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1 ***Vibrio* are a potential source of novel colistin-resistance** 2 **genes in European coastal environments**

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24 **Abstract**

25 Colistin is a widespread last resort antibiotic for treatment of multidrug-resistant bacteria.
26 The recent worldwide emergence of colistin resistance (Col-R) conferred by *mcr-1* in human
27 pathogens has raised concern, but the putative sources and reservoirs of novel *mcr* genes in
28 the marine environment remain underexplored. We observed a high prevalence of Col-R,
29 particularly in *Vibrio* isolated from European coastal waters by using a unique stock of
30 specific-pathogen-free (SPF) oysters as a bioaccumulator. The high sequence diversity found
31 in the *mcr/eptA* gene family was geographically structured, particularly for three novel *eptA*
32 gene variants, which were restricted to the Mediterranean (France, Spain) and occurred as
33 a *dgkA-eptA* operon controlled by the RstA/RstB two component system. By analyzing
34 29427 *Vibrionaceae* genome assemblies, we showed that this mechanism of intrinsic
35 resistance is prevalent and specific to the Harveyi clade, which includes strains of *Vibrio*
36 *parahaemolyticus* and *Vibrio alginolyticus* causing infections in humans. The operon
37 conferred colistin-resistance when transferred to sensitive non-*Vibrio* strains. While *mcr*-
38 and *arn*-based Col-R mechanisms were also identified, the widespread presence of *eptA* gene
39 variants in *Vibrio* suggests they play a key role in intrinsic resistance to colistin. Beyond these
40 ancient *eptA* gene copies having evolved with the *Vibrio* lineage, we also identified mobile
41 *eptA* paralogues that have been recently transferred between and within *Vibrio* clades. This
42 highlights *Vibrio* as a potential source of Col-R mechanisms, emphasizing the need for
43 enhanced surveillance to prevent colistin-resistant infections in coastal areas.

44

45 **Keywords.** Ocean, Shellfish, Bacteria, Antibiotic, Polymyxin, Resistance, *Vibrionaceae*.

46 **Introduction**

47 The excessive and inadequate use of antibiotics in human and veterinary medicine has led
48 to the spread of antimicrobial resistance genes (ARGs) by creating selective pressures
49 favoring the development of resistant bacteria¹. If this problem is not addressed, it is
50 estimated that by 2050, antimicrobial resistant bacteria (ARB) could lead to an annual loss
51 of approximately 10 million lives and limit options for effectively treating bacterial
52 infections².

53 The antibiotic properties of cationic cyclic antimicrobial peptides belonging to the group of
54 polymyxins (*e.g.* Colistin, polymyxin B) is well known, but the use of these peptides for
55 human therapy is limited due to strong side effects. For this reason, limited resistance is
56 reported in clinical settings, promoting the use of polymyxins as a last resort antibiotic for
57 treatments of multidrug-resistant infections³⁻⁵. Preservation of long-term effectiveness of
58 polymyxins is thus of primary importance for human health globally. Regrettably, the use of
59 colistin has increased as a growth promoter in poultry and swine farms. This has resulted in
60 the rapid spread of colistin resistance in Gram-negative bacteria that are clinically significant
61 on a global scale^{6,7}. The global spread of polymyxin-resistant bacteria in clinical and
62 environmental settings has become a major concern in the treatment of multidrug-resistant
63 pathogens in recent years.

64 Polymyxins bind to the negatively charged Lipid A component of lipopolysaccharides at the
65 outer membrane of Gram-negative bacteria^{5,8}, then they disrupt the structure of the outer
66 membrane, leading to the increase of cell permeability and subsequent cell death⁹.
67 Resistance to polymyxins by Gram-negative bacteria can rely on different mechanisms

68 leading to the reduction of Lipid A negative charges thus decreasing electrostatic
69 interactions with polymyxins¹⁰. In nature, the most frequently observed colistin resistance
70 genes are *eptA* and *pmrHFIJFKLM* (also referred to as *arnBCADTEF*). These genes are
71 chromosomally encoded and catalyze the addition of phosphoethanolamine (PEtN), or the
72 addition of a 4-amino-4-deoxy-L-arabinose (L-Ara4N) to the phosphate groups of lipids A¹¹.
73 In various bacterial genera the activation of the *eptA* and *arnBCADTEF* expression is
74 controlled by the PmrA/B and/or PhoP/Q two-component systems (TCS) (for review see¹²).
75 Additionally, a plasmid-mediated mechanism of resistance to colistin was discovered less
76 than 10 years ago and involves *mcr-1* (for mobile colistin resistance)⁶. It is a rare example of
77 a recent ARG capture and spread. The emergence and rapid spread of *mcr-1* among various
78 Gram-negative bacteria has alerted health organizations worldwide (Europe, Asia, North
79 America, and Africa)¹³, and the number of newly reported *mcr* genes is ever-growing since
80 2015¹⁴. Furthermore, recent research indicates that the majority of mobile colistin
81 resistance gene variants (*mcr1-9*) originate from environmental bacteria, particularly from
82 aquatic sources¹⁵. Similar to *eptA*, *mcr-1* encodes a phosphoethanolamine transferase (PET)
83 but its expression is not dependent on a regulatory system¹⁰.

84 The rapid spread of colistin resistance from environmental sources highlights the urgent
85 need to enhance our understanding of the prevalence and distribution of colistin-resistant
86 bacteria and their associated resistance genes in aquatic ecosystems. This calls for research
87 that emphasizes the interconnectedness of humans, animals, and the environment (*i.e.*, the
88 One-Health concept^{16,17}). However, the marine environment remains largely unexplored
89 regarding antimicrobial resistance (AMR), especially in Europe. Studying AMR in coastal
90 marine environments is particularly important because (i) coastal systems are highly

91 exposed to human contaminants that may select or co-select for ARGs, (ii) coastal systems
92 are highly interconnected through international trade, which favors the worldwide spread
93 of ARGs, (iii) human populations live along coasts and depend on marine environments as a
94 food source¹⁸, which increases the risk of transmission, and (iv) coastal waters and
95 sediments, wastewater discharge and marine aquaculture can act as sources/reservoirs of
96 ARGs and have contributed to localized increases in abundance of ARGs^{19,20}. Still, the study
97 of AMR in marine waters lags behind in comparison to other environments, and there is a
98 lack of understanding of the role these environments play in the global cycle of AMR.

99 The marine environment may harbor diverse ARGs, flourishing under human-induced
100 pressures. Almost all known variants of the *mcr* gene that have been reported thus far are
101 from aquatic bacteria from diverse environments¹⁵. Among them, aquatic bacteria from the
102 *Shewanella* genus are considered as a source of *mcr-4*¹⁵. Moreover, *mcr-1* was found recently
103 in colistin-resistant bacteria in marine coastal waters from Norway and Croatia, which may
104 constitute reservoirs^{21,22}. Some recent studies have also shown the role of variants of the
105 *eptA* gene in colistin resistance in strains of *Vibrio cholerae*²³, *Vibrio parahaemolyticus*²⁴
106 *Vibrio vulnificus*²⁵ and *Vibrio fisheri* (*Aliivibrio fisheri*)²⁶. Given the limited data available on
107 the frequency and distribution of colistin-resistant bacteria in marine environments, along
108 with the evidence of colistin-resistant *Vibrio* species with pathogenic potential in humans
109 and marine fauna²⁷, there is an urgent need to increase our understanding of the medical
110 and ecological significance of this phenomenon.

111 To further evaluate the potential role of the coastal environment in the emergence and
112 circulation of colistin resistance genes, we determined the prevalence of *mcr/eptA* genes in

113 culturable marine bacteria in coastal waters across Europe, as well as in *Vibrionaceae* in
114 general. We targeted three different European areas that are heavily impacted by
115 anthropogenic pollution from oyster farming and tourism: Sylt (Germany), the Ebro Delta
116 (Spain), and Thau Lagoon (France). We characterized the prevalence, diversity, and
117 distribution of *mcr/eptA* by using a unique stock of SPF-oysters as bioaccumulators that
118 were incubated in all sites for tracking AMR, as they concentrate bacteria from the
119 environment by filter feeding²⁸. Our data revealed an unexpected diversity of *eptA* in *Vibrio*,
120 with a clearly structured geographic distribution of *eptA* variants across Europe. Functional
121 genetic experiments were used to demonstrate the mechanisms of colistin resistance
122 conferred by the newly discovered *eptA* gene variants from the *Vibrio* Harveyi clade. The
123 discovery of highly diverse and prevalent mechanisms of colistin resistance as well as as
124 evidences of recent mobilization in *Vibrionaceae* highlights the underestimated risk of the
125 emergence of colistin resistance in European coastal environments and warrants further
126 investigation.

127

128 Results

129 Colistin-resistant bacteria are abundant in oysters in European coastal environments.

130 To estimate the prevalence of colistin resistance (Col-R) in culturable marine bacteria from
131 European coastal ecosystems, we immersed SPF-oysters in three sites either containing
132 natural beds (Sylt, Germany), or oyster farms (Ebro, Spain and Thau, France). After 2-3
133 weeks, marine bacteria isolated from the SPF-oysters, on either marine agar or Thiosulfate
134 Citrate Bile Salts Sucrose (TCBS) agar at 20°C or 37°C, were tested for Col-R using a
135 microtiter plate assay. Bacteria isolated on marine agar showed frequent resistance to
136 colistin with a minimal inhibitory concentration (MIC) > 5 µg/ml in Zobell medium, which
137 mimics the composition of seawater (Table 1). Out of 87 bacterial isolates from Thau
138 (France), 28 (32.1%) were Col-R. Most of these Col-R isolates (27/28) were isolated at 37°C.
139 In Ebro (Spain), 17/140 isolates were Col-R (12.1%) and most of them (11/17) were isolated
140 at 37°C. In Sylt (Germany), 37/51 isolates were Col-R (72.5%), and most of them (23/37)
141 were isolated at 37°C (Table 1, Fig. S1). Col-R phenotypes were even more prevalent among
142 bacteria isolated on TCBS (selective for *Vibrio*). Out of the 52 isolates obtained on TCBS from
143 Thau samples, 16 (30.8%) were Col-R. Among them, 11/16 were isolated at 20°C and 5/16
144 were isolated at 37°C. In Ebro, 11 out of 44 isolates (25%) were Col-R. Among them, 5/11
145 were isolated at 20°C and 6/11 were isolated at 37°C. In Sylt, most isolates (41/45; 91.1%)
146 were Col-R; all of them were isolated at 20°C (no bacterial isolates at 37°C) (Table 1). Overall,
147 our sampling highlighted a remarkable prevalence of colistin resistant isolates in culturable
148 bacteria accumulating in oysters immersed in European coastal waters, particularly in North
149 Germany (general linear model, $p = 1.5 \times 10^{-5}$). In France and Spain, the percentage was
150 particularly high in bacteria isolated at 37°C ($p=3.8 \times 10^{-5}$). There was also a clear effect of

151 the isolation on TCBS medium ($p= 7.7 \times 10^{-3}$), suggesting higher Col-R prevalence in *Vibrio*
152 (Fig. S1).

153

154 **High diversity of *mcr/eptA* genes in oysters in European coastal systems.** To capture
155 the diversity of *mcr/eptA* resistance genes circulating in culturable bacteria from European
156 marine coastal systems, pool-sequencing was performed on a total of 394 bacterial isolates
157 pooled by site and isolation conditions (culture medium, temperature) with an average
158 number of 4.10^7 reads per pool (99 to 149 strains per sampling site; see Table S1). A total of
159 53 complete unique nucleotide sequences related to *mcr/eptA* were found in the contigs
160 from the pool-sequencing after Prokka annotations (Table S1 and S2). Among them, 31 and
161 22 sequences were carried by bacteria isolated on either marine agar or TCBS, respectively
162 (Table S1). The diversity of the Mcr/EptA amino acid sequences was studied by
163 reconstructing their molecular phylogeny. Amino acid sequences deduced from the pool
164 sequencing were compared to the 104 Mcr-1 to -10 amino acid sequences present in the
165 CARD database. The whole set of sequences was also used to identify Mcr/EptA sequences
166 encoded in the 29427 *Vibrionaceae* genome assemblies found in GenBank. A total of
167 27921/29427 genomes encoded at least one Mcr/EptA, representing 4075 distinct amino
168 acid sequences among 31813 protein hits, which were included in the analysis. EptA and Mcr
169 sequences could clearly be distinguished based on the molecular phylogeny of their deduced
170 amino acid sequences (Fig. 1).

171 The EptA clade gathered a highly diversified set of sequences from *Vibrionaceae*, including
172 the four previously characterized EptA from *V. cholerae*²³, *V. parahaemolyticus*²⁴, *V.*

173 *vulnificus*²⁵ and *V. fisheri*²⁶ (Fig. 1). Most of the sequences from the pool-sequencing (42/53)
174 were assigned to EptA (83.87% and 77.27% of the sequences from bacteria isolated on
175 marine agar and on *Vibrio*-selective TCBS medium, respectively) (Fig. S2, Tables S3). They
176 were only found in *Vibrionaceae* (in genera *Vibrio*, *Photobacterium*, *Allivibrio*) based on
177 protein sequence similarity (more than 90% amino acid sequence identity over their full-
178 length sequence) (Table S4). Only a limited number of sequences (9/53) were assigned to
179 Mcr (Fig. 1, Table S4); they were found in more diverse genera including *Vibrio*,
180 *Photobacterium*, *Shewanella*, and *Pseudoalteromonas* (Fig. S2). These sequences encoding
181 Mcr protein variants were previously unreported and the majority clustered with Mcr-4 (Fig.
182 S2). They were far less abundant than sequences encoding EptA protein variants, both in
183 terms of sequence diversity and read counts (Fig 1, Fig. S2).

184

185 **EptA variants specific of the Harveyi and Splendidus clades are dominant in oysters.**

186 Four predicted EptA proteins (EptA-1 to -4) encoded by unique nucleotide sequences were
187 dominant in oysters, both in terms of sequence diversity (Fig. 1) and read counts (Fig. S2).
188 EptA-1 to -4 sequences harbored the catalytic threonine conserved in EptA orthologs
189 functionally characterized in *Vibrionaceae* (Fig. S3) as well as in EptA/Mcr proteins from
190 *Enterobacteriaceae*²³. EptA-1 to -3 clustered separately from EptA-4 in the Mcr/EptA
191 phylogeny (Fig. 1). Compared to previously characterized EptA variants, EptA-1 to -3 amino
192 acid sequences showed the maximum identity 80.9-83.6 % with VP_RS21300 from *V.*
193 *parahaemolyticus* and 69.9-72.9 % with VVM06_RS15980 from *V. vulnificus*. Only 58.2-58.7
194 % maximum identity was found with VCA1102 from *V. cholerae* and 42.5-44.5 % with

195 VF_A0210 from *V. fisheri*. EptA-4 was far more divergent with only 43.6 %, 55.2 %, 54.6 %
196 and 56.6 % maximum identity with VF_A0210, VCA1102, VP_RS21300 and VVM06_RS15980
197 respectively (Table S4).

198

199 EptA-1 (encoded by 10 unique nucleotide sequences), EptA-2 (3 unique nucleotide
200 sequences) and EptA-3 (encoded by 5 unique nucleotide sequences) clustered together on
201 the Mcr/EptA phylogenetic tree (Fig. 1). EptA-1 and EptA-2 showed the highest maximum
202 identity (87.2%), compared to a maximum of 78.6-78.8% between EptA-1/2 and EptA-3 (Fig.
203 1, Table S4). Moreover, their encoding genes shared a common, but previously undescribed
204 genomic environment, consisting of five conserved genes: *rstA-rstB*-Glycine zipper family
205 protein-*dgkA-eptA* (Fig. 2B, Table S3). This genomic environment was specific to the Harveyi
206 clade when considering the four genes upstream and downstream of the *mcr/eptA*
207 sequences in a *Vibrionaceae* phylogenetic tree, which was constructed from 23,642 genome
208 assemblies with sufficient quality to be included in the MLSA (Fig. 2A, Dataset 1). While the
209 *rstA-rstB*-Glycine zipper family protein-*dgkA-eptA* genomic environment was a clear
210 indicator of the assignment to the Harveyi clade, EptA polymorphism appeared to have
211 followed differentiation between species: EptA-1 was found in the species *V. harveyi*, *V.*
212 *campbelli*, *V. jasicida*, *V. owensii*, and *V. hyugaensis*; EptA-2 was found in *V. rotiferianus*; and
213 EptA-3 was found in *V. alginolyticus* (Fig. 2A). In addition, a very large cluster of EptA
214 sequences similar to VP_RS21300 (reference sequence not sampled in our pool sequencing),
215 which clustered with EptA-1 to -3 on the Mcr/EptA phylogenetic tree (Fig. 1), was found in
216 *V. parahaemolyticus* (Fig. 2A). Finally, an additional EptA sequence found in our pool

217 sequencing, which clustered close to EptA-3 (Fig. 1) and shared the same genomic
218 environment. It matched with *V. alfaciensis*, also belonging to the Harveyi clade (Table S3).
219 EptA-4 (encoded by 14 unique nucleotide sequences) was the other abundant EptA variant
220 found in our pool sequencing (Fig. 1), but it was a more divergent sequence with only 53.3-
221 53.6% maximum identity with EptA-1 to -3 (Fig. 1). Moreover, the *eptA-4* genomic
222 environment differed completely, lacking both *dgkA* and the *rstA/rstB* two-component
223 regulatory system (Fig. 2, Table S3). Within the *Vibrionaceae* phylogeny EptA-4 was specific
224 to the Splendidus clade (Fig. 2A).

225 Two EptA sequences almost identical to *V. fisheri* VF_A0210 (> 98.7 % identity) were carried
226 by bacterial isolates from our European sampling and clustered apart from the newly
227 discovered EptA-1 to -4 protein variants (Fig. 1) (Table S4, Fig.1). The remaining sequences
228 did not share any significant conservation of the sequence/synteny with other EptA variants
229 and were present in sequences of *Vibrio*, *Allivibrio* and *Photobacterium* outside the Harveyi
230 and Splendidus clades, as well as in the species *Halomonas* (Table S3).

231

232 **Existence of both ancient and mobile *eptA* paralogues in the Harveyi clade.** In order to
233 determine the potential risk of *eptA* gene transfer from *Vibrionaceae*, we analyzed the
234 genomic environment of *eptA* genes in 24,243 genomes. The majority of *eptA* genes were
235 single copy (Fig. S4) (19,118 / 24,243 genomes), located on chromosomes and we did not
236 detect any insertion sequences, phage fragments, or plasmid fragments in the proximity of -
237 4/+4 genes around these single copy *eptA* genes, supporting the ancient acquisition of *eptA*
238 genes in *Vibrio*. However, we also identified 2,543 genomes carrying 2 copies and 13

239 genomes carrying 3 copies of *eptA* (Fig. S4). Some of these additional copies showed sign of
240 recent mobilization as indicated by the presence of transposases/integrases near 137 *eptA*
241 genes from *Vibrionaceae* (0.57 % of *eptA* sequences) and 22 *eptA* genes predicted to be
242 carried on a plasmid (Dataset1). In species of the Harveyi clade, the conserved *eptA* copy (*e.g.*
243 *eptA*-1, -2 and -3) occurred in a *rstA-rstB*-glycine zipper-*dgkA-eptA* genomic environment
244 with no trace of mobile genetic elements in its close vicinity (Fig. 2-3). Some Harveyi species
245 such as *V. parahaemolyticus* also harbored a second copy of *eptA* in a distinct genomic
246 environment (*cytB-pepSY-eptA-dgkA*) with no evidence of mobility (Fig. 3). Remarkably,
247 additional *eptA* paralogues with transposases or integrases at their close vicinity (-4/+4
248 genes) were found in a *cytB-eptA-dgkA* genomic environment (Fig. 3). They showed much
249 closer similarity with *eptA* genes from other *Vibrio* species (*V. cholerae*, *V. anguillarum*) than
250 with the conserved *eptA* copy from the Harveyi clade (Fig. 3). These results strongly suggest
251 a recent mobilization of *eptA* paralogues between the Harveyi, Cholerae and Anguillarum
252 clades. Similar events of putative *eptA* mobility were also evidenced within the clade
253 Fluvialis where putative mobile paralogues were found in a *PAP2-dgkA-eptA* genomic
254 environment (Fig. 3). Altogether, our results highlight the existence in *Vibrionaceae* of an
255 ancient *eptA* gene acquisition having evolved with the *Vibrionaceae* lineage, and largely
256 conserved across *Vibrionaceae* species, as well as rarer and more recent *eptA* gene
257 mobilizations within and between *Vibrio* clades.

258

259 **EptA variants show distinct geographic distribution in Europe.** The most common EptA
260 protein variants identified in our sampling were ancient copies conserved in the Harveyi and

261 Splendidus clades and clearly clustered according to geographic locations (Fig. 1).
262 Specifically, a major Mediterranean cluster dominated by EptA-1-2-3 (Harveyi clade) was
263 observed, which includes sequences obtained from EBRO and THAU. It drives the significant
264 geographic structuring between the Mediterranean and North Sea sites (PERMANOVA all
265 sites: $F_{2,68} = 3.032$, $P < 0.001$, THAU vs. SYLT: $F_{1,34} = 3.108$, $P = 0.007$, EBRO vs. SYLT: $F_{1,50} =$
266 4.799 , $P < 0.001$, THAU vs. EBRO: $F_{1,52} = 0.900$, $P = 0.513$). Within this Mediterranean cluster
267 EptA-1 and EptA-3 were the more frequently detected variants (Fig. S2). Outside this
268 Mediterranean cluster, EptA-4 from the Splendidus clade was the other abundant EptA
269 variant (Fig.1, Fig. S2). However, geographical structuring within EptA-4 was much weaker
270 and no significant association with a given environment was found (PERMANOVA $F_{2,16} =$
271 2.888 , $P = 0.076$) (Fig. 1).

272

273 **EptA variants from Harveyi but not Splendidus clade are associated with intrinsic**
274 **colistin resistance.** To investigate the potential role of EptA variants from the Harveyi clade
275 in colistin resistance, we conducted whole genome sequencing (WGS) on nine randomly
276 chosen strains isolated from Thau displaying resistance to colistin. Among the selected
277 strains, three strains from the Harveyi clade, *Vibrio jasicida* TH21_20A_OE8, *Vibrio owensii*
278 TH21_37_OE7, and *Vibrio* sp. TH21_20A_OB7, possessed the *eptA-1* gene. Three other strains
279 also affiliated to the Harveyi clade, *V. alginolyticus* TH21_37A_OE12, *V. alginolyticus*
280 TH21_37_OE9, and *V. alginolyticus* TH21_37A_OE10, carried the *eptA-3* gene (Fig. 3, Table
281 S5). To enrich our collection, we explored genomes of *Vibrio* strains collected over the past
282 ten years in French oyster farms. We identified *eptA-1* and *eptA-2* in four and two strains of
283 the Harveyi clade, respectively, collected in Thau. We also included two *Vibrio* strains with

284 sequenced genomes and known pathogenic potential. The first was *V. parahaemolyticus*
285 strain IFVp22²⁹: it harbors the *eptA* gene variant from the species in the Harveyi *rstA-rstB*-
286 glycine zipper-*dgkA-eptA* genomic environment. The second, from outside the Harveyi clade,
287 was the zoonotic *V. vulnificus* CECT4999³⁰, which harbors a distinct *eptA* gene variant in a
288 *carR-caS-dgkA-eptA* genomic environment, where *carRS* (also known as *vprAB*) is
289 homologous to *rstAB*. Regarding strains carrying the *eptA-4* gene variant from the
290 Splendidus clade, we found the gene variant in four strains collected in Brest (French
291 Brittany), affiliated with *V. splendidus* and *V. crassostreae* (Fig. 4, Table S5).

292 Phenotypes of Col-R correlated perfectly with *eptA* variants and their associated genomic
293 environment, as observed by comparing strain phenotypes and single genome sequences for
294 the 16 strains harboring *eptA-1* to *-4* genes (isolated here from oysters) and the two
295 additional pathogenic *Vibrio* strains (Fig. 4). Thus, *Vibrio* strains with *eptA-1* to *-3* were
296 resistant to > 5 µg/ml colistin in Zobell medium, similar to the pathogenic strains *V.*
297 *parahaemolyticus* IFVp22 and *V. vulnificus* CECT4999. In contrast, strains carrying *eptA-4*
298 were susceptible to colistin at a concentration ≤ 1 µg/ml in the same conditions (Table S5,
299 Fig. 4), suggesting that *eptA-1* to *-3*, but not *eptA-4* confer resistance to colistin. Moreover,
300 the *dgkA* gene is consistently found adjacent to *eptA* in resistant strains (Fig. 4) and in the
301 Harveyi and Vulnificus clades in general (Fig. 4). In contrast, the *dgkA* gene is absent from
302 *eptA* genomic environment in the 4 colistin-susceptible strains harboring *eptA-4* (Fig. 4) and
303 in the Splendidus clade in general (Fig 2A). In strains of the Harveyi clade, we also noted the
304 conservation of the *rstA-rstB* two component signal transduction system (*carR-carS* in the
305 Vulnificus clade), located 1084-bp upstream of the *eptA* gene and 706-bp of the *dgkA* gene

306 (Fig. 2 & 4). A CDS encoding a potential glycine zipper protein separated *rstA-rstB* from the
307 *dgkA-eptA* operon in the Harveyi clade (Fig 2).

308

309 ***dgkA* is required for *eptA*-mediated colistin resistance in the Harveyi clade.**

310 The co-occurrence of *dgkA* and *eptA* in resistant strains of the Harveyi clade prompted us to
311 test their role in resistance to colistin. Gain of function assays were performed by cloning
312 genes of interest into the pBAD-TOPO expression vector used to transform a colistin-
313 susceptible strain of *E. coli*. Cloning was conducted in *E. coli* TOPO10. Basically, we cloned
314 the naturally occurring *dgkA-eptA-1* and *eptA-4* under the control of a pBAD promoter. In
315 addition, we cloned *eptA-1* alone and *dgkA* (from *eptA-1*) alone under the control of pBAD.
316 Among these four constructs, only *dgkA-eptA-1* increased *E. coli* TOPO10 resistance to
317 colistin (MIC > 16 µg/ml in Zobell medium) upon promotor induction. In contrast, the other
318 three constructs did not impact the resistance of *E. coli* TOPO10 to colistin (MIC = 0.25 µg/ml
319 in Zobell medium) (Table 2). The result was confirmed in Muller-Hinton (MHCA) medium
320 (European Committee on Antimicrobial Susceptibility Testing, EUCAST conditions) where
321 only *eptA-dgkA-1* expression increased the MIC of colistin from 0.5 to 4 µg/ml (Table 2). This
322 demonstrates that neither *eptA-1* nor *eptA-4* alone can confer resistance to colistin. Instead,
323 it shows that *dgkA* and *eptA-1* act together to confer Col-R, in agreement with the conserved
324 genomic environment of *eptA* gene variants 1, 2 and 3 isolated from Mediterranean coastal
325 environments.

326

327 ***rstA/B* controls colistin resistance mediated by *dgkA-eptA* in the Harveyi clade.** We
328 finally tested the potential role of the conserved *rstA/B* two-component system in the
329 expression of colistin resistance in Mediterranean *Vibrio* strains resistant to colistin. For
330 that, we performed *rstA* deletion by allelic exchange in *V. harveyi* strain Th15_F5-F11, which
331 harbors the conserved *rstA-rstB-glycine zipper-dgkA-eptA1* gene cluster and resists to 16
332 µg/ml colistin in Zobell medium (Table 2). The Col-R phenotype was significantly
333 compromised in the *rstA* deletion mutant, since growth was fully inhibited in the presence
334 of 2 µg/ml colistin (Table 2). Moreover, complementation with the pMRB-*rstA* plasmid was
335 sufficient to restore full growth at 16 µg/ml Colistin (Table 2). Similarly, in EUCAST
336 conditions, the resistance phenotype of the wild-type Th15_F5-F11 (MIC = 8 µg/ml in MHCA
337 medium), changed to susceptible in the *rstA* deletion mutant (MIC = 0.25 µg/ml in MHCA
338 medium) and was fully restored in the *rstA* complemented strain, but not in a
339 complementation control with *gfp* (Table 2). To demonstrate that the loss of the resistance
340 phenotype is due to an altered expression of *dgkA* and *eptA* in the *rstA* deletion mutant, we
341 quantified the transcripts of *dgkA* and *eptA* by RT-qPCR in the wild-type, mutant, and
342 complemented background. As anticipated, the expression of *dgkA* and *eptA* genes exhibited
343 a significant decrease in the *rstA* deletion mutant (Fig. 5). On the other hand, expression
344 levels were not significantly different between the wild-type *V. harveyi* Th15_F5-F11 strain
345 and its isogenic *rstA* mutant complemented with pMRB-*rstA* (Fig. 5). Such a functional
346 complementation was not observed with the pMRB-*gfp* control plasmid. This demonstrates
347 that *rstA/B* controls the Col-R phenotype of *V. harveyi* Th15_F5-F11 through the expression
348 of the *dgkA-eptA1* operon.

349

350 **Other mechanisms of colistin resistance were found in *Vibrio*.** While the *rstA-rstB-*
351 *glycine zipper-dgkA-eptA* gene cluster was conserved in 6 of the 9 sequenced colistin-
352 resistant isolates from Thau (66,6%), three resistant isolates from our European sampling
353 lacked both the *eptA* and *mcr* genes (*Vibrio sp.* TH21_20_OG1, TH21_20_OH4 and
354 TH21_20A_OC7). In these strains, we detected a well-known colistin resistance mechanism,
355 encoded by *arnBCADTEF* (transfer of L-arabinose onto Lipid A), along with the *phoP/Q* two-
356 component system known to regulate the expression of *arnABCDEFT* in a broad number of
357 bacterial species¹² (Fig. S5). These three isolates exhibited an average nucleotide identity
358 (ANI) of 91% with *Vibrio variabilis* strain CAIM1454, a marine bacterium previously isolated
359 from the cnidarian *Palythoa caribaeorum*³¹. Next to *phoP/Q* and *arnABCDEFT*, we further
360 detected a range of other orthologs of known colistin resistance genes, such as *pmrA/B* (=
361 *basS/R*), *crrA/B*, *soxR*, *tolC*, *kpnE*, *mprF*, and *ugd* genes in the pool sequencing libraries from
362 Thau, Sylt and Ebro¹². While we have not functionally characterized these genes, the high
363 prevalence of colistin resistance in the absence of *eptA-1-2-3* suggest the presence of a
364 substantial diversity of known and probably also novel resistance mechanisms.

365

366 **Discussion**

367 This study reveals that *eptA* genes, which encode phosphoethanolamine transferases, are
368 both abundant and diverse in culturable bacteria isolated from European coastal
369 environments and are widely distributed across *Vibrionaceae*. We specifically show that
370 ancient copies of *eptA* have evolved within distinct genomic environments specific to each
371 *Vibrio* clade and contribute to intrinsic Col-R in *Vibrionaceae*. In addition, a number of *eptA*

372 paralogues exhibit signatures of recent mobility within *Vibrio*, highlighting the need for
373 increased surveillance. These results fill a gap of knowledge. Indeed, while *eptA* genes had
374 previously been described in a number of *Vibrio* species for their role in conferring colistin
375 resistance, little was known about their distribution in coastal environments as well as in the
376 *Vibrionaceae* family.

377

378 In European coastal waters (France, Germany, Spain), *eptA* genes displayed high diversity,
379 with the most abundant variants found in *Vibrio* species belonging to the Harveyi and
380 Splendidus clades. Indeed, among *eptA* sequences, we identified four novel *eptA* gene
381 variants referred to as *eptA*-1 to -4 in this study. EptA-1 to -3 were expressed by colistin-
382 resistant strains of the Harveyi clade assigned to the species *V. alginolyticus*, *V. campbellii*, *V.*
383 *diabolicus*, *V. harveyi*, *V. jasicida*, *V. owensii*, and *V. rotiferianus*. In contrast EptA-4 was
384 expressed by susceptible strains of the Splendidus clade (species *V. splendidus* and *V.*
385 *crassostreae*). These two *Vibrio* clades naturally colonize oysters, and several species such as
386 *V. harveyi* and *V. crassostreae* cause infections in oysters^{32,33}. A remarkable contrast was
387 observed in the geographic distribution of *eptA* gene variants at a European scale, with the
388 active forms (*eptA*-1 to -3) only being detected in the Mediterranean area (Ebro, Spain and
389 Thau, France), suggesting an adaptive advantage responding to specific selection pressures
390 in that environment. However, since these genes were carried by *Vibrio* of the Harveyi clade,
391 which are adapted to warmer seawater temperatures, the distribution of these *eptA* variants
392 likely reflects the geographic range of *Vibrio* species along European coasts. This does not
393 rule out the possibility that environmental factors also select for *eptA*-mediated Col-R in the
394 Mediterranean coastal environments. No division was observed in the geographic

395 distribution of *eptA-4* (Splendidus clade), which were found in all three European
396 environments along with a number of unknown *mcr* genes. Our data also showed that two
397 weeks were sufficient for oysters to capture a number of Col-R genes specific to each
398 European environment. This finding has implications for the potential transfer of AMR
399 across Europe by oyster transport, a common and still unregulated practice in aquaculture,
400 which has been responsible for the spread at the European scale of oyster pathogens
401 including *Vibrio*³⁴.

402

403 In the Harveyi clade, we described a novel *eptA* genomic environment where *eptA* is co-
404 transcribed with *dgkA* under the control of the RstAB two-component signal transduction
405 system, whose environmental triggers remain unknown. Co-expression of the *dgkA-eptA-1*
406 operon was required for Col-R, in agreement with recent results indicating that *dgkA* is
407 needed for the *eptA* or *mcr-1*-mediated resistance to polymyxins in *E. coli*³⁵, as well as in
408 environmental isolates carrying *mcr-3* and *mcr-7*³⁶. The underlying mechanism involves the
409 detoxifying effect of DgkA, a diacylglycerol kinase that recycles diacylglycerol, a dead-end
410 metabolite of Mcr/EptA proteins, into useful precursor molecules. DgkA plays a crucial role
411 in Col-R by preventing the toxicity of Mcr/EptA by-products from inhibiting bacterial growth
412 (for review see ³⁷). We also showed that co-expression of *dgkA-eptA-1* is controlled by RstAB,
413 which enables bacteria to detect and respond to environmental fluctuations³⁸⁻⁴⁰ and triggers
414 adaptive responses for bacterial survival³⁹⁻⁴¹. Until now in *Vibrio*, RstAB had been shown to
415 control motility, adhesion, biofilm formation and haemolytic activity⁴². Whereas in
416 *Photobacterium damsela* RstAB would not control the *eptA*-mediated resistance to
417 colistin⁴³, the homologous TCS *carRS* controls *eptA* expression and Col-R in *V. vulnificus*²⁵.

418 Unlike in *Harveyi*, the conserved *eptA-4* copy carried by *Vibrio* of the Splendidus clade, which
419 has evolved in a completely distinct genomic environment lacking *dgkA*, appears to have lost
420 its ability to confer colistin resistance, as also reported for *eptA* from the classical *V. cholerae*
421 strain 0395²³. The conservation of this gene in the Splendidus clade may indicate that EptA
422 proteins have evolved different specificities/functions along the *Vibrio* phylogeny, although
423 we cannot rule out the possibility that the gene was not activated under our experimental
424 conditions.

425

426 Ancient copies of *eptA* were found widely distributed in *Vibrionaceae* suggesting they
427 contribute significantly to intrinsic colistin resistance in these bacteria. First, from our
428 phylogenetic studies on nearly 30,000 *Vibrionaceae* genomes, we showed that only a very
429 limited number of species (*e.g. V. natriegens*) have lost this gene during evolution. Second,
430 despite sequence identities as low as 43% across the *Vibrio* phylogeny, many EptA variants
431 retain a conserved role in conferring colistin resistance, as shown here for EptA-1 from the
432 *Harveyi* clade in the EUCAST conditions, and reported elsewhere for species of the *Vibrio*
433 clades *Cholerae*, *Vulnificus*, and *Fisheri*^{23, 25, 26}. This gives some hints about the functional
434 role and selective pressures encountered by this gene in nature. In the marine environment,
435 various microorganisms, including bacteria produce cationic lipopeptides. Thus, species of
436 *Pseudoalteromonas* are members of the oyster microbiota and the lipopeptides they produce
437 (called alterins) are structurally and functionally similar to polymyxins⁴⁴. Moreover, marine
438 animals such as oyster⁴⁵ and squid²⁶ produce cationic antimicrobial (lipo)peptides and
439 proteins as a mechanism of immune defense and to control their microbiota. Like
440 polymyxins and alterins, some of them target the lipopolysaccharide of Gram-negative

441 bacteria⁴⁶. Not surprisingly, Lipid A modifications have evolved as defense mechanisms
442 against cationic antimicrobial peptides, both in pathogens and commensals to circumvent
443 the antimicrobial response of their animal host and competing members of the host
444 microbiome⁴⁷. The importance of *eptA*-mediated colistin resistance in the success of host
445 colonization was clearly demonstrated in the squid symbiont *V. fischeri*²⁶. An almost identical
446 gene (>98% sequence identity) was sampled two times here in oysters from Thau and Ebro.
447 It is likely that bacterial species that live in close association with marine animals like
448 oysters, such as *Vibrio* clade Harveyi³², have also developed such resistance mechanisms. We
449 also demonstrated here that conserved copies of *eptA* have evolved within *Vibrio* species in
450 specific genomic environments, further supporting the hypothesis of an ancient acquisition
451 of the *eptA* gene in *Vibrio* phylogenetic history and little to no interspecific horizontal gene
452 transfer since the differentiation of *Vibrio* species. The diversity of genomic environments
453 observed for *eptA* variants is specific to *Vibrio* clades. As we showed here for *V. harveyi*, these
454 genomic contexts are key determinants of *EptA* expression and activity. The presence in
455 many *eptA* genomic contexts of clade-specific two-component regulatory systems suggests
456 that *eptA* expression responds to distinct environmental triggers in different *Vibrio* clades.
457
458 Importantly, we also evidenced recent mobility events for certain *eptA* paralogues. Indeed,
459 beyond the ancient *eptA* copy inherited by the majority of *Vibrionaceae* species, we found
460 that a number of species from the clades Harveyi, Cholerae, Anguillarum and Fluvialis harbor
461 mobile *eptA* paralogues. Their genomic environment differs from the clade-specific ancient
462 *eptA* copy, indicating they are likely expressed in response to distinct environmental signals.
463 Although representing less than 1% of the *eptA* genes identified in *Vibrio*, the mobile *eptA*

464 paralogues are surrounded by transposases and integrases and show signs of recent mobility
465 both at an intra-clade and at an inter-clade level. Remarkably they co-occur with a *dkgA* gene
466 in their direct neighborhood, suggesting functionality. This deserves particular attention
467 since such mobile genetic elements significantly increase the risk of environmental capture,
468 as this happened for the *mcr-1* gene carried by a plasmid and now circulating in human
469 pathogens.

470

471 **Conclusion**

472 Our exploration of the genetic basis of Col-R focused on *mcr/eptA* genes, which have a
473 putative origin in aquatic environments and currently raise concerns in a clinical context
474 worldwide^{48,49}. We found a high diversity of *eptA* variants, but only a limited number of *mcr*
475 variants in bacteria isolated from European coastal waters. An ancient *eptA* gene copy was
476 highly prevalent in *Vibrio* supporting a key role in *Vibrio* intrinsic resistance to colistin
477 including strains from species responsible for most human pathologies (*V. cholerae*, *V.*
478 *parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*). Most often, these species harbor a *dgkA-eptA*
479 operon under the control of a two-component regulatory system (RstAB in the Harveyi clade
480 or its homolog CarRS in the Vulnificus clade). In European coastal environments, the newly
481 described Harveyi *eptA* genomic environment was found in Mediterranean strains of *V.*
482 *alginolyticus* and *V. harveyi*, which thrive in warm seawaters. The chromosomal location of
483 *eptA*, the absence of mobile genetic elements at the vicinity of the *dgkA-eptA* genes and the
484 evidence that the *eptA* gene polymorphism has evolved with the *Vibrio* lineage argue in favor
485 of a low risk of horizontal gene transfer from *Vibrio* to other bacterial genera. However, the
486 identification of mobile *eptA* paralogues in *Vibrio* genomes should warn us on a risk of

487 mobilization outside *Vibrionaceae*. Further studies will be needed to characterize the risk
488 associated to other Col-R mechanisms present in oyster associated bacteria. In the context
489 of global warming, where *Vibrio* are already causing an increasing number of human disease
490 cases in Europe⁵⁰, we expect colistin-resistant *Vibrio* species of the Harveyi clade to
491 proliferate. A correct understanding of the spread of Col-R and of ecological factors
492 interfering with it in coastal areas, and of its persistence and selection in the oyster
493 pathobiome is fundamental for the preservation of oyster farming as sustainable
494 aquaculture in oceans exposed to global warming ²⁰.

495

496 **Methods**

497 **Animals**

498 Specific-pathogen free (SPF) diploid oysters were produced during Spring 2021 from 166
499 wild genitors at the Ifremer hatchery of Argenton (PHYTNESS Ifremer research unit, France),
500 as described in Petton (2011, 2015). Oysters were transferred after 6 weeks to the Ifremer
501 nursery of Bouin (EMMA Ifremer research unit, France), where they were maintained under
502 controlled biosecured conditions with filtered and UV-treated seawater enriched in
503 phytoplankton (*Skeletonema costatum*, *Isochrysis galbana*, and *Tetraselmis suecica*). Before
504 transfer to the field, the SPF status of the animals was confirmed by (i) the absence of OsHV-
505 1 DNA detection by qPCR, (ii) a low *Vibrio* load (~10 CFU/mg of oyster tissue) determined
506 by isolation on selective culture medium (TCBS agar). The absence of World Organisation for
507 Animal Health (WOAH) listed parasites (*Bonamia* sp., *Marteilia* sp., *Perkinsus* sp., *Mikrocytos*
508 sp. and *Haplosporidium* sp.) was confirmed on histological sections of 114 oysters by an
509 independent laboratory (LABOCEA, France). Oysters were observed to remain free of any
510 abnormal mortality until use.

511

512 **Study Area, Sample Collection and Microbial Isolation**

513 The same SPF oyster batches (*i.e.* oysters with identical life history traits) were deployed at
514 the juvenile stage (6 months old) in three different European locations used for oyster
515 culture: SYLT in Germany (N 55° 1' 42.539', E 8° 26' 1.953'), THAU lagoon (N 43°26.058', E
516 003°39.878') in France, and EBRO delta (N 40°37.106880', E: 0°37.318320') in Spain. Two
517 to three weeks exposures in the environment were performed at the end of the year 2021
518 (THAU: Bouzigues, from 4/10/2021 to 18/10/2021, EBRO: Alfacs bay, from 8/10/2021 to

519 25/10/2021, and SYLT: Königshafen, from 22/12/2021 to 10/01/2022). No oyster
520 mortalities were recorded during the field exposures. After being exposed to their respective
521 environments, 60 oysters were collected at each sampling site. Tissues were homogenized
522 in artificial seawater (ASW: 400 mM NaCl, 20 mM KCl, 5 mM MgSO₄, 2 mM CaCl₂) using an
523 ultra-Turrax apparatus⁵¹. The homogenates were pooled and plated in replicates on TCBS
524 agar (Difco™) agar plates for *Vibrio* isolation and on marine agar (Difco™) as a non-selective
525 agar for marine bacteria. Duplicate plates were incubated at 20°C or 37°C. Bacteria were
526 isolated after 24-48h (Table S1). After colony purification on Zobell medium (ASW
527 supplemented with 0.4% bactopectone and 10% yeast extract, pH 7.8), a glycerol stock of
528 each isolate was conserved at -80°C. Up to 48 isolates were conserved per condition of
529 isolation (TCBS 20°C, marine agar 20°C, TCBS 37°C, marine agar 37°C).

530

531 **DNA extraction, pool-sequencing, and whole genome sequencing.**

532 Bacterial colonies isolated from oyster flesh were cultured overnight in liquid Zobell medium
533 at either 20°C or 37°C, according to their conditions of isolation. For pool-sequencing,
534 bacterial cultures were pooled in equal amounts according to geographic site, culture
535 medium and temperature of isolation. DNA was extracted with the NucleoSpin Tissue kit for
536 DNA from cells and tissue (Macherey-Nagel). For single genome sequencing, DNA was
537 extracted from cultures with the MagAttract HMW DNA Kit (QIAGEN, France). The extracted
538 DNA was quantified using a Qubit High Sensitivity Assay Kit (Life Technologies, Carlsbad,
539 USA), and sequencing was carried out at the Bio-Environment platform (University of
540 Perpignan Via Domitia) using the Nextera XT DNA Library Prep Kit (Illumina) according to
541 the manufacturer's instructions, with 1 ng of DNA. The quality of the libraries was checked

542 using a High Sensitivity DNA chip (Agilent) on a Bioanalyzer. Sequencing was performed on
543 a NextSeq 550 instrument (Illumina) in 2x150 paired-end mode, resulting in an average
544 mean reads of 45,000,000 bp for pool sequencing, and 243 Mb for whole genomes (mean
545 coverage 47X).

546

547 **Bioinformatics Analysis tools**

548 **Pool-sequencing** - FastQC was used to check the quality of reads, followed by trimming
549 using Trimmomatic V-0.38⁵² to trim leading/trailing bases with quality scores below 30. The
550 recovered reads were assembled into contigs using MEGAHIT V-1.2.9⁵³. The Meta-marc tool
551 (database model 3) was then used to identify the contigs carrying the *eptA/mcr* resistance
552 genes (Fig. S6). The contigs recovered carrying the *eptA/mcr* resistance–genes were
553 annotated using Prokka and predicted coding sequences was specifically re-analyzed for
554 colistin resistance genes using Meta-marc. Sequences annotated as *eptA* and *mcr* according
555 to Meta-marc and Prokka, were validated by BlastP against the NCBI and the CARD database
556 V- 3.2.7. Subsequently, we eliminated incomplete *eptA* and *mcr* sequences (*i.e.* partial
557 sequences missing a 5' and/or 3' region) from our analysis. Full length gene sequences were
558 translated *in silico* and the resulting amino acid sequences were aligned by MAFFT V-7.407
559 (<https://ngphylogeny.fr/tools/>). A phylogenetic tree was generated through maximum
560 likelihood analysis of deduced EptA/Mcr amino acid sequences using PhyML V-3.0
561 (<https://ngphylogeny.fr/tools/>) with the WAG model and 100 bootstrap replicates. Pool-
562 sequencing raw data and complete *eptA* gene sequences were deposited on GenBank under
563 accession numbers SAMN37810832 to SAMN37810840 and OR578979 to OR579029,
564 respectively.

565

566 **Whole genome sequencing of single bacterial isolates** - The quality assessment and reads
567 trimming steps were performed as described for pool-sequencing. The obtained reads were
568 then assembled into contigs using Spades V-3.15.4 within the Galaxy Europe platform⁵⁴.
569 Default parameters were used ('Isolate', 'Automatic k-mer selection, Phred quality offset
570 adjustment, and coverage cutoff in the assembly of individual bacterial genomes). In order
571 to detect the presence of the *eptA* or *mcr* genes, the assembled genomes were annotated in
572 MAGe (<https://mage.genoscope.cns.fr/>). When feasible, a taxonomic affiliation was assigned
573 to the selected isolates at the species level using Average Nucleotide Identity (ANI) and
574 DNA:DNA hybridization (dDDH) percentages. This analysis was conducted utilizing Defast
575 (DDBJ Fast Annotation and Submission Tool) available at <https://dfast.ddbj.nig.ac.jp/>, in
576 conjunction with genome clustering tools through MAGe and the reference strain genomes
577 obtained from Type Strain Genome Server (TYGS) (<https://tygs.dsmz.de/>). Raw reads and
578 genome assemblies were deposited on ENA under project number PRJEB67316.

579

580 **Genetic diversity of detected *eptA* and *mcr* genes, and their genomic environment** - In
581 order to investigate the diversity of the detected *eptA/mcr* sequences, we conducted a
582 comparative analysis of the amino acid sequences encoded by genes found in the assembled
583 pool-sequencing data. First to determine a sequence identity cut-off enabling the
584 discrimination of Mcr variants, we used a total of 177 *mcr* sequences sourced from NCBI and
585 CARD databases whose deduced amino acid sequences showed homology with Mcr-1-10
586 (Table S3). Next, Mcr/EptA sequences were searched using diamond blastp (v2.1.9.163)⁵⁵

587 against a collection 29244 *Vibrionaceae* assemblies (GenBank July 2023) using 30% identity
588 and 50% coverage as selection criteria. Multiple proteins alignment was then performed
589 using FAMSA (v2.2.2)⁵⁶ and trimmed using trimAI with gappyout method (v1.5)⁵⁷. A
590 phylogenetic tree was constructed using fasttree with LG model (v2.1.11)⁵⁸ and visualization
591 was done using iTOL⁵⁹.

592 Additionally, we examined the genomic environment of all *eptA/mcr* genes from the 29244
593 *Vibrionaceae* assemblies. Neighbor genes from +4 to -4 were clustered with the diamond
594 cluster module. After filtering out incomplete environments, i.e. those which lacked genes at
595 +4 or -4 due to contig breaks, the different genomic environments were positioned on a
596 *Vibrionaceae* phylogenetic tree. For that, a multilocus sequence analysis of *Vibrionaceae*
597 genomes was performed based on 8 genes recommended by *Vibrio* Clade 3.0⁶⁰, namely *ftsZ*,
598 *gapA*, *mreB*, *rpoA*, *topA*, *gyrB*, *pyrH*, *recA*. Alignment of the 8 concatenated nucleic sequences
599 was done with halign (v3.0.0)⁶¹. A phylogenetic tree was constructed with fasttree with a GTR
600 model. iTOL was used for visualization. ISfinder (<https://isfinder.biotoul.fr/blast.php>),
601 PlasmidHunter⁶² and MetaPhinder-2.1 (<https://cge.food.dtu.dk/services/MetaPhinder/>)
602 were used to search the insertion sequences, mobile elements and bacteriophage sequences
603 within a 25,000 bp average region surrounding the *eptA* gene.

604

605 **Data normalization and statistical analysis** - Bowtie2 V-2.5.1⁶³ was employed with its
606 default settings to align reads to the gene sequences of interest. This approach provides read
607 counts for each identified sequence (Table S4). To normalize the number of reads of each
608 identified sequence, the following formula was used:

Normalized data= (mapped reads of each sequence / total assembly reads) x 10^{609}

610 Furthermore, we investigated the geographical structuring of molecular diversity in EptA
611 and Mcr sequences by analyzing the distance matrix underlying the amino acid sequences
612 phylogeny (see above). This involved conducting pairwise comparisons of distances within
613 and between sites using a permutational analysis of variance (PERMANOVA). The
614 PERMANOVA was implemented in the R package *vegan*, utilizing the *adonis2* function.
615 (<https://github.com/vegandevs/vegan>). Initially, we conducted an analysis of variation
616 using all sites collectively. Subsequently, we performed pairwise comparisons to detect low-
617 level structuring.

618

619 **Colistin susceptibility testing**

620 MICs of colistin were determined against *Vibrio* isolates and recombinant *E. coli* strains
621 constructed in the present study following the microdilution assay from the EUCAST V14.0
622 guidelines. Colistin sulfate (Thermo Fisher) corrected for activity units was tested in the
623 range of 0.125 to 16 $\mu\text{g/ml}$, at 35°C, in cation-adjusted MHCA. Each well was seeded with
624 $5 \cdot 10^5$ CFU/ml. MIC values are expressed as the lowest colistin concentration tested that
625 causes 100% of growth inhibition after a 18h incubation. In parallel, we performed MIC
626 determination in Zobell medium, which mimics the seawater composition. For quality
627 control (QC), we used *Escherichia coli* O6 (ATCC 25922) with the colistin QC MIC range
628 provided by EUCAST v14.0 (0.25-1 $\mu\text{g/ml}$). In the absence of colistin breakpoint for *Vibrio*,
629 we used EUCAST breakpoints for *Enterobacterales* resistance in tables V14.0 (MIC > 2
630 $\mu\text{g/ml}$).

631 For a rapid screening of Col-R on 139 bacterial isolates from the Thau lagoon, France, 184
632 from Ebro, Spain and 96 from Sylt, Germany and control *Vibrio* strains from previous studies
633 (see Tables S5-S6), we used the same liquid broth inhibition assay with fixed colistin
634 concentrations (5 µg/ml). Screening was performed in Zobell medium at 20°C or 37°C,
635 according to conditions of isolation. Assays were performed in duplicate wells. Strains were
636 considered resistant when duplicate wells grew in the presence of 5 µg/ml colistin (*i.e.* 2.5
637 fold the clinical breakpoint) after a 18h incubation.

638

639 **Cloning *dgkA* and *eptA* genes**

640 Gene variant *eptA*-1 and the operon *dgkA-eptA*-1 were amplified by PCR from colistin
641 resistance strain *Vibrio owensii* Th15_Z_G08 (Table S5). The *eptA*-4 variant was amplified
642 from *Vibrio splendidus* 7T7_2. Primer sets used for gene amplification are listed in Table S6.
643 PCR were performed in a 25 µl total volume under the following conditions: initial
644 denaturation at 95°C for 30 s, followed by 35 cycles of 95°C for 5 s, 69°C for 30 s, and 72°C
645 for 30 s and by a final extension at 72°C for 2 min. Amplicons were cloned in the pBAD-TOPO
646 expression vector (Invitrogen, France) under the control of the pBAD inducible promoter
647 and transformed into *E. coli* TOP10 competent cells according to manufacturer's
648 instructions. Recombinant colonies were tested for the presence of specific *eptA* and *dgkA*
649 genes by standard PCR and by whole plasmid sequencing using Oxford Nanopore
650 Technologies sequencers (Eurofins, France).

651

652

653 **Heterologous expression of *dgkA* and *eptA* genes in *E. coli***

654 Recombinant *E. coli* TOP10 carrying *eptA-1*, *dgkA-eptA-1* or *eptA-4* in the pBAD-TOPO vector
655 were tested for colistin resistance. Basically, bacterial cells were cultured at 37°C in Luria-
656 Bertani (LB) broth in the presence of 2 % arabinose to induce the pBAD promoter.
657 Recombinant bacteria were considered colistin-resistant if they were able to grow in LB
658 containing 5 µg/ml colistin in the microtiter plate assay described above.

659

660 **Generation of mutants in the regulation systems *rstA/B***

661 The *Vibrio* strain TH15_F5_F11 carrying *dgkA-eptA-1* preceded by *rstA/B* (Col-R) was grown
662 at 37°C in LB or LB-agar (LBA) + 0.5 M NaCl. *E. coli* strains were grown at 37°C in LB broth
663 and on LB medium for cloning and conjugation experiments. Chloramphenicol (Cm, at 5 or
664 25 µg/ml for *Vibrio* and *E. coli*, respectively), thymidine (0.3 mM) and diaminopimelate
665 (0.3 mM) were added as supplements when necessary. Induction of the P_{BAD} promoter was
666 achieved by the addition of 0.2% L-arabinose to the growth medium and, conversely, was
667 repressed by the addition of 1% D-glucose where indicated. All plasmids used or constructed
668 in the present study are described in Table S5. Gene deletion was performed by allelic
669 exchange using the pSW7848T suicide plasmid^{64,65}. To this end, two ≈500 bp fragments
670 flanking the target gene were amplified (Table S6), cloned into pSW7848T as previously
671 described⁶⁶, and transferred by conjugation from *E. coli* as donor to *Vibrio* as recipient.
672 Subsequently, the first and second recombination's leading to pSW7848T integration and
673 elimination were selected on Cm/glucose and arabinose-containing media, respectively.
674 Deletion mutants were screened by PCR using external primers flanking the target gene. For
675 the complementation experiments, the gene was cloned into the stable pMRB plasmid,

676 resulting in constitutive expression from a P_{LAC} promoter⁶⁷. Conjugations between *E. coli* and
677 *Vibrio* were performed at 37°C⁶⁴.

678

679 **RT-qPCR**

680 In this study, we employed the DirectZol RNA Miniprep kit (R2051) provided by
681 ZymoResearch to extract total RNA from Trizol conserved samples obtained from both wild
682 and mutant strains, following the manufacturer's instructions. The extraction process was
683 performed in duplicate for each condition at two different growth stages, specifically the
684 exponential and stationary phases. To eliminate any genomic DNA contamination, the RNA
685 was treated with DNase I. To determine the concentration of the total RNA, we used a
686 NanoDrop spectrophotometer from ThermoFisher Scientific. The cDNA was produced using
687 M-MLV Reverse Transcriptase M1302 (Sigma-Aldrich, France) with 1 µg of extracted RNA.
688 Real-time quantitative PCR (qPCR) was performed at the MGX platform in Montpellier. The
689 MGX platform employed the Light-Cycler 480 System from Roche. The primers used for qPCR
690 are listed in Supporting Information (Table S6). To analyze the relative expression levels, we
691 employed the $2^{-\Delta\Delta C_q}$ method developed by Pfaffl in 2001⁶⁸. For normalization, two genes
692 (6PFK (VS_2913) and CcmC (VS_0852)) were chosen due to their constitutive expression
693 across various conditions in both the RNAseq and qRT-PCR analyses⁶⁹. For our own study,
694 we designed and validated specific primers for these genes on *Vibrio harveyi* Th15_F5_F11
695 (Table S6).

696

697

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714

715 **Declaration of competing interest.** The authors declare that they have no known
716 competing financial interests or personal relationships that could have appeared to influence
717 the work reported in this paper.

718

719 **Author contributions.**

720 Conceptualization: S.J., B.V., T.M.A., M.C., W.K.M., D.D.G.

721 Methodology: S.J., B.V., B.C., A.B.K., G.D., A.M., P.B., L.Y, T.P., F.D., A.G., V.L., C.G., D.C.A., K.H.,

722 L.E., C.G., R.O., P.J., E.J.M., M.D., C.G., T.E., T.M.A., M.C., W.K.M., D.D.G.

723 Investigation: S.J., B.V., B.C., A.B.K., G.D., A.M., P.B., L.Y, T.P., F.D., A.G., V.L., C.G., D.C.A., K.H., L.E.,

724 C.G., R.O., P.J., E.J.M., C.G., T.E., T.M.A., M.C., W.K.M., D.D.G.

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726 C.G., R.O., P.J., E.J.M., C.G., T.E., T.M.A., M.C., W.K.M., D.D.G.

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728 Administration: A.B.K., V.L, W.K.M., D.D.G.

729 Supervision: A.B.K., W.K.M., C.G., D.D.G.

730 Writing– original draft: S.J., D.D.G.

731 Writing – review & editing: S.J., B.V., B.C., A.B.K., G.D., A.M., P.B., L.Y, T.P., F.D., A.G., V.L., C.G.,

732 D.C.A., K.H., L.E., C.G., R.O., P.J., E.J.M., M.D., C.G., T.E., T.M.A., M.C., W.K.M., D. D.G.

733

734 **Ethics approval**

735 The animal (oyster *Crassostrea gigas*) testing followed all regulations concerning animal

736 experimentation. The authors declare that the use of genetic resources fulfill the French

737 regulatory control of access and EU regulations on the Nagoya Protocol on Access and

738 Benefit-Sharing (TREL2302365S/750, ABSCH-IRCC-FR-266230-1).

739

740 **Data availability**

741 Targeted gene sequences (*eptA*) and pool-sequencing raw data were deposited at GenBank
742 under accession numbers OR578979 to OR579029 and SAMN37810832 to SAMN37810840,
743 respectively. Genome raw data and assemblies were deposited at the European Nucleotide
744 Archive (ENA) under project accession no. PRJEB67316 (ERR12116510 to ERR12116518)
745 and are available on MicroScope plateforme MaGe (“Magnifying Genomes”)
746 <https://mage.genoscope.cns.fr/>.

747

748 **Code availability.** Software’s and databases used in this paper are openly available in Galaxy
749 Open platform hosted by our institute <https://galaxy-datarmor.ifremer.fr/>. Specific source
750 codes for antimicrobial resistance gene identification are also available on GitHub
751 <https://github.com/lakinsm/meta-marc>.

752

753

Table 1. Colistin-resistant (Col-R*) bacterial isolates across European oyster farms.**

Sampling site	Isolation medium	No. isolates (20°C; 37°C)	No. Col-R isolates (20°C; 37°C)	% Col-R isolates
THAU	Marine agar	87 (41; 46)	28 (1; 27)	32.1
EBRO	Marine agar	140 (96; 44)	17 (6;11)	12.1
SYLT	Marine agar	51 (19;32)	37 (14;23)	72.5
THAU	TCBS	52 (47; 5)	16 (11; 5)	30.8
EBRO	TCBS	44 (28; 16)	11 (5; 6)	25.0
SYLT	TBCS	45 (45; 0)	41 (41; 0)	91.1

754 * Col-R, MIC > 5 µg/ml in Zobell medium

755 ** bacteria isolated on TCBS or Marine agar medium

756

Table 2. Role of *eptA* variants, *dgkA* and *rstA* in resistance to colistin.

MICs were determined by the microdilution assay in the range of 0.125 - 16 μ M colistin.

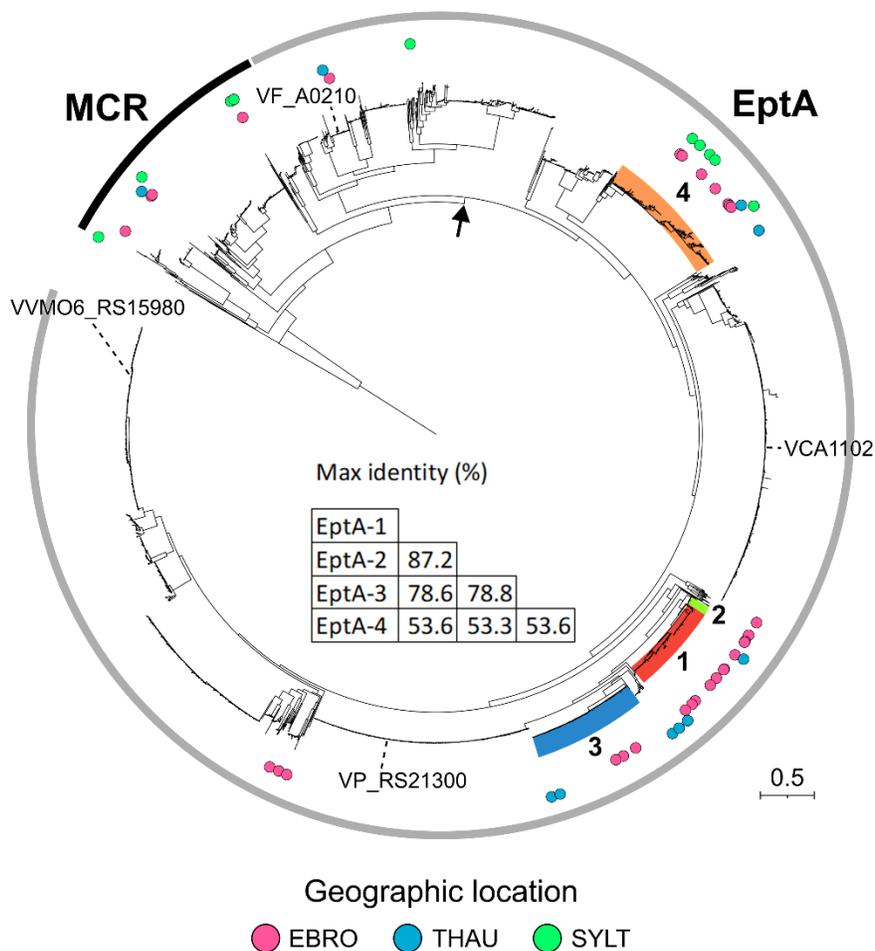
Strain	Genotype	MIC (μ g/ml)	
		MHCA	Zobell
<i>E. coli</i> TOP10	wild type	0.5	0.25
<i>E. coli</i> IHPE 10466	TOP10 pBAD-TOPO- <i>eptA-1</i> *	0.5	0.25
<i>E. coli</i> IHPE 10471	TOP10 pBAD-TOPO- <i>dgkA</i> *	0.5	0.25
<i>E. coli</i> IHPE 10469	TOP10 pBAD-TOPO- <i>dgkA-eptA-1</i> *	4	> 16
<i>E. coli</i> IHPE 10467	TOP10 pBAD-TOPO- <i>eptA-4</i> **	0.5	0.25
<i>V. harveyi</i> IHPE 390 (Th15_F5-F11)	wild type <i>rstA-rstB-glycine zipper-dgkA-eptA-1</i>	8	> 16
<i>V. harveyi</i> IHPE 10451	Th15_F5-F11 Δ <i>rstA</i>	0.5	2
<i>V. harveyi</i> IHPE 10453	Th15_F5-F11 Δ <i>rstA</i> pMRB- <i>rstA</i>	8	> 16
<i>V. harveyi</i> IHPE 10452	Th15_F5-F11 Δ <i>rstA</i> pMRB- <i>gfp</i>	0.5	2
<i>E. coli</i> O6 (ATCC 25922)		1	1

757 * cloned from *V. owensii* Th15_Z_G08 (IHPE 303)

758 ** cloned from *V. tasmaniensis* 7T7_2 (IHPE 20)

759

760

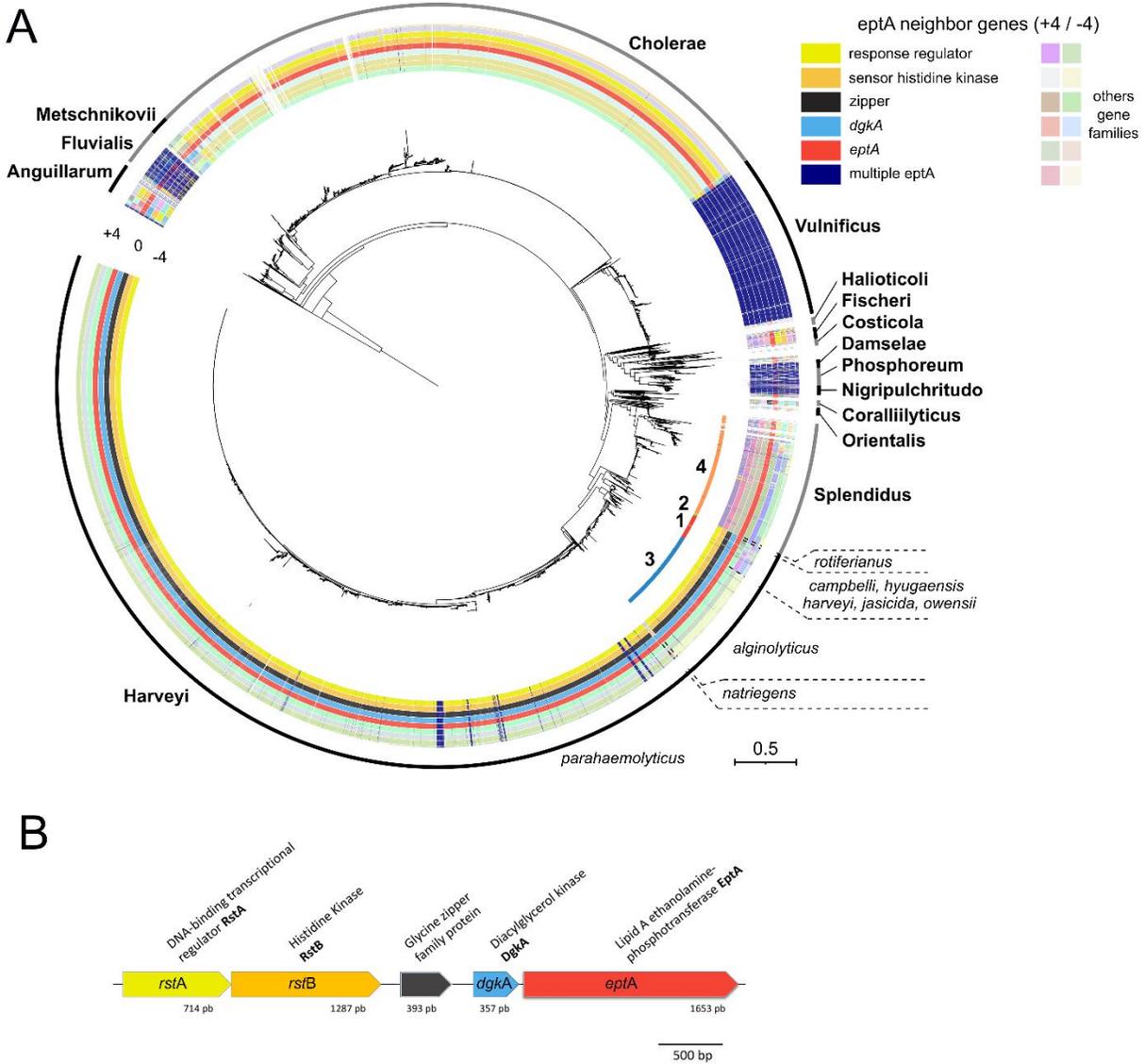


761

762 **Fig. 1. Clustered distribution of *mcr/eptA* gene variants across European coastal**
 763 **environments.** The detected EptA and Mcr variants from the present study are included in
 764 a phylogenetic tree together with 4075 distinct Mcr/EptA sequences found in 27921
 765 *Vibrionaceae* assemblies carrying *mcr/eptA* genes. Sequences used as reference are Mcr-1 to
 766 10 sequences found in the CARD database as well as 4 EptA sequences from functionally
 767 characterized EptA variants (VP_RS21300, VVMO_RS15980, VCA1102 and VF_A0210). A
 768 phylogenetic tree generated from deduced EptA/Mcr amino acid sequences was constructed
 769 using fasttree with LG model and visualization was done using iTOL. Corresponding protein
 770 sequences were obtained from the pool-sequencing of bacteria isolated both on Marine agar

771 and TCBS medium in three European regions. See Fig. S2 for an illustration including
772 conditions of isolation and read counts. The node that separates Mcr (outer black arc) from
773 EptA sequences (outer grey arc) is indicated by an arrow. Numbers refer to the EptA-1 (red),
774 EptA-2 (green), EptA-3 (blue) and EptA-4 (orange) variants described in the present study.
775 Maximum identity between newly identified EptA protein variants is displayed. The
776 geographic origin of each sequence is represented in by dots colored pink (Ebro), green
777 (Sylt), and blue (Thau).

778



779

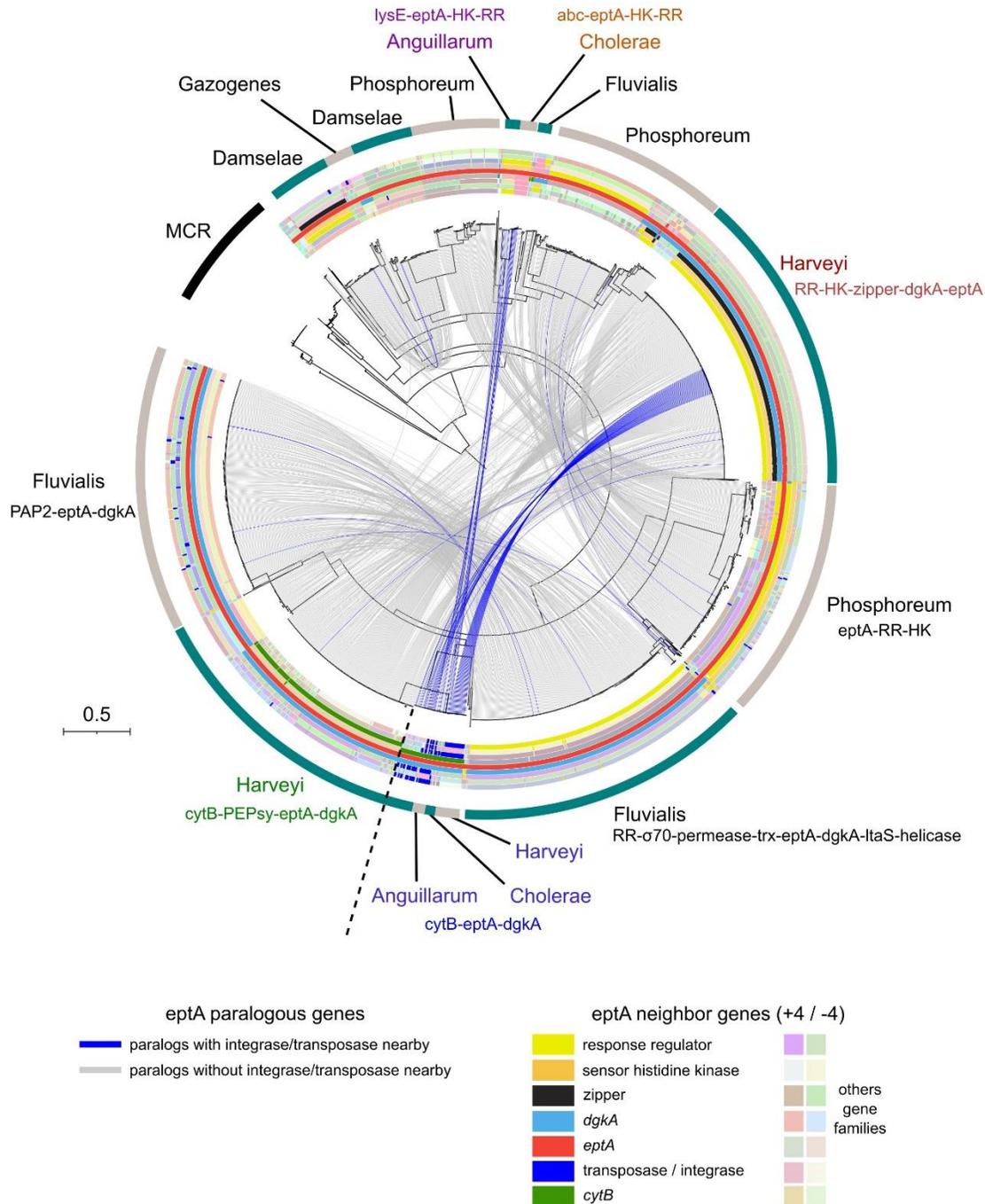
780 **Fig. 2. The genomic environment of *ep*tA** gene variants has evolved with the phylogeny**
 781 **of *Vibrionaceae*.**

782 **(A)** Distribution of *mcr/ep*tA** genomic environments (-4/+4 genes) along an MLST of
 783 *Vibrionaceae*. The *Vibrionaceae* phylogenetic tree was based on 8 polymorphic genes *ftsZ*,
 784 *gapA*, *mreB*, *rpoA*, *topA*, *gyrB*, *pyrH*, *recA* according to *Vibrio* Clade 3.0⁶⁰. Numbers on the
 785 inner circle indicate the phylogenetic positioning of EptA-1, EptA-2, EptA-3, and EptA-4
 786 variants identified in the present study (Table S2). Outer circles show genes in the genomic

787 environment of *mcr/eptA* genes (red) with the following color code: response regulator
788 (yellow), sensor histidine kinase (orange), *dgkA* (light blue), glycine zipper family protein
789 (grey). Other conserved gene families, identified through diamond clustering, are shown
790 using different light colors. When > 1 *mcr/eptA* gene copy were found in *Vibrio* genomes,
791 their genomic environments were not displayed (dark blue).

792 **(B)** Newly discovered genetic environment in the Harveyi clade. A *rstA/rstB* (response
793 regulator/histidine kinase) two component system is located upstream *dkgA* and *eptA*. A
794 gene encoding a glycine zipper family protein separates *rstA-rstB* from *dkgA-eptA*. The figure
795 is based on an *eptA-1* sequence.

796



797

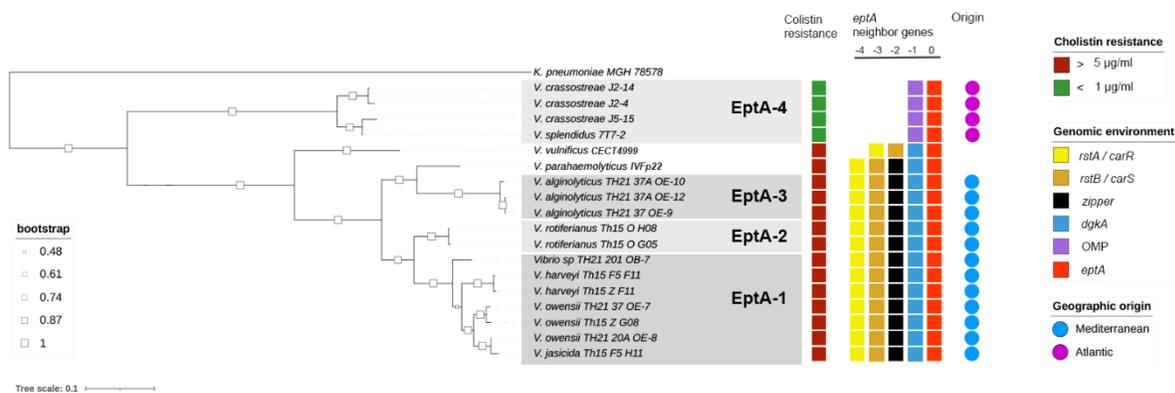
798 **Fig. 3. Evidence of gene mobilization in *eptA* paralogues from *Vibrionaceae***

799 Phylogeny of *eptA* paralogues for genomes containing multiple *eptA* genes, focusing on the

800 distribution of *eptA* genomic environments (-4/+4 genes). The *eptA* phylogenetic tree was

801 constructed using FastTree with the LG model, and visualization was performed with iTOL.
802 To simplify the figure, *V. vulnificus*, which contains two conserved *eptA* paralogues (see Fig.
803 S4) was not included. All paralogues are connected by inner grey lines, while inner blue
804 lines indicate paralogues associated with integrase/transposase in the -4/+4 environ-
805 ments. The outer circles represent genes in the genomic environment of *eptA* genes (in
806 red), with the following color coding: response regulators (yellow), sensor histidine ki-
807 nases (orange), *dgkA* (light blue), glycine zipper family proteins (grey), transposases/inte-
808 grases (dark blue), and cytochrome B family proteins (dark green). Other conserved gene
809 families identified through diamond clustering are depicted in various light colors. The
810 outer circle indicates the Sawabe clade⁶⁰ and provides an environmental summary.

811



812

813

814 **Fig. 4. Colistin resistance correlates with EptA polymorphism and *eptA* genomic**

815 **environment.** A phylogenetic tree of EptA amino acid sequences was generated through

816 maximum likelihood analysis of deduced EptA/Mcr amino acid sequences using PhyML v-

817 3.0 (<https://ngphylogeny.fr/tools/>) with the WAG model and 100 bootstrap runs. Red and

818 green empty squares indicate strains resistant to 5 µg/ml or susceptible to 1 µg/ml colistin,

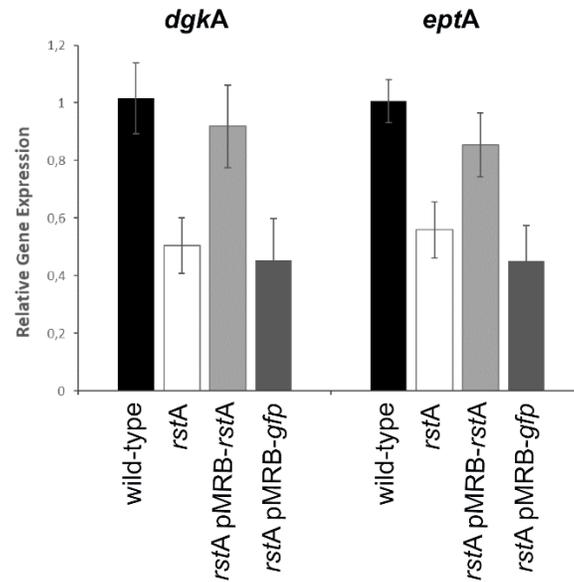
819 respectively, as phenotyped in Zobell medium. Colored plain squares indicate conserved

820 genes present at the vicinity of *eptA* (red): *rstA / carR* (yellow), *rstB / carS* (orange), *dgkA*

821 (light blue), glycine zipper family protein (grey), *opa* (purple). Numbers indicate the position

822 of the upstream genes relative to EptA.

823



824

825

826 **Fig. 5. *dgkA* and *eptA* gene expression is controlled by *rstA*.** Expression of *dgkA* and *eptA*
827 genes was quantified in wild-type *V. harveyi* Th15_F5-F11 and its *rstA* isogenic mutant. The
828 *rstA*-deletion mutant and the mutant complemented with a pMRB-*gfp* plasmid showed a
829 significant decrease in expression for *dgkA* (estimate = -0.511 ± 0.173 , $t = -2.963$, $p = 0.021$
830 and estimate = -0.561 ± 0.193 , $t = -2.910$, $p = 0.023$) and *eptA* (estimate = -0.446 ± 0.136 , $t =$
831 -3.271 , $p = 0.014$ and estimate = -0.553 ± 0.153 , $t = -3.623$, $p = 0.008$), whereas
832 complementation with *rstA* restored wild type expression levels (*dgkA* estimate = $-0.0978 \pm$
833 0.172 , $t = -0.567$, $p = 0.589$ and *eptA* estimate = -0.151 ± 0.136 , $t = -1.109$, $p = 0.304$). Data
834 were normalized using two housekeeping genes.

835

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