

1 **Closely related *Vibrio alginolyticus* strains encode an identical repertoire of**
2 **prophages and filamentous phages**

3 Cynthia Maria Chibani^{1,2}, Robert Hertel², Michael Hoppert³, Heiko Liesegang², Carolin
4 Charlotte Wendling^{4,5*}

5

6 **Institutional Affiliation**

7 1. Institut für Allgemeine Mikrobiologie, Christian-Albrechts-Universität zu Kiel, Am Botanischen Garten 1-9,
8 24118 Kiel, Germany. E-mail: cchibani@ifam.uni-kiel.de

9 2. Institute of Microbiology and Genetics, Department of Genomic and Applied Microbiology, Georg-August-
10 University, 37077 Göttingen, Germany. E-mail: rhertel@gwdg.de, hlieseg@gwdg.de

11 3. Institute for Microbiology and Genetics, University of Göttingen, Göttingen, Germany. E-mail:
12 mhoppet@gwdg.de

13 4. GEOMAR, Helmholtz Centre for Ocean Research, Kiel, Düsternbrookerweg 20, 24105 Kiel

14 5. ETH Zürich, Institute of Integrative Biology, Universitätstrasse 16, CHN D 33, 8092 Zürich, Switzerland

15

16 *Corresponding author: Dr. Carolin Charlotte Wendling; carolin.wendling@env.ethz.ch

17

18 **Keywords:** Vibriophages, filamentous phages, *Vibrio* virulence, prophages, Zot, Inoviridae

19

20 **Abbreviations:** HGT: horizontal gene transfer, MGE: mobile genetic element, CT: cholera
21 toxin, Zot: zona occludens toxin, Ace: accessory cholera enterotoxin, TEM: transmission
22 electron microscopy, MCMC: Markov chain Monte Carlo

23

24 **Data statement:** All supporting data have been provided within the article or through
25 supplementary data files. Four supplementary tables and six supplementary figures are
26 available with the online version of this article.

27 **Abstract**

28 Filamentous vibriophages represent a massive repertoire of virulence factors which can be
29 transferred across species boundaries, leading to the emergence of deadly pathogens. All
30 filamentous vibriophages that were characterized until today were isolated from human
31 pathogens. Considering frequent horizontal gene transfer among vibrios, we predict that other
32 environmental isolates, including non-human pathogens also carry filamentous phages, of
33 which some may encode virulence factors.

34 The aim of this study was to characterize the phage repertoire, consisting of prophages and
35 filamentous phages, of a marine pathogen, *Vibrio alginolyticus*. To do so, we sequenced eight
36 different *V. alginolyticus* strains, isolated from different pipefish and characterised their
37 phage repertoire using a combination of morphological analyses and comparative genomics.

38 We were able to identify a total of five novel phage regions (three different *Caudovirales*
39 and two different *Inoviridae*), whereby only those two loci predicted to correspond to
40 filamentous phages (family *Inoviridae*) represent actively replicating phages. Unique for this
41 study was that all eight host strains, which were isolated from different eukaryotic hosts have
42 identical bacteriophages, suggesting a clonal expansion of this strain after the phages had
43 been acquired by a common ancestor. We further found that co-occurrence of two different
44 filamentous phages leads to within-host competition resulting in reduced phage replication by
45 one of the two phages. One of the two filamentous phages encoded two virulence genes (Ace
46 and Zot), homologous to those encoded on the *V. cholerae* phage CTXΦ. The coverage of
47 these zot-encoding phages correlated positively with virulence (measured in controlled
48 infection experiments on the eukaryotic host), suggesting that this phages is an important
49 virulence determinant.

50

51

52 **Impact statement:**

53 Many bacteria of the genus *Vibrio*, such as *V. cholerae* or *V. parahaemolyticus* impose a
54 strong threat to human health. Often, small viruses, known as filamentous phages encode
55 virulence genes. Upon infecting a bacterial cell, these phages can transform a previously
56 harmless bacterium into a deadly pathogen. While filamentous phages and their virulence
57 factors are well-characterized for human pathogenic vibrios, filamentous phages of marine
58 vibrios, pathogenic for a wide range of marine organisms, are predicted to carry virulence
59 factors, but have so far not been characterized in depth. Using whole genome sequencing and
60 comparative genomics of phages isolated from a marine fish pathogen *V. alginolyticus*, we
61 show that also environmental strains harbour filamentous phages that carry virulence genes.
62 These phages were most likely acquired from other vibrios by a process known as horizontal
63 gene transfer. We found that these phages are identical across eight different pathogenic *V.*
64 *alginolyticus* strains, suggesting that they have been acquired by a common ancestor before a
65 clonal expansion of this ecotype took place. The phages characterized in this study have not
66 been described before and are unique for the Kiel *V. alginolyticus* ecotype.

67

68 **Data Summary:**

- 69 1. The GenBank accession numbers for all genomic sequence data analysed in the
70 present study can be found in Table S1.
- 71 2. All phage regions identified by PHASTER analysis of each chromosome and the
72 respective coverage of active phage loci are listed in Table S2.
- 73 3. GenBank files were deposited at NCBI for the two actively replicating filamentous
74 phages VALGΦ6 (Accession number: MN719123) and VALGΦ8 (Accession
75 number: MN690600)

76 4. The virulence data from the infection experiments have been deposited at
77 PANGAEA: Accession number will be provided upon acceptance of the manuscript.

78 **Introduction**

79 Bacteriophages contribute significantly to bacterial adaptation and evolution. In particular
80 through bacterial lysis and subsequent killing, phages impose a strong selection pressure on
81 their bacterial hosts. However, phages can also transfer genetic material to neighbouring cells
82 via horizontal gene transfer (HGT) thereby increasing bacterial genome plasticity (1). In
83 addition, many phages, in particular temperate and filamentous phages, often carry virulence
84 genes (2, 3). When such filamentous phages integrate into the bacterial chromosome, they
85 can alter the phenotype of their bacterial host, resulting in increased bacterial virulence,
86 through a process known as lysogenic conversion (3).

87 One of the best-known examples of lysogenic conversion is the transformation of non-
88 toxigenic *Vibrio cholerae* into deadly pathogens via the filamentous CTX Φ phage, that
89 carries the cholera toxin (CT) (3). Since this first description of lysogenic conversion in 1996,
90 several other filamentous phages of which many carry bacterial virulence factors have been
91 discovered in particular for the genus *Vibrio*. For instance, two phages VfO4K68 and
92 VfO3K6 that carry the zona occludens toxin (Zot) and the accessory cholera enterotoxin
93 (Ace), have been isolated from *V. parahaemolyticus* (4, 5). Zot and Ace are particularly
94 common among vibriophages isolated from human pathogens, such as *V. cholerae* and *V.*
95 *parahaemolyticus* (6-8) but are also present in prophage-like elements of non-human
96 pathogens such as *V. coralliilyticus* (9) and *V. anguillarum* (10), suggesting frequent HGT
97 among different vibrio species (11).

98 Other environmental vibrios, which cause severe diseases, not only in marine animals but
99 also in humans, include for instance: *V. splendidus*, *V. tubiashii* and *V. alginolyticus* (12-15).
100 While virulence of these vibrio species is often attributed to multiple factors, such as
101 temperature and host immunity (16), the phage repertoire and any phage-encoded virulence
102 factors of environmental isolates are often not well characterized. One reason might be that

103 only long-read sequencing data which allow us to generate fully-closed genomes, are suitable
104 to reliably identify integrated phages. Another reason might be a research bias towards
105 human pathogens. There are 196 closed *Vibrio* genomes (as of October 2019), of which more
106 than 50% comprise human pathogens such as *V. cholerae* (51 genomes), *V. parahaemolyticus*
107 (33 genomes), and *V. vulnificus* (18 genomes), while all other environmental isolates are
108 represented with fewer than 10 genomes per species. Additionally, to our knowledge only 17
109 filamentous vibriophages have so far been described in detail of which all were isolated from
110 human pathogens, i.e. *V. cholerae* (11 phages) and *V. parahaemolyticus* (5 phages). Some of
111 these 17 filamentous phages, encode at least one virulence factor. Other filamentous
112 vibriophages, which do not encode virulence factor, are still able to transfer them to other
113 strains by means of specialized transduction (17, 18). Recombination between filamentous
114 vibriophages can further result in hybrid phages, which are then able to vertically transmit
115 toxins to other vibrios (17, 19). Indeed, some filamentous vibriophages can infect distantly
116 related species (20) and phage-mediated horizontal transfer of virulence genes seems to be a
117 dynamic property among environmental vibrios (21). Thus, the low number of well-described
118 filamentous vibriophages is concerning and it is essential that we start to characterize
119 filamentous phages and their virulence factors in environmental *Vibrio* isolates. We predict,
120 that also filamentous phages isolated from environmental vibrios, may contain virulence
121 factors responsible for disease outbreaks in marine eukaryotes. Indeed, a closer look at more
122 than 1,800 *Vibrio* genome sequences, covering 64 species revealed that 45% harboured
123 filamentous phages which encoded Zot-like proteins (22). Even though a detailed
124 characterization of these phages is missing, this study suggests that also filamentous
125 vibriophages of non-human pathogens contain virulence genes in (22).

126 The aim of the present study was to identify prophages and filamentous phages (both types
127 will also be referred to as non-lytic phages in the present study) isolated from closely related

128 *V. alginolyticus* strains and to characterize their virulence potential. *V. alginolyticus*, a
129 ubiquitous marine opportunistic pathogen can cause mass mortalities in shellfish, shrimp and
130 fish, resulting in severe economic losses worldwide (23-25). Additionally, wound infections
131 and fatal septicaemia in immunocompromised patients caused by *V. alginolyticus* have been
132 reported in humans (26). In contrast to the classical human pathogenic vibrios, only little is
133 known about *V. alginolyticus* phages and their potential role in its virulence. By combining
134 morphological and comparative genomic analyses we identified and characterized three
135 prophages and two novel filamentous phages from eight different environmental *V.*
136 *alginolyticus* isolates of which one filamentous phage encoded virulence genes homologues
137 to those found in *V. cholerae* and *V. parahaemolyticus*.
138

139 **Methods:**

140 *Vibrio alginolyticus* strains used in the present study were isolated either from the gut or
141 the gills of six different pipefish (*Syngnathus typhle*) in the Kiel Fjord in 2012 (27) and have
142 been shown to cause mortality in juvenile pipefish ((28), and this study). Using a
143 combination of PacBio and Illumina sequencing we generated eight closed bacterial genomes
144 of the host bacteria as described in (28).

145

146 **Phage isolation and sequencing**

147 *Prophage induction*

148 We induced filamentous phages from all nine *V. alginolyticus* strains using Mitomycin C
149 (Sigma), for details see (28), with some modifications: bacteria were grown in liquid
150 Medium101 (Medium101: 0.5% (w/v) peptone, 0.3% (w/v) meat extract, 3.0% (w/v) NaCl in
151 MilliQ water) at 250 rpm and 25 °C overnight. Cultures were diluted 1:100 in fresh medium
152 at a total volume of 20ml and grown for another 2h at 250 rpm and 25 °C to bring cultures
153 into exponential growth before adding Mitomycin C at a final concentration of 0.5 µg/ml.
154 Afterwards, samples were incubated for 4 h at 25 °C at 230 rpm. After 4 h, lysates were
155 centrifuged at 2500 g for 5 min. The supernatant was sterile filtered using 0.45 µm pore size
156 filter (Sarstedt, Nümbrecht, Germany). We added lysozyme from chicken egg white
157 (10µg/ml, SERVA Heidelberg, Germany) to disrupt the cell walls of potentially remaining
158 host cells, RNase A (Quiagen, Hilden, Germany) and DNase I (Roche Diagnostics,
159 Mannheim, Germany) at a final concentration of 10 µg/ml to remove free nucleic acids and
160 remaining host cells as described in (29). After incubation at 25°C for 16 hours phage
161 particles were sedimented by ultracentrifugation using a Sorvall Ultracentrifuge OTD50B
162 with a 60Ti rotor applying 200,000 g for 4 hours. The supernatant was discarded and the
163 pellet was dissolved in 200 µl TMK buffer, and directly used for DNA isolation.

164

165 *Prophage DNA extraction*

166 DNA isolation was performed using a MasterPure DNA Purification kit from Epicenter
167 (Madison, WI, USA). We added 200 µl 2x T&C-Lysis solution containing 1 µl Proteinase K
168 to the phage suspensions and centrifuged the samples for 10 min at 10,000 g. The supernatant
169 was transferred to a new tube, mixed with 670 µl cold isopropanol and incubated for 10 min
170 at – 20°C. DNA precipitation was performed by centrifugation for 10 min at 17,000 g and
171 4°C. The DNA pellet was washed twice with 150 µl 75% ethanol, air-dried and re-suspended
172 in DNase free water.

173

174 *Prophage sequencing*

175 dsDNA for library construction was generated from viral ssDNA in a 50 µl reaction. The
176 reaction was supplemented with 250 ng viral ss/DNA dissolved in water, 5 pmol random
177 hexamer primer (#SO142, Thermo Scientific), 10 units Klenow Fragment (#EP0051, Thermo
178 Scientific) and 5 µmol dNTPs each (#R0181, Thermo Scientific) and incubated at 37°C for 2
179 hours. The reaction was stopped by adding 1 µl of a 0.5M EDTA pH 8 solution. The
180 generated DNA was precipitated by adding 5 µl of a 3M sodium acetate pH 5.2 and 50 µl
181 100% Isopropanol to the DNA solution, gently mixing and chilling for 20 min at -70°C. DNA
182 was pelleted via centrifugation at 17,000 g, 4°C for 10 min. Pellets were washed twice with
183 70% ethanol. Remaining primers and viral ss/DNA were removed in a 50 µl reaction using
184 10 units S1 nuclease (#EN0321, Thermo Scientific) for 30 min at 25°C. S1 nuclease was
185 inactivated through addition of 1µM 0.5M EDTA pH 8 and incubation for 10 min at 70°C.
186 Consequently ds/DNA was precipitated as described above and resolved in pure water.
187 Presence of ds/DNA was verified via TAE gel electrophoresis in combination with an

188 ethidium bromide staining and visualization via UV-light. NGS libraries were generated with
189 the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, USA), and the
190 sequencing was performed on an Illumina GAII sequencer (Illumina, San Diego, USA). All
191 generated reads were checked for quality using the programs FastQC (30) and Trimmomatic
192 (31).

193

194 **Transmission electron** Electron microscopy was carried out on a Jeol 1011 electron
195 microscope (Eching, Germany). Negative staining and transmission electron microscopy
196 (TEM) of phage-containing particles was performed as described previously (29, 32).
197 Phosphotungstic acid dissolved in pure water (3%; pH 7) served as staining solution.

198

199 **Genomic analysis:**

200 *Prediction of phage regions:* All host genomes were scanned with PHASTER (33) to
201 identify prophage like elements in each chromosome. Predicted prophage regions were
202 further analysed using Easyfig (34) for pairwise phage sequence comparisons and synteny
203 comparisons with an *E*-value cut-off of $1e-10$. We used SnapGene Viewer (v. 4.3.10) to
204 generated circular genomes including the predicted prophage regions from each strain.

205

206 *Annotation:* Annotation was performed using Prokka v1.11 (35) which was applied using
207 prodigal for gene calling (36), *Vibrio* as the genus reference (--genus *Vibrio* option) and a
208 comprehensive *Inoviridae* vibriophage protein database as a phage features reference
209 database. Reference *Inoviridae* vibriophages used for the reference protein fasta database are
210 listed in (Supplementary material, Table S3).

211 *Prediction of active phage regions:* All reads from phage DNA have been mapped using
212 bowtie2 (37) to the corresponding reference *V. alginolyticus* genome. The generated mapping

213 files were analysed using TraV (38) to visualize phage DNA derived coverage within the
214 genomic context. Increased coverage was exclusively observed in genomic regions that have
215 been identified by PHASTER (33) as phage regions. This was used as an indication for active
216 prophages.

217 To estimate the relative phage production of each active phage locus we estimated the
218 coverage of each locus relative to the coverage of the chromosome. Deeptools v.3.3.0 (39)
219 was used to compute read coverage which was normalized using the RPKM method as
220 follows: $RPKM(\text{per bin}) = \text{number of reads per bin} / (\text{number of mapped reads (in millions)} * \text{bin length (kb)})$. The length of the bin used is 1kb.

222

223 *Comparative genomic analysis:* We used the MUSCLE algorithm implemented in
224 AliView v. 1.15 (40) to conduct whole genome alignments within all phage-groups that
225 showed a high similarity based on Easyfig. Additionally, we performed alignments of the
226 flanking regions by comparing five genes located upstream and five genes located
227 downstream of each integrated phage.

228 To investigate the phylogenetic relationship of *Vibrio* phage VALGΦ6 and *Vibrio* phage
229 VALGΦ8 with other well-studied filamentous phages we generated a phylogenetic tree based
230 on the major coat protein (pVIII) of 20 well-characterized filamentous phages, which
231 determined the structure of the virion coat. This protein is the most abundant protein present
232 in all filamentous phages (41) and commonly used to infer phylogenetic relationships
233 between filamentous phages. After alignment of the protein sequences using MUSCLE (42),
234 we constructed a phylogenetic tree using the Bayesian Markov chain Monte Carlo (MCMC)
235 method as implemented in MrBayes version 3.2.5 (43, 44). The TN93 (45) model plus
236 invariant sites (TN93 + I), as suggested by the Akaike information criterion (AIC) given by
237 jModelTest (46), was used as statistical model for nucleotide substitution. The MCMC

238 process was repeated for 10^6 generations and sampled every 5000 generations. The first
239 2000 trees were deleted as burn-in processes and the consensus tree was constructed from the
240 remaining trees. Convergence was assured via the standard deviation of split frequencies
241 (<0.01) and the potential scale reduction factor (PSRF \sim 1). The resulting phylogenetic tree
242 and associated posterior probabilities were illustrated using FigTree version 1.4.2
243 (<http://tree.bio.ed.ac.uk/software/figtree/>). We used *Propionibacterium phage B5* which is a
244 phage preying on a gram-positive bacterium as an outgroup.

245 We additionally compared the predicted phage regions from the present study with
246 potential phage regions from all other so far published fully closed *V. alginolyticus* genomes
247 (Table S4). To do so, we identified potential phage regions on each chromosome using
248 PHASTER (33) and compared those with the phage regions from the present study using
249 Easyfig (34).

250

251 *Analysis of virulence factors:* We found that one of the active filamentous phages (i.e.
252 *Vibrio* phage VALG Φ 6) contains the virulence cassette comprising the Zot and the Ace
253 proteins, which is frequently found in vibriophages and responsible for severe gastro-
254 intestinal diseases (47, 48). To compare these two proteins with other Zot and Ace proteins
255 isolated from various vibriophages we generated protein alignments using AliView (40) and
256 examined the presence of Walker A and Walker B motifs in *Vibrio* phage VALG Φ 6 Zot
257 proteins. We further used the TMHMM Server (<http://www.cbs.dtu.dk/services/TMHMM/>)
258 to confirm the presence of a transmembrane domain typically found in the Zot protein.

259

260

261

262 *Infection experiments:*

263 We performed a controlled infection experiment to estimate the virulence of the eight
264 sequenced strains on juvenile pipefish (a detailed description of methods and the statistical
265 analysis can be found in (28). Briefly, we fed 9-12 juvenile pipefish per tank in using
266 triplicate tanks with *Artemia* nauplii which were previously exposed to $\sim 10^9$ CFU/ml or
267 seawater as control. Twenty-four hours post infection, each fish was killed and bacterial load
268 was determined as colony forming units (CFU/ml) as in (28).

269

270 GenBank files were deposited at NCBI for the two actively replicating filamentous phages
271 VALGΦ6 (Accession number: MN719123) and VALGΦ8 (Accession number: MN690600)

272

273 **Results**

274 1. General overview

275 We sequenced the bacterial DNA of eight closely related *Vibrio alginolyticus* strains as
276 well as the DNA extracted from the supernatant of mitomycin C treated liquid cultures of
277 each strain. Within the eight sequenced *V. alginolyticus* strains we discovered three different
278 prophage regions each of which could be assigned to the family *Caudovirales* and two
279 different regions that were assigned to filamentous phages (family *Inoviridae*). From the
280 sequenced supernatant we could only identify filamentous phages but no head-tail phages,
281 suggesting that in the present strains, filamentous phages are the only active replicating
282 phages. To locate the exact positions of the induced prophages, we performed a PHAGE-seq
283 experiment (29). In control experiments, the complete procedure has been applied without
284 mitomycin C where the reference genomes were sequenced using Illumina technology. Both
285 experiments revealed an increased coverage exclusively at *Inoviridae* loci (Supplementary
286 material Figure S1). This indicates that induced and non-induced cultures produce
287 comparable amounts of particles encoded by the same filamentous phage. As a further
288 control total DNA without DNase A treatment resulted in a coverage increased by the factor
289 of 100-100,000 at the loci encoding filamentous phages compared to the average
290 chromosomal coverage. We thus conclude that the cultures produced a permanent amount of
291 phage particle protected ssDNA independent of the induction from mitomycin C.

292

293 2. Caudovirales

294 Whole genome comparison between the eight sequenced *V. alginolyticus* strains revealed
295 the presence of three different prophage regions belonging to the family *Caudovirales*, none
296 of which generated phage particles nor protein protected DNA in the experimental settings
297 used in this study (Figure 1). Thus, a more thorough classification based on morphological

298 characterization was not possible. We further did not find regions of increased coverage for
299 these three *Caudovirales* regions (Supplementary material Figure S1) on the bacterial
300 chromosomes indicating that these phages were neither actively replicating in uninduced
301 bacterial cultures nor able to switch to the lytic cycle upon induction with mitomycin C. We
302 could not identify sequence similarities between these three different *Caudovirales* phages,
303 suggesting that they are genetically distinct phages. However, each of the three *Caudovirales*
304 phages was 100% identical across all eight strains where they all have the same integration
305 site (Figure 2).

306

307 *Vibrio* phage VALGΦ1: The genome of *Vibrio* phage VALGΦ1 is composed of a 33.3kb
308 DNA molecule with a GC content of 46.06 % and no tRNAs. The total open reading frames
309 (ORFs) is 22, with 10 ORFs assigned to one of five functional groups typical for phages
310 (Replication, Assembly, Structural proteins, Integration, Lysis) and 12 ORFs to hypothetical
311 proteins (Figure 1). All ORFs were orientated in the same direction. Even though *Vibrio*
312 phage VALGΦ1 could not be found in induced and uninduced supernatants, it is predicted to
313 be intact according to PHASTER. *Vibrio* phage VALGΦ1 is exclusively found on
314 Chromosome 1, where it has a unique integration site, which is identical across all eight
315 sequenced strains. The phage genome as well as the flanking regions (five genes upstream
316 and five genes downstream of the integrated phage) showed 100% sequence similarity across
317 all eight sequenced strains, suggesting that the phage is highly conserved across host-strains.
318 Comparative genomic analysis between *Vibrio* phage VALGΦ1 and ten closest hits on NCBI
319 reveals that the two closest related phages are FDAARGOS_105 integrated on chromosome 1
320 of *V. diabolicus* with a query cover of 77% and a similarity of 94.68% followed by an
321 uncharacterized region on chromosome 1 of *V. alginolyticus* ATCC 33787 with a query cover

322 of 57% and a similarity of 96.13%. These low query covers suggest that *Vibrio* phage
323 VALGΦ1 is a novel bacteriophage.

324 *Vibrio* phage VALGΦ2: The genome of *Vibrio* phage VALGΦ2 is 26.3 kb with a GC
325 content of 49.37 %, no tRNAs and a total of 29 ORFs, with 22 assigned to one of five
326 functional phage-related groups and seven hypothetical proteins (Figure 1). All ORFs were
327 orientated in the same direction. *Vibrio* phage VALGΦ2 is predicted to be questionable by
328 PHASTER, suggesting that it does not contain sufficient prophage genes to be considered a
329 complete functional phage. Even though, *Vibrio* phage VALGΦ2 has a unique integration
330 site on chromosome 2 across all eight strains, the upstream region is not identical across
331 strains. In contrast, the downstream region of *Vibrio* phage VALGΦ2 is identical across all
332 strains and has a length of 2584 bp followed by another prophage, identical across all
333 sequenced strains and, identified as *Vibrio* phage VALGΦ2b. Due to their incompleteness
334 and the short gap between these two phages we refer to them as a *Caudovirales* complex
335 consisting of *Vibrio* phage VALGΦ2 and *Vibrio* phage VALGΦ2b.

336

337 *Vibrio* phage VALGΦ2b: *Vibrio* phage VALGΦ2b is predicted to be incomplete by
338 PHASTER, suggesting that it may represent a cryptic phage. The genome of *Vibrio* phage
339 VALGΦ2 is 26.5 kb long, with zero tRNAs and a GC content of 48.32%. Of the 20 identified
340 OFRs, 12 could be assigned to phage-functional groups and eight as hypothetical proteins.
341 *Vibrio* phage VALGΦ2 contains an ORF assigned as MarR family transcriptional regulator
342 accompanied with a transposase 3749 bp upstream.

343

344 3. Inoviridae

345 Phage morphology: We determined the morphology of all active phages from every strain
346 using a transmission electron microscope (TEM, see Supplementary material, Figure S2).

347 According to the International Committee on Taxonomy of Viruses (ICTV), all phage
348 particles were identified as filamentous phages.

349 Phage genomics: Within the eight sequenced *V. alginolyticus* strains we could identify two
350 different filamentous phages, i.e. *Vibrio* phage VALGΦ6 and *Vibrio* phage VALGΦ8. Both
351 phages contain single-stranded ssDNA genomes of 8.5 and 7.3 kbp in size and a GC content
352 of 44.6% and 46.3%, respectively. ORFs were mostly orientated in a single direction,
353 whereas the transcription regulator was transcribed in the reverse direction (Figure 3). Both
354 phages showed similar functional genes (typical for *Inoviridae*), which could be roughly
355 grouped into three functional modules: Replication, assembly or structural proteins (41).
356 *Vibrio* phage VALGΦ6 and *Vibrio* phage VALGΦ8 share relatively little homology, except
357 for proteins involved in DNA replication (Figure 3).

358 *Vibrio* phage VALGΦ6 can be found exclusively on chromosome 2 in all eight strains, has
359 a unique integration site and is identical across all strains. In contrast, *Vibrio* phage VALGΦ8
360 can only be found in five out of the eight strains and has a more diverse life-style. It can
361 integrate on chromosome 2 (strain K04M3, K04M5, K10K4), chromosome 1 (Strain K05K4,
362 K10K4) or exists extra-chromosomally (strain K04M1 without an intrachromosomal copy or
363 in strain K05K4 with an intrachromosomal copy, Figure 4). When integrated on chromosome
364 2, *Vibrio* phage VALGΦ8 is always located directly behind *Vibrio* phage VALGΦ6,
365 sometimes resulting in multi-phage cassettes (Figure 2). When integrated on chromosome 1,
366 *Vibrio* phage VALGΦ8 is orientated in a reverse order as on chromosome 2. In strain K10K4,
367 *Vibrio* phage VALGΦ8 is found on both chromosomes.

368

369 Phage activity: All loci predicted to correspond to filamentous phages represent actively
370 replicating phages. We conclude this from several lines of evidence. First, we were able to
371 detect filamentous phages in TEM pictures of all cultures (see Supplementary material,

372 Figure S2). Second, phage particles isolated from induced and uninduced cultures contained
373 exclusively DNA that matched at these phage loci (Supplementary material, Figure S1, Table
374 S2). Differences in coverage values of loci corresponding to both filamentous phages we
375 found that the production of phage particles varies across phage regions and strains
376 (Supplementary material, Table S2). For strains that did not contain *Vibrio* phage VALGΦ8,
377 we found that all regions encoding *Vibrio* phage VALGΦ6 had on average a 100000x higher
378 coverage relative to the coverage of the chromosome. However, the presence of *Vibrio* phage
379 VALGΦ8 reduced the coverage of *Vibrio* phage VALGΦ6 encoding regions by 10 – 1000x,
380 but only when *Vibrio* phage VALGΦ8 was integrated on chromosome 2, not when it existed
381 exclusively extrachromosomal or had an additional copy on chromosome 1.

382 Phylogeny: Whole genome alignment (Figure 3) and phylogenetic comparisons (Figure 5)
383 based on the major coat protein (pVIII) suggest that *Vibrio* phage VALGΦ6 and *Vibrio* phage
384 VALGΦ8 group closely with other known filamentous vibriophages. Overall, filamentous
385 vibriophages group more closely with class II phages of *Pseudomonas* and *Xanthomonas* and
386 form a distinct cluster from class I filamentous coliphages. *Vibrio* phage VALGΦ6 shares
387 more sequence homology with VfO4K68 and VfO3K6, both isolated from *V.*
388 *parahaemolyticus* (Figure 3). *Vibrio* phage VALGΦ8 shares more sequence homology with
389 VF33 also isolated from *V. parahaemolyticus*. Blastn comparisons using whole phage
390 genomes suggest that *Vibrio* phage VALGΦ6 and *Vibrio* phage VALGΦ8 are different from
391 other bacteriophages described until today. For *Vibrio* phage VALGΦ6 the two closest hits
392 were the two *V. parahaemolyticus* phages VfO4k68 and VfO3k6 with query covers of 66%
393 and 75% and similarity values of 94.65% for each phage. The closest hits for *Vibrio* phage
394 VALGΦ8 were the two *V. parahaemolyticus* phages Vf12 and Vf13 with a query cover of
395 88% and a similarity of 94.65%.

396

397 To compare all non-lytic phages between strains from the present study and other *V.*
398 *alginolyticus* isolates we used PHASTER to predict prophages from all available closed non-
399 Kiel *V. alginolyticus* genomes and found a total of 14 predicted prophage regions (Table S4).
400 Comparisons between those uncharacterized vibriophages and phages from the present study
401 revealed that *Vibrio* phage VALGΦ6 and the *Caudovirales* cassette consisting of *Vibrio*
402 phage VALGΦ2 and 2b is unique to the Kiel *alginolyticus* system. In contrast, we found
403 integrated *Inoviridae* with high similarity to *Vibrio* phage VALGΦ8 in four of the six non-
404 Kiel *V. alginolyticus* strains and one integrated *Caudovirales* that shared high similarity with
405 *Vibrio* phage VALGΦ1 and had the same attL/ attR sequence, i.e. CGTTATTGGCTAAGT
406 (Figure 2). Despite having two, respectively one, unique integration site within the Kiel
407 isolates, phages from the non-Kiel isolates with high similarity to *Vibrio* phage VALGΦ8 and
408 *Vibrio* phage VALGΦ1 were mostly integrated on different positions in the respective
409 chromosomes (Figure 2). All other uncharacterized phages did not contain functional genes
410 typical for *Inoviridae* suggesting that no other filamentous phage is present in the non-Kiel
411 strains. In contrast to the Kiel strains, where most phages were integrated on chromosome 2,
412 only two out of the non-Kiel strains had prophages on chromosome 2 and one strain
413 FDAARGOS_108 did not have a single prophage in its genome. Overall, the typical phage
414 composition consisting of *Vibrio* phage VALGΦ6, *Vibrio* phage VALGΦ2 and 2b on
415 chromosome 2 together with *Vibrio* phage VALGΦ1 on chromosome 1 is unique for our
416 system and has not been found elsewhere.

417

418 Multi-phage-cassettes

419 We found multi-phage cassettes on chromosome 2 in two strains, i.e. K04M3 and K04M5.
420 While the cassette in K04M5 consists of *Vibrio* phage VALGΦ6 followed by *Vibrio* phage
421 VALGΦ8, K04M3 has two multi-phage cassettes. The first cassette consists of *Vibrio* phage

422 VALGΦ6, followed by a tandem repeat of two identical *Vibrio* phage VALGΦ8 regions, the
423 second cassette, which is located 10389 bp downstream of the first cassette is identical to the
424 one identified in K04M5. Even though *Vibrio* phage VALGΦ6 is identical across all strains,
425 the second replicate in K04M3, which represents the start of the second multi-phage cassette,
426 misses the transcription regulator and has major deletions, particularly affecting assembly
427 and structural proteins (Supplementary material, Figure S4).

428

429 Virulence of Kiel *V. alginolyticus* ecotypes

430 Comparative genomic analysis between virulence factors commonly encoded on
431 filamentous phages revealed that only *Vibrio* phage VALGΦ6 contains the virulence cluster
432 containing Ace and Zot. In contrast no known virulence factors could be found on *Vibrio*
433 phage VALGΦ8 and the described *Caudovirales*. Sequence comparisons of Zot proteins
434 encoded on different vibrios revealed that the *Vibrio* phage VALGΦ6 encoded Zot is highly
435 similar to Zot genes encoded on other closely related *Vibrio* species from the *harveyi* clade
436 (such as *V. parahaemolyticus* or *V. campbellii*, Figure S5). Even though we found less
437 similarity between the *Vibrio* phage VALGΦ6 encoded Zot protein and CTXΦ-encoded Zot
438 proteins, we found two conserved motifs (Walker A and B, common among human
439 pathogens), which were at the N-terminal side of the Zot proteins (Figure S5). In addition, we
440 found a transmembrane domain in the *Vibrio* phage VALGΦ6 encoded Zot protein (Figure
441 S6), suggesting that similar to the CTXΦ-encoded Zot, the *Vibrio* phage VALGΦ6 encoded
442 Zot is also a transmembrane protein.

443 Controlled infection experiments on juvenile pipefish revealed differences in total
444 bacterial load among strains, a proxy for virulence. We found that those strains, that only
445 encode *Vibrio* phage VALGΦ6 (i.e. K01M1, K06K5 and K08M3) were the most virulent
446 (Figure 6). Whereas strains with a reduced coverage of *Vibrio* phage VALGΦ6 encoding

447 regions were by far the least virulent strains, in particular strain K04M5, where we also
448 observed the strongest reduction in coverage compared to *Vibrio* phage VALGΦ8-free
449 strains.

450

451

452 Discussion

453 We present five new non-lytic phages (comprising filamentous phages and prophages)
454 isolated from eight different *Vibrio alginolyticus* strains. Using a combination of whole
455 genome sequencing, comparative genomic analyses, and transmission electron microscopy
456 we found three distinct phage regions belonging to the family *Caudovirales* and two distinct
457 regions corresponding to actively replicating filamentous phages. Based on comparative
458 genomic analyses we conclude that all five phages described in the present study are novel
459 bacteriophages. Our main findings are that (1) closely related *V. alginolyticus* isolates, which
460 were isolated from different eukaryotic hosts have identical bacteriophages, which are unique
461 for this ecotype, (2) filamentous phages can have different life-styles and are able to suppress
462 each other, and (3) horizontal gene transfer (HGT) of *Vibrio* phage VALGΦ6 containing the
463 virulence cluster comprising zona occludens toxin (Zot) and accessory cholera enterotoxin
464 (Ace) may have led to the emergence of pathogenicity of the Kiel *V. alginolyticus* ecotype.

465

466 *Closely related V. alginolyticus isolates, which were isolated from different eukaryotic hosts*
467 *share unique but identical bacteriophages*

468 Four of the five described phages in this study (i.e. *Vibrio* phage VALGΦ1 on
469 chromosome 1, the *Caudovirales* complex consisting of *Vibrio* phage VALGΦ2 and
470 VALGΦ2b as well as the filamentous *Vibrio* phage VALGΦ6 of chromosome 2) were
471 present in all eight sequenced strains, had the same integration site and no variation in
472 flanking regions on the chromosome (only exception: upstream region of *Vibrio* phage
473 VALGΦ2). Only the filamentous *Vibrio* phage VALGΦ8 was not present in all strains,
474 existed in two different life-styles (intra- and extrachromosomal) and had different
475 integration sites (possible recombination with both chromosomes). All eight strains have no
476 core genomic variation and sequence variation is mainly attributable to differences in mobile

477 genetic elements (MGEs), such as plasmids and presence/ absence of *Vibrio* phage VALGΦ8
478 (49). Comparative genomic analyses across a wider range of *V. alginolyticus* isolates
479 indicated that the phage repertoire of the Kiel *alginolyticus* ecotype is unique and cannot be
480 found elsewhere. Thus, we hypothesize that the identical prophage composition in this
481 ecotype together with the identical integration sites and flanking regions suggests that these
482 phages may have been acquired from a common ancestor before a clonal expansion of the
483 Kiel *alginolyticus* ecotype took place. Under this scenario we predict that these five
484 prophages are increasing the fitness of this ecotype in the present habitat and are thus
485 maintained by selection.

486 The sampling design, spanning two different organs (gills or gut) from six different
487 pipefish allows us not only to look at the phage composition of closely related bacteria across
488 eukaryotic hosts but also within eukaryotic hosts. We found more similarity within pipefish
489 Nr. 4 (strains K04M1, K04M3 and K04M5) than across all six pipefish: First, all three strains
490 contained *Vibrio* phage VALGΦ8, and second, the only two multi-phage cassettes were
491 found in strains K04M3 and K04M5, both isolated from pipefish Nr. 4. It is tempting to
492 speculate that the high prevalence of *Vibrio* phage VALGΦ8 relative to all eight sequenced
493 strains is a result of the close proximity between strains inside the gut, which favours the
494 rapid horizontal spread of *Vibrio* phage VALGΦ8. Future experiments would be needed to
495 study the likelihood for *Vibrio* phage VALGΦ8 to establish successful chronic infections and
496 the circumstances which favour the different life-styles (extra- or intra-chromosomal) and
497 integration sites (chromosome 1 or chromosome 2).

498

499 *Filamentous phages differ in their life-style*

500 While *Vibrio* phage VALGΦ6 was exclusively found at one integration site across all
501 eight sequenced strains (exceptions: the multi-phage cassettes in strains K04M3 and

502 K04M5), *Vibrio* phage VALGΦ8 had different integration sites on both chromosomes and
503 existed intra-and extrachromosomal. We identified one, respectively two extrachromosomal
504 closed circular contigs within the assembly of strains K04M1 and K05K4 representing
505 multimers of *Vibrio* phage VALGΦ8 (Figure 4). This indicates the presence of
506 extrachromosomal phage replicons in two out of the eight sequenced *V. alginolyticus*
507 genomes. Filamentous phages typically multiply via the rolling circle replication (RCR)
508 mechanism (41). Considering that K05K4 contains another copy of *Vibrio* phage VALGΦ8
509 integrated on chromosome 1 and that the extrachromosomal contigs contain two, respectively
510 three copies of *Vibrio* phage VALGΦ8 (Figure 4), we hypothesise that the K05K4
511 extrachromosomal contigs represent RCR intermediates of the integrated *Vibrio* phage
512 VALGΦ8. However, to confirm or falsify this hypothesis experiments using knock-out
513 versions of the intrachromosomal copy of *Vibrio* phage VALGΦ8 in strain K05K4 have to be
514 performed which are beyond the scope of this study. In contrast, K04M1 does not contain an
515 intrachromosomal version of *Vibrio* phage VALGΦ8 and the extrachromosomal contig of
516 K04M1 only consists of one phage replicon (Figure 4). This suggests that *Vibrio* phage
517 VALGΦ8 is able to establish a chronic extra-chromosomal infection without the need of an
518 intrachromosomal copy.

519

520 *Within-host competition can lead to the reduction of phage producing particles*

521 *Vibrio* phage VALGΦ1 has been predicted to be complete, but we did not find phage
522 particles of head-tail phages in the supernatant nor did we detect any DNA-sequences in phage
523 particles that map to its region in the chromosome under lab-conditions. Without a proof-of
524 principle which would again require knock-out versions of the filamentous phages, we can
525 only speculate that the *Caudovirales Vibrio* phage VALGΦ1 is suppressed by the two actively
526 replicating filamentous phages. Indeed, within-host competition between different

527 *Caudovirales* has been found in other systems, for instance in *Bacillus licheniformis* (29).
528 Alternatively, *Vibrio* phage VALGΦ1 might be not induced within the conditions of our
529 experimental set up or could have been wrongly predicted to be complete by the software but
530 is, however not able to actively replicate suggesting prophage domestication, which has also
531 been predicted for *Vibrio* phage VALGΦ2 and 2b. When head-tail phages switch from the
532 lysogenic to the lytic cycle they always kill their host. Selection for a strict repression of the
533 lytic life cycle of prophage inactivation should thus be strong (50). Indeed, bacterial genomes
534 have numerous defective prophages and prophage-derived elements (51, 52), which
535 presumably originate from pervasive prophage domestication (50). By domesticating
536 prophages, bacteria can evade the risk of getting lysed but are still able to maintain beneficial
537 accessory genes, in the present case for instance the Mar family proteins encoded on the
538 defective *Vibrio* phage VALGΦ2b, which encode transcriptional regulators involved in the
539 expression of virulence, stress response and multi-drug resistance (53, 54). Another case of
540 within-host competition between phages is the 10-x reduced coverage of *Vibrio* phage
541 VALGΦ6 in strains where both filamentous phages were present. Again, we hypothesise, that
542 one phage, in this case *Vibrio* phage VALGΦ8, negatively affects the replication of another
543 phage, here *Vibrio* phage VALGΦ6. As above, to verify or falsify this hypothesis, knock-out
544 versions of strains containing both filamentous phages would be required, as have been used
545 in (29). Within-host competition is common among different head-tail prophages within the
546 same host leading to strong selection for short lysis time (55). However, to the best of our
547 knowledge, nothing is known about within-host competition among filamentous phages and
548 whether filamentous phages are able to suppress each other's replication. However, studies on
549 the classical biotype *V. cholerae* where CTXΦ was present as an array of two truncated, fused
550 prophages found, that even though the cholera toxin is expressed, no viral particles are
551 produced (56). Deficiencies in the array-structure and not mutations affecting individual

552 CTX Φ genes have been suggested to be responsible for the absence of phage particle
553 production. Similarly, we found that the strongest reduction in coverage for *Vibrio* phage
554 VALG Φ 6 encoding regions, where *Vibrio* phage VALG Φ 6 was present as part of a multi-
555 phage cassette, containing arrays of two or more adjacent filamentous phages (strain K04M3
556 and strain K04M5). As we also did not find any genomic differences among the different
557 regions encoding for *Vibrio* phage VALG Φ 6, it is tempting to speculate that similar
558 deficiencies in array structure are causing the coverage reduction of *Vibrio* phage VALG Φ 6
559 encoding regions. Alternatively, suppression of one phage by another could be the result of
560 methylation leading to a less efficient or even inactivate phage particle production. Future
561 studies unravelling within-host interactions of filamentous phages should elucidate whether
562 such within-host competitions can influence the dynamics and evolutionary trajectories of
563 filamentous phages.

564

565 *HGT of Vibrio phage VALG Φ 6 containing the virulence cluster comprising Zot and Ace may*
566 *have led to the emergence of the pathogenic Kiel V. alginolyticus ecotype*

567 Filamentous phages are the most recognized vibriophages and present in almost every
568 *Vibrio* genome sequenced to date (for a detailed overview see (57)). Filamentous phages
569 isolated from the Kiel *V. alginolyticus* ecotype share more homology with filamentous
570 phages isolated from *V. parahaemolyticus* than with other non-Kiel *V. alginolyticus* strains.
571 This suggests a constant movement of filamentous phages between different *Vibrio* species
572 without losing the ability to replicate in the old host(s). Indeed, some filamentous
573 vibriophages have a very broad host range (20) and movement of vibriophages is not
574 uncommon (58-60). If filamentous phages are able to establish a chronic infection in the new
575 host, this movement of phages across species boundaries will facilitate horizontal gene
576 transfer (HGT), which plays a significant role in the evolution of vibrios (57).

577 HGT also contributes substantially to the emergence of pathogenic vibrios from non-
578 pathogenic environmental populations (57). For instance, CTX Φ is able to transduce the
579 cholera toxin (CT) from *V. cholera* to *V. mimicus* leading to the emergence of a pathogenic *V.*
580 *mimicus* form (58, 59). Many vibriophages contain virulence genes responsible for severe
581 gastro-intestinal diseases (47, 48). For instance, almost 80% of clinical *V. parahaemolyticus*
582 strains contain filamentous phages, encoding the zona occludens toxin (Zot) (22). Also, non-
583 human pathogens, such as *V. coralliilyticus* and *V. anguillarum* contain prophage-like
584 elements encoding Zot, suggesting frequent horizontal gene transfer (HGT) of Zot via
585 prophages among vibrios (11). In the present study, we found one filamentous phage, i.e.
586 *Vibrio* phage VALG Φ 6, that contains the virulence cluster comprising Ace and Zot, which
587 are common among vibrios (61). Considering the high homology between *Vibrio* phage
588 VALG Φ 6 and phages isolated from *V. parahaemolyticus* this might represent another
589 example where the movement of a filamentous phages across species boundaries leads to the
590 transfer of virulence factors possibly being responsible for the pathogenicity of Kiel *V.*
591 *alginolyticus* ecotypes. Controlled infection experiments revealed a close link between
592 virulence and coverage of the region encoding for *Vibrio* phage VALG Φ 6. Strains, for which
593 we observed a strong reduction in the coverage for the region encoding for *Vibrio* phage
594 VALG Φ 6 caused a reduced infection load compared to strains, with a high coverage for this
595 locus. This suggests that the low coverage may result in a reduced number of viral particles
596 and potentially a reduced production of both toxins, which may ultimately result in lower
597 virulence. We are aware, that to be able to ultimately prove that Ace and Zot encoded on
598 *Vibrio* phage VALG Φ 6 are causing the virulence of our isolates we would need a strain that
599 does not contain *Vibrio* phage VALG Φ 6 for further experiments.

600

601 Conclusion

602 By characterizing two novel filamentous vibriophages isolated from environmental strains
603 we increase our knowledge about filamentous vibriophages, which is as of October 2019
604 heavily biased towards human pathogens. We show that also non-human pathogenic vibrios
605 represent a reservoir of filamentous phages, which can contain virulence factors and
606 potentially move between species leading to the emergence of pathogens. We want to
607 encourage future studies on the phage-repertoire and their virulence factors of other non-
608 human pathogenic vibrios. By looking at a wider range of *Vibrio* species we will then
609 considerably expand our knowledge on the types of MGEs in *Vibrio* and in particular how
610 they influence the virulence and evolution of this species.

611 **Author statements:**

612 **Authors and contributors:** Methodology: RH, Conceptualisation: CCW, HL. Validation:
613 CCW, HL. Formal analysis: CCW, CMC. Data curation: HL. Writing-Original Draft
614 Preparation: CCW. Writing-Review and Editing: CMC, RH, MH, HL, CCW. Visualisation:
615 MH, CCW. Supervision: HL, CCW. Project administration: CCW. Funding: CCW.

616 **Conflict of interest:** The authors declare there are no conflicts of interest.

617 **Funding information:** This project was funded by a DFG grant [WE 5822/ 1-1] within the
618 priority programme SPP1819 and a grant from the Cluster of Excellence “The Future
619 Ocean”, given to CCW.

620 **Ethics approval:** Approval for using pipefish during infection experiments was given by the
621 Ministerium für Landwirtschaft, Umwelt und ländliche Räume des Landes Schleswig-
622 Holstein.

623 **Consent for publication:** This work does not need any consent for publication.

624 **Acknowledgements:** We thank Jelena Rajkov, and Olivia Roth for useful comments on a
625 previous version of this manuscript.

626

627

628 **Data bibliography:**

629 The accession numbers of the eight *Vibrio alginolyticus* genomes analysed in the present
630 study are provided in Supplementary information, Table S1.

631

632 The accession numbers of the two newly discovered filamentous phages are provided in the
633 manuscript, see Data statement.

634

635 The accession numbers of all other filamentous phage genomes used for comparative
636 genomics in the present study are provided in Supplementary information, Table S3 and
637 Figure 5.

638

639

640 References

- 641 1. Iguchi A, Iyoda S, Terajima J, Watanabe H, Osawa R. Spontaneous recombination
642 between homologous prophage regions causes large-scale inversions within the Escherichia
643 coli O157 : H7 chromosome. *Gene*. 2006;372:199-207.
- 644 2. Wagner PL, Waldor MK. Bacteriophage control of bacterial virulence. *Infect Immun*.
645 2002;70(8):3985-93.
- 646 3. Waldor MK, Mekalanos JJ. Lysogenic conversion by a filamentous phage encoding
647 cholera toxin. *Science*. 1996;272(5270):1910-4.
- 648 4. Lan SF, Huang CH, Chang CH, Liao WC, Lin IH, Jian WN, et al. Characterization of
649 a New Plasmid-Like Prophage in a Pandemic *Vibrio parahaemolyticus* O3:K6 Strain. *Appl*
650 *Environ Microb*. 2009;75(9):2659-67.
- 651 5. Chan B, Miyamoto H, Taniguchi H, Yoshida S. Isolation and genetic characterization
652 of a novel filamentous bacteriophage, a deleted form of phage f237, from a pandemic *Vibrio*
653 *parahaemolyticus* O4:K68 strain. *Microbiol Immunol*. 2002;46(8):565-9.
- 654 6. Faruque SM, Comstock L, Kaper JB, Albert MJ. Distribution of Zonula-Occludens
655 Toxin (Zot) Gene among Clinical Isolates of *Vibrio-Cholerae*-O1 from Bangladesh and
656 Africa. *J Diarrhoeal Dis Res*. 1994;12(3):222-4.
- 657 7. Kurazono H, Pal A, Bag PK, Nair GB, Karasawa T, Mihara T, et al. Distribution of
658 Genes Encoding Cholera-Toxin, Zonula Occludens Toxin, Accessory Cholera-Toxin, and El-
659 Tor Hemolysin in *Vibrio-Cholerae* of Diverse Origins. *Microb Pathogenesis*. 1995;18(3):231-
660 5.
- 661 8. Khouadja S, Suffredini E, Baccouche B, Croci L, Bakhrouf A. Occurrence of
662 virulence genes among *Vibrio cholerae* and *Vibrio parahaemolyticus* strains from treated
663 wastewaters. *Environ Monit Assess*. 2014;186(10):6935-45.
- 664 9. Weynberg KD, Voolstra CR, Neave MJ, Buerger P, van Oppen MJH. From cholera to
665 corals: Viruses as drivers of virulence in a major coral bacterial pathogen. *Scientific reports*.
666 2015;5.
- 667 10. Castillo D, Alvise PD, Xu R, Zhang F, Middelboe M, Gram L. Comparative Genome
668 Analyses of *Vibrio anguillarum* Strains Reveal a Link with Pathogenicity Traits. *mSystems*.
669 2017;2(1).
- 670 11. Castillo D, Perez-Reytor D, Plaza N, Ramirez-Araya S, Blondel CJ, Corsini G, et al.
671 Exploring the Genomic Traits of Non-toxigenic *Vibrio parahaemolyticus* Strains Isolated in
672 Southern Chile. *Front Microbiol*. 2018;9:161.
- 673 12. Balcazar JL, Gallo-Bueno A, Planas M, Pintado J. Isolation of *Vibrio alginolyticus*
674 and *Vibrio splendidus* from captive-bred seahorses with disease symptoms. *Antonie Van*
675 *Leeuwenhoek*. 2010;97(2):207-10.
- 676 13. Gómez-León J, Villamil L, Lemos M, Novoa B, Figueras A. Isolation of *Vibrio*
677 *alginolyticus* and *Vibrio splendidus* from Aquacultured Carpet Shell Clam (*Ruditapes*
678 *decussatus*) Larvae Associated with Mass Mortalities. *Appl Environ Microb*. 2005;71(1):98-
679 103.
- 680 14. Lacoste A, Jalabert F, Malham S, Cueff A, Gelebart F, Cordevant C, et al. A *Vibrio*
681 *splendidus* strain is associated with summer mortality of juvenile oysters *Crassostrea gigas*
682 in the Bay of Morlaix (North Brittany, France). *Dis Aquat Organ*. 2001;46(2):139-45.
- 683 15. Hada HS, West PA, Lee JV, Stemmler J, Colwell RR. *Vibrio tubiashii* Sp-Nov, a
684 *Pathogen of Bivalve Mollusks*. *Int J Syst Bacteriol*. 1984;34(1):1-4.
- 685 16. Wendling CC, Wegner KM. Relative contribution of reproductive investment,
686 thermal stress and *Vibrio* infection to summer mortality phenomena in Pacific oysters.
687 *Aquaculture*. 2013;412-413:88-96.

- 688 17. Campos J, Martinez E, Izquierdo Y, Fando R. VEJ phi, a novel filamentous phage of
689 *Vibrio cholerae* able to transduce the cholera toxin genes. *Microbiol-Sgm*. 2010;156:108-15.
- 690 18. Campos J, Martinez E, Suzarte E, Rodriguez BL, Marrero K, Silva Y, et al. VGJ phi,
691 a novel filamentous phage of *Vibrio cholerae*, integrates into the same chromosomal site as
692 CTX phi. *J Bacteriol*. 2003;185(19):5685-96.
- 693 19. Campos J, Martinez E, Marrero K, Silva Y, Rodriguez BL, Suzarte E, et al. Novel
694 type of specialized transduction for CTX phi or its satellite phage RS1 mediated by
695 filamentous phage VGJ phi in *Vibrio cholerae*. *J Bacteriol*. 2003;185(24):7231-40.
- 696 20. Wendling CC, Goehlich H, Roth O. The structure of temperate phage-bacteria
697 infection networks changes with the phylogenetic distance of the host bacteria. *Biol Lett*.
698 2018;14(11).
- 699 21. Munro J, Oakey J, Bromage E, Owens L. Experimental bacteriophage-mediated
700 virulence in strains of *Vibrio harveyi*. *Dis Aquat Organ*. 2003;54(3):187-94.
- 701 22. Castillo D, Kauffman K, Hussain F, Kalatzis P, Rorbo N, Polz MF, et al. Widespread
702 distribution of prophage-encoded virulence factors in marine *Vibrio* communities. *Scientific*
703 *reports*. 2018;8.
- 704 23. Gonzalez-Escalona N, Blackstone GM, DePaola A. Characterization of a *Vibrio*
705 *alginolyticus* strain, isolated from Alaskan oysters, carrying a hemolysin gene similar to the
706 thermostable direct hemolysin-related hemolysin gene (*trh*) of *Vibrio parahaemolyticus*. *Appl*
707 *Environ Microbiol*. 2006;72(12):7925-9.
- 708 24. Lee KK, Yu SR, Yang TI, Liu PC, Chen FR. Isolation and characterization of *Vibrio*
709 *alginolyticus* isolated from diseased kuruma prawn, *Penaeus japonicus*. *Lett Appl Microbiol*.
710 1996;22(2):111-4.
- 711 25. Zhang DL, Manos J, Ma XR, Belas R, Karaolis DKR. Transcriptional analysis and
712 operon structure of the *tagA-orf2-orf3-mop-tagD* region on the *Vibrio* pathogenicity island in
713 epidemic *V-cholerae*. *Fems Microbiol Lett*. 2004;235(1):199-207.
- 714 26. Hormansdorfer S, Wentges H, Neugebauer-Buchler K, Bauer J. Isolation of *Vibrio*
715 *alginolyticus* from seawater aquaria. *Int J Hyg Environ Health*. 2000;203(2):169-75.
- 716 27. Roth O, Keller I, Landis SH, Salzburger W, Reusch TB. Hosts are ahead in a marine
717 host-parasite coevolutionary arms race: innate immune system adaptation in pipefish
718 *Syngnathus typhle* against *Vibrio phylotypes*. *Evolution*. 2012;66(8):2528-39.
- 719 28. Wendling CC, Piecyk A, Refardt D, Chibani C, Hertel R, Liesegang H, et al.
720 Tripartite species interaction: eukaryotic hosts suffer more from phage susceptible than from
721 phage resistant bacteria. *BMC Evol Biol*. 2017;17(98).
- 722 29. Hertel R, Rodriguez DP, Hollensteiner J, Dietrich S, Leimbach A, Hoppert M, et al.
723 Genome-Based Identification of Active Prophage Regions by Next Generation Sequencing in
724 *Bacillus licheniformis* DSM13. *Plos One*. 2015;10(3).
- 725 30. Andrews S. FastQC: a quality control tool for high throughput sequence data 2010
726 [Available from: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>].
- 727 31. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina
728 sequence data. *Bioinformatics*. 2014;30(15):2114-20.
- 729 32. Willms IM, Hoppert M, Hertel R. Characterization of *Bacillus Subtilis* Viruses
730 *vB_BsuM-Goe2* and *vB_BsuM-Goe3*. *Viruses*. 2017;9(6).
- 731 33. Arndt D, Grant JR, Marcu A, Sajed T, Pon A, Liang Y, et al. PHASTER: a better,
732 faster version of the PHAST phage search tool. *Nucleic Acids Res*. 2016;44(W1):W16-21.
- 733 34. Sullivan MJ, Petty NK, Beatson SA. Easyfig: a genome comparison visualizer.
734 *Bioinformatics*. 2011;27(7):1009-10.
- 735 35. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics*.
736 2014;30(14):2068-9.

- 737 36. Hyatt D, Chen GL, Locascio PF, Land ML, Larimer FW, Hauser LJ. Prodigal:
738 prokaryotic gene recognition and translation initiation site identification. *Bmc*
739 *Bioinformatics*. 2010;11:119.
- 740 37. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods*.
741 2012;9(4):357-9.
- 742 38. Dietrich S, Wiegand S, Liesegang H. TraV: a genome context sensitive transcriptome
743 browser. *PLoS One*. 2014;9(4):e93677.
- 744 39. Ramirez F, Dundar F, Diehl S, Gruning BA, Manke T. deepTools: a flexible platform
745 for exploring deep-sequencing data. *Nucleic Acids Res*. 2014;42(Web Server issue):W187-
746 91.
- 747 40. Larsson A. AliView: a fast and lightweight alignment viewer and editor for large
748 datasets. *Bioinformatics*. 2014;30(22):3276-8.
- 749 41. Mai-Prochnow A, Hui JG, Kjelleberg S, Rakonjac J, McDougald D, Rice SA. 'Big
750 things in small packages: the genetics of filamentous phage and effects on fitness of their
751 host'. *Fems Microbiol Rev*. 2015.
- 752 42. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high
753 throughput. *Nucleic Acids Res*. 2004;32(5):1792-7.
- 754 43. Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, et al.
755 MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large
756 Model Space. *Syst Biol*. 2012;61(3):539-42.
- 757 44. Huelsenbeck JP, Ronquist F. MRBAYES: Bayesian inference of phylogenetic trees.
758 *Bioinformatics*. 2001;17(8):754-5.
- 759 45. Tamura K, Nei M. Estimation of the Number of Nucleotide Substitutions in the
760 Control Region of Mitochondrial-DNA in Humans and Chimpanzees. *Mol Biol Evol*.
761 1993;10(3):512-26.
- 762 46. Posada D, Buckley TR. Model selection and model averaging in phylogenetics:
763 advantages of akaike information criterion and bayesian approaches over likelihood ratio
764 tests. *Syst Biol*. 2004;53(5):793-808.
- 765 47. Trucksis M, Galen JE, Michalski J, Fasano A, Kaper JB. Accessory cholera
766 enterotoxin (Ace), the third toxin of a *Vibrio cholerae* virulence cassette. *Proc Natl Acad Sci*
767 *U S A*. 1993;90(11):5267-71.
- 768 48. Perez-Reytor D, Jana V, Pavez L, Navarrete P, Garcia K. Accessory Toxins of *Vibrio*
769 *Pathogens and Their Role in Epithelial Disruption During Infection*. *Frontiers in*
770 *Microbiology*. 2018;9.
- 771 49. Chibani C, Roth O, Liesegang H, Wendling CC. Comparative genomic analysis
772 reveals that the mobilome of *V. alginolyticus* plays a major role in its niche adaptation. *in*
773 *prep*.
- 774 50. Bobay LM, Touchon M, Rocha EPC. Pervasive domestication of defective prophages
775 by bacteria. *P Natl Acad Sci USA*. 2014;111(33):12127-32.
- 776 51. Casjens S. Prophages and bacterial genomics: what have we learned so far? *Mol*
777 *Microbiol*. 2003;49(2):277-300.
- 778 52. Canchaya C, Fournous G, Brussow H. The impact of prophages on bacterial
779 chromosomes. *Mol Microbiol*. 2004;53(1):9-18.
- 780 53. Grove A. MarR family transcription factors. *Curr Biol*. 2013;23(4):R142-3.
- 781 54. Ellison DW, Miller VL. Regulation of virulence by members of the MarR/SlyA
782 family. *Curr Opin Microbiol*. 2006;9(2):153-9.
- 783 55. Refardt D. Within-host competition determines reproductive success of temperate
784 bacteriophages. *Isme J*. 2011;5(9):1451-60.

- 785 56. Davis BM, Moyer KE, Boyd EF, Waldor MK. CTX prophages in classical biotype
786 *Vibrio cholerae*: functional phage genes but dysfunctional phage genomes. *J Bacteriol.*
787 2000;182(24):6992-8.
- 788 57. Hazen TH, Pan L, Gu JD, Sobecky PA. The contribution of mobile genetic elements
789 to the evolution and ecology of *Vibrios*. *Fems Microbiology Ecology.* 2010;74(3):485-99.
- 790 58. Boyd EF, Moyer KE, Shi L, Waldor MK. Infectious CTX Phi, and the vibrio
791 pathogenicity island prophage in *Vibrio mimicus*: Evidence for recent horizontal transfer
792 between V-mimicus and V-cholerae. *Infection and Immunity.* 2000;68(3):1507-13.
- 793 59. Faruque SM, Rahman MM, Asadulghani, Nasirul Islam KM, Mekalanos JJ.
794 Lysogenic conversion of environmental *Vibrio mimicus* strains by CTXPhi. *Infect Immun.*
795 1999;67(11):5723-9.
- 796 60. Ruby EG, Urbanowski M, Campbell J, Dunn A, Faini M, Gunsalus R, et al. Complete
797 genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. *Proc*
798 *Natl Acad Sci U S A.* 2005;102(8):3004-9.
- 799 61. Park JH, Cho YJ, Chun J, Seok YJ, Lee JK, Kim KS, et al. Complete genome
800 sequence of *Vibrio vulnificus* MO6-24/O. *J Bacteriol.* 2011;193(8):2062-3.
- 801 62. Marvin DA, Symmons MF, Straus SK. Structure and assembly of filamentous
802 bacteriophages. *Prog Biophys Mol Bio.* 2014;114(2):80-122.

803

804

805

806

807 **Figure legends**

808

809 **Figure 1** Genomic maps of *Vibrio* phage VALGΦ1 (top), *Vibrio* phage VALGΦ2 (bottom left) and *Vibrio* phage VALGΦ2b
810 (bottom right). ORFs are color-coded according to predicted function: red: replication, green: assembly, blue: structural
811 proteins, yellow: integration, purple: lysis, orange: accessory genes, grey: hypothetical proteins.

812

813 **Figure 2** Whole Chromosome alignment with prophage regions in coloured boxes/ arrows. Active prophages
814 are marked in red. Blocks of the same colour indicate phage-types, purple: *Vibrio* phage VALGΦ1, light blue:
815 *Vibrio* phage VALGΦ2, *Vibrio* phage VALGΦ2b complex, dark green: *Vibrio* phage VALGΦ6, light green:
816 *Vibrio* phage VALGΦ8, dark blue: multi-phage complex containing sequences of *Vibrio* phage VALGΦ6 and
817 *Vibrio* phage VALGΦ8, grey: unknown phages.

818

819 **Figure 3** Genomic maps of *Vibrio* phage VALGΦ6 (second from top) and *Vibrio* phage VALGΦ8 (third from top) in
820 comparison to VfO4K68 (top) and VF33 (bottom). ORFs are color-coded according to predicted function: red: replication,
821 green: assembly, blue: structural proteins, grey: hypothetical proteins. High homologous sequences are indicated by dark
822 grey and low homologous sequences by light grey. pI – pX correspond to known filamentous phage proteins and putative
823 homologues.

824

825 **Figure 4** Extrachromosomal contigs of (a) Strain K04M1 and (b) Strain K05K4 with the 2-replicon containing
826 contig (left) and the 3-replicon containing contig (right). ORF-coding and protein names as in Figure 3.

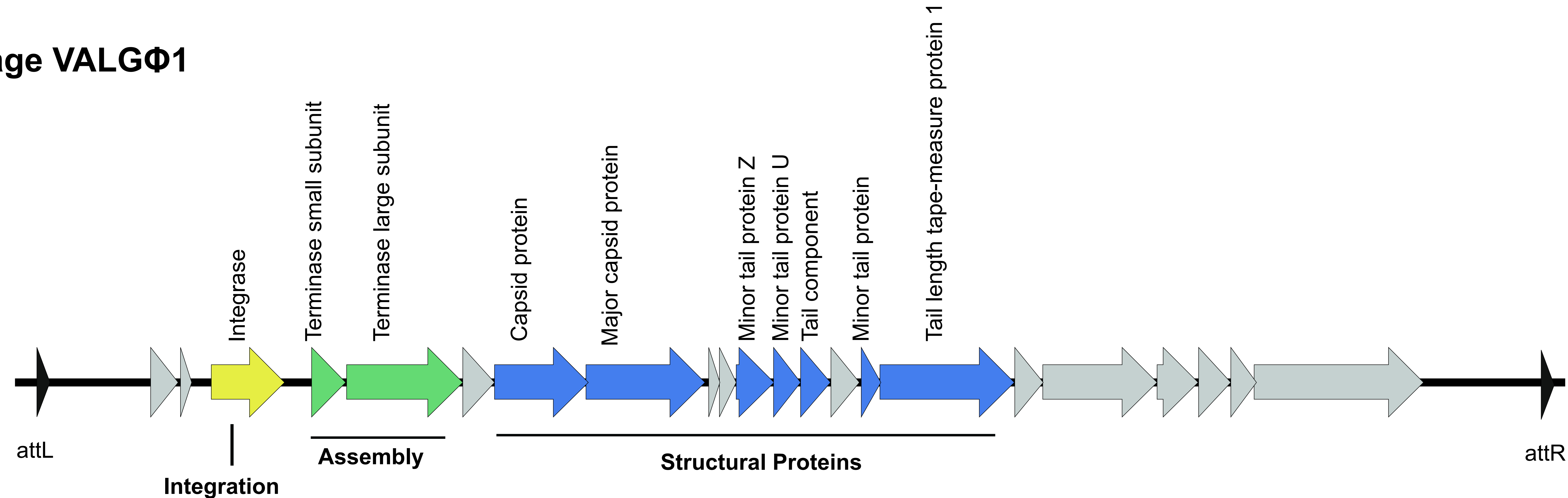
827

828 **Figure 5** Phylogenetic tree based on the major coat protein (pVIII) alignment highlighting the position of *Vibrio*
829 phage VALGΦ6 and *Vibrio* phage VALGΦ8 relative to other filamentous phages. The corresponding NCBI
830 accession numbers for the different phages are denoted in brackets, ena accession numbers are indicated with an
831 *, uniprot accession numbers of the major coat protein are indicated with **. For outgroup *Propionibacterium*
832 *phage B5* was used. Class I and Class II phages represent clusters according to (62).

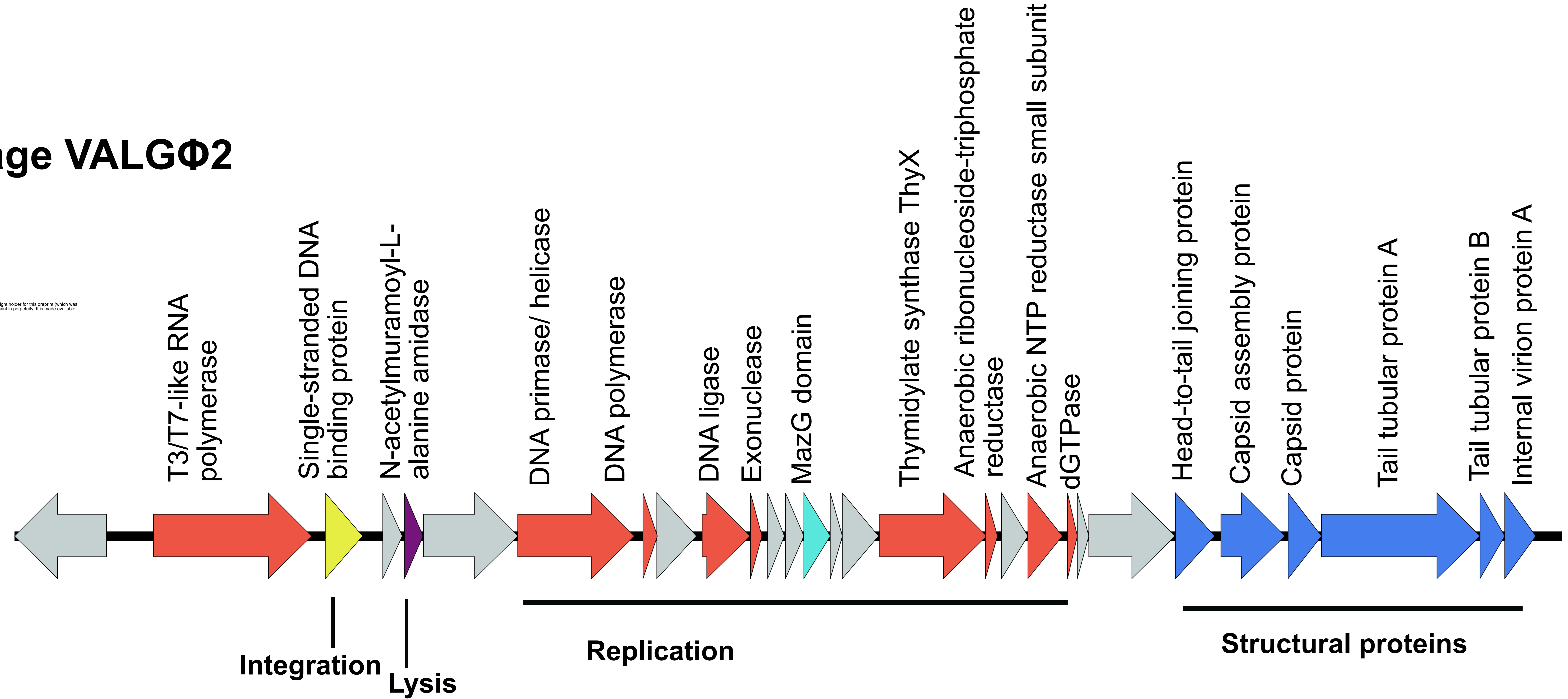
833

834 **Figure 6** Virulence of all eight sequenced strains (x-axis), measured as bacterial load (CFU/ml).

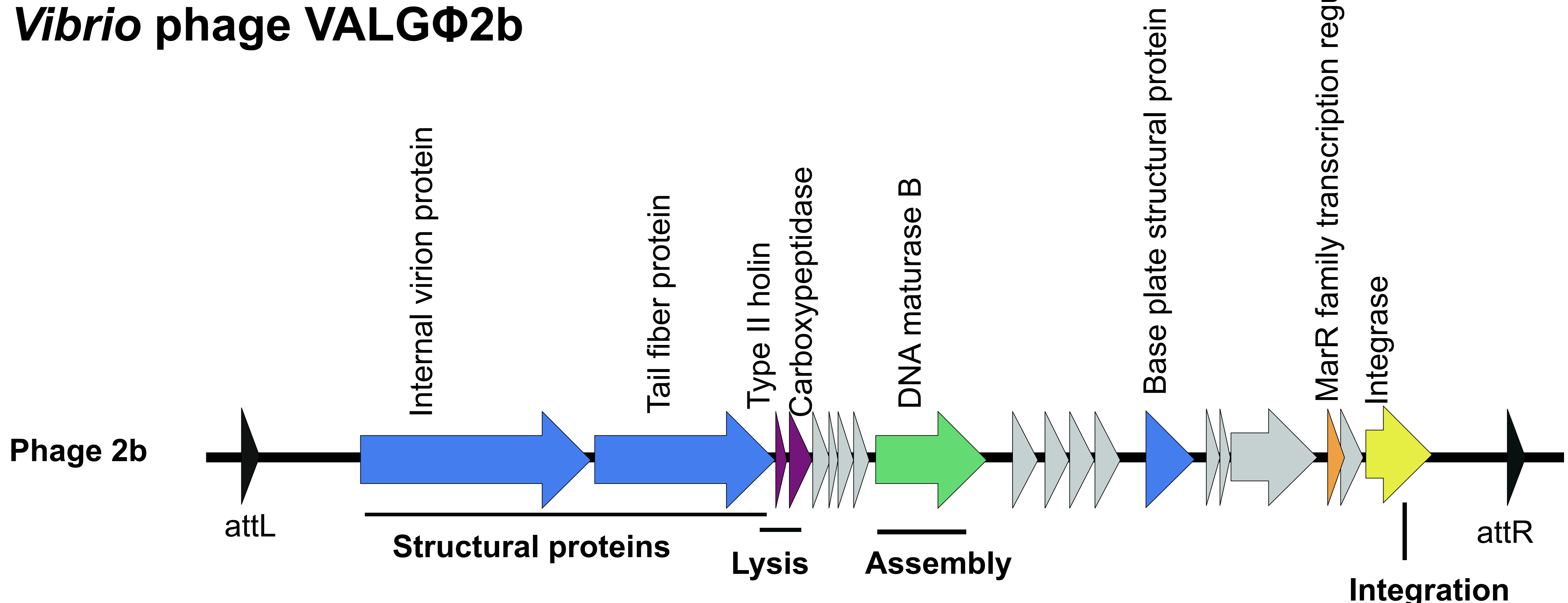
Vibrio phage VALGΦ1



Vibrio phage VALGΦ2



Vibrio phage VALGΦ2b



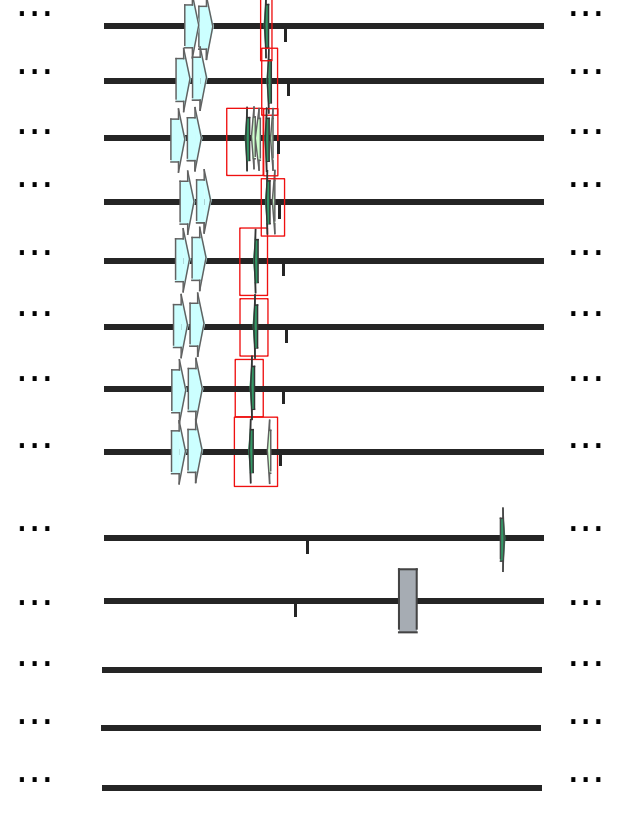
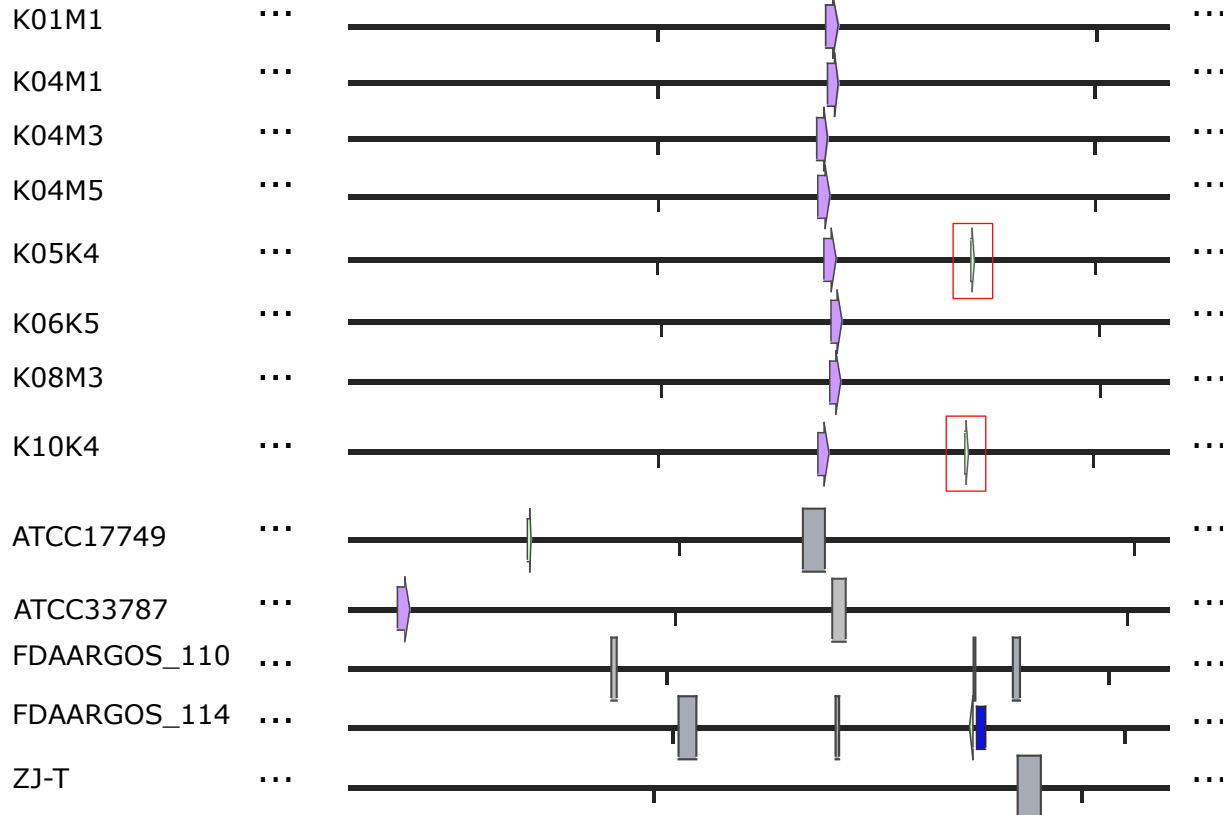
Strain


Chromosome 1

Chromosome 2


0 1,000,000 2,000,000


0 1,000,000 1,500,000




 *Vibrio* phage VALGΦ1

 *Vibrio* phage VALGΦ2 and 2b

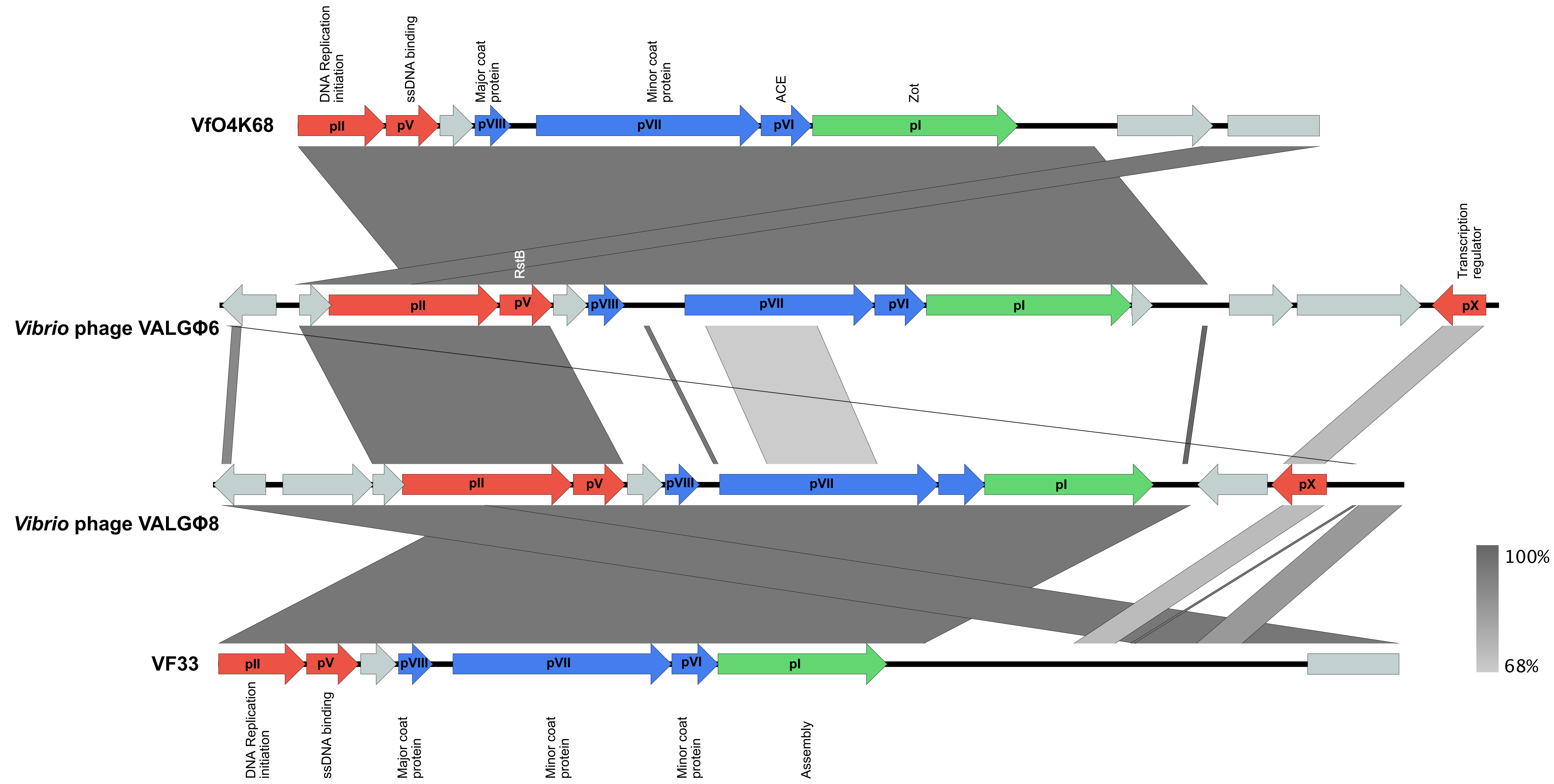
 *Vibrio* phage VALGΦ6

 *Vibrio* phage VALGΦ8

 active phage

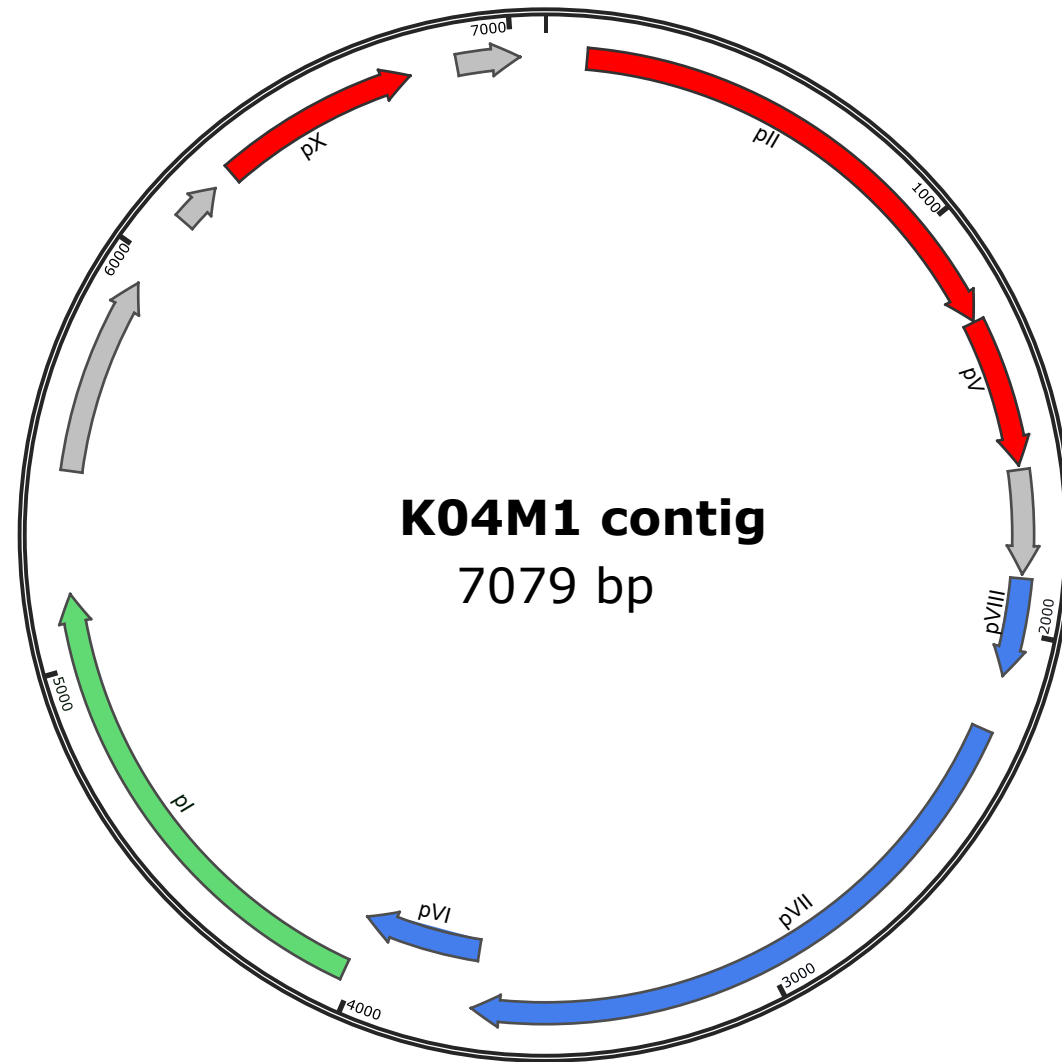
 unknown phage

 Phage 6/ Phage 8 Mix

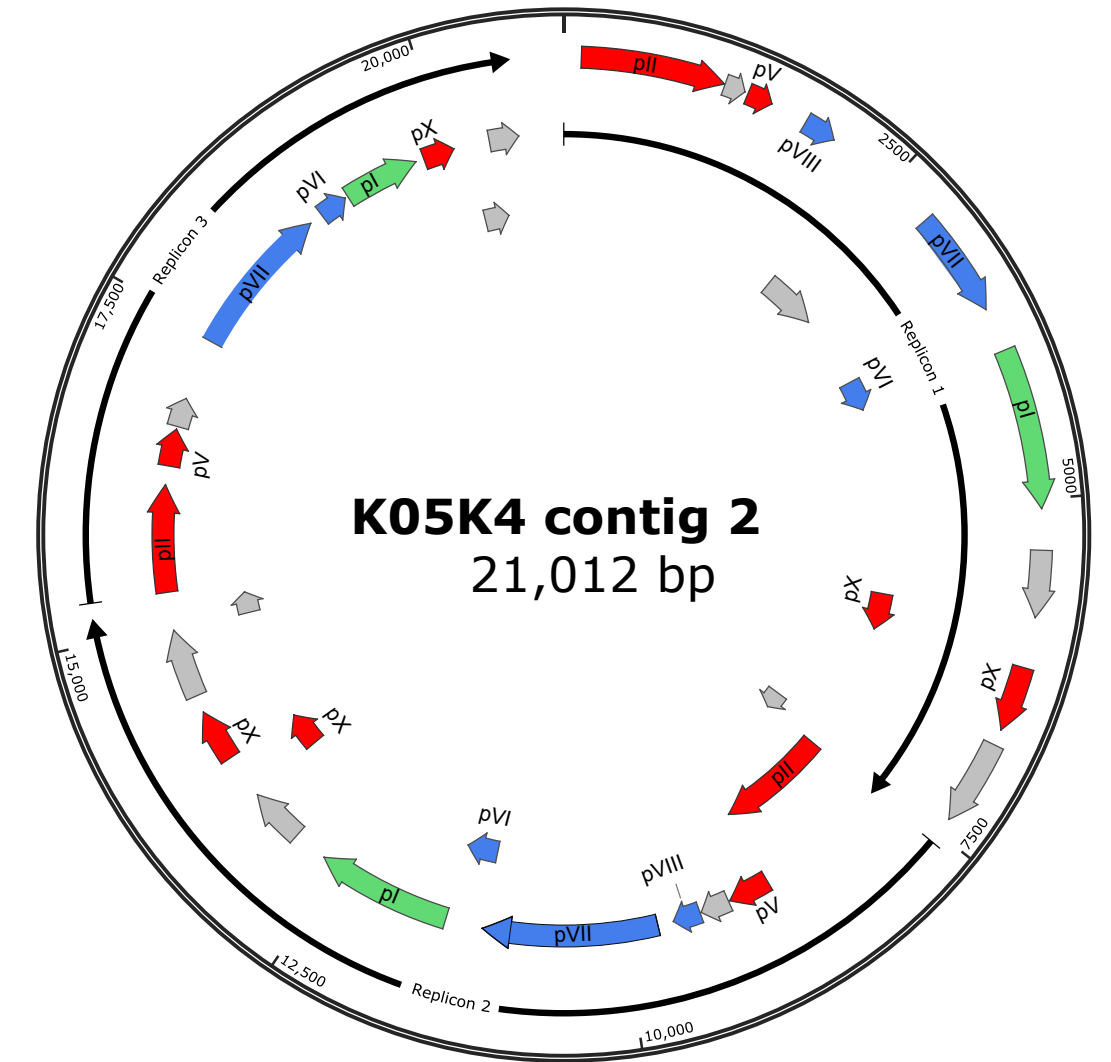
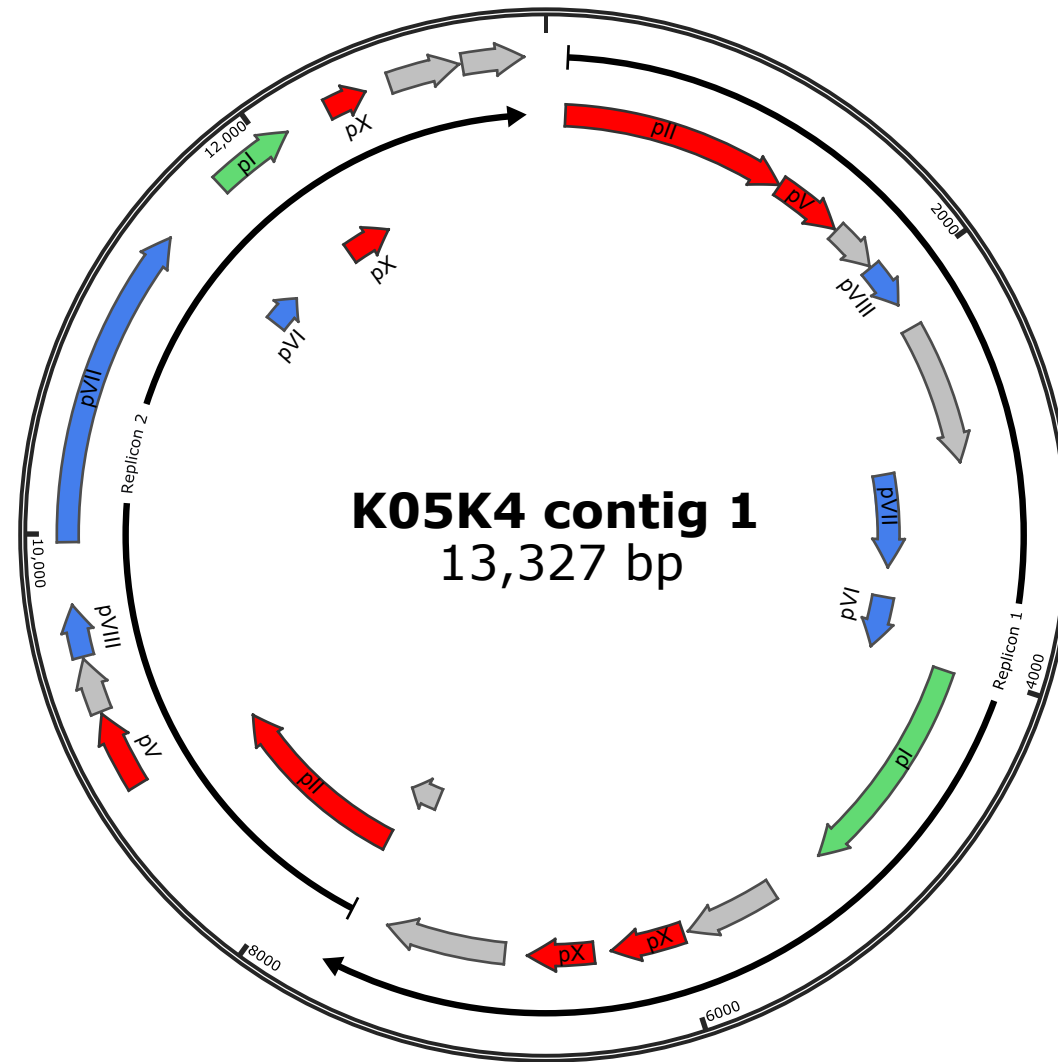


bioRxiv preprint doi: <https://doi.org/10.1101/2021.03.10.432801>; this version posted November 20, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

(a)



(b)



Host strain:

Escherichia coli
Propriobacterium
Pseudomonas
Thermus thermophilus
Vibrio alginolyticus
Vibrio cholerae
Vibrio parahaemolyticus
Xanthomonas

