyme activation at 95°C for 10 min,
441 followed by 40 cycles of denaturation (95°C, 10 s), hybridization (60°C, 20 s) and elongation
442 (72°C, 25 s). A subsequent melting temperature curve of the amplicon was performed to
443 verify the specificity of the amplification. Absolute quantification of bacterial DNA copies
444 were estimated by comparing the observed Cq values to a standard curve of the amplification
445 product cloned into the pCR2.1-TOPO vector.

446
447 *Genome sequencing, assembly and annotation.* *V. crassostreae* DNAs were sequenced by the
448 Joint Genome Institute using 300 bp library and HiSeq2000 illumina sequencing technology
449 or at the BioMicro Center at the MIT using Nextera FLEX for library and NextSeq 80PE for
450 sequencing. Contigs were assembled de novo using Spades 3.11⁴⁰. Computational prediction
451 of coding sequences and functional assignments were performed using the automated
452 annotation pipeline implemented in the MicroScope platform⁴¹.

453 *Core genome phylogeny.* The proteome of each isolates was compared by performing a Blastp
454 all-vs-all. Silix⁴² was used to reconstruct protein families based on 80% of reciprocal length of
455 alignment and an identity of at least 80% for *V. crassostreae*. Protein sequences of each
456 family were first aligned with Muscle, filtered using Gblocks with relaxed parameters⁴³ and
457 concatenated. Phylogenetic reconstruction was done using RAxML version 8³⁹ on this
458 concatemer using an LG model of evolution, a gamma model and default parameters.

459 *Comparative genomics.* The ANI-value of genomes was determined using pyani
460 (<https://github.com/widdowquinn/pyani>). The phylogenetic profile method implemented in
461 the MicroScope platform⁴¹ was used to identify putative phage resistance genes and regions.
462 To this aim we searched for genes present in all strains resistant to phage (80% identities on
463 80% coverage) and absent from sensitive strains. The same approach was used to estimate
464 specific genes of *Vibrio* in pairwise genome comparisons.

465

466 **Phage isolation, identification and genome analysis.**

467 *Isolation and generation of high titer stocks.* We used the methods previously described by
468 Kauffman and coll³⁸. Briefly isolation of phages was performed by directly plating on a
469 bottom agar plate (1.5% agar, in MB) 100 μL of an overnight bacterial culture, 20 μL of
470 seawater flocculate (equivalent to 20 mL of seawater containing viruses) or 20 μL of oyster
471 homogenate and 2.5 ml molten top agar (55 °C, 0.4% agar, in MB) to form host lawns in
472 overlay and allow for plaque formation. After incubation for 48h at room temperature (RT), a
473 maximum of six plaques per morphotype was archived. Plaque plugs were first eluted in 500
474 μl of MB for 24 h, 0.2- μm filtered to remove bacteria, and re-isolated three times on the
475 sensitive host for purification before storage at 4°C and, after supplementation of 25%
476 glycerol at -80°C. High titer stocks ($>10^9$ PFU/ml) were generated by confluent lysis in agar
477 overlays.

478 *Electron microscopy.* Following concentration on centrifugal filtration devices (Millipore,
479 amicon Ultra centrifugal filter, Ultracel 30K, UFC903024), 20 μl of the phage concentrate
480 were adsorbed for 10 min to a formvar film on a carbon-coated 300 mesh copper grid (FF-300
481 Cu formvar square mesh Cu, delta microscopy). The adsorbed samples were negatively
482 contrasted with 2% Uranyl acetate (EMS, Hatfield, PA, USA). Imaging was performed using
483 a Jeol JEM-1400 Transmission Electron Microscope equipped with an Orious Gatan camera.
484 at the platform MERIMAGE (Station Biologique de Roscoff, France).

485 *DNA extraction, sequencing, assembly and annotation.* Phage DNA extractions were
486 performed from high titer suspensions using the MasterPure™ Complete DNA and RNA
487 Purification Kit (Epicentre), according to the manufacturer's instructions. Alternatively, DNA

488 was extracted following a previously described protocol³⁸. Phage suspensions were
489 concentrated on centrifugal filtration devices (30 kDa Millipore Ultra Centrifugal Filter,
490 Ultracel UFC903024) and washed with 1/100 MB to decrease salt concentration. The
491 concentrates were treated for 30 min at 37°C with 10µL of DNase (Promega) and 2,5µL of
492 RNase (Macherey-Nagel) at 1000 unit and 3,5mg/mL, respectively. These nucleases were
493 inactivated by adding EDTA (20 mM, pH8). DNA extraction encompassed a first step of
494 protein lysis (0.02 M EDTA pH 8.0, 0.5 mg/ml proteinase K, 0.5% sodium dodecyl sulfate)
495 for 30 min incubation at 55°C, a phenol chloroform extraction and an ethanol precipitation.
496 DNA was visualized by agarose gel electrophoresis (0.7% agarose, 50 Volt, overnight at 4°C)
497 and quantified using QuBit. Phages were sequenced by the Biomics platform at the Pasteur
498 Institute using NextSeq Illumina technology. The assembly, annotation and comparative
499 analysis were performed as described above for *V. crassostreae* genome.

500 *Phage clustering*. The phage proteome was used to reconstruct a network showing the shared
501 families using the force-directed layout implemented in Cytoscape¹⁹

502

503 **Host range determination.**

504 *Single-phage-by-single-host host range infection assay*. Host range assays were carried out
505 using a robot hosted at EligoBioscience (Paris, France) or manually using an electronic
506 multichannel pipette by spotting 5 µL of the phage suspension normalized at 2×10^5 PFU/ml
507 (10^3 PFU/spot) on the agar overlay inoculated with the tested host. Plates were incubated
508 overnight at room temperature and plaque formation was observed after 24 hours. Spot assays
509 were performed in duplicate and positive interactions were confirmed in a third experiment.

510 *Classification of host sensitivity*. To explore the sensitivity of bacteria, 10-fold serial dilutions
511 of phages ($1-10^{-7}$ PFU) were prepared and 5 µL drop spots of each dilution were pipetted onto
512 bacterial host lawns. For some spot tests, turbid plaques were observed for the highest
513 concentrations of phage lysates. To determine whether the bacterial host was sensitive,
514 partially sensitive or insensitive but impaired, we explored the titer of the phage on a given
515 bacteria compared to the maximum titer observe (i.e. with the host used to produce the
516 phage). A total of 5µL of serial phage dilutions was mixed with 100 µL of an overnight host
517 culture and 2,5 ml top agar to form host lawns in overlay and plaques were counted after
518 24hours. In sensitive and partially sensitive hosts plaques were obtained using 1-10 and 10^5 -
519 10^6 PFU respectively. In resistant but impaired host no plaque was observed using up to 10^7
520 PFU.

521 *Phage adsorption*. Phage adsorption experiments were performed as previously described⁴⁴.
522 Phages were mixed with exponentially growing cells (OD0.3; 10^7 CFU/mL) at a MOI of 0.01
523 and incubated at RT without agitation. At 0, 15 and 30 minutes, 250 µL of the culture was
524 transferred in a 1.5 mL tube containing 50 µL of chloroform and centrifuged at 14,000 rpm
525 for 5 min. The supernatant was 10-fold serially diluted and drop spotted onto a fresh lawn of a
526 sensitive host to quantify the remaining free phage particles.

527

528 **Molecular microbiology.**

529 *Strains and plasmids*. All plasmids and strains used or constructed in the present study are
530 described in Table S6 and S7. *V. crassostreae* isolates were grown in Luria-Bertani (LB), or
531 LB-agar (LBA) +0.5 M NaCl at RT. *Escherichia coli* strains were grown in LB or on LBA at
532 37°C. Chloramphenicol (5 or 25µg/ml for *V. crassostreae* and *E. coli*, respectively),
533 thymidine (0.3 mM) and diaminopimelate (0.3 mM) were added as supplements when
534 necessary. Induction of the P_{BAD} promoter was achieved by the addition of 0.2% L-arabinose
535 to the growth media, and conversely, was repressed by the addition of 1% D-glucose.
536 Conjugation between *E. coli* and *Vibrio* were performed at 30°C as described

537 previously⁴⁵ with the exception that we used TSB-2 (Tryptic Soy Broth supplemented with
538 1.5% NaCl) instead of LB for mating and selection.

539 *Clonings.* All clonings in pSW7848T were performed using herculase II fusion DNA
540 polymerase (Agilent) for PCR amplification and the Gibson Assembly Master Mix (New
541 England Biolabs, NEB) according to the manufacturer instructions. Before cloning in
542 pSW23T, a PCR fragment was amplified using GoTaq DNA polymerase (Promega) and
543 subcloned in a TOPO cloning vector (Invitrogen). The plasmid miniprep was digested with
544 *EcoRI* (NEB) and the insert was cloned in pSW23T. The phage specific region was amplified
545 using the herculase, digested with *ApaI* and *XbaI* and cloned in PSU18T- P_{BAD} instead of the
546 *gfp* gene. All clonings were first confirmed by digesting plasmid minipreps with specific
547 restriction enzymes and second by sequencing the insert (Macrogen).

548 *Vibrio mutagenesis.* Gene inactivation was performed by cloning an internal region of the
549 target gene in the suicide plasmid pSW23T⁴⁶. After conjugative transfer, selection of the
550 plasmid-borne drug marker (Cm^R) resulted from integration of pSW23T in the target gene by
551 a single crossing-over. Region deletion was performed by cloning 500bp fragments flanking
552 the region in the pSW7848T suicide plasmid⁴⁵. This pSW23T derivative vector encodes the
553 *ccdB* toxin gene under the control of an arabinose-inducible and glucose-repressible promoter,
554 P_{BAD} ⁴⁵. Selection of the plasmid-borne drug marker on *Cm* and glucose resulted from
555 integration of pSW7848T in the genome. The second recombination leading to pSW7848T
556 elimination was selected and arabinose media.

557 *Phage mutagenesis.* $P5_{blue}$ phage was engineered using double crossing over with a plasmid
558 carrying regions of homology (438 and 156 bp) to the phage genome $P5_{red}$. A 3745bp region
559 of the phage $P5_{red}$ (44E38.1) was amplified by PCR and cloned in a replicative plasmid ($P15A$
560 *oriV*; Cm^R) under the control of the conditional P_{BAD} promoter. Selection of the
561 transformants on *Cm* + Glucose 1% prevented the expression of toxic phage genes. This
562 plasmid was transferred by conjugation to a $V5_{blue}$ strain (28_O_24). Plate lysates were
563 generated by mixing 500 μ l of an overnight culture of the transconjugant with the $P5_{blue}$ phage
564 (66E30.1) and plating in 7.5 ml agar overlay. After the development of a confluent lysis of
565 lawns, the lysate was harvested by addition of 10 mL of MB, shredding of the agar overlay
566 and stored ON at 4°C for diffusion of phage particles. The lysates were next centrifuged, the
567 supernatant filtered through 0.2 μ m filter and stored at 4°C. Recombinant phages were
568 enriched by infecting the $P5_{red}$ (29_0_45) derivative Δ retron in agar overlays. Recombinant
569 phages were screened by PCR using a primer set targeting the $P5_{red}$ specific gene
570 (PODOV008_V2_p0019 in 44E30.1) and single plaque as template. The recombination was
571 further confirmed by sequencing genes that are polymorphic between between $P5_{red}$ and $P5_{blue}$
572 phages.

573 To isolate mutant phages that escape Ec48 defense, $P5_{red}$ or $P5_{red-PAPS}$ phages were plated on
574 $V5_{red}$ wild type or Δ Dnd derivative using the double-layer plaque assay. Plaques were
575 obtained only using the $P5_{red-PAPS}$ as viral source and 10 single plaques were picked for re-
576 isolation. The region (from p0024 to p0032 in 66E30.1, 5.3kb) that shows polymorphism
577 between the $P5_{red}$ and $P5_{blue}$ phages was PCR amplified sequenced. Reads were aligned to the
578 ancestor genome.

579 580 **Time shift analysis.**

581 To characterize coevolutionary dynamics between *V. crassostreae* and its phages, we
582 examined how phage infectivity varied with the time shift between *V. crassostreae* and phage
583 isolates^{32,47}. This was done for 1 to 15 *V. crassostreae* colonies per sampling day (median 3
584 colonies), and 1 to 46 47 phage strains (median 6 strains), for a total of 254,974687 cross-
585 inoculations. Infectivity was a binary variable coding whether the phage can infect or not the
586 bacterial isolate. The mean infectivity as a function of time shift category peaked around the

587 present and declined as phages were inoculated on bacterial strains of the past and the future
588 (Fig. S19a). This pattern is characteristic of fluctuating selection dynamics, whereby phage
589 populations are maximally adapted to their contemporary bacterial populations. We fitted to
590 this pattern a smooth unimodal relationship (proportional to the density of a skew-normal
591 distribution) by least-squares (Fig. S19b).

592 It is difficult to statistically test for the significance of this pattern, as it emerges from non-
593 independent combinations of *V. crassostreae* and phage strains, and with limited sampling of
594 diverse populations at each time point that can generate spurious temporal fluctuations. As a
595 simple approach, we compared the difference in infectivity of contemporary vs. non-
596 contemporary combinations. Phages could infect contemporary bacteria in 156211/1,843392
597 combinations (frequency: 0.11105), and non-contemporary bacteria in 12151188/243,14915
598 combinations (frequency: 0.049524). The difference was significant according to a test based
599 on binomial probabilities ($p = 0.0085799$ for the null hypothesis that the true frequency is the
600 same for the two groups).

601

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751 **Author contributions**

752 FLR and DB conceived of the project. FLR wrote the paper with contributions of DP, MB,
753 YL, FB, MW, FAH, KK, MP, DB and SG. DP performed phage-vibrio interaction
754 experiments with assistance from SC, RBC and EL. MB performed the *in silico* analyses with
755 assistance from KK. YL and FLR performed the genetics. SL performed the electronic
756 microscopy analyses. DP, YL, SC, AJ, BP and FLR established the times series sampling.
757 MKW, FLR and JD isolated the phage and vibrio collections from Sylt. FAH and MP
758 performed and funded part of the vibrio sequencing. FB and SG designed, FB performed the
759 time-shift analysis. FLR supervised the project and secured funding.

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761 **Competing interests**

762 Authors declare no competing interests.

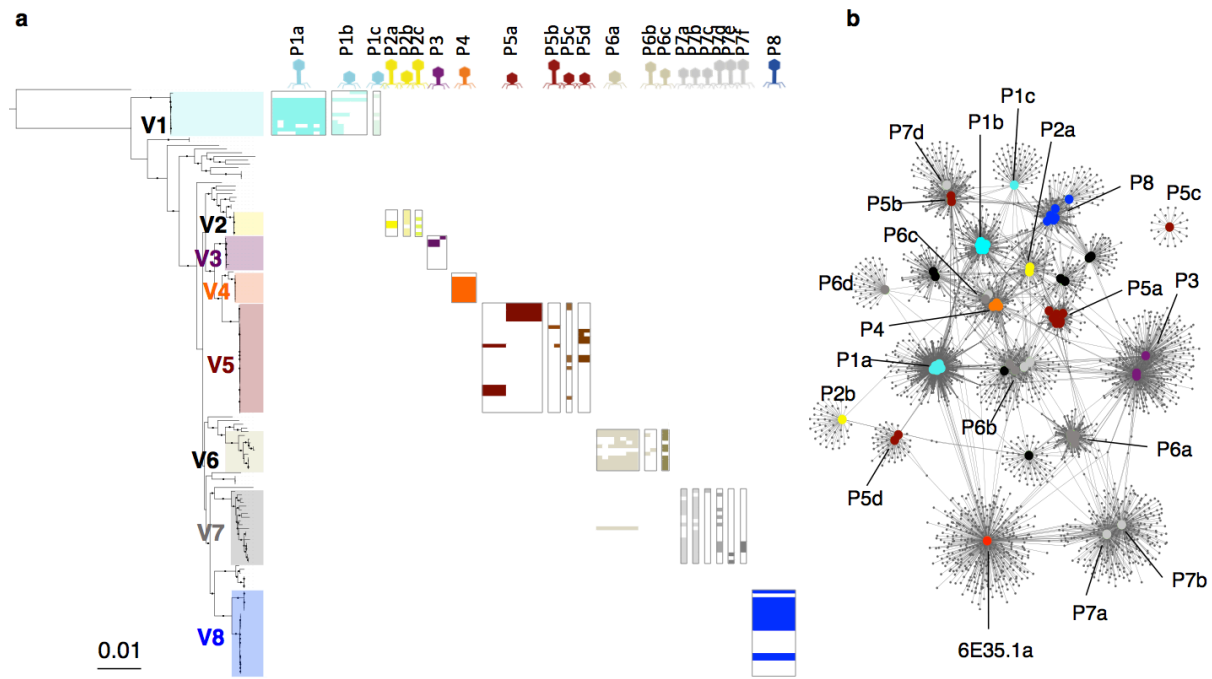
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764 **Data and materials availability**

765 New genomes used in this work have been deposited under the NCBI BioProject with accession
766 numbers presented in Table S3 and S4. All data, code, and materials are available upon request.

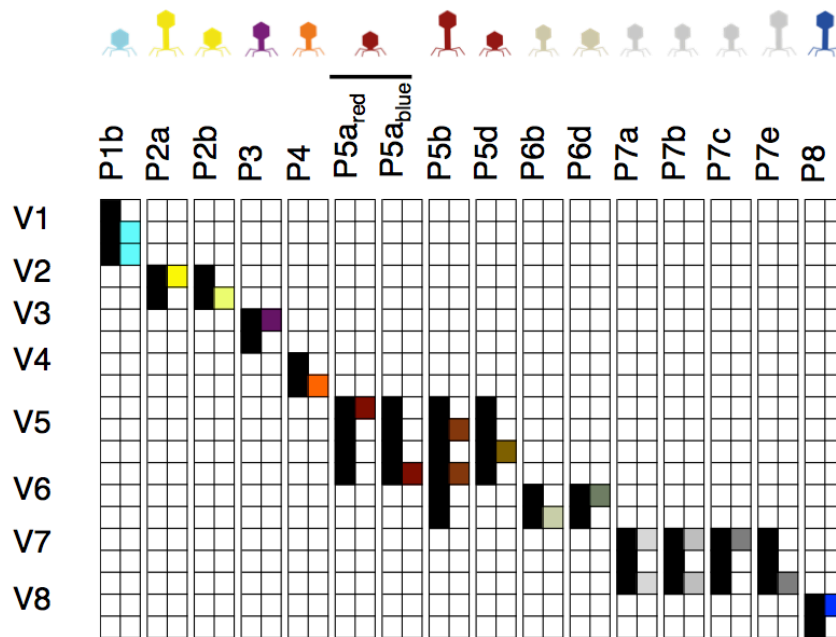
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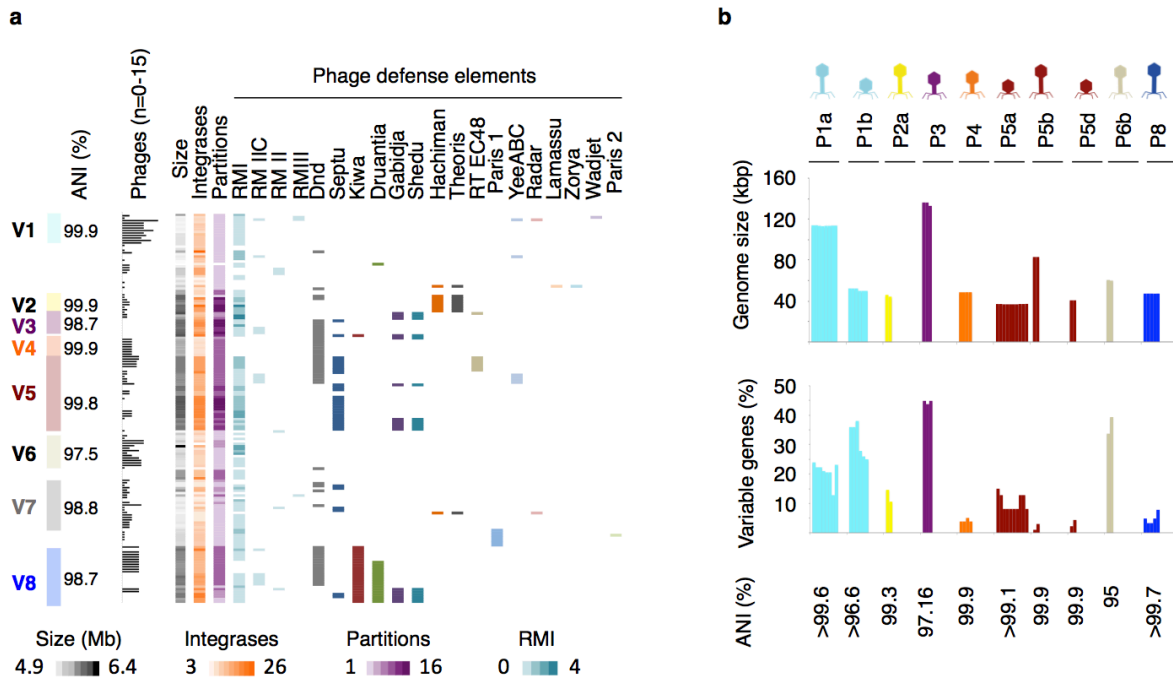
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Figure 1. The modularity of the phage-vibrio infection network involves phylogenetic clade within *V. crassostreae* and genomic cluster of phages. a, Host range matrix for assay of genome-sequenced phages on genome-sequenced hosts. Rows represent *Vibrio* strains ordered by a Maximum likelihood core genome phylogeny of 157 *V. crassostreae* isolates and *V. gigantis* strain 43_P_281 as an out-group (2498 genes). Clades (V1 to V8) are labeled with different colors. Columns represent phages (n=76) ordered by genomic clusters as defined in b. *Vibrio* killing by each phage is represented by colored squares. Phage morphotypes are indicated by specific icons for siphoviruses (long tail), myoviruses (medium tail) and podoviruses (short tail). **b**, Phage genome forms clusters. The network was integrated with 2,486 genes family from 76 phages and revealed clustering of phages with genomes (large circles) linked by common genes (small grey circles), %id aa>30% and >80% coverage. The color of each phage genome refers to the clade assignment of the host they kill.



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Figure 2. Graphic summary of adsorption and killing assays. Bacteria and phage belonging to a specific cluster are arrayed in rows and columns, respectively. Positive and negative adsorptions are represented by black and white squares respectively. Positive and negative killings are represented by colored and white squares respectively. For phage cluster P5a, two phages (P5a_{red} and P5a_{blue}) that differ in their host range are shown. All 320 adsorption assays were performed twice (Fig. S9).



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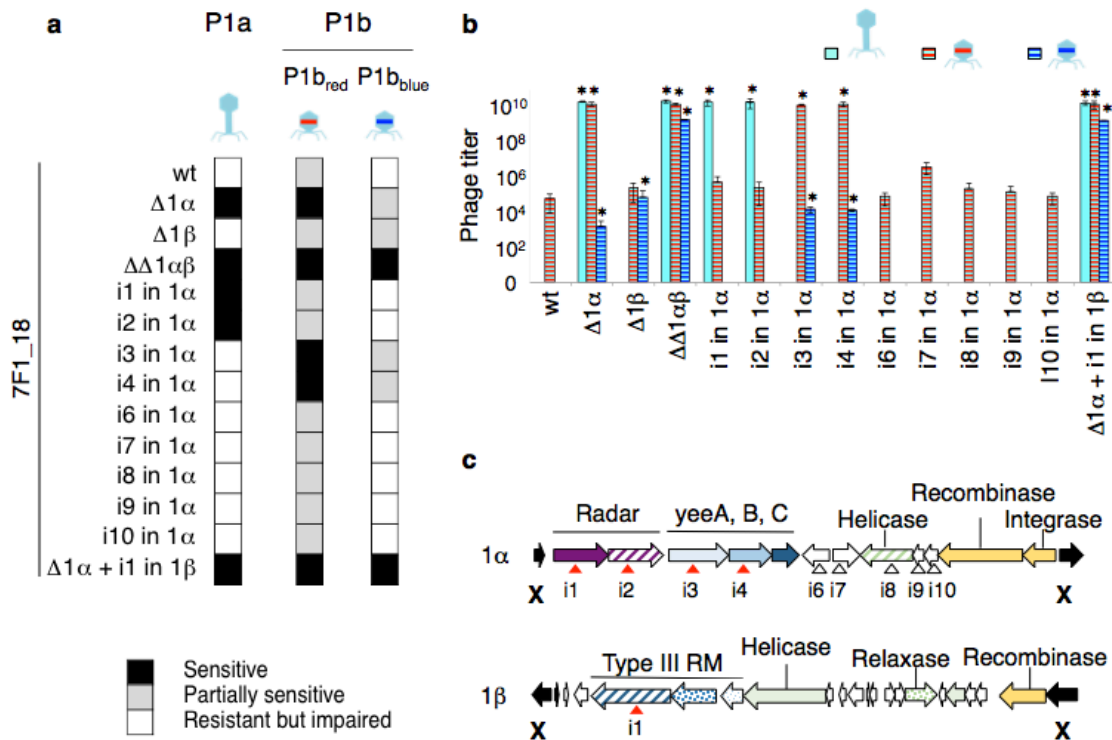
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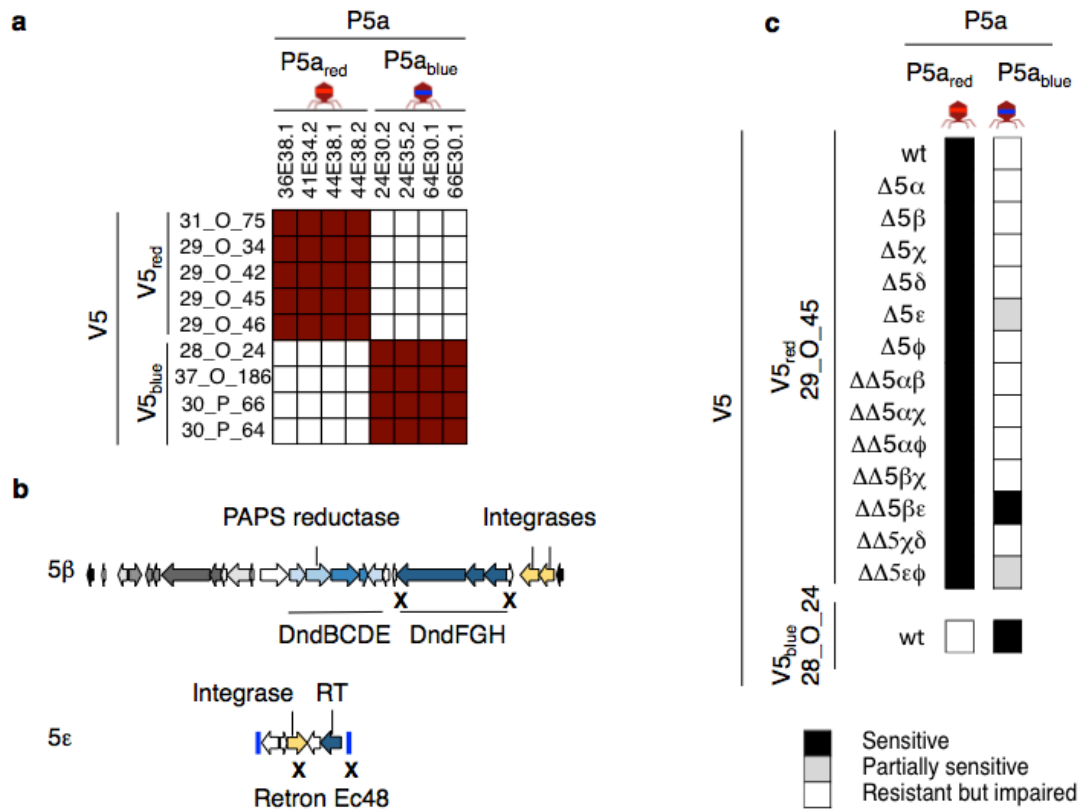
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Figure 3. Bacteria and phage flexible genome diversity is indicative of host defense and phage anti-defense interplay. **a**, *V. crassostreae* core genome phylogeny forms clades (V1 to V8 Fig. 1) with specific properties. The ANI value between strains within each clade is indicated. The bar graph indicates the number of phages out of 76 that kill each strain. Columns indicate the genome size (grey); the number of integrases (orange); the number of partition systems (purple); the number of restriction modification systems (blue); the presence of phage defense elements describes by Wang et al., Gao et al., Millman et al., Doron et al., Rousset et al.^{10,11,22,24,48} **b**, intra-cluster comparative genomics indicating the genome size, the percentage of variable genes and the ANI value based on the core genes. Phage morphotypes are indicated on the top by specific icons for siphoviruses (long tail), myoviruses (medium tail) and podoviruses (short tail).



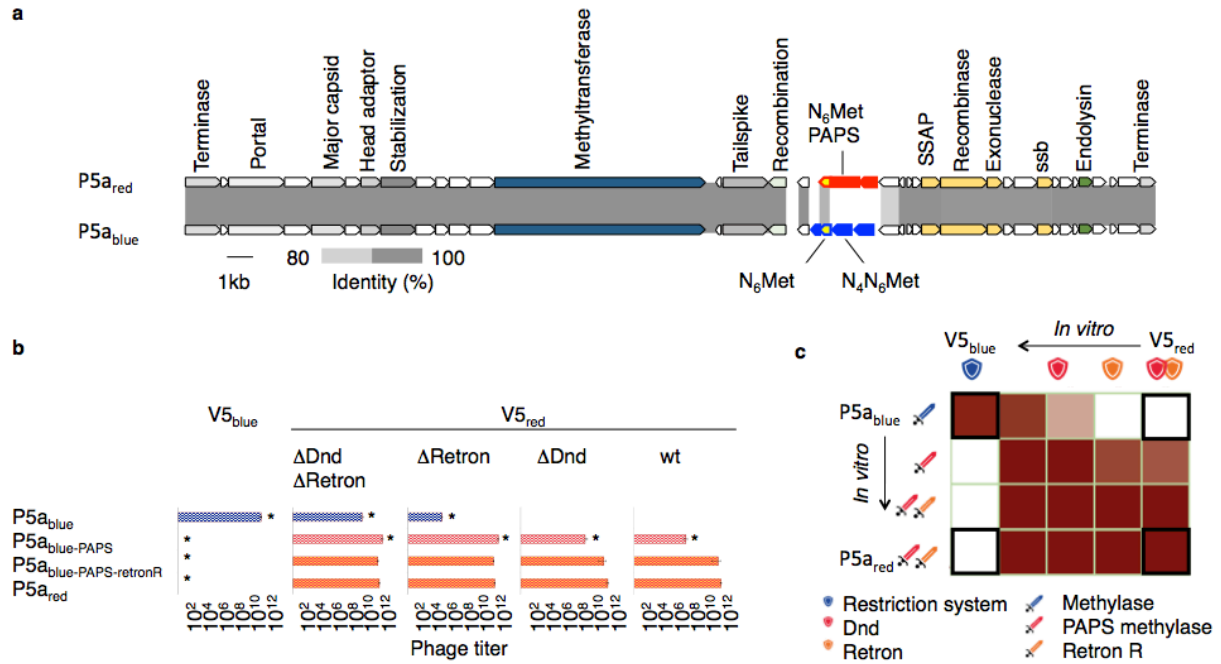
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Figure 4. Antiphage elements identified in a strain from *V. crassostreae* clade V1, 7F1_18. **a**, Summary of wild type susceptibility to different phages and changes in susceptibility to the same phages after defense regions deletions or single gene inactivation (see complete results in extended data). **b**, Efficiency of plating (EOP) using representative phages from cluster P1a, P1b_{red} and P1b_{blue} on the wild type (wt) 7F1_18 host strain and its derivatives. All experiments (a and b) were performed twice and showed that phage reproduction strongly depended on the specific combination of phage and gene knock-out ($F_{26,42} = 205.20$, $p < 0.001$, * above bars show significant differences for each phage compared to the wt strain). **c**, Gene diagrams of defense regions specific to the 7F1_18. X indicates the 500bp flanking sequence cloned in a suicide plasmid to delete the region by double recombination (see method). Triangles indicate the integration site of a suicide plasmid by single recombination, when conferring a modification in sensitivity in red triangle, when no phenotype in white.



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829 **Figure 5. Phage defense elements identified in vibrio V5_{red}, and P5_{red}.** **a**, Modularity of the
 830 interactions between vibrios from clade V5 and phage from cluster P5a. **b**, Gene diagrams of
 831 two regions specific to the V5_{red} strain 29_0_45 and demonstrated to be involved in resistance
 832 to phage P5a_{blue}. X indicates the 500bp flanking sequence cloned in a suicide plasmid to
 833 delete the region by double recombination (see Methods). Blue lines indicate the end of a
 834 contig. **c**, Summary of the changes in susceptibility to phage observed for defense regions
 835 deletions (see complete results in extended data). All experiments were performed twice.
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839 **Figure 6. Bacterial defense and phage anti-defense interplay.** **a**, Alignment of P5_{red} and
840 P5_{blue} phage genomes showing that gene synteny and content are highly conserved. Only two
841 (red) and four (in blue) genes are found specifically in P5_{red} and P5_{blue} phage. **b**, Number of
842 PFU/ml obtained after vibrio V5_{blue} wild type, V5_{red} wild type and derivatives infection by
843 phage P5_{blue} wild type, P5_{red} wild type and derivatives. All experiments were performed twice
844 and showed that phage reproduction depended on the combination of phage and host derivatives
845 ($F_{12,20} = 801.49$, $p < 0.001$, asterisk * show significant differences of each phage derivative
846 compared to the V5_{red} wildtype, wt). **c**, Graphic summary of the results. Bold framed indicate
847 the production of phages in combination of phage and host isolated from nature. The other
848 combination results from laboratory manipulation of phage and/or vibrios (in vitro). Colored
849 shields represent anti-phage defense systems acquired by the host (immunity). Colored
850 swords represent the phage anti-defense systems (escape). In the cross matrix, a white square
851 indicates that the phage cannot be produced by the host. Gradients of maroon indicate a
852 production of 10^{11} – 10^4 PFU/ml depending on the combination of phage and host tested, as
853 detailed in (a). Exchanging anti-restriction systems allows P5_{blue}-PAPS infection of a new host,
854 V5_{red}, but the evolved phage is maladapted to the ancestral host, V5_{blue}. When the phage
855 escapes retron defense, P5_{blue}-PAPS-retronR was similarly infectious to V5_{red} wild type and all
856 derivatives. Indeed P5_{blue}-PAPS-retronR evolved a P5_{red} phenotype.
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