

1 Parallel evolution of two clades of a major Atlantic endemic *Vibrio parahaemolyticus* pathogen
2 lineage by independent acquisition of related pathogenicity islands

3

4 Feng Xu^{1,2,3}, Narjol Gonzalez-Escalona⁴, Kevin P. Drees^{1,2}, Robert P. Sebra⁵, Vaughn S.
5 Cooper^{1,2,*}, Stephen H. Jones^{1,6}, and Cheryl A. Whistler^{1,2#}

6

7 Running Title: parallel evolution of ST631 *Vibrio parahaemolyticus*

8

9 ¹Northeast Center for Vibrio Disease and Ecology, University of New Hampshire, Durham, NH;

10 ²Department of Molecular, Cellular and Biomedical Sciences, University of New Hampshire,

11 Durham, NH; ³Genetics Graduate Program, University of New Hampshire, Durham, NH;

12 ⁴Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park,

13 MD; ⁵Icahn Institute and Department of Genetics & Genomic Sciences, Icahn School of

14 Medicine at Mount Sinai, New York, NY; and ⁶Department of Natural Resources and the

15 Environment, University of New Hampshire, Durham, NH, USA.

16

17 *Current address: Microbiology and Molecular Genetics, University of Pittsburgh School
18 of Medicine, Pittsburgh, PA

19

20 #Corresponding author e-mail: cheryl.whistler@unh.edu

21 **ABSTRACT**

22 Shellfish-transmitted *Vibrio parahaemolyticus* infections have recently increased from
23 locations with historically low disease incidence, such as the Northeast United States (US). This
24 change coincided with a bacterial population shift towards human pathogenic variants occurring
25 in part through the introduction of several Pacific native lineages (ST36, ST43 and ST636) to
26 near-shore areas off the Atlantic coast of the Northeast US. Concomitantly, ST631 emerged as a
27 major endemic pathogen. Phylogenetic trees of clinical and environmental isolates indicated that
28 two clades diverged from a common ST631 ancestor, and in each of these clades, a human
29 pathogenic variant evolved independently through acquisition of distinct *Vibrio* pathogenicity
30 islands (VPaI). These VPaI differ from each other and bear little resemblance to hemolysin-
31 containing VPaI from isolates of the pandemic clonal complex. Clade I ST631 isolates either
32 harbored no hemolysins, or contained a chromosome I-inserted island we call VPaI β that
33 encodes a type three secretion system (T3SS2 β) typical of *Trh* hemolysin-producers. The more
34 clinically prevalent and clonal ST631 clade II had an island we call VPaI γ that encodes both *tdh*
35 and *trh* and that was inserted in chromosome II. VPaI γ was derived from VPaI β but with some
36 additional acquired elements in common with VPaI carried by pandemic isolates, exemplifying
37 the mosaic nature of pathogenicity islands. Genomics comparisons and amplicon assays
38 identified VPaI γ -type islands containing *tdh* inserted adjacent to the *ure* cluster in the three
39 introduced Pacific and most other emergent lineages. that collectively cause 67% of Northeast
40 US infections as of 2016.

41

42 **IMPORTANCE**

43 The availability of three different hemolysin genotypes in the ST631 lineage provided a
44 unique opportunity to employ genome comparisons to further our understanding of the processes
45 underlying pathogen evolution. The fact that two different pathogenic clades arose in parallel
46 from the same potentially benign lineage by independent VPaI acquisition is surprising
47 considering the historically low prevalence of community members harboring VPaI in waters
48 along the Northeast US Coast that could serve as the source of this material. This illustrates a
49 possible predisposition of some lineages to not only acquire foreign DNA but also to become
50 human pathogens. Whereas the underlying cause for the expansion of *V. parahaemolyticus*
51 lineages harboring VPaIγ along the US Atlantic coast and spread of this element to multiple
52 lineages that underlies disease emergence is not known, this work underscores the need to define
53 the environment factors that favor bacteria harboring VPaI in locations of emergent disease.

54

55 INTRODUCTION

56 *Vibrio parahaemolyticus* is an emergent pathogen capable of causing human gastric
57 infections when consumed, most often with contaminated shellfish (1, 2). Some human
58 pathogenic *V. parahaemolyticus* variants evolve from diverse non-pathogenic communities
59 through horizontal acquisition of *Vibrio* pathogenicity islands (VPaI) (3-5). Gastric pathogenic *V.*
60 *parahaemolyticus* typically harbor islands with at least one of two types of horizontally acquired
61 hemolysin genes (*tdh* and *trh*) that are routinely used for pathogen discrimination even though
62 their role in disease appears modest (6-11). Most pathogenic *V. parahaemolyticus* isolates also
63 carry accessory type three secretion systems (T3SS) that translocate effector proteins that
64 contribute to host interaction (12-14). Two evolutionarily divergent horizontally-acquired
65 accessory systems (T3SS2α or T3SS2β) contribute to human disease and are genetically linked

66 to hemolysin genes (two *tdh* genes with T3SS2 α , and *trh* with T3SS2 β) in contiguous but distinct
67 islands (4, 15-17). The first described *tdh*-harboring island [called by several different names
68 including Vp-PAI (15), VPAl-7 (4), and *tdh*VPA (17)] from an Asian pandemic strain called
69 RIMD 2210366 is fairly well-characterized (4, 5, 13, 18, 19). In contrast, islands containing
70 T3SS2 β linked to *trh* and a urease (*ure*) cluster, which confers a useful diagnostic phenotype,
71 [where similar islands are described by others as Vp-PAI_{TH3966} (16), or *trh*VPA(17, 20)] have
72 received only modest attention. Pathogenic variants harboring both *tdh* and *trh* are increasingly
73 associated with disease in North America (21-26), and yet, to our knowledge, the exact
74 configuration of hemolysin-associated VPAl(s) in isolates that contain both *tdh* and *trh* have not
75 yet been described [although see (20)]. Thus it is unclear how virulence loci and islands in these
76 emergent pathogen lineages carrying both hemolysins evolved and spread.

77 The expanding populations of *V. parahaemolyticus* have increased infections even in
78 temperate regions previously only rarely impacted by this pathogen and where most
79 environmental isolates harbor no known virulence determinants (27). A related complex of Asia-
80 derived pandemic strains, most often identified as serotype O3:K6 and also known as sequence
81 type (ST) 3 (based on allele combinations of seven housekeeping genes) causes the most disease
82 globally (28). An unrelated Pacific native lineage called ST36 (also described as serotype
83 O4:K12) currently dominates infections in North America, including from the Northeast United
84 States (US) (21, 26, 29). The introduction of ST36 into the Atlantic Ocean by an unknown route
85 precipitated a series of outbreaks from Atlantic shellfish starting in 2012 (29, 30). Prior to 2012,
86 residential lineages contributed to low but increasing sporadic infection rates on the Northeast
87 US coast (<https://www.cdc.gov/vibrio/surveillance.html>, 2017) (21), with ST631 emerging as the
88 major lineage that is endemic to near-shore areas of the Atlantic Ocean bordering North America

89 (the northwest Atlantic Ocean) (31). However, we previously identified a single ST631 isolate
90 lacking hemolysins (21, 27) suggesting this pathogen lineage may have recently evolved through
91 VPaI acquisition.

92 The goal of our study was to understand the genetic events and changing population
93 context for the evolution of the ST631 pathogenic lineage. We conducted whole and core
94 genome phylogenetic analysis of three environmental and 39 clinical ST631 isolates along with
95 isolates from other emergent lineages from the region, which revealed two ST631 clades of
96 common ancestry, from which human pathogens evolved in parallel. The single clade I clinical
97 isolate acquired a *recA* gene insertion previously seen associated with Asian lineages, and had a
98 VPaI that is typical of isolates harboring *trh* in the absence of *tdh*. In contrast, isolates from the
99 clonal ST631 clade II that dominates Atlantic-derived ST631 infections (31) had a related but
100 distinct VPaI. This VPaI contained a *tdh* gene and four associated hypothetical protein encoding
101 genes inserted within, not next to, an existing *ure-trh-T3SS2β* island in close proximity to the
102 *ure* cluster. Nearly all emergent resident and invasive lineages, including all three Pacific
103 lineages (ST36, ST636 and ST43) contained islands that similarly had a *tdh* gene inserted within
104 the VPaI in an identical location adjacent to the *ure* cluster providing a mechanism for
105 simultaneous acquisition of both hemolysins with T3SS2β.

106

107 **RESULTS**

108 **Atlantic endemic ST631 and several invasive lineages harboring both the *tdh* and *trh*** 109 **hemolysin genes are clinically prevalent in four reporting Northeast US States.**

110 Ongoing analysis of clinical isolates revealed that even as the Pacific-derived ST36
111 lineage continued to dominate infections (50%), the endemic (autochthonous) ST631 lineage

112 accounted for 14% of infections (Table 1). Concurrently, a limited number of other lineages
113 contributed individually to fewer infections ($\leq 3\%$ each), among which were two lineages that
114 have caused infections in the Pacific Northwest in prior decades: ST43 and ST636 (22, 23).
115 ST43 and ST636 only recently (2013 and 2011 respectively) (21) have been linked to product
116 harvested from waters along the Northeast US coast, and also caused infections in subsequent
117 years. As is common among US clinical isolates, pathogenic isolates of all the aforementioned
118 lineages harbor both the *tdh* and *trh* hemolysin genes (Table 1). Among environmental isolates,
119 ST34 and ST674 are the most frequently recovered pathogen lineages but these caused
120 comparatively few infections (Table 1). ST34 was first reported from the environment in 1998,
121 from both the Gulf of Mexico and near-shore areas of MA, and was also recovered in NH in
122 2012 (21) suggesting it is an established resident in the region. ST674 which was first reported
123 from an infection in Virginia in 2007 (32) was first recovered from the local environment in
124 2012 (www.pubmlst.org/vparahaemolyticus) (21). Notably even though all four ST674
125 environmental isolates, like ST34, harbored both hemolysin genes, the single ST674 clinical
126 isolate (MAVP-21) lacked hemolysins (Table 1) (21). The decrease in clinical prevalence of *trh*-
127 harboring Atlantic endemic ST1127, which caused no infections in the last three years, coincided
128 with the increase in clinical prevalence of all three Pacific-derived lineages which harbor both
129 hemolysins. Notably, very few other clinical isolates harbored *trh* in the absence of *tdh* and
130 clinical isolates containing only *tdh* (i.e. ST1725) were extremely rare (Table 1). Concurrent with
131 this shift in composition of clinical lineages that includes multiple Pacific-derived lineages,
132 hemolysin producers have increased in relative abundance in nearshore areas of the region,
133 where historically these represented $\sim 1\%$ of all isolates (27). Since 2012, hemolysin producers
134 have been recovered more frequently, and in the last two years their proportion has increased by

135 up to an order of magnitude (comprising as much as 10%) in some regional shellfish associated
136 populations (data not shown).

137

138 **A single clinical ST631 lineage isolate with an unusual *recA* allele harbors *trh* in the**
139 **absence of *tdh***

140 Employing ST631-specific marker-based assays (see methods), we identified two
141 additional 2015 environmental isolates (one from NH and one from MA) and one additional
142 2011 local-source clinical isolate (MAVP-R) (21) with a hemolysin profile (*trh*⁺ without *tdh*)
143 that is atypical of the ST631 lineage (Table 1). Although analysis of the seven-housekeeping
144 gene allele combination confirmed the environmental isolates were indeed ST631, MAVP-R was
145 not ST631 based on only one locus: *recA*. Examination of the *recA* locus of MAVP-R uncovered
146 a large insertion within the ancestral ST631 *recA* gene (allele recA21;
147 www.pubmlst.org/vparahemolyticus) incorporating an intact but different *recA* gene into the
148 locus [allele recA107(33)] and fragmenting the ancestral gene (Fig. 1). The insertion in the
149 ancestral *recA* gene in MAVP-R is identical to one observed in the *recA* locus of two Hong Kong
150 isolates (isolates S130 and S134) and similar to the one in isolate 090-96 (ST189a) isolated in
151 Peru but believed to have originated in Asia (33).

152

153 **ST631 forms two divergent clades**

154 The existence of three different hemolysin profiles (Table 1) among all available ST631
155 draft genomes suggested there could be more than one ST631 lineage. Therefore we evaluated
156 whole genome maximum likelihood (ML) phylogenies of select ST631 isolates and all other
157 lineages causing two or more infections reported in four Northeast US States to evaluate whether

158 there was more than one ST631 lineage (Table 1) (Fig. 2). The phylogenetic tree showed that
159 ST631 isolates, regardless of their hemolysin genotype, clustered together but they formed two
160 distinct clades, indicative of common ancestry (Fig. 2). Clade I harbored either *trh* or no
161 hemolysins and consisted of all three environmental isolates which were from MA and NH, and
162 the single clinical isolate MAVP-R, whereas clade II consisted of all other isolates all of which
163 harbor both hemolysins. The two distinct ST631 clades shared 85% of their DNA in common
164 and displayed polymorphisms in $\leq 12\%$ of the shared DNA content. The most closely related
165 sister lineage to ST631 was formed by *trh*-harboring ST1127 isolates that have been exclusively
166 reported from clinical sources in the Northeast US (21).

167 We next evaluated the relationships of all available ST631 isolate genomes at NCBI and
168 sequenced by us (Supplemental Table 1) using a custom core genome multi-locus sequence
169 typing (cgMLST) method as previously described (31). Minimum spanning trees built from core
170 genome loci from 42 ST631 isolates indicated that only 390 loci varied between the most closely
171 related isolate of clade I (MAVP-L) and clade II (G6928) (Fig. 3). The most distantly related
172 isolates within clade I (G149 and MAVP-R) exhibited 80 core genome loci differences whereas
173 clade II is clonal with only 51 variant loci between the most divergent isolates: clinical isolate
174 09-4436 and environmental isolate S487-4, both reported from PEI Canada (Fig. 3) (31).

175

176 **Each ST631 clade independently acquired a distinct pathogenicity island positioned on
177 different chromosomes**

178 Given the variation in ST631, comparisons between these isolates could elucidate the
179 events that led not only to the evolution of two pathogenic clades but also address unresolved
180 questions about the unique configurations and contents of pathogenicity islands in western

181 Atlantic Ocean emergent lineages. The physical proximity of *tdh* with the *ure* cluster and *trh*,
182 and the co-occurrence of *tdh* with T3SS2 β reported in many *tdh*⁺/*trh*⁺ clinical isolates suggested
183 *tdh* could be harbored within or next to the same pathogenicity island harboring *trh* in at least
184 some lineages as was previously suggested (20, 24, 34).

185 To identify the location and determine the architecture of the pathogenicity elements
186 harboring hemolysin genes, we generated high quality annotated genomes for the clade I ST631
187 isolate MAVP-R and clade II ST631 isolate MAVP-Q (both reported in 2011 from MA)
188 employing PacBio sequencing. The pathogenicity island regions in these isolates genomes were
189 extracted, aligned, and the contents compared with pathogenicity island harboring two *tdh* genes
190 [previously called Vp-PAI (15), VPaI-7 (4) and *tdh*VPA(17)] from RIMD 2210366 and Vp-
191 PAI_{TH3996} (16) [also called *trh*VPI (17)] harboring *trh* (Supplemental Table 2). This comparison
192 revealed that MAVP-R harbored a pathogenicity island typical of *trh*-containing isolates that
193 includes a linked *ure* cluster and T3SS2 β that is orthologous, with the exception of few unique
194 regions, with Vp-PAI_{TH3996} (16) (Supplemental Table 2 and Fig. 4). Because the lack of
195 convention in uniformly naming syntenous islands that distinguish them from distinctive and yet
196 functionally analogous islands can impede communication, we hereafter will consistently
197 reference the same island by a common descriptive name regardless of isolate lineage. Hereafter
198 we will refer to islands sharing the same general configuration to that in MAVP-R by the name
199 VPaI β , and refer to *tdh*-containing islands similar to that described in strain RIMD 2210366 by
200 the name VPaI α , regardless of bacterial isolate background. We adopted this simplified
201 nomenclature in reference to the version of the key virulence determinant carried in the islands
202 (T3SS2 α and T3SS2 β) in the two already described island types. This scheme importantly
203 accommodates naming of additional uniquely-configured islands as they are identified. As noted

204 previously (16, 17, 20), VPaI β is dissimilar to VPaI α in most gene content with ~ 78 ORFs
205 unique to VPaI β (where the number of identified ORFs used for comparison can differ slightly
206 depending on which annotation program is applied) (Supplemental Table 2, Fig. 4). Even so,
207 VPaI β had many homologous genes of varying sequence identity (n=~38 ORFs, excluding *tdh*
208 homology with *trh*) when compared to VPaI α (Supplemental Table 2, Fig. 4)(4, 5, 16).
209 Identification of some homologs required that we relax matching to 50% such as for the
210 divergent, but homologous T3SS2 α and T3SS2 β genes encoding the apparatus, chaperones, and
211 some shared effectors (Supplemental Table 2). No homolog of the T3SS2 α effector gene *vopZ*
212 was identified, but a single ORF whose deduced protein sequence bears only 27% identity with
213 VopZ is located in its place (Fig. 2 and Supplemental Table 2). VPaI β from strain TH3996 and
214 VPaI α from pandemic strain RIMD 2210633 are inserted in an identical location in chromosome
215 II adjacent to an Acyl-CoA hydrolase-encoding gene. In contrast the VPaI β s in MAVP-R,
216 ST1127 isolate MAVP-25, and Asia-derived AQ4037 are in chromosome I, in each case in the
217 same insertion location identified for strain AQ4037 (17).

218 MAVP-Q contained both *tdh* and *trh* within the same contiguous unique VPaI (hereafter
219 called VPaI γ) that shared features with both VPaI α and VPaI β (Fig. 4, Supplemental Table 2).
220 Specifically, VPaI γ had a core that with few exceptions was orthologous in content and
221 syntenous with VPaI β from MAVP-R (Fig. 4) with only a few exceptions. VPaI γ displays high
222 conservation with VPaI α near its 3' end, as has been described in other draft *tdh*⁺*trh*⁺ harboring
223 genomes (20) as well as in the VPaI β island of strain TH3996, although the presence of this
224 element may not be typical of VPaI β (e.g. it is absent in the islands from AQ4037 (17), MAVP-
225 R and MAVP-25). The VPaI γ also contained a *tdh* gene homologous to *tdh2* (also called *tdhA*)
226 from VPaI α (98.6%) near its 5' end but not at the 5' terminus of the island (Fig. 4). Rather, the

227 DNA flanking both sides of the *tdh* gene in VPaI γ was conserved in VPaI β of MAVP-R and
228 absent from VPaI α , (Fig. 4). Analysis of 300 genomes of *V. parahaemolyticus* (representing a
229 minimum of 28 distinct sequence types) of sufficient quality for analysis confirmed that the
230 module of four hypothetical proteins preceding the *tdh2* homolog was present only in *trh*-
231 harboring genomes, but not in genomes harboring *tdh* in the absence of *trh* (i.e. VPaI α
232 containing genomes), providing evidence that the *tdh* gene was acquired horizontally by
233 insertion into, not next to, an existing VPaI β , perhaps through activity of the adjacent transposase
234 gene (11) (Supplemental Table 3, Supplemental fig. 1, and data not shown). Like with VPaI α
235 from RIMD 2210633, and VPaI β of TH3996, VPaI γ of clade II ST631 is located in a conserved
236 location of chromosome II, adjacent to an Acyl-CoA hydrolase-encoding gene.

237 The final environmental ST631 clade I isolate that lacked hemolysins, G149, had no
238 VPaI α , β or γ elements in its genome. Close examination of the DNA corresponding to the VPaI
239 insertion sites in either chromosome revealed no remnants of these islands in either chromosomal
240 location indicating this isolate likely never acquired a pathogenicity island (Supplemental Fig. 2
241 and data not shown). Because clade I isolate G149 lacked these islands, this could be the
242 ancestral state of the ST631 lineage (21).

243

244 **Most clinically prevalent isolates from the Northeast US harbor similar contiguous**
245 **pathogenicity islands containing *tdh* inserted in the same location of their VPaI**

246 We next asked which isolates from other lineages likely residing within the mixed
247 population with ST631 in near-shore areas of the Northeast US harbored islands of similar
248 structure to VPaI γ that contain both hemolysin genes. Assembly of short-read sequences into
249 contigs that cover the full length of VPaI which is necessary for comparative analysis of entire

250 island configuration was impeded by the fact that homologous transposase sequences and other
251 sequences were repeated multiple times throughout the island. Therefore, we determine whether
252 other lineages harboring both hemolysin genes harbor *tdh* in the same island location, between
253 the conserved VPaI β / γ module of four hypothetical proteins (to the left or 5' of *tdh*) and the *ure*
254 cluster (to the right or 3' of *tdh*) (Fig. 4) by combining bioinformatics analysis of sequenced
255 genomes with amplicon assays (Supplemental Fig. 1). First we analyzed assembled draft
256 genomes for *tdh* co-occurrence and proximity with the four adjacent hypothetical protein-
257 encoding genes that are absent in VPaI β but present in VPaI γ (See Methods). Every emergent
258 pathogenic lineage of the Northeast US (Table 1) harboring both *tdh* and *trh* carried homologous
259 DNA corresponding to all four hypothetical proteins adjacent to the *tdh* gene in a contiguous
260 segment (Supplemental Table 3). To determine whether *tdh* was also adjacent to the *ure* cluster
261 in these same isolates we next designed specific flanking primers and amplified the unique
262 juncture between the *tdh*-containing transposon associated module and the *ure* cluster for all
263 clinical isolates harboring both *tdh* and *trh* (See Methods) (Supplemental Fig. 1). The results
264 were congruent with our bioinformatics assessment (Supplemental Table 3), and demonstrated
265 that isolates from all emergent pathogenic lineages harboring both hemolysins have *tdh* inserted
266 in close proximity to an *ure* cluster in a configuration similar to VPaI γ from MAVP-Q (Fig. 5,
267 Table 1). This confirmed that these isolates harboring both hemolysins harbor *tdh* within, and not
268 next to, the same VPaI thereby facilitating simultaneous acquisition of both hemolysin genes.

269

270 **DISCUSSION**

271 Even preceding the increased illnesses from Pacific-invasive lineages, two different
272 clades of the predominant endemic Atlantic lineage of pathogenic *V. parahaemolyticus*, ST631

273 (31) evolved and contributed to a rise in sporadic illnesses in the four reporting Northeast US
274 States (Table 1, Fig. 2 & 3). Several lines of evidence support the interpretation of parallel
275 pathogen evolution. The two lineages exhibit differences in both clinical and environmental
276 prevalence suggesting the pathogenic variants of each clade have not evolved the same degree of
277 virulence (Table 1). Pathogenic members in each lineage also acquired different pathogenicity
278 islands with different hemolysin gene content (Fig. 2 & 3). Although it was a formal possibility
279 that ST631 clade II evolved from clade I by independent horizontal acquisition of *tdh* into its
280 existing VPAl β , it is notable that other resident and even invasive lineages now in the Atlantic
281 harbor VPAl γ with *tdh* and four additional co-occurring ORFs inserted into the same location of
282 the island, suggesting a common evolutionary origin of this hybrid-type island (Fig. 4 and
283 Supplemental Fig. 1). Finally, each of the two clades harbor VPAl insertions on different
284 chromosomes: the less clinically prevalent ST631 clade I contains three isolates that harbor
285 VPAl β in chromosome I (Fig. 3) and a single environmental isolate lacking any island (Table 1,
286 supplemental Fig. 2), whereas the clonal ST631 clade II isolates all harbor VPAl γ on
287 chromosome II.

288 Given that several other resident lineages harbor similar β and γ -type VPAl, pathogens in
289 each clade could have acquired their islands from the reservoir of resident bacteria already
290 circulating in the Atlantic even before the presume arrival of invasive Pacific lineages. Several
291 well-documented members of the Gulf of Mexico *V. parahaemolyticus* population (35-37) may
292 also have expanded their range through movement of ocean currents and could be the source for
293 these VPAl (Table 1, Fig. 5). But historically, hemolysin producers were extremely rare in near
294 shore areas of the Atlantic US coast (25) and represented only about ~1% of isolates in an
295 estuary of NH as of a decade ago (27) limiting the potential for interacting partners or sources for

296 acquired VPaI. Given this historical context, it is remarkable that two different clades from the
297 same lineage independently acquired different VPaI—which for clade II ST631 occurred prior to
298 2007—well before the recent shift in abundance of hemolysin producers.

299 The parallel evolution of two different lineages through lateral DNA acquisition alludes
300 to the possibility that as-yet-undefined attributes may increase the chances of acquisition or
301 prime some bacterial lineages (such as ST631) to more readily acquire and maintain genetic
302 material or become pathogenic upon island acquisition. Even though the ecological niche in
303 which horizontal island acquisition took place is unknown, it is conceivable that co-colonization
304 of hosts or substrates favorable to the growth of ST631 and hemolysin producers may have
305 facilitated island movement. Certainly, association of bacteria with specific marine substrates
306 such as chitinous surfaces of plankton that also induce a natural state of competence could
307 promote lateral transfer through close contact between the progenitors of the pathogenic
308 subpopulation of each clade and island donors (3, 38, 39). Alternatively, conjugative plasmids or
309 transducing phage could have been the agents of island delivery. The finding that the only
310 clinical clade I isolate, MAVP-R, also harbors a second horizontal insertion in its *recA* locus that
311 matched one previously found in Asia-derived strains (33) indicates it acquired more than one
312 segment of foreign DNA during its evolution as a pathogen (Fig. 1) further illustrating that
313 mechanisms that facilitate DNA transfer and acquisition may both have been at play. It also
314 suggests that horizontal transfer of DNA from introduced bacteria not yet detected in the Atlantic
315 could add to the genetic material available for pathogen evolution from Atlantic Ocean
316 populations. The more detailed molecular epidemiological, comparative genomics, and
317 functional analyses necessary to assess the impact of introduced pathogens on resident Atlantic

318 lineages are warranted given this evidence and the documented introduction of multiple Pacific-
319 derived lineages in the region (Table 1).

320 There has been some consideration of the roles of human virulence determinants in
321 ecological fitness, but the natural context of pathogenic *V. parahaemolyticus* evolution is still
322 unknown (40-42). Whereas *tdh* and T3SS2 α each may promote growth when bacteria are under
323 predation, isolates that carry *trh*-containing islands (which likely also have T3SS2 β) do not
324 derive similar benefits from their islands (43). This is surprising considering the islands encode
325 several homologous effectors (Fig. 4 and Supplemental Table 2) that don't have an established
326 role in enteric disease but they could alternatively or additionally mediate eukaryotic cell
327 interactions with natural hosts thereby promoting environmental fitness (13, 14). But these
328 islands also lack homologous open for the VPaI α effector that is most closely associated with
329 enteric disease: *vopZ* (11) (Fig. 4 and Supplemental Table 2). The general lack of knowledge of
330 unique T3SS2 β effectors and other gene function in these islands (Fig. 4 and Supplemental Table
331 2) even with regard to enteric disease, limits comparative analysis with the well-studied and
332 functionally defined VPaI α which could elucidate the bases for pathogen evolution. The higher
333 clinical prevalence of clade II ST631 than clade I which has also been recovered on more than
334 one occasion from the environment (Table 1) could indicate that VPaI γ confers greater virulence
335 potential than VPaI β , perhaps owing to the presence of *tdh*, a known virulence factor (1, 7, 44).
336 However, the resident community members in both the Pacific and the Atlantic Ocean that
337 harbor *tdh* and T3SS2 α comparatively rarely cause human infections (21-23). The unique
338 environmental conditions that underlie pathogen success from northern latitudes that favors
339 bacteria with VPaI β and VPaI γ including two different ST631 lineages suggests the shared
340 content of these islands could confer abilities that are distinct from VPaI α which could underlie

341 the repeated acquisition and maintenance of these related islands by so many different lineages
342 now present in near-shore areas of the Northeast US.

343

344 MATERIALS AND METHODS

345 Bacteria isolates, media and growth conditions.

346 *V. parahaemolyticus* clinical isolates for this study were provided by cooperating public
347 health laboratories in Massachusetts, New Hampshire, Maine, and Connecticut whereas a select
348 number of environmental isolates were enriched from estuarine substrates as described (21).
349 Detailed information about these isolates was described previously (31) and listed in
350 Supplemental Table 1. Isolates were routinely cultured in Heart Infusion (HI) media
351 supplemented with NaCl at 37°C as described (21).

352

353 Whole genome sequencing, assembly, annotation and sequence type identification.

354 Genomic DNA was extracted using the Wizard Genomic DNA purification Kit (Promega,
355 Madison WI USA) or by organic extraction (21). The quality genomic DNA was determined by
356 spectrophotometric measurements by NanoDrop (ThermalFisher, Waltham MA USA). Libraries
357 for DNA sequencing were prepared using a high-throughput Nextera DNA preparation protocol
358 (45) using an optimal DNA concentration of 2ng/μl. Genomic DNA was sequenced using an
359 Illumina – HiSeq2500 device at the Hubbard Center for Genome Studies at the University of
360 New Hampshire, using a 150bp paired-end library. *De novo* assembly was performed using the
361 A5 pipeline (46), and the assemblies annotated with Prokka1.9 using the "genus" option and
362 selecting "Vibrio" for the reference database (47). The sequence types were subsequently
363 determined using the SRST2 pipeline (48). The sequence type of each genome was determined

364 when using *V. parahaemolyticus* as the database (<https://pubmlst.org/vparahaemolyticus/>). For
365 most isolates where the combination of each allele was not found in the database representing
366 novel sequence types, the genome was submitted for a new sequence type designation
367 (www.pubmlst.org/vparahaemolyticus).

368 Isolates MAVP-Q and MAVP-R were sequenced using the Pacific Biosciences RSII
369 technology. Using between 3.7-5.3 µg DNA, the library preparation and sequencing was
370 performed according to the manufacturer's instructions (Pacific Biosciences, Menlo Park CA,
371 USA) and reflects the P5-C3 sequencing enzyme and chemistry for MAVP-Q isolate and the P6-
372 C4 configuration for MAVP-R. The mass of double-stranded DNA was determined by Qubit
373 (Waltham, MA USA) and the sample diluted to a final concentration of 33 µg / µL in a volume
374 of 150 µL elution buffer (Qiagen, Germantown MD USA). The DNA was sheared for 60
375 seconds at 4500 rpm in a G-tube spin column (Covaris, Woburn MA USA) which was
376 subsequently flipped and re-spun for another 60 seconds at 4500 rpm resulting in a ~20,000 bp
377 DNA verified using a DNA 12000 Bioanalyzer gel chip (Agilent, Santa Clara, CA USA). The
378 sheared DNA isolate was then re-purified using a 0.45X AMPure XP purification step (Beckman
379 Coulter, Indianapolis IN USA). The DNA was repaired by incubation in DNA Damage Repair
380 solution. The library was again purified using 0.45X Ampure XP and SMRTbell adapters ligated
381 to the ends of the DNA at 25°C overnight. The library was treated with an exonuclease cocktail
382 (1.81 U/µL Exo III 18 and 0.18 U/µL Exo VII) at 37°C for 1 hour to remove un-ligated DNA
383 fragments. Two additional 0.45X Ampure XP purifications steps were performed to remove
384 <2000 bp molecular weight DNA and organic contaminant.

385 Upon completion of library construction, samples were validated using an Agilent
386 DNA 12000 gel chip. The isolate library was subjected to additional size selection to the range

387 of 7,000 bp – 50,000 bp to remove any SMRTbells < 5,000 bp using Sage Science Blue Pippin
388 0.75% agarose cassettes to maximize the SMRTbell sub-read length for optimal *de*
389 *novo* assembly. Size-selection was confirmed by Bio-Analysis and the mass was quantified using
390 the Qubit assay. Primer was then annealed to the library (80°C for 2 minute 30 followed by
391 decreasing the temperature by 0.1°/s to 25°C). The polymerase-template complex was then
392 bound to the P5 or P6 enzyme using a ratio of 10:1 polymerase to SMRTbell at 0.5 nM for 4
393 hours at 30°C and then held at 4°C until ready for magbead loading, prior to sequencing. The
394 magnetic bead-loading step was conducted at 4°C for 60-minutes per manufacturer's guidelines.
395 The magbead-loaded, polymerase-bound, SMRTbell libraries were placed onto the RSII machine
396 at a sequencing concentration of 110-150 pM and configured for a 180-minute continuous
397 sequencing run. Long read assemblies were constructed using HGAP version 2.3.0 for *de novo*
398 assembly generation. Further, hybrid assemblies were generated and error corrected with
399 illumina raw reads using Pilon v1.20 (49).

400

401 **Lineage-specific marker-based assays**

402 To more rapidly identify ST631 isolates from clinical and environmental collections we
403 developed PCR-amplicon assays to unique gene content in ST631. Whole genome comparisons
404 were performed on MAVP-Q (a ST631 clinical isolate), G149 (a ST631 environmental isolate),
405 MAVP-26 (ST36), RIMD2210633 (ST3), and AQ4037 (ST96) (Supplemental Fig. 3). A total of
406 26 distinct genomic regions, each greater than 1kb in size, were present in MAVP-Q but absent
407 in other comparator genomes, including environmental ST631 that lacks hemolysins (G149)
408 (Supplemental Fig. 3). Within a large genomic island ~37.6 Kb in length with an integrase at one
409 terminus and an overall lower GC content (40.6% compared to 45.8% for the genome) a single

410 ORF homologous to restriction endonucleases (AB831_06355) that was restricted to clinical
411 ST631 isolates in our collection and publicly available draft genomes (n=693)
412 (<http://www.ncbi.nlm.nih.gov/genome/691>, 2017) was selected as a suitable amplicon target. The
413 distribution of this locus was further analyzed using the BLAST algorithm by a query against the
414 nucleotide collection, the non-redundant protein sequences, and against the genus *Vibrio* (taxid:
415 662), excluding *V. parahaemolyticus* (taxid: 691), using the default settings for BLASTn (50).
416 Similar approaches were applied to identify ST631 diagnostic loci inclusive of the single
417 environmental isolate (G149), which identified a hypothetical protein encoding region
418 (AB831_06535) (ST631env). Oligonucleotide primers were designed to amplify the diagnostic
419 regions including AB831_06355 using primers ST631end F
420 (5'AGTCATCAGGTAGAGAGTTAGAGGA3') and ST631endR
421 (5'TCTTCGTTACCATAGTATGAGCCA3') which produces an amplicon of c.a. 494bp, and
422 AB831_06535 using primers ST631envF (5'TGGGCGTTAGGCTTG3') and ST631-envR
423 (5'GGGCTTCTACGACTTCTGCT3') producing an amplicon of 497bp.

424 Amplification of diagnostic loci was evaluated in individual assays using genomic DNA
425 from positive and negative controls: MAVP-Q and G149 (ST631), G4186 (ST34), G3578
426 (ST674), and MAVP-M (ST1127), MAVP-26 (ST36) and G61 (ST1125). Amplification of
427 specific sequence types were performed with Accustart enzyme mix on purified DNA. Cycling
428 was performed with an initial denaturation at 94°C for 3 min., followed by 30 cycles of a
429 denaturation at 94°C for 1min, annealing at 55°C for 1 min, and amplification at 72°C for 30s
430 with a final elongation at 72°C for 5 min. The primer pairs only produced amplicons from
431 template DNA from ST631 and each was the expected size (data not shown, and Supplemental
432 Fig. 3). Amplicon assays were applied to 208 clinical isolates from the Northeast US States (ME,

433 NH, MA and CT) and 1140 environmental isolates collected from 2015-2016 from NH and MA.
434 These assays identified all known ST631 clinical isolates with 100% specificity and also
435 identified an additional 7 *tdh*⁺*trh*⁺ clinical isolates (ST631*end* and ST631*env* positive), and two
436 environmental (ST631*end* negative and ST631*env* positive) isolates from our archived collection.
437 Each, with the exception of MAVP-R, was subsequently confirmed to be ST631 by seven-locus
438 MLST (www.pubmlst.org).

439

440 **Examination of *recA* allele and adjacent sequences**

441 The PacBio sequenced genome of MAVP-R, contig 000001 (Accession No.
442 MPPP00000000) that contained the *recA* gene, was annotated using PROKKA1.9 (47). The
443 sequences of *recA* and its surrounding DNA was then compared to the contig containing *recA*
444 region from isolate S130 (AWIW01000000), S134 (AWIS01000000), 090-96 (JFFP01000036)
445 (33) and MAVP-Q (Accession No. MDWT00000000). The map of *recA* region of the five
446 isolates was illustrated using Easyfig (51).

447

448 **Core genome SNP determination and phylogenetic analysis**

449 Whole genome phylogenies were constructed with single nucleotide polymorphisms
450 (SNPs) identified from draft genomes using kSNP3 to produce aligned SNPs in FASTA format
451 (52). A maximum likelihood (ML) tree was then built from the FASTA file using raxMLHPC
452 with model GTRGAMMA and the -f option, and 100 bootstraps (53). Since there were no
453 differences among the clade II ST631 isolates we used a subset representing geographic and
454 temporal span of isolation.

455 Minimum spanning tree (MST) analysis was built based on core gene SNPs produced
456 from a cluster analysis. The cluster analysis of ST631 was performed using a custom core
457 genome multi-locus sequence type (cgMLST) analysis using RidomSeqSphere+software v3.2.1
458 (<http://www.ridom.de.seqsphere>, Ridom GmbH, Münster, Germany) as previously described
459 (31). Briefly, the software first defines a cgMLST scheme using the target definer tool with
460 default settings using the PacBio generated MAVP-Q genome as the reference. Then, five other
461 *V. parahaemolyticus* genomes (BB22OP, CDC_K4557, FDA_R31, RIMD2210633, and UCM-
462 V493) were used for comparison with the reference genome to establish the core and accessory
463 genome genes. Genes that are repeated in more than one copy in any of the six genomes were
464 removed from the analysis. Subsequently, a task template was created that contains both core and
465 accessory genes. Each individual gene locus from MAVP-Q was assigned allele number 1. Then
466 each ST631 isolate genome assembly was queried against the task template, where any locus that
467 differed from the reference genome or any other queried genome was assigned a new allele
468 number. The cgMLST performed a gene-by-gene analysis of all core genes (excluding accessory
469 genes) and identified SNPs within different alleles to establish genetic distance calculations.

470

471 **Configuration and distribution of VPaIs**

472 The VPaI sequence from the PacBio sequenced genomes of MAVP-Q and MAVP-R
473 were identified by comparison with the published RIMD2210633 VPaI-7 (NC_004605 region
474 between VPA1312 – VPA1395) and VPaI_{TH3996} (AB455531) (16). Identification of the complete
475 MAVP-Q VPaIy and genomic junctures in chromosome II was done by comparison with the
476 same region of chromosome II in MAVP-R and G149 (which lack an island in this location)
477 using Mauve (54). In a reciprocal manner, the absence of an island in chromosome I in MAVP-Q

478 and G149 was assessed by comparison with chromosome I of MAVP-R. MAVP-Q VPaI γ
479 (MF066646) and MAVP-R VPaI β (MF066647) were then extracted as a single contiguous
480 sequence and annotated using Prokka 1.9. Gene content and order of the VPaI elements in
481 MAVP-Q, MAVP-R and RIMD2210633 were then illustrated by Easyfig (51). Roary (55) was
482 then employed to determine homologs among VPaIs based on each island's annotated sequences
483 with identity set at 50%. Identification of the genome locations of VPaI β in ST1127 isolate
484 MAVP-M (accession number GCA_001023155) and for VPaI γ in AQ4037 (accession number
485 GCA_000182365) (17) was also done using Mauve (54).

486 To examine the distribution of the VPaI γ in all publicly available draft genomes
487 (<https://www.ncbi.nlm.nih.gov/genome/genomes/691>, 2016) and genomes from archived
488 regional isolates, whole draft genome sequences were aligned to a 6,118 bp subsequence of the
489 MAVP-Q VPaI with NASP version 1.0.2 (56) (<https://pypi.python.org/pypi/nasp/1.0.2>, 2017).
490 This subsequence spanned the unique juncture of the four conserved hypothetical proteins
491 (AB831_22090, AB831_22095, AB831_22100, AB831_22105) with the adjacent inserted *tdh*
492 (AB831_22110, c.a. 2549 bp upstream of *ure* cluster)(Supplemental Fig. 1). Percent coverage of
493 the reference sequence was used to determine whether each genome harbored only the four
494 hypothetical proteins, only a *tdh* gene, or the entire module including the fusion of the four genes
495 with *tdh* (Supplemental Fig. 1 and Supplemental Table 3). The sequence type of each genome
496 harboring the fused element characteristic of VPaI γ was then determined using the SRST2
497 pipeline (48). Where sequencing reads were not available as the input for SRST2, they were
498 simulated from assemblies using an in-house Python script
499 (<https://github.com/kpdrees/fasta2reads>).

500 A PCR amplification approach was developed and applied to survey the presence of *tdh*
501 adjacent to the *ure* gene cluster. Primers were designed to conserved sequences of the 3' end of
502 *tdh* (PIHybF8: 5'GCCAACATGGATATAAATAAAAATGA3') and the 5' end of *ureG*
503 (*tdhUreGrev5*: 5'GACAAAGGTATGCTGCCAAAGTG3') as determined by gene alignments,
504 which when used together produced a 2631 bp amplicon of the insertion juncture when used with
505 MAVP-Q as a template (Supplemental Fig. 4). Amplification was performed on purified DNA
506 with Accustart enzyme mix, with an initial denaturation at 94°C for 3 min., followed by 30
507 cycles of a denaturation at 94°C for 1 min, annealing at 61°C for 1min, and amplification at 72°C
508 for 2.5 min, with a final elongation at 72°C for 5 min. This amplification was performed in
509 parallel with a diagnostic multiplex PCR amplification of *tdh*, *trh* and *tlh* using published
510 methods (10, 57) to investigate the co-occurrence of VPAl γ with both hemolysin encoding genes
511 in representative isolates of various clinically prevalent sequence types. Amplicons were
512 visualized using a 1.2% agarose gel in TAE buffer (Supplemental Fig. 4).

513

514 **Nucleotide sequence accession numbers.**

515 The accession number of Pacific Biosciences sequenced genome for MAVP-Q is
516 MDWT00000000, and for MAVP-R is MPPP00000000. The accession number of Illumina
517 sequenced draft genome for G6928 is MPPN00000000, for MA561 is MPPM00000000 and for
518 G149 is MPPO00000000. Detailed information about all other ST631 isolate draft genomes were
519 described previously (31) and are listed in Supplemental Table 1. The accessions for the short
520 reads for the remaining sequenced genomes are listed in Supplemental Table 4. The accession
521 number of VPAl β from MAVP-R is MF066647 and the accession number of VPAl γ from MAVP-
522 Q is MF066646.

523

524 ACKNOWLEDGEMENTS

525

526 We are grateful for clinical isolates and wish to thank specifically: Jana Ferguson and Tracy
527 Stiles of the Massachusetts Department of Public Health, and M. Hickey and C. Schillaci from
528 the Massachusetts Department of Marine Fisheries; J.K. Kanwit of the Maine Department of
529 Marine Resources and A. Robbins from the Maine Department of Health and Human Services;
530 and Laurn Mank from the Connecticut Department of Public Health Laboratory, and K.
531 DeRosia-Banick, Connecticut Department of Agriculture, Bureau of Aquaculture. Assistance
532 with genome sequencing was provided by W. K. Thomas, and technical assistance provided by J.
533 Lemaire, K. Hartman, C. Hallee, M. Malanga, S. Ilyas, J. Hall, J. Sevigny, M. Dillon, K. Flynn,
534 A. Goupil, J. Means, R. Foxall, E. DaSilva, and M.S. Pankey. Partial funding for this work was
535 provided by the USDA National Institute of Food and Agriculture (Hatch projects NH00574,
536 NH00609 [accession number 233555], and NH00625 [accession number 1004199]). Additional
537 funding was provided by the National Oceanic and Atmospheric Administration College Sea
538 Grant program and grants R/CE-137, R/SSS-2, and R/HCE-3. Support was also provided
539 through the National Institutes of Health (1R03AI081102-01), the National Science Foundation
540 (EPSCoR IIA-1330641), and the National Science Foundation (DBI 1229361 NSF MRI). N.G.-
541 E. was funded through the FDA Foods Science and Research Intramural Program. Feng Xu and
542 Cheryl A. Whistler declare a potential conflict of interest in the form of a pending patent
543 application (U.S. patent application 62/128,764). This is Scientific Contribution Number 2722
544 for the New Hampshire Agricultural Experiment Station.

545

546 **REFERENCES**

547 1. **Hiyoshi H, Kodama T, Iida T, Honda T.** 2010. Contribution of *Vibrio*
548 *parahaemolyticus* virulence factors to cytotoxicity, enterotoxicity, and lethality in mice.
549 *Infect Immun* **78**:1772-1780.

550 2. **Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M-A, Roy SL, Jones**
551 **JL, Griffin PM.** 2011. Foodborne illness acquired in the United States—major
552 pathogens. *Emerg Infect Dis* **17(1)**:7-15.

553 3. **Hazen TH, Pan L, Gu J-D, Sobecky PA.** 2010. The contribution of mobile genetic
554 elements to the evolution and ecology of *Vibrios*. *FEMS Microbiol Ecol* **74**:485-499.

555 4. **Hurley CC, Quirke A, Reen FJ, Boyd EF.** 2006. Four genomic islands that mark post-
556 1995 pandemic *Vibrio parahaemolyticus* isolates. *BMC Genomics* **7**:104
557 DOI:110.1186/1471-2164-1187-1104.

558 5. **Boyd EF, Cohen AL, Naughton LM, Ussery DW, Binnewies TT, Stine OC, Parent**
559 **MA.** 2008. Molecular analysis of the emergence of pandemic *Vibrio parahaemolyticus*.
560 *BMC Microbiol* **8**:110.

561 6. **Kishishita M, Matsuoka N, Kumagai K, Yamasaki S, Takeda Y, Nishibuchi M.** 1992.
562 Sequence variation in the thermostable direct hemolysin-related hemolysin (*trh*) gene of
563 *Vibrio parahaemolyticus*. *Appl Environ Microbiol* **58**:2449-2457.

564 7. **Honda T, Ni Y, Miwatani T, Adachi T, Kim J.** 1992. The thermostable direct
565 hemolysin of *Vibrio parahaemolyticus* is a pore-forming toxin. *Can J Microbiol* **38**:1175-
566 1180.

567 8. **Park K-S, Ono T, Rokuda M, Jang M-H, Iida T, Honda T.** 2004. Cytotoxicity and
568 enterotoxicity of the thermostable direct hemolysin-deletion mutants of *Vibrio*
569 *parahaemolyticus*. *Microbiol Immunol* **48**:313-318.

570 9. **Shirai H, Ito H, Hirayama T, Nakamoto Y, Nakabayashi N, Kumagai K, Takeda Y, Nishibuchi M.** 1990. Molecular epidemiologic evidence for association of thermostable
571 direct hemolysin (TDH) and TDH-related hemolysin of *Vibrio parahaemolyticus* with
572 gastroenteritis. *Infect Immun* **58**:3568-3573.

574 10. **Panicker G, Call DR, Krug MJ, Bej AK.** 2004. Detection of pathogenic *Vibrio* spp. in
575 shellfish by using multiplex PCR and DNA microarrays. *Appl Environ Microbiol*
576 **70**:7436-7444.

577 11. **Nishibuchi M, Kaper JB.** 1995. Thermostable direct hemolysin gene of *Vibrio*
578 *parahaemolyticus*: a virulence gene acquired by a marine bacterium. *Infect Immun*
579 **63**:2093.

580 12. **Park K-S, Ono T, Rokuda M, Jang M-H, Okada K, Iida T, Honda T.** 2004.
581 Functional characterization of two type III secretion systems of *Vibrio parahaemolyticus*.
582 *Infect Immun* **72**:6659-6665.

583 13. **Broberg CA, Calder TJ, Orth K.** 2011. *Vibrio parahaemolyticus* cell biology and
584 pathogenicity determinants. *Microb Infect* **13**:992-1001.

585 14. **Zhang L, Orth K.** 2013. Virulence determinants for *Vibrio parahaemolyticus* infection.
586 *Curr Opin Microbiol* **16**:70-77.

587 15. **Makino K, Oshima K, Kurokawa K, Yokoyama K, Uda T, Tagomori K, Iijima Y, Najima M, Nakano M, Yamashita A.** 2003. Genome sequence of *Vibrio*

589 *parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*. The Lancet
590 **361**:743-749.

591 16. **Okada N, Iida T, Park K-S, Goto N, Yasunaga T, Hiyoshi H, Matsuda S, Kodama T, Honda T.** 2009. Identification and characterization of a novel type III secretion system in
592 trh-positive *Vibrio parahaemolyticus* strain TH3996 reveal genetic lineage and diversity
593 of pathogenic machinery beyond the species level. *Infect Immun* **77**:904-913.

594 17. **Chen Y, Stine OC, Badger JH, Gil AI, Nair GB, Nishibuchi M, Fouts DE.** 2011.
595 Comparative genomic analysis of *Vibrio parahaemolyticus*: serotype conversion and
596 virulence. *BMC Genomics* **12**:1.

597 18. **Zhou X, Gewurz BE, Ritchie JM, Takasaki K, Greenfeld H, Kieff E, Davis BM, Waldor MK.** 2013. *vopZ* A *Vibrio parahaemolyticus* T3SS effector mediates
598 pathogenesis by independently enabling intestinal colonization and inhibiting TAK1
599 activation. *Cell Reports* **3**:1690-1702.

600 19. **Hubbard TP, Chao MC, Abel S, Blondel CJ, zur Wiesch PA, Zhou X, Davis BM, Waldor MK.** 2016. Genetic analysis of *Vibrio parahaemolyticus* intestinal colonization.
601 Proc Nat Acad Sci USA **113**:6283-6288.

602 20. **Ronholm J, Petronella N, Leung CC, Pightling A, Banerjee S.** 2016. Genomic
603 Features of Environmental and Clinical *Vibrio parahaemolyticus* Isolates Lacking
604 Recognized Virulence Factors Are Dissimilar. *Appl Environ Microbiol* **82**:1102-1113.

605 21. **Xu F, Ilyas S, Hall JA, Jones SH, Cooper VS, Whistler CA.** 2015. Genetic
606 characterization of clinical and environmental *Vibrio parahaemolyticus* from the
607 Northeast USA reveals emerging resident and non-indigenous pathogen lineages. *Name:*
608 *Front Microbiol* **6**:272.

609
610
611

612 22. **Banerjee SK, Kearney AK, Nadon CA, Peterson C-L, Tyler K, Bakouche L, Clark**
613 **CG, Hoang L, Gilmour MW, Farber JM.** 2014. Phenotypic and genotypic
614 characterization of Canadian clinical isolates of *Vibrio parahaemolyticus* collected from
615 2000 to 2009. *J Clin Microbiol* **52**:1081-1088.

616 23. **Turner JW, Paranjpye RN, Landis ED, Biryukov SV, González-Escalona N, Nilsson**
617 **WB, Strom MS.** 2013. Population structure of clinical and environmental *Vibrio*
618 *parahaemolyticus* from the Pacific Northwest coast of the United States. *PLoS ONE*
619 **8(2):e55726**

620 24. **Jones JL, Lüdeke CH, Bowers JC, Garrett N, Fischer M, Parsons MB, Bopp CA,**
621 **DePaola A.** 2012. Biochemical, serological, and virulence characterization of clinical and
622 oyster *Vibrio parahaemolyticus* isolates. *J Clin Microbiol* **50**(7):2343-2352.

623 25. **DePaola A, Ulaszek J, Kaysner CA, Tenge BJ, Nordstrom JL, Wells J, Puhr N,**
624 **Gendel SM.** 2003. Molecular, serological, and virulence characteristics of *Vibrio*
625 *parahaemolyticus* isolated from environmental, food, and clinical sources in North
626 America and Asia. *Appl Environ Microbiol* **69**:3999-4005.

627 26. **Haendiges J, Timme R, Allard MW, Myers RA, Brown EW, Gonzalez-Escalona N.**
628 2015. Characterization of *Vibrio parahaemolyticus* clinical strains from Maryland (2012–
629 2013) and comparisons to a locally and globally diverse *V. parahaemolyticus* strains by
630 whole-genome sequence analysis. *Front Microbiol* **6**:125

631 27. **Ellis CN, Schuster BM, Striplin MJ, Jones SH, Whistler CA, Cooper VS.** 2012.
632 Influence of seasonality on the genetic diversity of *Vibrio parahaemolyticus* in New
633 Hampshire shellfish waters as determined by multilocus sequence analysis. *Appl Environ*
634 *Microbiol* **78**:3778-3782.

635 28. **Nair GB, Ramamurthy T, Bhattacharya SK, Dutta B, Takeda Y, Sack DA.** 2007.
636 Global dissemination of *Vibrio parahaemolyticus* serotype O3: K6 and its serovariants.
637 Clin Microbiol Rev **20**:39-48.

638 29. **Martinez-Urtaza J, Baker-Austin C, Jones JL, Newton AE, Gonzalez-Aviles GD, DePaola A.** 2013. Spread of Pacific Northwest *Vibrio parahaemolyticus* strain. N Engl J Med **369**:1573-1574.

641 30. **Newton AE, Garrett N, Stroika SG, Halpin JL, Turnsek M, Mody RK, Division of Foodborne W, Environmental D.** 2014. Notes from the field: Increase in *Vibrio parahaemolyticus* infections associated with consumption of Atlantic coast shellfish—2013. MMWR Morb Mortal Wkly Rep **63**:335-336.

645 31. **Xu F, Gonzalez-Escalona N, Haendiges J, Myers RA, Ferguson J, Stiles T, Hickey E, Moore M, Hickey JM, Schillaci C.** 2017. Sequence type 631 *Vibrio parahaemolyticus*, an emerging foodborne pathogen in North America. J Clin Microbiol **55**:645-648.

648 32. **Lüdeke CH, Gonzalez-Escalona N, Fischer M, Jones JL.** 2015. Examination of clinical and environmental *Vibrio parahaemolyticus* isolates by multi-locus sequence typing (MLST) and multiple-locus variable-number tandem-repeat analysis (MLVA). Frontiers in microbiology **6**:564

652 33. **González-Escalona N, Gavilan RG, Brown EW, Martinez-Urtaza J.** 2015. Transoceanic spreading of pathogenic strains of *Vibrio parahaemolyticus* with distinctive genetic signatures in the recA gene. PloS one **10**:e0117485.

655 34. **Park K-S, Suthienkul O, Kozawa J, Yamaichi Y, Yamamoto K, Honda T.** 1998. Close proximity of the *tdh*, *trh* and *ure* genes on the chromosome of *Vibrio parahaemolyticus*. Microbiology **144**:2517-2523.

658 35. **Johnson C, Flowers A, Young V, Gonzalez-Escalona N, DePaola A, Noriea III N, Grimes D.** 2009. Genetic relatedness among *tdh*+ and *trh*+ *Vibrio parahaemolyticus* cultured from Gulf of Mexico oysters (*Crassostrea virginica*) and surrounding water and sediment. *Microb Ecol* **57**:437-443.

662 36. **González-Escalona N, Martinez-Urtaza J, Romero J, Espejo RT, Jaykus L-A, DePaola A.** 2008. Determination of molecular phylogenetics of *Vibrio parahaemolyticus* strains by multilocus sequence typing. *J Bacteriol* **190**:2831-2840.

665 37. **Ellingsen BA, Olsen JS, Granum PE, Rorvik LM, González-Escalona N.** 2013. Genetic characterization of *trh* positive *Vibrio* spp. isolated from Norway. *Front Cell Infect Microbiol* **3**:107.

668 38. **Chen Y, Dai J, Morris JG, Johnson JA.** 2010. Genetic analysis of the capsule polysaccharide (K antigen) and exopolysaccharide genes in pandemic *Vibrio parahaemolyticus* O3: K6. *BMC Microbiol* **10**:1.

671 39. **Meibom KL, Blokesch M, Dolganov NA, Wu C-Y, Schoolnik GK.** 2005. Chitin induces natural competence in *Vibrio cholerae*. *Science* **310**:1824-1827.

673 40. **Takemura AF, Chien DM, Polz MF.** 2014. Associations and dynamics of *Vibrionaceae* in the environment, from the genus to the population level. *Front Microbiol* **5**:38.

675 41. **Lovell CR.** 2017. Ecological fitness and virulence features of *Vibrio parahaemolyticus* in estuarine environments. *Appl Microbiol Biotechnol* **101**:1781-1794.

677 42. **Johnson CN.** 2013. Fitness factors in vibrios: a mini-review. *Microb Ecol* **65**:826-851.

678 43. **Matz C, Nouri B, McCarter L, Martinez-Urtaza J.** 2011. Acquired type III secretion system determines environmental fitness of epidemic *Vibrio parahaemolyticus* in the interaction with bacterivorous protists. *PloS one* **6**:e20275.

681 44. **Nishibuchi M, Kaper JB.** 1985. Nucleotide sequence of the thermostable direct
682 hemolysin gene of *Vibrio parahaemolyticus*. *J Bacteriol* **162**:558-564.

683 45. **Baym M, Kryazhimskiy S, Lieberman TD, Chung H, Desai MM, Kishony R.** 2015.
684 Inexpensive multiplexed library preparation for megabase-sized genomes. *PloS one*
685 **10**:e0128036.

686 46. **Tritt A, Eisen JA, Facciotti MT, Darling AE.** 2012. A5. An integrated pipeline for *de*
687 *novo* assembly of microbial genomes. *PLoS ONE* **7**:e42304.

688 47. **Seemann T.** 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics*.
689 **30**:2068-9

690 48. **Inouye M, Conway TC, Zobel J, Holt KE.** 2012. Short read sequence typing (SRST):
691 multi-locus sequence types from short reads. *BMC Genomics* **13**:338.

692 49. **Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, Cuomo CA,**
693 **Zeng Q, Wortman J, Young SK.** 2014. Pilon: an integrated tool for comprehensive
694 microbial variant detection and genome assembly improvement. *PloS one* **9**:e112963.

695 50. **Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden**
696 **TL.** 2009. BLAST+: architecture and applications. *BMC Bioinformatics* **10**:421.

697 51. **Sullivan MJ, Petty NK, Beatson SA.** 2011. Easyfig: a genome comparison visualizer.
698 *Bioinformatics* **27**:1009-1010.

699 52. **Gardner SN, Slezak T, Hall BG.** 2015. kSNP3. 0: SNP detection and phylogenetic
700 analysis of genomes without genome alignment or reference genome. *Bioinformatics*
701 **31**:2877-8.

702 53. **Stamatakis A.** 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic
703 analyses with thousands of taxa and mixed models. *Bioinformatics* **22**:2688-2690.

704 54. **Darling AC, Mau B, Blattner FR, Perna NT.** 2004. Mauve: multiple alignment of
705 conserved genomic sequence with rearrangements. *Genome Res* **14**:1394-1403.

706 55. **Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MT, Fookes M,**
707 **Falush D, Keane JA, Parkhill J.** 2015. Roary: rapid large-scale prokaryote pan genome
708 analysis. *Bioinformatics* **31**:3691-3693.

709 56. **Sahl JW, Lemmer D, Travis J, Schupp J, Gillece J, Aziz M, Driebe E, Drees K,**
710 **Hicks N, Williamson C.** 2016. The Northern Arizona SNP Pipeline (NASP): accurate,
711 flexible, and rapid identification of SNPs in WGS datasets. *Microb Genom* **2**:e000074

712 57. **Whistler CA, Hall JA, Xu F, Ilyas S, Siwakoti P, Cooper VS, Jones SH.** 2015. Use of
713 Whole-Genome Phylogeny and Comparisons for Development of a Multiplex PCR Assay
714 To Identify Sequence Type 36 *Vibrio parahaemolyticus*. *J Clin Microbiol* **53**:1864-1872.

715 58. **Jolley KA, Chan M-S, Maiden MC.** 2004. mlstdbNet—distributed multi-locus sequence
716 typing (MLST) databases. *BMC Bioinformatics* **5**:86.

717 59. **Alikhan N-F, Petty NK, Zakour NLB, Beatson SA.** 2011. BLAST Ring Image
718 Generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics* **12**:402

719

720

721 Table 1: Clinical and environmental prevalence of emergent Northeast US *V. parahaemolyticus*
 722 lineages with associated virulence features.

Sequence type ^a	Northeast US States ^b		MLST Database ^c		Hemolysin genotype	VPaI type ^d
	Clinical	Environmental	Clinical	Environmental		
3	2	0	217	33	<i>tdh</i> ⁺	α
36	91	1	58	5	<i>tdh</i> ⁺ <i>trh</i> ⁺	γ
631	24	0	12	0	<i>tdh</i> ⁺ <i>trh</i> ⁺	γ
	1 ^e	2	0	0	<i>trh</i> ⁺	β
	0	1	0	0	neither	absent
43	5	0	17	4	<i>tdh</i> ⁺ <i>trh</i> ⁺	γ
636	4	0	2	0	<i>tdh</i> ⁺ <i>trh</i> ⁺	γ
1127	4	0	0	0	<i>trh</i> ⁺	β
110	3	0	0	1	<i>tdh</i> ⁺ <i>trh</i> ⁺	γ
34/324	2	2	4	19	<i>tdh</i> ⁺ <i>trh</i> ⁺	γ
674	0	4	1	20	<i>tdh</i> ⁺ <i>trh</i> ⁺	γ
	1	0	0	0	neither	absent
308	2	0	0	2	<i>tdh</i> ⁺ <i>trh</i> ⁺	γ
12	2	0	0	4	<i>trh</i> ⁺	β
162	2	0	1	1	neither	absent
194	2	0	1	0	neither	absent
809	2	0	0	1	<i>trh</i> ⁺	β
1716	2	0	0	0	<i>trh</i> ⁺	β
1123	1	1	0	0	<i>trh</i> ⁺	β
8	1	0	13	5	<i>trh</i> ⁺	β
23	1	0	0	3	<i>tdh</i> ⁺ <i>trh</i> ⁺	γ
749	1	0	1	0	<i>tdh</i> ⁺ <i>trh</i> ⁺	γ
1295	1	0	0	1	neither	absent
134	1	0	1	0	neither	absent
741	1	0	0	1	neither	absent
98	1	0	0	1	<i>trh</i> ⁺	β
1205	1	0	0	1	neither	absent
1561	1	0	0	0	neither	absent
1717	1	0	0	0	neither	absent
1725	1	0	0	0	<i>tdh</i> ⁺	α

723 ^a Some clinical isolates had insufficient sequencing coverage to determine sequence type and included
 724 eight *tdh*⁺*trh*⁺ isolates, one *tdh*⁺ isolate, four *trh*⁺ isolates, and 11 isolates without hemolysins, some of
 725 which were from wound infections. Two wound infection isolates lacking hemolysins were of known
 726 sequence types and are not listed above.

727 ^b Data generated from all available gastric infection clinical and environmental isolates four reporting
 728 Northeast US States including ME, NH, MA, and CT between 2010 and 2016.

729 ^c <http://pubmlst.org/vparahaemolyticus>, 2017 (36, 58)

730 ^d Presence of the VPaIy architecture was determined by PacBio genome sequencing of isolate MAVP-Q
 731 and MAVP-26, whereas for other isolates, identification of VPaI type was determined through illumina
 732 genome sequencing, PCR amplification and Sanger sequencing.

733 ^e This single isolate harbors a *recA* allele (allele 21) typical of ST631 fused to allele 107 through an
 734 insertion event, generating a hybrid allele previously described (33).

735

736

737 Figure 1. Schematic of a horizontally acquired insertion in the *recA*-encoding region of MAVP-R.
738 Sequences of the *recA* gene and flanking region from MAVP-Q (reference ST631 genome),
739 MAVP-R, Asia-derived isolates S130/S134 and Peru-derived isolate 090-96 were extracted and
740 aligned. Open reading frames designated with arrows and illustrated by representative colors to
741 highlight homologous and unique genes. The % similarity between homologs is illustrated by
742 grey bars.

743

744 Figure 2. Phylogenetic relationships of *V. parahaemolyticus* lineages and identification of
745 distinct ST631 clades. An ML phylogeny of representative *V. parahaemolyticus* genomes of
746 clinical isolates causing two or more infections was built on whole genome SNPs identified by
747 reference-free comparisons as described in the methods. The branch length represents the
748 number of nucleotide substitutions per site. Numbers at nodes represent percent bootstrap
749 support where unlabeled nodes had bootstraps of less than 70.

750

751 Figure 3. Minimum spanning tree relationships among clade I and clade II ST631. A cgMLST
752 core gene-by-gene analysis (excluding accessory genes) was performed and SNPs were
753 identified within different alleles. The numbers above the connected lines (not to scale) represent
754 SNP differences. The isolates are colored based on different hemolysin genotypes as labeled.

755

756 Figure 4. Comparisons of the pathogenicity islands containing hemolysins and T3SS2.
757 Sequences of VPaI were extracted from select genomes and aligned. VPaI α was derived from
758 ST3 strain RIMD2210633, VPaI γ was derived from ST631 clade II isolate MAVP-Q, and VPaI β
759 was derived from ST631 clade I isolate MAVP-R. ORFs are depicted in defined colors and

760 similarities ($\geq 75\%$) among ORFs are illustrated in grey blocks. Homologs between VPaI α and
761 VPaI β/γ (50–75% identity) are named and listed in Supplemental Table 2.

762

763 Figure 5. Distribution of VPaI γ in emergent pathogen lineages. The presence of *tdh*, *trh* and
764 VPaI γ along with positive control *tlh* was determined by PCR amplification using gene-specific
765 primers and visualized on a 1.2% agarose gel. The order from left to right is 1kb+ ladder, ST3
766 (MAVP-C), ST36 (MAVP-26), ST631 CII (clade II isolate MAVP-Q), ST631 CI (clade I
767 isolates MAVP-R and G149), ST43 (MAVP-71), ST636 (MAVP-50), ST1127 (MAVP-M),
768 ST110 (MAVP-46), ST34 (CTVP19C), ST324 (MAVP-14), and ST674 (CT4291, MAVP21).
769 The corresponding sizes of the ladder fragments are as labeled to the left and the identity of the
770 amplicons listed to the right of the gel image.

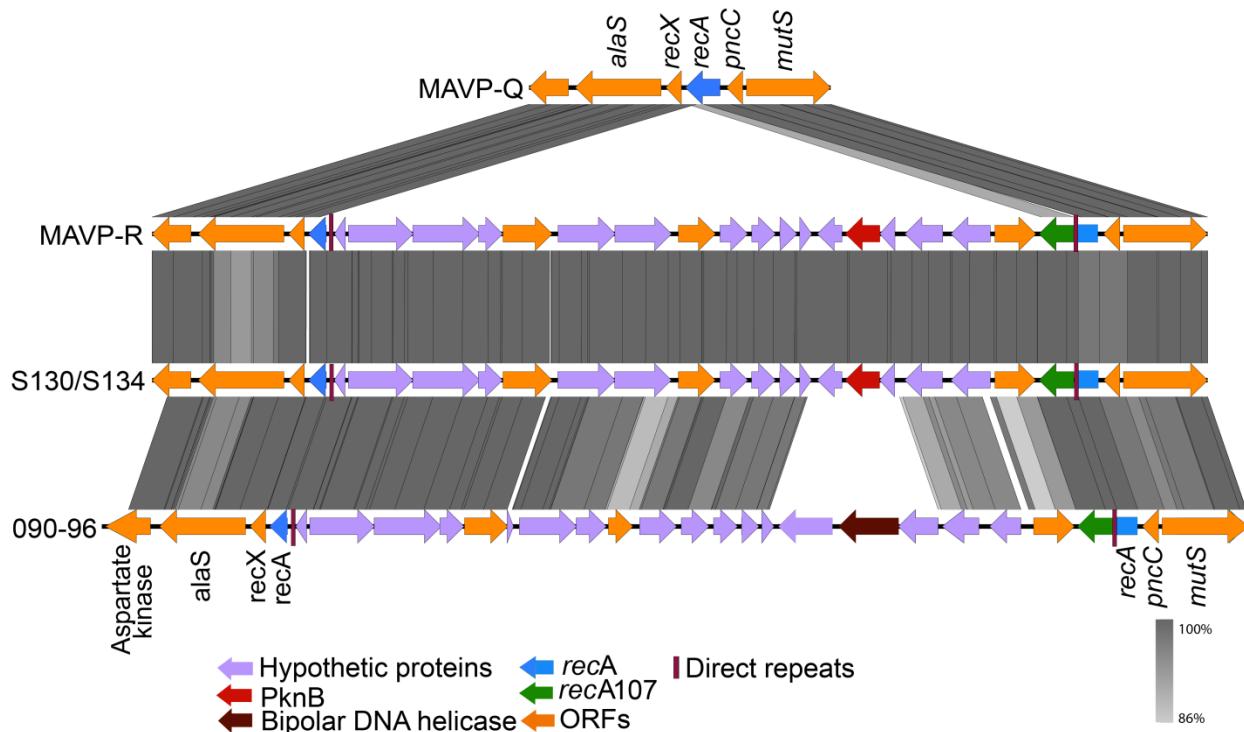


Figure 1. Schematic of a horizontally acquired insertion in the *recA*-encoding region of MAVP-R. Sequences of the *recA* gene and flanking region from MAVP-Q (reference ST631 genome), MAVP-R, Asia-derived isolates S130/S134 and Peru-derived isolate 090-96 were extracted and aligned. Open reading frames designated with arrows and illustrated by representative colors to highlight homologous and unique genes. The % similarity between homologs is illustrated by grey bars.

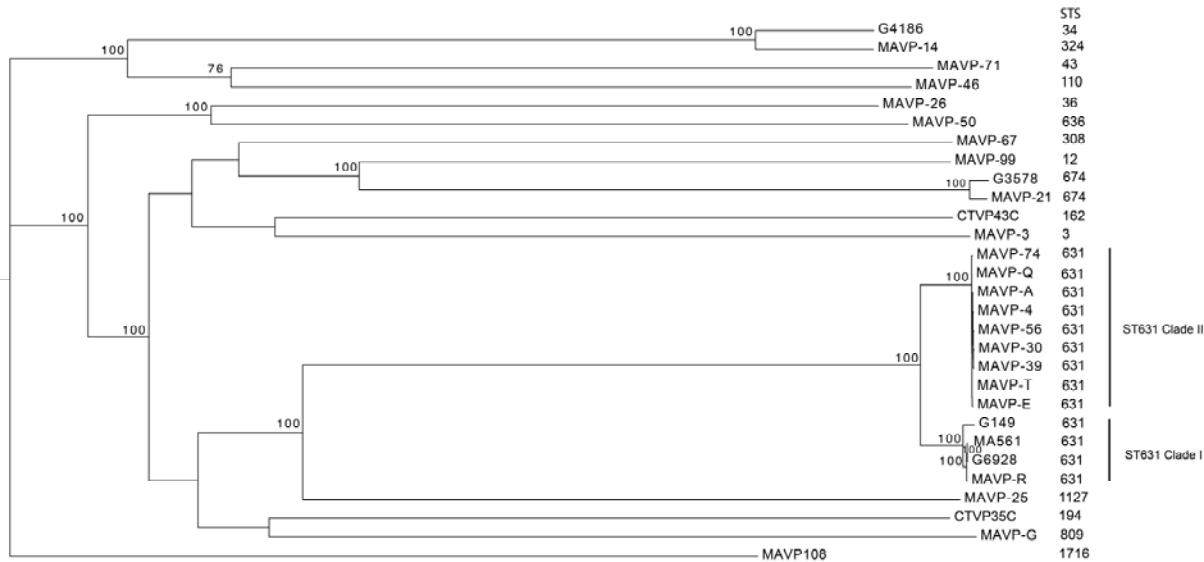


Figure 2. Phylogenetic relationships of *V. parahaemolyticus* lineages and identification of distinct ST631 clades. An ML phylogeny of representative *V. parahaemolyticus* genomes of clinical strains causing two or more infections was built on whole genome SNPs identified by reference-free comparisons as described in the methods. The branch length represents the number of nucleotide substitutions per site. Numbers at nodes represent percent bootstrap support where unlabeled nodes had bootstraps of less than 70.

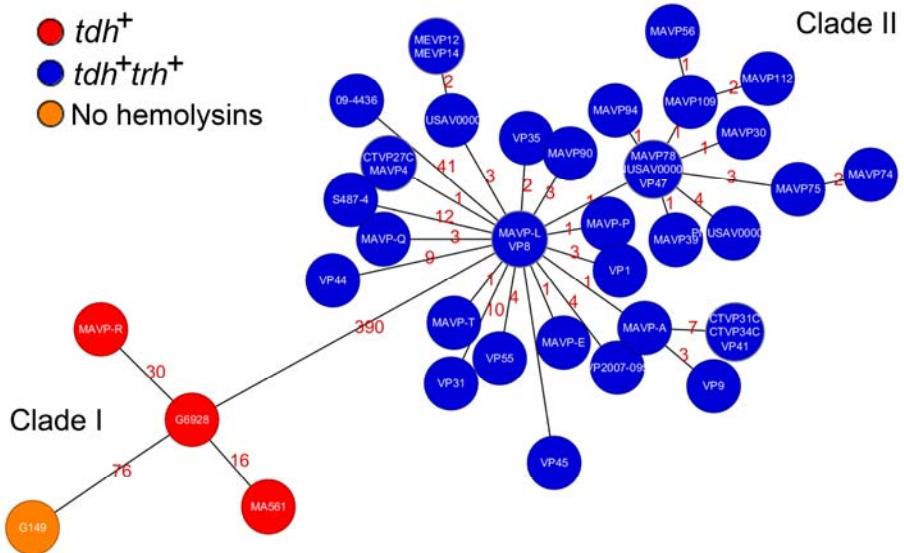


Figure 3. Minimum spanning tree relationships among clade I and clade II ST631. A core gene-by-gene analysis (excluding accessory genes) was performed and SNPs were identified within different alleles. The numbers above the connected lines (not to scale) represent SNP differences. The isolates are colored based on different hemolysin genotypes as labeled.

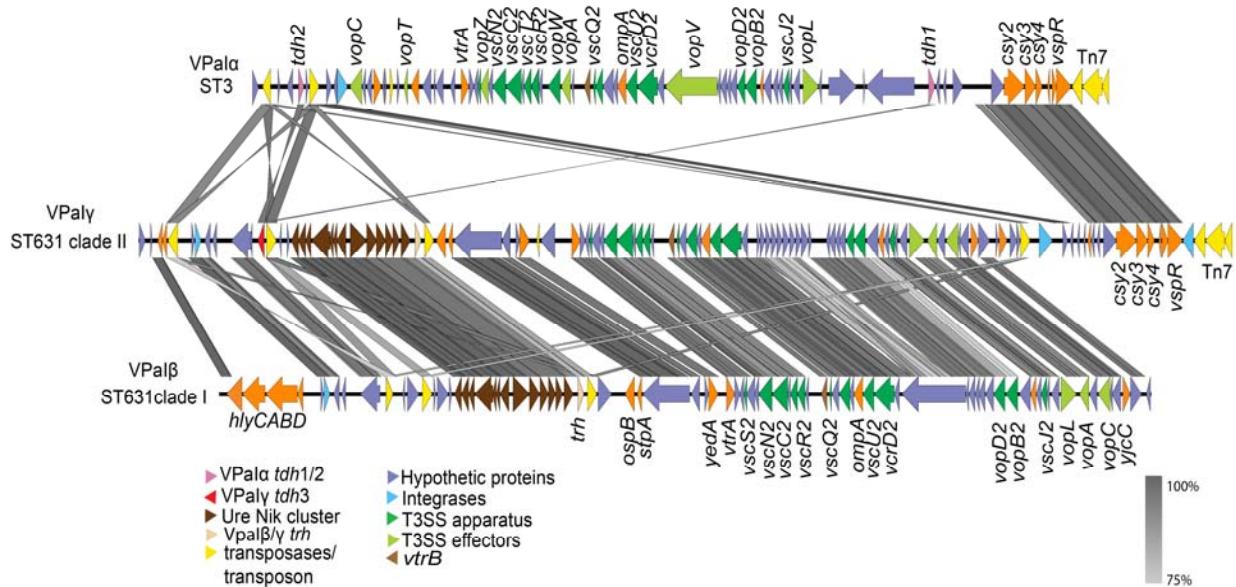


Figure 4. Comparisons of the pathogenicity islands containing hemolysins and T3SS2. Sequences of VPAl were extracted from select genomes and aligned. VPAl α was derived from ST3 strain RIMD2210633, VPAl γ was derived from ST631 clade II isolate MAVP-Q, and VPAl β was derived from ST631 clade I isolate MAVP-R. ORFs are depicted in defined colors and similarities ($\geq 75\%$) among ORFs are illustrated in grey blocks. omologs between VPAl α and VPAl β/γ ($50\% - 75\%$ identity) are named and listed in supplemental table 2.

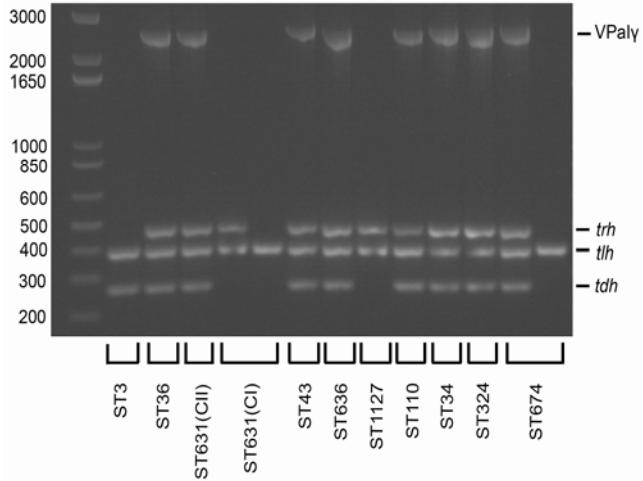


Figure 5. Distribution of VPaly in emergent pathogen lineages. The presence of *tdh*, *trh* and VPaly along with positive control *tlh* was determined by PCR amplification using gene-specific primers and visualized on a 1.2% agarose gel. The order from left to right is 1kb+ ladder, ST3 (MAVP-C), ST36 (MAVP-26), ST631 CII (clade II isolate MAVP-Q), ST631 CI (clade I isolates MAVP-R and G149), ST43 (MAVP-71), ST636 (MAVP-50), ST1127 (MAVP-M), ST110 (MAVP-46), ST34 (CTVP19C), ST324 (MAVP-14), and ST674 (CT4291, MAVP21). The corresponding sizes of the ladder fragments are as labeled to the left and the identity of the amplicons listed to the right of the gel image.