

1 **Genome-wide biases in the rate and molecular spectrum of spontaneous**
2 **mutations in *Vibrio cholerae* and *Vibrio fischeri***

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38 V.C. wrote the paper.

39

ABSTRACT

40 The vast diversity in nucleotide composition and architecture among bacterial
41 genomes may be partly explained by inherent biases in the rates and spectra of
42 spontaneous mutations. Bacterial genomes with multiple chromosomes are relatively
43 unusual but some are relevant to human health, none more so than the causative agent
44 of cholera, *Vibrio cholerae*. Here, we present the genome-wide mutation spectra in wild-
45 type and mismatch repair (MMR) defective backgrounds of two *Vibrio* species, the low-
46 GC% squid symbiont *V. fischeri* and the pathogen *V. cholerae*, collected under
47 conditions that greatly minimize the efficiency of natural selection. In apparent contrast
48 to their high diversity in nature, both wild-type *V. fischeri* and *V. cholerae* have among
49 the lowest rates for base-substitution mutations (bpsms) and insertion-deletion
50 mutations (indels) that have been measured, below 10^{-3} /genome/generation. *V. fischeri*
51 and *V. cholerae* have distinct mutation spectra, but both are AT-biased and produce a
52 surprising number of multi-nucleotide indels. Furthermore, the loss of a functional MMR
53 system caused the mutation spectra of these species to converge, implying that the
54 MMR system itself contributes to species-specific mutation patterns. Bpsm and indel
55 rates varied among genome regions, but do not explain the more rapid evolutionary
56 rates of genes on chromosome 2, which likely result from weaker purifying selection.
57 More generally, the very low mutation rates of *Vibrio* species correlate inversely with
58 their immense population sizes and suggest that selection may not only have
59 maximized replication fidelity but also optimized other polygenic traits relative to the
60 constraints of genetic drift.

61

INTRODUCTION

62 In 2000, the complete genome sequence of *Vibrio cholerae*, the first bacterial
63 genome with multiple chromosomes to be completed, was published in *Nature* to
64 acclaim (Heidelberg et al. 2000). Deemed a “treasure trove” for microbiology and
65 genomics researchers (Waldor and RayChaudhuri 2000), the *V. cholerae* genome
66 revealed several intriguing asymmetries between the chromosomes. The larger
67 chromosome 1 (chr1) is approximately 3 Mb in size and contains most essential genes
68 and the pathogenicity elements required to cause the disease cholera, whereas the
69 smaller chromosome 2 (chr2) is approximately 1 Mb and contains few essential genes
70 but many undefined genes acquired by horizontal transfer (Heidelberg et al. 2000;
71 Cooper et al. 2010). The plasmid-like origin of replication on chr2 implies that it
72 originated as a megaplasmid that became entrapped by the translocation of essential
73 genes, and the greater variation in gene content among strains on chr2 has led many to
74 speculate that the small chromosome somehow confers an evolutionary advantage in
75 varied environments, perhaps by being enriched for conditionally useful traits (Schoolnik
76 and Yildiz 2000). Indeed, this two-chromosome structure is found throughout the
77 *Vibrionaceae* family, suggesting an uncertain evolutionary advantage in their aquatic
78 habitats.

79 Subsequent studies of the evolution of *Vibrio* genomes have shown that not only
80 is chr2 more variable in its content, its conserved genes tend to evolve more rapidly
81 (Cooper et al. 2010). These elevated evolutionary rates likely stem from a gene dosage
82 bias between chr1 and chr2 that emerges from their replication timing. Specifically,
83 replication of chr2 occurs later than chr1, leading to synchronous termination of

84 replication of both chromosomes, even though chr1 is larger (Egan and Waldor 2003;
85 Duigou et al. 2006; Rasmussen et al. 2007). The earlier replication of two-thirds of chr1
86 generates a gene dosage bias that can be greatly compounded during rapid growth in
87 which multiple replication forks are active (Stokke et al. 2011). Consequently, genes
88 replicated early tend to be expressed more, experience greater purifying selection, and
89 hence evolve more slowly (Cooper et al. 2010; Morrow and Cooper 2012). Effects of
90 translocations between chromosomes also support this model: genes moving between
91 chromosomes tend to exhibit altered expression rates that match their new neighbors
92 (Morrow and Cooper 2012), and orthologs found on secondary chromosomes are
93 predisposed to more rapid evolution even when found in related genomes with a single
94 chromosome (Cooper et al. 2010).

95 While these comparative-genomic studies suggest that replication timing and its
96 effects on gene dosage may influence the strength of purifying selection (and hence the
97 fate of genetic variation), evidence also exists that replication timing positively
98 correlates, albeit weakly, with mutation rates (the origin of variation) in all domains of life
99 (Stamatoyannopoulos et al. 2009; Chen et al. 2010; Agier and Fischer 2012;
100 Martincorena et al. 2012). Mutation rates may be non-uniform because of greater rates
101 of damage, asymmetric nucleotide pools, structural differences affecting polymerase
102 fidelity, biases in gene expression or replication, or variation in the effectiveness of DNA
103 repair (Sharp et al. 1989; Zhang and Mathews 1995; Mira and Ochman 2002; Cooper et
104 al. 2010; Lee et al. 2012). If late-replicated genes experience both weaker purifying
105 selection and greater mutation rates relative to early-replicated genes, selection could

106 therefore act on genome organization to influence both the origin and fate of genetic
107 variation.

108 The goal of this study was to examine how mutation rates and spectra vary within
109 and among chromosomes based on existing models of replication timing in two species
110 of *Vibrio*, *V. cholerae* and *V. fischeri*. Both species are globally significant and are often
111 compared to one another because of their marine habitats and similar genome
112 structures, but contrasting roles in pathogenesis and symbiosis (Goldberg and Murphy
113 1983; Thompson et al. 2004; Ruby et al. 2005). We also evaluated how the mismatch
114 repair (MMR) system influenced the rates and spectra of spontaneous mutations in both
115 organisms by removing the *mutS* gene from each genome. Our approach used the well-
116 documented method of mutation accumulation (MA) paired with whole-genome
117 sequencing (WGS) (Behringer and Hall 2016). Using the MA-WGS method, a single
118 clonal ancestor is used to initiate many replicate lineages that are passaged through
119 hundreds of single cell bottlenecks before each being subjected to WGS. The
120 bottlenecking regime minimizes the efficiency of natural selection on mutations, and
121 when the genome sequences are compared, a nearly unbiased picture of the natural
122 mutation rates and spectra of the ancestor is revealed. A growing body of MA-WGS
123 studies in microbes have revealed some general properties of spontaneous mutation
124 (Lynch et al. 2008; Denver et al. 2009; Ossowski et al. 2010; Lee et al. 2012; Sung et al.
125 2012; Schrider et al. 2013; Heilbron et al. 2014; Long et al. 2014; Zhu et al. 2014; Dillon
126 et al. 2015; Long et al. 2015; Sung et al. 2015; Dettman et al. 2016), but unique
127 properties of the spontaneous mutation rates and spectra in many taxa have revealed

128 the value of conducting detailed MA-WGS studies in a more comprehensive collection
129 of species, particularly those with multiple chromosomes.

130 Here, we performed four detailed MA-WGS experiments on both wild-type and
131 MMR-deficient strains of *V. fischeri* and *V. cholerae*. We identified a total of 439 wild-
132 type mutations and 5990 $\Delta mutS$ mutations distributed across both chromosomes of *V.*
133 *fischeri* and *V. cholerae*, producing the first genome-wide perspective of the bpsm and
134 indel biases in wild-type and MMR-deficient strains of these two significant bacterial
135 species. Mutation rates were non-uniform among genome regions and varied in
136 patterns that appear to be associated with replication timing. Both genomes also
137 exhibited distinct base-substitution mutation spectra that correlated with their different
138 %GC content and also experienced a surprising number of multi-nucleotide indel
139 mutations. Remarkably, the loss of MMR caused both mutation spectra to converge
140 towards similar patterns dominated by transition mutations and small indels, which
141 suggests that variation in the MMR system may be partly responsible for producing
142 differences in genome composition.

143

144

RESULTS

145 The two ancestral genomes for this study share the two-chromosome
146 architecture common to *Vibrio* but differ in other important aspects. The genome of *V.*
147 *fischeri* strain ES114, which was completely sequenced previously, harbors two
148 chromosomes of 2.90 Mb and 1.33 Mb that differ slightly in their %GC content (38.96%
149 for chr1 and 37.02% for chr2). In addition, *V. fischeri* ES114 has a 45.85 Kb plasmid
150 with a %GC content of 38.44% (Ruby et al. 2005). The genome of *V. cholerae* strain

151 2740-80, which was previously deposited as a draft assembly, was closed and polished
152 for this study using a combination of PacBio sequencing, Illumina sequencing, and
153 consensus assemblies (see Methods). This *V. cholerae* genome includes a 2.99 Mb
154 primary chromosome (chr1) and a 1.10 Mb secondary chromosome (chr2), with %GC
155 contents of 47.85% and 46.83%, respectively. Four MA experiments were conducted
156 with these strains using daily single-cell bottlenecks produced by isolation-streaking on
157 agar plates, which limits the efficiency of natural selection to purge deleterious and
158 enrich beneficial mutations. For the two wild-type (wt) experiments, *V. fischeri* ES114
159 (*Vf*-wt) and *V. cholerae* 2740-80 (*Vc*-wt) colonies were used to found 75 MA lineages,
160 each of which was propagated for 217 days. For the two mutator (mut) experiments, *V.*
161 *fischeri* ES114 (*Vf*-mut) and *V. cholerae* 2740-80 (*Vc*-mut) strains lacking a *mutS* gene
162 were used to found 48 MA lineages, each of which was propagated for 43 days. The
163 properties of each of these experiments, the observed mutation spectra, and the lack of
164 genetic parallelism suggest that few of these mutations were subject to the biases of
165 natural selection (Supplemental Text). Summary parameters of each MA experiment,
166 including the numbers of mutations identified in all final isolates, generations of growth,
167 and overall mutation rates are summarized in Table 1. Owing to the fitness cost of
168 increasing mutational load, generations of growth per day declined over the course of
169 each MA experiment, particularly in the mutator lineages (Figure S1).

170

171 **Wild-type base-substitution mutation rates and spectra.** The wild-type base-
172 substitution mutation rates for these *Vibrio* species are among the lowest per-generation
173 rates that have been observed in any organism (Figure S2). The bpsm rate for *V.*

174 *fischeri* is $2.07 (0.207) \cdot 10^{-10}$ /bp/generation (SEM), which is approximately twice the
175 rate of bpsm in *V. cholerae*, $1.07 (0.094) \cdot 10^{-10}$ /bp/generation (SEM). These per-site
176 bpsm rates correspond to genome-wide bpsm rates of 0.0009 /genome/generation for
177 *V. fischeri* and 0.0004 /genome/generation for *V. cholerae*. (Table 1). Bpsm rates differ
178 somewhat between the chromosomes of *V. fischeri*, with chr2 experiencing significantly
179 greater rates than chr1 ($\chi^2 = 4.799$, d.f. = 1, $p = 0.028$), but no difference was found
180 between the chromosomes of *V. cholerae* ($\chi^2 = 0.556$, d.f. = 1, $p = 0.456$) (Figure 1A).
181 The increased bpsm rate on chr2 of *V. fischeri* cannot be explained by the relative
182 nucleotide contents of the chromosomes (%GC: chr1, 39.0%; chr2, 37.0%). In fact,
183 when we account for AT-biased mutation in *V. fischeri*, the difference between the
184 expected and observed bpsms between the two chromosomes increased ($\chi^2 = 5.747$,
185 d.f. = 1, $p = 0.017$). Correcting for AT-biased mutation in *V. cholerae* (%GC: chr1,
186 47.9%; chr2, 46.8%) revealed no significant difference between the bpsm rates of chr1
187 and chr2 ($\chi^2 = 0.123$, d.f. = 1, $p = 0.726$).

188 Base-substitution mutation spectra in both *V. fischeri* and *V. cholerae* were
189 significantly AT-biased (*Vf*: $\chi^2 = 108.090$, d.f. = 1, $p < 0.0001$; *Vc*: $\chi^2 = 28.744$, d.f. = 1,
190 $p < 0.0001$) (Figure 1C). Interestingly, the AT-bias is stronger in *V. fischeri*, which has
191 the lower genome wide %GC-content. However, consistent with previous MA studies of
192 bacteria (Lind and Andersson 2008; Lee et al. 2012; Sung et al. 2012; Dillon et al. 2015;
193 Sung et al. 2015; Dettman et al. 2016), AT-mutation bias alone fails to explain realized
194 genome-wide %GC-contents. For *V. fischeri*, the expected %GC-content under
195 mutation-drift equilibrium is 0.202 ± 0.034 (SEM), 0.182 lower than the genome-wide
196 %GC-content (0.384). This AT-bias is generated by high relative bpsm rates of both

197 G:C > A:T transitions and G:C > T:A transversions, but it is the relative G:C > T:A
198 transversion rate that is especially high in comparison to MA studies in other species.
199 With a rate of $9.19 \cdot 10^{-9}$ /bp/generation, G:C > T:A transversions occur at a higher rate
200 than any other bpsm, generating a transition/transversion ratio (T_S/T_V) of 0.851.
201 Similarly, the expected %GC content of *V. cholerae* under mutation-drift equilibrium is
202 0.281 ± 0.044 (SEM), 0.195 lower than the genome-wide %GC content (0.476).
203 However, in *V. cholerae*, the AT-bias is generated mostly by G:C > A:T transitions
204 rather than G:C > T:A transversions, resulting in a T_S/T_V of 1.59.

205 To test whether the bpsm spectra on chr1 were significantly different from those
206 on chr2, we used the conditional bpsm rates (corrected for %GC-content) of chr1 to
207 generate expected ratios for each bpsm and compared these with the observed bpsm
208 spectra on chr2 (Figure 1C). Neither the overall bpsm spectra of *V. fischeri* or *V.*
209 *cholerae* varied significantly between chromosomes (*Vf*: $\chi^2 = 7.797$, d.f. = 5, $p = 0.168$;
210 *Vc*: $\chi^2 = 6.516$, d.f. = 5, $p = 0.259$). However, the G:C > T:A transversion rate was
211 significantly higher on chr2 of *V. fischeri* (Welch's two tailed t-test; $t = 2.348$, $df =$
212 71.952 , $p = 0.022$) and the A:T > G:C transition rate was significantly lower on chr2 of *V.*
213 *cholerae* (Welch's two tailed t-test; $t = -2.155$, $df = 95.749$, $p = 0.034$) (Figure 1C).
214 Interestingly, late replicating regions of chr1 in *V. fischeri* (the terminal 1,330,333 bp,
215 equal to the size of chr2) also had elevated G:C > T:A transversion rates, and late
216 replicating regions of chr1 in *V. cholerae* (terminal 1,101,931 bp, equal to the size of
217 chr2) had reduced A:T > G:C transition rates relative to early replicating regions, though
218 neither of these intra-chromosomal differences were significant (Welch's two tailed t-
219 test; *Vf* G:C>T:A T_V : $t = 1.789$, $df = 81.645$, $p = 0.077$; *Vc* A:T > G:C T_S : $t = -0.720$, $df =$

220 95.489, $p = 0.473$). Bpsm rates of other mutation types in late replicating regions of chr1
221 do not conform to those on chr2 (Table S1), suggesting that not all bpsms are
222 influenced by replication timing.

223
224 **Wild-type insertion-deletion mutation rates.** Indels occurred at approximately one-
225 fifth the rate of bpsm and occurred predominantly in simple sequence repeats (SSRs).
226 Cumulative indel mutation rates for *V. fischeri* and *V. cholerae* were $5.68 (0.691) \cdot 10^{-11}$
227 /bp/generation and $1.71 (0.337) \cdot 10^{-11}$ /bp/generation (SEM), respectively (Table 1).
228 These rates correspond to genome-wide indel rates of 0.0002 /genome/generation for
229 *V. fischeri* and 0.00007 /genome/generation for *V. cholerae* (Table 1). The indel spectra
230 of both species were biased towards deletions, with deletions occurring at
231 approximately twice the rate of insertions in *V. fischeri* ($\chi^2 = 4.267$, d.f. = 1, $p = 0.039$),
232 and three times the rate of insertions in *V. cholerae* ($\chi^2 = 6.546$, d.f. = 1, $p = 0.011$)
233 (Figure 1). Indels were also significantly more frequent on chr1 in *V. fischeri* ($\chi^2 = 9.066$,
234 d.f. = 1, $p = 0.003$) and slightly more common on chr1 in *V. cholerae* ($\chi^2 = 0.123$, d.f. =
235 1, $p = 0.726$), though not significantly so (Figure 1B).

236 In contrast with previous reports from bacterial MA-WGS studies, we find many
237 multi-nucleotide indels in both the *Vf*-wt and *Vc*-wt MA experiments. In *Vf*-wt, only
238 20.00% of indels involved the insertion or deletion of a single nucleotide, while 36.36%
239 of the *Vc*-wt indels involved a single nucleotide. Furthermore, the distribution of the
240 number of indels identified for each length below 10-bps demonstrates that short multi-
241 nucleotide indels were relatively common, particularly in *V. fischeri* (Figure 2).

242 More strikingly, in *V. fischeri*, the indel rates of SSRs scaled positively with the
243 repeat unit length and the number of repeated units. 41 of the 60 indels (68.33%)
244 observed in *V. fischeri* occurred in SSRs containing three or more repeated units, which
245 is significantly more than we expect based on the frequency of bases in SSRs in the *V.*
246 *fischeri* genome ($\chi^2 = 82.915$, d.f. = 1, $p < 0.0001$). When SSRs are categorized by their
247 repeat unit length (mono-, di-, tri-nucleotide, etc.), SSRs with longer repeat units
248 experience elevated mutation rates (Figure 3). Importantly, these rates vary over orders
249 of magnitude and their observed frequencies differ significantly from the null expectation
250 derived from their target sizes ($\chi^2 = 2.120 \cdot 10^5$, d.f. = 8, $p < 0.0001$). SSR indel rates
251 also scale positively with the number of repeats in the SSR and again differ significantly
252 from the null expectation derived from their genome target (Chi-square test, repeat
253 numbers 3-10; $\chi^2 = 5.590 \cdot 10^2$, d.f. = 7, $p < 0.0001$). Lastly, a few SSRs appear to be
254 especially mutagenic because the same locus mutated independently in multiple
255 lineages (Supplementary Dataset 2). We cannot ascertain whether similar SSR biases
256 exist in *V. cholerae* because only 22 indels were observed and only 8 of those were in
257 SSRs with three or more repeats (36.36%). However, the elevated occurrence of indels
258 in SSRs in *V. cholerae* is consistent with our observations in *V. fischeri* ($\chi^2 = 4.547$, d.f.
259 = 1, $p = 0.033$).

260

261 **Effects of losing DNA mismatch repair.** The deletion of the *mutS* gene results in a
262 faulty MMR system and is expected to increase the rates and alter the spectra of bpsms
263 and indels. In *V. fischeri*, $\Delta mutS$ produced a 317-fold increase in the bpsm rate and a
264 102-fold increase in the indel rate (Table 1). This $\Delta mutS$ mutation also eliminated

265 differences between chromosomes in both bpsm and indel rates observed in the *Vf*-wt
266 lineages (bpsm: $\chi^2 = 0.109$, d.f. = 1, $p = 0.741$; indels: $\chi^2 = 2.076$, d.f. = 1, $p = 0.150$). In
267 *V. cholerae*, the *mutS* deletion produced an 85-fold increase in the bpsm rate and a
268 142-fold increase in the indel rate (Table 1). However, in this case, slightly more bpsm
269 occurred on chr1 in *Vc-mut* lineages than expected (bpsm: $\chi^2 = 4.540$, d.f. = 1, $p =$
270 0.0331 ; indels: $\chi^2 = 0.041$, d.f. = 1, $p = 0.840$).

271 Losing functional mismatch repair caused the bpsm spectra of both *V. fischeri*
272 and *V. cholerae* to change dramatically and converge (Figure 4). Despite the
273 significantly different bpsm spectra between wild-type species ($\chi^2 = 32.788$, d.f. = 5, $p <$
274 0.0001), the loss of *mutS* caused *Vf*-mut and *Vc*-mut lineages to accumulate a
275 remarkably similar spectrum of bpsms ($\chi^2 = 10.178$, d.f. = 5, $p = 0.070$). Specifically,
276 both sets of mutator lines were dominated by A:T > G:C and G:C > T:A transitions,
277 which would yield significantly more %GC-rich genomes of 0.482 ± 0.016 (SEM) in *Vf*-
278 mut and 0.475 ± 0.011 (SEM) in *Vc*-mut at mutation-drift equilibrium. In addition, while
279 *V. fischeri* continues to have a slight deletion bias with defective MMR, insertions
280 actually occur at a slightly higher rate than deletions in the *Vc*-mut lineages, although
281 not significantly so (*Vf*-mut: $\chi^2 = 6545$, d.f. = 1, $p = 0.011$; *Vc*-mut: $\chi^2 = 0.297$, d.f. = 1, p
282 $= 0.586$). Lastly, as with the wild-type lineages, the bpsm spectra do not vary
283 significantly between chromosomes for either MMR deficient species (*Vf*-mut: $\chi^2 =$
284 8.929 , d.f. = 1, $p = 0.112$; *Vc*-mut: $\chi^2 = 5.239$, d.f. = 1, $p = 0.387$).

285 While multi-nucleotide indels were relatively common in the wild-type MA lines,
286 the vast majority of indels involved a single nucleotide in both the *Vf*-mut (93.62%) and
287 *Vc*-mut (85.66%) lines (Figure 2). The *mutS* deletion increased the single-nucleotide

288 indel rate 473-fold but the multi-nucleotide indel rate only 13-fold in *V. fischeri*. Similar
289 patterns were seen in *V. cholerae*, where $\Delta mutS$ increased the single-nucleotide indel
290 rate 334-fold and the multi-nucleotide indel rate only 64-fold. Most of the increase in the
291 multi-nucleotide mutation rate resulted from di- and tri-nucleotide indels that were rarely
292 observed in the wild-type MA experiments (Figure 2). Yet all indels detected in the
293 mutator MA experiments up to 10-bps in length were also significantly over-represented
294 in mutator MA experiments (Table S2), implying that *mutS* and the MMR system in
295 general are involved in the repair of all indel mutations up to 10-bps in length.

296 Most single-nucleotide indels in *Vf*-mut and *Vc*-mut lines occurred in
297 homopolymeric runs. The single-nucleotide indel mutation rate in both *Vf*-mut and *Vc*-
298 mut correlates positively with the length of the homopolymer (Figure 5) and differs
299 significantly from the null expectation derived from the genome target size (Chi-square
300 test, repeat numbers 3-11; *Vf*-mut: $\chi^2 = 3.189 \times 10^4$, d.f. = 8, $p < 0.0001$; *Vc*-mut: $\chi^2 =$
301 6.850×10^4 , d.f. = 8, $p < 0.0001$). Because indels in SSRs with longer repeated units
302 were rarely observed in the mutator lineages, we cannot confirm whether the length of
303 the repeated unit in an SSR also correlates positively with its indel rate in either of the
304 mutator lineages.

305
306 **Genomic distribution of spontaneous mutations.** To this point we have only
307 discussed inter-chromosomal differences in mutation rates between two autonomously
308 replicating chromosomes. However, the distribution of bpsms and indels in *V. fischeri*
309 and *V. cholerae* may also vary among regions within these circular chromosomes. We
310 analyzed the genome-wide distribution of bpsms by dividing each chromosome into 100

311 kb intervals extending bi-directionally from the origin of replication. Despite apparent
312 intra-chromosome variation in the bpsm rate of both wild-type ancestors (Figure 6A,B),
313 the observed number of bpsms in 100kb intervals does not differ significantly from
314 random expectations based on the number of analyzed sites (*Vf*-wt Chr1: $\chi^2 = 31.254$,
315 d.f. = 29, $p = 0.354$; *Vf*-wt Chr2: $\chi^2 = 17.185$, d.f. = 15, $p = 0.308$; *Vc*-wt Chr1: $\chi^2 =$
316 29.607 , d.f. = 29, $p = 0.434$; *Vc*-wt Chr2: $\chi^2 = 12.825$, d.f. = 11, $p = 0.305$). However,
317 bpsms were not distributed evenly on chr1 in either of the mutator experiments (*Vf*-mut:
318 $\chi^2 = 132.970$, d.f. = 29, $p < 0.0001$; *Vc*-mut: $\chi^2 = 102.420$, d.f. = 29, $p < 0.0001$),
319 resulting in a mirrored wave-like pattern of bpsm rates on the right and left replichores
320 (Figure 6A,B). Indeed, we find a significant positive relationship between the bpsm rates
321 on the right replichore and concurrently replicated regions on the left replichore in both
322 genomes (Linear regression; *Vf*-mut: $F = 10.98$, $df = 13$, $p = 0.0060$, $r^2 = 0.46$; *Vc*-mut: F
323 $= 6.76$, $df = 13$, $p = 0.0221$, $r^2 = 0.34$). In contrast to chr1, the observed distribution of
324 bpsms on chr2 of the *V. fischeri* and *V. cholerae* mutator lineages does not differ from a
325 null, constant rate among regions (*Vf*-mut: $\chi^2 = 17.459$, d.f. = 15, $p = 0.292$; *Vc*-mut: χ^2
326 $= 10.984$, d.f. = 11, $p = 0.445$), suggesting that bpsm rates are more consistent across
327 chr2 than they are on chr1 (Figure 6A, B).

328 Indels occur predominantly in SSRs, which are not uniformly distributed across
329 the genome, and are thus also expected to vary significantly between genome regions.
330 This variation was seen in all experiments except *Vc*-wt, likely because only 22 indels
331 were detected in this experiment (Figure 6 C,D) (*Vf*-wt: $\chi^2 = 83.079$, d.f. = 43, $p <$
332 0.0001 ; *Vc*-wt: $\chi^2 = 42.224$, d.f. = 41, $p = 0.418$; *Vf*-mut: $\chi^2 = 96.383$, d.f. = 43, $p <$
333 0.0001 ; *Vc*-mut: $\chi^2 = 157.240$, d.f. = 41, $p < 0.0001$). However, indel rates were not

334 conserved on opposing replichores of either chromosome and were rather dispersed
335 throughout the genomes.

336

337

DISCUSSION

338 **Causes and effects of high genetic diversity within *Vibrio***

339 The unique and broadly significant biology of both *V. cholerae* and *V. fischeri*
340 motivated this comprehensive study of their mutational processes in the near-absence
341 of natural selection. *V. cholerae* is one of the most significant human pathogens,
342 infecting 3-5 million people and causing approximately 100,000 deaths annually from
343 side effects of the profuse diarrhea caused by toxigenic strains (Lozano et al Lancet
344 2012). *V. cholerae* also lives in a broad range of aquatic habitats associated with
345 zooplankton and is often highly abundant, demonstrating that only a small number of
346 strains are capable of causing cholera. This ecological diversity is borne out in the
347 genetic diversity seen in sequenced genomes, namely a high nucleotide diversity at
348 silent sites ($\pi_s = 0.110$) (Sung et al. 2016). This nucleotide diversity implies that the
349 effective population size (N_E) of this species is $\approx 4.78 \times 10^8$ individuals.

350 *V. fischeri*, in apparent contrast, is well known as a beneficial symbiont of sepiolid
351 squid and monocentrid fishes, in which it produces light that enables the host to evade
352 predators by counter-illumination (Soto et al. 2014). The hosts it colonizes are
353 distributed in different oceans around the world, but it can also be found as a free-living
354 member of the ocean bacterioplankton (Soto et al. 2014). Here also the ecological
355 diversity is borne out by high genetic diversity at a number of loci, with estimated $\pi_s =$
356 0.067 and an inferred N_E of $\approx 1.62 \times 10^8$ (Wollenberg and Ruby 2012). For both species,

357 and for the *Vibrionaceae* more generally, what forces lead to such high genetic diversity
358 within species?

359 Both mutation and horizontal-gene transfer (HGT) are expected to contribute to
360 *Vibrio* biodiversity, but evidence exists that mutation is the primary force driving
361 diversification within *Vibrio* clades (Thompson et al. 2004; Sawabe et al. 2009; Vos and
362 Didelot 2009). It has therefore been tempting to invoke high mutation rates in *Vibrio*
363 species to explain their high genetic diversity. However, we show here that both bpsm
364 and indel rates in *V. fischeri* and *V. cholerae* are low, even for bacteria (Figure S2). In
365 fact, *V. cholerae* has one of the lowest recorded genome-wide rates of bpsms (0.0004
366 /genome/generation) and indels (0.00007 /genome/generation) of any bacterial species
367 (Sung et al. 2016). We suggest that the high genetic diversity and low mutation rates in
368 these *Vibrio* species can be reconciled by the drift-barrier hypothesis, which states
369 generally that any trait, including replication fidelity, may be refined by natural selection
370 only to the point at which further improvement becomes overwhelmed by the power of
371 genetic drift (Lynch 2010; Lynch 2011; Sung et al. 2012; Sung et al. 2016). Natural
372 selection is most powerful in large populations of organisms with genomes composed of
373 a high amount of coding sequence. Although *Vibrio* genomes are not exceptionally
374 large, most sites are coding and their effective population sizes are among the highest
375 recorded (Wollenberg and Ruby 2012; Sung et al. 2016). Thus, both high amounts of
376 coding sequence and high effective population size increase the ability of natural
377 selection to reduce both bpsm and indel rates (Lynch 2010; Lynch 2011; Sung et al.
378 2012), yet yield enormous allelic diversity at any given time in both of these *Vibrio*
379 species (Thompson et al. 2004; Vos and Didelot 2009).

380 If the extremely low mutation rates of these *Vibrio* species are a product of
381 powerful selection enabled by massive populations to refine the machinery of DNA
382 replication, it follows that many other heritable traits may have also been optimized in
383 these organisms. One of the best known *Vibrio* traits is their extremely rapid growth
384 rates, with several species achieving doubling times of 10-15 minutes (Brenner et al.
385 2005). Growth rate is a broad polygenic character requiring the coordination of
386 essentially all systems, including ribosome synthesis, catabolic and anabolic pathways,
387 and of course, replication. *Vibrio* species are also typified by having genomes with two
388 chromosomes, which provide two origins of replication that could conceivably accelerate
389 DNA replication. This genome architecture has clearly been refined by selection for
390 rapid growth: not only does the experimental reduction of the *V. cholerae* genome from
391 two chromosomes to one decrease growth rates by 25-40% (Val et al. 2012), the early-
392 replicating chr1 is enriched relative to chr2 in genes required for rapid growth relative
393 (Cooper et al. 2010). Other *Vibrio* traits (metabolism, regulatory capacity in fluctuating
394 environments, stress tolerance) in addition to their high-fidelity replication and rapid
395 growth may also conceivably have been exceptionally refined by selection. Put more
396 broadly, the drift-barrier hypothesis may be extended and applied to many traits,
397 rendering the prediction that DNA replication fidelity and the optimality of many other
398 phenotypes may be positively correlated – the lower the mutation rate, the more
399 selectively optimized the polygenic trait.

400

401 **Mutation rate and spectra variation among genome regions**

402 This hypothesis that *Vibrio* replication has been exquisitely optimized relative to
403 other bacterial genomes because of their high coding content and high effective
404 population sizes does not mean that all regions of *Vibrio* genomes have been
405 equivalently refined relative to one another. Both bpsm and indel rates and spectra
406 varied among genomic regions of *V. fischeri* and *V. cholerae*, which may systematically
407 affect genome composition. In the *Vf*-wt lines, both G:C > A:T transitions and G:C > T:A
408 transversions occurred at higher rates on chr2, although only the rate of G:C > T:A
409 transversions was significantly higher (Figure 1C). Using experiment-wide estimates of
410 each conditional bpsm rate in *V. fischeri*, we estimate that the %GC-content should be
411 0.184 in the absence of selection and recombination. Yet, the %GC-content of chr2 is
412 expected to be 0.156 at mutation-drift equilibrium, 0.047 lower than expectations for
413 chr1 (0.203). The actual %GC content of chr2 of the *V. fischeri* ES114 genome is also
414 lower than chr1 (chr1: 0.390; chr2: 0.370) suggesting that bpsm biases on these
415 chromosomes have contributed to this pattern. Even stronger biases differentiating the
416 chromosomes are seen in the *Vc*-wt lines, driven by significantly higher A:T > G:C
417 transition rates on chr1 and by non-significant increases in G:C > A:T and G:C > T:A
418 rates on chr2 (Figure 1C). These spectra predict %GC-contents of 0.293 for chr1 and
419 0.201 for chr2 at mutation-drift equilibrium and likely contribute to the lower realized
420 %GC content of chr2 in *V. cholerae* 2740-80 (chr1: 0.479; chr2: 0.468). Overall, these
421 findings suggest that bpsm pressures contribute to genome-wide and intra-genome
422 variation in %GC contents, but indel biases (Dillon et al. 2015), selection (Hershberg
423 and Petrov 2010; Hildebrand et al. 2010), and/or biased gene conversion (Hershberg

424 and Petrov 2010; Lassalle et al. 2015) must also contribute to the realized %GC
425 contents in *V. fischeri* and *V. cholerae*.

426 Prior MA studies have reported that the majority of indels involve the loss or gain
427 of a single nucleotide and occur predominantly in simple-sequence repeats (SSRs),
428 where the number of repeated units scales positively with the indel rate (Xu et al. 2000;
429 Lee et al. 2012; Long et al. 2014; Dettman et al. 2016). These SSRs have gained
430 attention not only because they vary sufficiently in repeat number among strains to
431 enable rapid genotyping (van Belkum et al. 1998; Danin-Poleg et al. 2007; Ghosh et al.
432 2008), but also because in some species they associate with variable heritable
433 expression of genes related to host colonization and disease (Moxon et al. 1994; Field
434 et al. 1999; Moxon et al. 2006), begging the question of whether these mutation-prone
435 sequences have indirectly evolved to enable this plasticity.

436 In *V. fischeri*, the insertion-deletion rates in SSRs correlated positively with both
437 the number of repeated units and the length of the repeated unit (Figure 2). While the
438 former bias is consistent with a number of previous studies (Xu et al. 2000; Lee et al.
439 2012; Long et al. 2014; Dettman et al. 2016), the positive correlation between the length
440 of the repeated unit in an SSR and its indel rate has not been reported in previous MA-
441 WGS experiments. One possible reason for this discrepancy might be an increased
442 occurrence of SSRs with longer repeats in the *V. fischeri* ES114 genome (Ruby et al.
443 2005), which we find to be highly mutagenic (Figure 3). There are 100 SSRs of three or
444 more units in the *V. fischeri* ES114 genome where the repeated unit is at least 4-bps in
445 length. A second possibility is that larger indels have gone undetected by prior MA-
446 WGS analyses focused on MMR-deficient strains, in which single-nucleotide indels are

447 evidently more common (Supplementary Dataset 2). Further, longer indels, especially
448 those in SSRs, are subject to increased false-negative rates due to limitations in the
449 ability of short-read sequencing to resolve them. The majority of multi-nucleotide indels
450 that we identified were supported with very low consensus in the initial alignments
451 because of reads that only partly covered the SSR. Only when we filtered out reads that
452 were not anchored by bps on both sides of the SSR did we achieve high consensus for
453 these indels (Supplementary Dataset 2). It will be interesting to apply these sensitive
454 detection methods for long SSR-associated indels to future experiments to see whether
455 other species also experience elevated indel rates in SSRs with longer repeat units. We
456 emphasize that this experiment also demonstrates that the loss of MMR shifts the
457 spectrum of indel mutations from multi-nucleotide indels in SSRs with longer repeated
458 units towards single nucleotides in homopolymeric runs, a shift with potentially broad
459 phenotypic consequences.

460 Our study adds to the theory that SSRs may generate localized hyper-mutation in
461 coding regions that may serve as “contingency loci,” enabling potentially beneficial
462 population-level variation in both function and expression of the affected genes (van
463 Belkum et al. 1998; Moxon et al. 2006). These experiments, particularly those
464 conducted in a $\Delta mutS$ background, identified clusters of frameshift mutations found
465 within genes that could yield adaptive mutant phenotypes in *Vibrio* populations (Table
466 2). Some in fact have previously been reported to be hypermutable in experimental
467 studies of bacterial function. In *V. cholerae*, *tcpH* is an important co-regulator that links
468 temperature and pH to control expression of the major virulence regulon (Carroll et al.
469 1997). During experimental infections of the rabbit model, *tcpH* frameshift mutations

470 occurred at a frequency of 10^{-4} owing to mutations in the same poly-G tract that
471 accumulated in our experiment and they enhanced fitness in culture, which the authors
472 speculated could reflect a benefit during dispersal from the host into the environment
473 (Carroll et al. 1997). Another locus, *mshQ*, encodes a pilus-associated adhesion
474 protein, in which mutants have reported to contribute to the rapid transition between
475 opaque and translucent colonies in *Vibrio* species (Enos-Berlage et al. 2005). These
476 colony types associate with different dynamics of biofilm formation in the laboratory and
477 may reflect different capacity for attachment to natural substrates or other cells,
478 facilitating biofilm differentiation (Yildiz and Visick 2009). Yet another mutagenic
479 homopolymeric tract potentially generating population heterogeneity in biofilm
480 production was found in *vpsT* in *V. fischeri*, a transcriptional activator that regulates
481 polysaccharide production (Yildiz and Visick 2009). Other hypermutable
482 mononucleotide tracts were observed in 14 other loci in the *V. cholerae* and *V. fischeri*
483 $\Delta mutS$ lines and were enriched in genes responsible for biofilm production, including
484 putative diguanylate cyclases that affect the regulation of biofilm traits via the
485 messenger molecule cyclic diguanylate and (Yildiz and Visick 2009). Taken together,
486 this study shows that despite inherently very low mutation rates, *Vibrio* populations likely
487 harbor genetic variation at SSR's that affect traits in which plasticity may be beneficial –
488 such as virulence or adherence mechanisms – and this is especially the case for
489 populations with defective mismatch repair.

490

491 **The role of mismatch repair in genome evolution**

492 The mismatch repair pathway is a primary DNA repair pathway in organisms
493 across the tree of life (Kunkel and Erie 2005), but strains lacking MMR are common in
494 nature (Hazen et al. 2009), chronic infections (Hall and Henderson-Begg 2006; Mena et
495 al. 2008; Oliver 2010; Marvig et al. 2013), or long-term evolution experiments
496 (Sniegowski et al. 1997). Loss of a functional MMR system can elevate mutation rates
497 anywhere from 5 to 1000-fold, depending on both the defective component of the
498 pathway and the genetic background (Lyer et al. 2006; Long et al. 2015; Reyes et al.
499 2015). The primary proteins involved in the MMR pathway in bacteria include the MutS
500 protein, which binds mismatches to initiate repair, the MutL protein, which coordinates
501 multiple steps of MMR synthesis, and the MutH protein, which nicks the unmethylated
502 strand to remove the replication error (Kunkel and Erie 2005). The removal of the *mutS*
503 gene in this study resulted in a 317-fold increase in the bpsm rate and a 102-fold
504 increase in the indel rate in *V. fischeri* ES114. The removal of the *mutS* gene in *V.*
505 *cholerae* had a less dramatic effect on the bpsm rate (85-fold increase), but a more
506 dramatic effect on the indel rate (142-fold increase). Overall, this suggests that MMR is
507 more central to the repair of bpsms in *V. fischeri*, while it is more important for the repair
508 of indels in *V. cholerae*.

509 Despite the relatively wide range in the consequences of losing a functional MMR
510 system for bpsm rates, the resulting bpsm spectra were remarkably similar in *V. fischeri*
511 and *V. cholerae*. Specifically, unlike the wild-type experiments, the bpsm spectra of the
512 *Vf-mut* and *Vc-mut* MA experiments were not significantly different, as both were
513 dominated by G:C > A:T and A:T > G:C transitions. Furthermore, the vast majority of
514 indels in the both the *Vf-mut* and *Vc-mut* experiments involve only a single-nucleotide,

515 most of which occurred in homopolymers, where their rates scaled positively with the
516 length of the homopolymer (Figure 5). These observations are consistent with previous
517 reports in other bacterial MA experiments using MMR-deficient strains (Lee et al. 2012;
518 Long et al. 2014; Sung et al. 2015; Dettman et al. 2016), but reveal distinct mutation
519 biases from genotypes with functional MMR. In fact, the strong site-specific biases in
520 the mutation spectra generated by the loss of MMR (Table 2) may help to explain the
521 prevalence of mutator alleles in environmental and clinical settings, despite the
522 inevitable fitness costs of the mutational load. Couce et al. have found that mutator
523 alleles can modify the distribution of fitness effects of individual beneficial mutations by
524 enriching a specific spectrum of spontaneous mutations, and impact the evolutionary
525 trajectories of different strains (Couce et al. 2013; Couce et al. 2015), although the
526 generality of how mutators influence adaptive dynamics requires further study.

527 The loss of MMR also helps reveal mutation biases associated with the
528 replicative polymerase that cannot be observed using the low number of mutations
529 generated in wild-type MA-WGS experiments (Lee et al. 2012; Sung et al. 2015;
530 Dettman et al. 2016). A mirrored wave-like pattern of bpsm rates on opposing
531 replichores has now been observed in multiple MMR-deficient species studied by MA-
532 WGS, although the exact shape and strength of the pattern varies between species
533 (Foster et al. 2013; Long et al. 2014; Dettman et al. 2016). We find the same mirrored
534 wave-like pattern of bpsm rates on the opposing replichores of chr1 in MMR-deficient *V.*
535 *fischeri* and *V. cholerae* (Figure 6A, B) and show that bpsm rates of concurrently
536 replicating regions of this chromosome are significantly correlated in both genomes.
537 This suggests that bpsm rates are impacted by genome location and that regions

538 replicated at similar times on opposing replichores experience similar bpsm rates, at
539 least in MMR-deficient strains. However, we do not observe any significant variation in
540 the bpsm rates on chr2 (Figure 6A, B). We suggest that bpsm rates are less variable on
541 chr2 because of their delayed replication. Specifically, chr2 replication is not initiated
542 until a large portion of chr1 has already been replicated (Egan and Waldor 2003; Duigou
543 et al. 2006; Rasmussen et al. 2007), which means that chr2 is not replicated during the
544 primary peaks in the bpsm rate on the opposing replichores of chr1, and thus
545 experience more consistent bpsm rates across the chromosome. These patterns of
546 variation in mutation rates among genome regions suggest that it is conceivable that
547 selection may act to position some genes to avoid regions of higher mutation pressure,
548 especially in populations of the size and diversity of *Vibrio* species.

549

550 **Concluding Remarks**

551 Mutation-accumulation experiments paired with whole-genome sequencing
552 enable an unprecedented view of genome-wide mutation rates and spectra and reveal
553 the underlying biases of spontaneous mutation. These underlying biases can explain
554 why some genome regions evolve more rapidly than others, why the coding content of
555 different genome regions varies, and perhaps how clonal populations may generate
556 adaptively diverse progeny. The primary properties of *V. fischeri* and *V. cholerae*
557 spontaneous mutation that we have identified in this MA-WGS study are: (a) base-
558 substitution and insertion-deletion mutation rates are low, consistent with other bacterial
559 species; (b) base-substitution mutation biases vary between chromosomes, but don't
560 fully explain their realized %GC contents; (c) both the length of repeat units and the

561 number of repeated units in simple-sequence repeats correlate positively with the
562 insertion-deletion rate of the SSR; (d) loss of a proficient mismatch repair system has
563 inconsistent effects on base-substitution and indel mutation rates in different taxa, but
564 consistently generates convergent mutation spectra that are dominated by transitions
565 and short indels; and (e) base-substitution mutations in strains deficient in mismatch
566 repair vary in a mirrored wave-like pattern on opposing replichores on chromosome 1,
567 but variation is limited on chromosome 2. As we uncover properties of spontaneous
568 mutation in diverse microbes, we can continue to assess the generality of mutational
569 biases and more accurately evaluate the role of mutation bias in molecular evolution.

570

571

MATERIALS AND METHODS

572 **Bacterial strains and culture conditions.** The two wild-type MA experiments were
573 founded from a single clone derived from *V. fischeri* ES114 and *V. cholerae* 2740-80,
574 respectively. All MA experiments with *V. fischeri* were carried out on tryptic soy agar
575 plates supplemented with NaCl (TSAN) (30 g/liter tryptic soy broth powder, 20 g/liter
576 NaCl, 15 g/liter agar) and were incubated at 28°. Frozen stocks of each MA lineage
577 were prepared at the end of the experiment by growing a single colony overnight in 5ml
578 of tryptic soy broth supplemented with NaCl (TSBN) (30 g/liter tryptic soy broth powder,
579 20 g/liter NaCl) at 28° and freezing in 8% DMSO at -80°. For *V. cholerae*, all MA
580 experiments were carried out on tryptic soy agar plates (TSA) (30 g/liter tryptic soy broth
581 powder, 15 g/liter agar) and were incubated at 37°. Similarly, frozen stocks were
582 prepared by growing a single colony from each lineage overnight in 5ml of tryptic soy

583 broth (TSB) (30 g/liter tryptic soy broth powder) at 37° and were stored in 8% DMSO at -
584 80°.

585 Mutator strains of *V. fischeri* ES114 and *V. cholerae* 2740-80 were generated by
586 replacing the *mutS* gene in each genome with an erythromycin resistance cassette, as
587 described previously (Datsenko and Wanner 2000; Heckman and Pease 2007; Val et al.
588 2012). Briefly, we used splicing by overlap extension (PCR-SOE) to generate two
589 erythromycin resistance cassettes, one of which was flanked by \approx 750 bps of the
590 upstream and downstream regions of the *mutS* gene in *V. fischeri* ES114, while the
591 second was flanked by \approx 750 bps of the upstream and downstream regions of the *mutS*
592 gene in *V. cholerae* 2740-80 (Heckman and Pease 2007). Both the *V. fischeri* ES114
593 and *V. cholerae* 2740-80 Δ *mutS* fragments were then cloned into the R6K γ -ori-based
594 suicide vector pSW7848, which contains a *ccdB* toxin gene that is arabinose-inducible
595 and glucose-repressible (P_{BAD}) (Val et al. 2012). Both of these pSW7848 plasmids,
596 henceforth referred to as pSW7848-*Vf* Δ *mutS* and pSW7848-*Vc* Δ *mutS*, were
597 transformed into *Escherichia coli* pi3813 chemically competent cells and stored at -80°
598 (Datsenko and Wanner 2000).

599 Conjugal transfer of the pSW7848-*Vf* Δ *mutS* and pSW7848-*Vc* Δ *mutS* plasmids
600 was performed using a tri-parental mating with the *E. coli* pi3813 cells as the donors
601 (Val et al. 2012), *E. coli* DH5 α -pEVS104 as the helper (Stabb and Ruby 2002), and *V.*
602 *fischeri* ES114 and *V. cholerae* 2740-80 as the respective recipients. For *V. fischeri*
603 ES114, the chromosomally inserted pSW7848-*Vf* Δ *mutS* plasmid resulting from a
604 crossover at the Δ *mutS* gene was selected on LBS plates (Graf et al. 1994) containing
605 1% glucose and 1ug/ml chloramphenicol at 28°. Selection for loss of the plasmid

606 backbone from a second recombination step was then performed on LBS plates
607 containing 0.2% arabinose at 28°, which induces the P_{BAD} promoter of the *ccdB* gene
608 and ensures that all cells that have not lost the integrated plasmid will die (Val et al.
609 2012). For *V. cholerae*, the chromosomally inserted pSW7848-*Vc* Δ *mutS* plasmid was
610 selected on LB plates (Sambrook et al. 1989) containing 1% glucose and 5ug/ml
611 chloramphenicol at 30°. Selection for loss of the plasmid backbone was performed on
612 LB plates with 0.2% arabinose at 30°. Replacement of the *mutS* gene in *V. fischeri*
613 ES114 and *V. cholerae* 2740-80 were verified by conventional sequencing, and *V.*
614 *fischeri* ES114 Δ *mutS* and *V. cholerae* 2740-80 Δ *mutS* were used to found the two
615 mutator MA experiments, under identical conditions to those described above for the
616 wild-type experiments.

617

618 **Ancestral reference genomes.** Prior to this study, the genome of *V. fischeri* ES114
619 was already in completed form and annotated, consisting of three contigs representing
620 chr1, chr2, and the 45.85 Kb plasmid (Ruby et al. 2005). Further, the location of the *oriC*
621 on both chromosomes was available in dOriC 5.0, a database for the predicted *oriC*
622 regions in bacterial and archaeal genomes (Gao et al. 2013). Fortunately, the *oriC*
623 region on both chromosomes had been placed at coordinate zero, allowing us to
624 proceed with this *V. fischeri* ES114 reference genome for all subsequent *V. fischeri*
625 analyses. In contrast, when we initiated our MA experiment the *V. cholerae* 2740-80
626 genome was still in draft form, consisting of 257 scaffolds with unknown chromosome
627 association. Therefore, to reveal inter-chromosomal variation and assess the effects of
628 genome location on bpsm and indel rates, we used single molecule, real-time (SMRT)

629 sequencing to generate a complete assembly separated into the two contigs of *V.*
630 *cholerae* 2740-80.

631 The Pacific Biosciences RSII sequencer facilitates the completion of microbial
632 genomes by producing reads of multiple kilobases (kb) that extend across repetitive
633 regions and allow whole-genomes to be assembled at a relatively limited cost (Koren
634 and Phillippy 2015). Genomic DNA (gDNA) was prepared using the Qiagen Genomic-
635 Tip Kit (20/G) from overnight cultures of *V. cholerae* 2740-80 grown in LB at 37° using
636 manufacturer's instructions. Importantly, this kit uses gravity filtration to purify gDNA,
637 which limits shearing and increases the average fragment size of the resulting gDNA
638 sample. Long insert library preparation and SMRT sequencing was performed on this *V.*
639 *cholerae* 2740-80 gDNA at the Icahn School of Medicine at Mount Sinai according to
640 the manufacturer's instructions, as described previously (Beaulaurier et al. 2015).
641 Briefly, libraries were size selected using Sage Science Blue Pippin 0.75% agarose
642 cassettes to enrich for long-reads, and were assessed for quantity and insert size using
643 an Agilent DNA 12,000 gel chip. Primers, polymerases, and magnetic beads were
644 loaded to generate a completed SMRTbell library, which was run in a single SMRT cell
645 of a Pacific Biosciences RSII sequencer at a concentration of 75 pM for 180 minutes.

646 As expected, our long insert SMRT sequencing library generated mostly long
647 reads, with an average sub-read length of 8,401 bps and an N50 of 11,480 bps. We
648 used the hierarchical genome-assembly process workflow (HGAP3) to generate a
649 completed assembly of *V. cholerae* 2740-80 and polished our assembly using the
650 Quiver algorithm (Chin et al. 2013). The resultant assembly consisted of two contigs
651 representing chr1 and chr2, with an average coverage of 128x. We annotated this

652 assembly using prokka (v1.11), specifying *Vibrio* as the genus (Seemann 2014). We
653 then identified the location of the *oriC* on both contigs using Ori-finder, which applies
654 analogous methods to those used by dOriC 5.0 to identify *oriC* regions in bacterial
655 genomes (Gao and Zhang 2008; Gao et al. 2013). Of course, these *oriC* regions were
656 not located at coordinate zero of the *V. cholerae* 2740-80 reference genome, so we re-
657 formatted the reference genome to place each *oriC* region at the beginning of the chr1
658 and chr2 contigs, then stitched the contigs back together and re-polished the genome
659 using Quiver. Prokka was then run a second time to update the location of all genes,
660 and this re-formatted *V. cholerae* 2740-80 genome was used as the ancestral reference
661 genome for all subsequent *V. cholerae* analyses.

662

663 **MA-WGS Process.** For the two wild-type MA experiments, seventy-five independent
664 lineages were founded by single cells derived from a single colony of *V. fischeri* ES114
665 and *V. cholerae* 2740-80, respectively. Each of these lineages was then independently
666 propagated every 24 hours onto fresh TSAN for *V. fischeri* and fresh TSA for *V.*
667 *cholerae*. This cycle was then repeated for a total of 217 days. For the two mutator MA
668 experiments, forty-eight independent lineages were founded and propagated as
669 described above from a single colony each of *V. fischeri* ES114 $\Delta mutS$ and *V. cholerae*
670 2740-80 $\Delta mutS$, respectively. However, because of their higher mutation rates, these
671 lineages were only propagated for a total of 43 days. At the conclusion of the four MA
672 experiments, each lineage was grown overnight in the appropriate liquid broth at the
673 appropriate temperature (see above), and stored at -80° in 8% DMSO.

674 Daily generations were estimated monthly for the wild-type lineages and bi-
675 monthly for the mutator lineages by calculating the number of viable cells in a
676 representative colony from 10 lineages per MA experiment following 24 hours of growth.
677 During each measurement, the representative colonies were placed in 2 ml of
678 phosphate buffer saline (80 g/liter NaCl, 2 g/liter KCl, 14.4 g/liter Na₂HPO₄ • 2H₂O, 2.4
679 g/liter KH₂PO₄), serially diluted, and spread plated on TSAN or TSA for *V. fischeri* and
680 *V. cholerae*, respectively. These plates were then incubated for 24 hours at 28° or 37°,
681 and the daily generations per colony were calculated from the number of viable cells in
682 each representative colony. The average daily generations were then calculated for
683 each time-point using the ten representative colonies, and the total generations elapsed
684 between each measurement were calculated as the product of the average daily
685 generations and the number of days before the next measurement. The total of number
686 of generations elapsed during the duration of the MA experiment per lineage was then
687 calculated as the sum of these totals over the course of each MA study (Figure S1).

688 At the conclusion of each of the four MA experiments, gDNA was extracted using
689 the Wizard Genomic DNA Purification Kit (Promega) from 1 ml of overnight culture
690 (TSBN at 28° for *V. fischeri*; TSB at 37° for *V. cholerae*) inoculated from 50
691 representative stored lineages for *Vf*-wt and *Vc*-wt experiments, and all 48 stored
692 lineages for the *Vf*-mut and *Vc*-mut experiments. For the wild-type MA experiments,
693 gDNA from the ancestral *V. fischeri* ES114 and *V. cholerae* 2740-80 strains was also
694 extracted. All libraries were prepared using a modified Illumina Nextera protocol
695 designed for inexpensive library preparation of microbial genomes (Baym et al. 2015).
696 Sequencing of the *Vf*-wt and *Vc*-wt lineages and their respective ancestors was

697 performed using the 101-bp paired-end Illumina HiSeq platform at the Beijing Genome
698 Institute (BGI), while sequencing of the *Vf*-mut and *Vc*-mut lineages was performed
699 using the 151-bp paired-end Illumina HiSeq platform at the University of New
700 Hampshire Hubbard Center for Genomic Studies.

701 Our raw fastQ reads were analyzed using fastQC, and revealed that 48 *Vf*-wt
702 lineages, 49 *Vc*-wt lineages, 19 *Vf*-mut lineages, and 22 *Vc*-mut lineages were
703 sequenced at sufficient depth to accurately identify bpsm and indel mutations. Our
704 failure to successfully sequence a high proportion of *Vf*-mut and *Vc*-mut lineages was
705 mostly generated by a poorly normalized library, leading to limited sequence data for
706 several of the mutator lineages. For the successfully sequenced lineages, all reads
707 were mapped to their respective reference genomes with both the Burrows-Wheeler
708 Aligner (BWA) (Li and Durbin 2009) and Novoalign (www.novocraft.com). The average
709 depth of coverage across the successfully sequenced lineages of each MA experiment
710 was 100x for *Vf*-wt, 96x for *Vc*-wt, 124x for *Vf*-mut, and 92x for *Vc*-mut.

711
712 **Base-substitution mutation identification.** For all four MA experiments, bpsms were
713 identified as described previously in an MA experiment with *Burkholderia cenocepacia*
714 (Dillon et al. 2015). Briefly, we used SAMtools to convert the SAM alignment files from
715 each lineage to mpileup format (Li et al. 2009), then in-house perl scripts to produce the
716 forward and reverse read alignments for each position in each line. A three-step
717 process was then used to detect putative bpsms. First, pooled reads across all lines
718 were used to generate an ancestral consensus base at each site in the reference
719 genome. This allows us to correct for any differences that may exist between the

720 reference genomes and the ancestral colony of each our MA experiments. Second, a
721 lineage specific consensus base was generated at each site in the reference genome
722 for each individual MA lineage using only the reads from that line. Here, a lineage
723 specific consensus base was only called if the site was covered by at least two forward
724 and two reverse reads and at least 80% of the reads identified the same base.
725 Otherwise, the site was not analyzed. Third, each lineage specific consensus base that
726 was called was compared to the overall ancestral consensus of the MA experiment and
727 a putative bpsm was identified if they differed. This analysis was carried out
728 independently with the alignments generated by BWA and Novoalign, and putative
729 bpsms were considered genuine only if both pipelines independently identified the bpsm
730 and they were only identified in a single lineage.

731 Using relatively lenient criteria for identifying lineage specific consensus bases,
732 we were able to analyze the majority of the genome in all of our lineages, but increase
733 our risk of falsely identifying bpsms at low coverage sites. Therefore, we generated a
734 supplementary dataset for all genuine bpsms identified in this study, which includes the
735 read coverage and consensus at each site where a bpsm was identified
736 (Supplementary Dataset 1). We do not see clusters of bpsms at the lower limits of our
737 coverage or consensus requirements. In fact, the vast majority of bpsms in all of four
738 MA experiments were covered by more than 50 reads and were supported by more
739 than 95% of the reads that covered the site. Furthermore, we verified that none of the
740 bpsms that we identified were present in the ancestral *V. fischeri* ES114 and *V.*
741 *cholerae* 2740-80 strains that we sequenced, so we are confident that nearly all of the

742 bpsms identified in this study were spontaneous bpsms that arose during the mutation
743 accumulation experiments.

744

745 **Insertion-deletion mutation identification.** All indels identified in this study were also
746 identified using similar requirements to those described in our MA experiment with *B.*
747 *cenocepacia* (Dillon et al. 2015), with a few modifications. First, we extracted all putative
748 indels from the BWA and Novoalign alignments for each lineage under the requirements
749 that the indel was covered by at least two forward and two reverse reads, and 30% of
750 those reads identified the exact same indel (size and motif). All putative indels that were
751 independently identified by both BWA and Novoalign, where 80% of the reads identified
752 the exact same indel were considered genuine. Next, we noticed that nearly all putative
753 indels that had been identified with less than 80% consensus were in SSRs. Therefore,
754 for indels where only 30-80% of the reads identified the exact same indel, we parsed
755 out only reads that had bases on both the upstream and downstream region of the SSR
756 (if the indel was in an SSR), and on both the upstream and downstream region of the
757 indel itself (if the indel was not in an SSR). Using only this subset of reads, we
758 reassessed the number of reads that identified the exact same indel (size and motif),
759 and considered these initially low confidence putative indels genuine if more than 80%
760 of these sub-reads identified the exact same indel. In addition, we passaged our
761 alignment output through the pattern-growth algorithm PINDEL to identify any large-
762 scale genuine indels using paired-end information (Ye et al. 2009). Here, we required a
763 total of 20 reads, with at least 6 forward and 6 reverse reads, and 80% of the reads to
764 identify the exact same indel for the indel to be considered genuine. This summative

765 collection of genuine indels was then compared to the analysis of the ancestral *V.*
766 *fischeri* ES114 and *V. cholerae* 2740-80 strains, and any indels that were identified in
767 their corresponding ancestor, or more than 50% of the analyzed lineages from the
768 corresponding MA experiment were excluded from subsequent analyses.

769 Our initial filters for indels were even more lenient than those for bpsms, which
770 could have led to false positive indel identification in the putative indel phase. However,
771 we subsequently required at least 80% consensus for all genuine indels identified in this
772 study among reads that had bases covering both the upstream and downstream regions
773 of putative indels that were not in an SSR. For genuine indels that were in an SSR, we
774 required at least 80% consensus among reads that had bases covering both the
775 upstream and downstream regions of the SSR. Further, we verified that all indels
776 identified were not present in our ancestral *V. fischeri* ES114 or *V. cholerae* 2740-80
777 strains and were not identified in more than 50% of the other lineages analyzed in the
778 same MA experiment. As with our bpsms, we generated a supplementary dataset
779 containing all genuine indels analyzed in this study, which includes read coverage and
780 consensus of each site where an indel was identified, as well as the read coverage and
781 consensus among reads with bases covering both the upstream and downstream
782 regions of the indel or SSR if the initial consensus among reads covering the indel was
783 below 80% (Supplementary Dataset 2). Thus, we are confident that nearly all indels
784 identified in this study were genuine spontaneous indels that arose during the mutation
785 accumulation process.

786

787 **Mutation-rate analyses.** Overall bpsm and indel rates were calculated for each lineage
788 using the equation: $\mu = m/nT$, where μ represents the mutation rate, m represents the
789 number of mutations observed, n represents the number of ancestral sites analyzed,
790 and T represents the total number of generations elapsed per lineage. Conditional bpsm
791 rates for each lineage were calculated using the same equation, but with m representing
792 the number of bpsms of the focal bpsm type, and n representing the number analyzed
793 ancestral sites that could generate the focal bpsm type. All summative bpsm and indel
794 rates presented for each MA experiment were calculated as the average mutation rate
795 across all analyzed lineages, while summative standard errors were calculated as the
796 standard deviation of the mutation rate across all lines (s), divided by the square root of
797 the total number of lines in the corresponding MA experiment (N): $SE_{pooled} = s/\sqrt{N}$.

798 For our analysis of bpsm and indel rates within chromosomes, we divided each
799 chromosome into 100 kb intervals, starting at the origin of replication and extending bi-
800 directionally to the replication terminus. Bpsm rates in each interval were measured by
801 dividing the total number of bpsm or indel mutations from this study, by the product of
802 the total number of sites analyzed in each interval across all lines and the number of
803 generations per line, using the same formula described above for genome wide
804 mutation rates: $\mu = m/nT$. Because none of the chromosomes were exactly divisible by
805 100 kb, the final intervals on each replicore were always less than 100 kb, but their
806 mutation rates were calibrated to the number of bases analyzed.

807 Potential effects of selection on observed mutations were assessed as follows.
808 For each MA experiment, the expected ratio of coding to non-coding mutations was
809 determined directly from each ancestral reference genome, and the expected ratio of

810 synonymous to nonsynonymous bpsms was calculated from each ancestral reference
811 genome, after accounting for codon usage and %GC content of synonymous and
812 nonsynonymous sites.

813

814 **Data availability.** Separate Bioprojects have been generated on NCBI for all
815 sequencing data that pertains to these *V. fischeri* (PRJNA256340) and *V. cholerae*
816 (PRJNA256339) MA-WGS projects. Under these Bioprojects, Illumina DNA sequences
817 for all the *V. fischeri* and *V. cholerae* MA-WGS lines are available under the following
818 Biosamples: *Vf*-wt = SAMN05366916, *Vc*-wt = SAMN05366910, *Vf*-mut =
819 SAMN05366917, *Vc*-mut = SAMN05366911. The completed *V. cholerae* 2740-80
820 genome assembly generated in this study is also available under the Biosample
821 SAMN05323685. All strains are available upon request.

822

823 **Statistical analyses.** All statistical analyses were performed in R Studio Version
824 0.99.489 using the Stats analysis package (R Development Core Team 2013).

825

826

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832

TABLES

Table 1. Parameters and observed mutations in the four mutation accumulation experiments.

MA Lines	Sequenced Lines	Gen. per line	Gen. total	No. of bpsm	No. of indels	Bpsm rate per nucleotide ^a	Bpsm rate per genome ^b	Indel rate per nucleotide ^a	Indel rate per genome ^b
Vf_wt	48	5187	248976	219	60	2.07×10^{-10}	8.85×10^{-4}	5.68×10^{-11}	2.43×10^{-4}
Vc_wt	49	6453	316197	138	22	1.07×10^{-10}	4.38×10^{-4}	1.71×10^{-11}	6.98×10^{-5}
Vf_mut	19	810	15390	4313	382	6.57×10^{-8}	2.81×10^{-1}	5.82×10^{-9}	2.49×10^{-2}
Vc_mut	22	1254	27588	1022	273	9.09×10^{-9}	3.72×10^{-2}	2.43×10^{-9}	9.93×10^{-3}

^aBpsm and indel mutation rates/nucleotide/generation are calculated as the number of observed mutations, divided by the product of sites analyzed and number of generations per lineage. The above estimates represent the average rate across all sequenced lineages.

^bBpsm and indel mutation rates/genome/generation are calculated by multiplying the mutation rate/nucleotide/generation in each lineage by the genome size. The above estimates represent the average rate across all sequenced lineages.

Table 2. Simple sequence repeats (SSRs) in *Vibrio cholerae* and *Vibrio fischeri* found in coding regions to be mutagenic and associated with gene products whose expression could benefit from population-level plasticity.

Organism	Locus tag	SSR	Annotation	Function (*putative)	PMID
<i>Vibrio cholerae</i> 2740-80	vc274080_02054	9c	<i>tcpC</i>	Toxin-coregulated pilus, outer membrane lipoprotein	
	vc274080_02058	7c	<i>tcpH</i>	Regulator of virulence cascade, associated with phase variation	9350866
	vc274080_02440	8c	<i>mshQ</i>	MSHA pili biogenesis, associated with biofilm transition	15686562
	vc274080_03677	9c	<i>tagE</i>	Cell wall biosynthesis*	
	vc274080_01185	9g	--	hypothetical	
	vc274080_01566	7c	--	CheY-like sensor histidine kinase/response regulator*	
	vc274080_01721	9c	--	Diguanylate cyclase*	
	vc274080_01742	11c	--	Putative lipoprotein*	
	vc274080_02184	7c	--	Beta-hexosaminidase*	
<i>Vibrio fischeri</i> ES114	VF_0194	10a	<i>wbjE</i>	group 1 glycosyl transferase	
	VF_0587	11g	<i>mltA</i>	murein transglycosylase A	
	VFA_0866	3taa	<i>tcpF</i>	toxin coregulated pilus biosynthesis protein TcpF	
	VFA_0867	8t	<i>tcpE</i>	toxin coregulated pilus biosynthesis protein TcpE	
	VFA_1130	9t		Hypothetical protein*	
	VF_1200	9t		Diguanylate cyclase*	
	VF_1672	7c	<i>menF</i>	Isochorismate synthase 2	
	VF_1689	9t	<i>sprE</i>	Proteolytic adapter for RpoS degradation by ClpXP	19767441
	VF_2408	9t	<i>vpsT</i>	DNA-binding transcriptional activator	17071756

844
845

FIGURE LEGENDS

846 **Figure 1.** Wild-type base-substitution (bpsm) and insertion-deletion (indel) mutation
847 rates and spectra for the two chromosomes of *Vibrio fischeri* and *Vibrio cholerae*; error
848 bars indicate one standard error of the mean. (A and B) Overall bpsm and indel
849 mutation rates per base-pair per generation. (C) Conditional bpsm and indel rates per
850 conditional base-pair per generation, estimated by dividing the number of observed
851 mutations by the product of the analyzed sites capable of producing a given mutation
852 and the number of generations of mutation accumulation in each lineage.

853
854 **Figure 2.** Relative frequency of insertion-deletion mutations (indels) of different lengths
855 observed in the wild-type (wt) and mismatch repair deficient (mut) strains of *Vibrio*
856 *fischeri* (A) and *Vibrio cholerae* (B). Note that the overall indel rates of Vf-mut and Vc-
857 mut are 102-fold and 142-fold higher than the wild-type rates, respectively, but it is the
858 relative frequencies of different indel lengths that are shown here. The increase in the
859 number of single-nucleotide indels in mutator lines is significant (Vf-mut: $\chi^2 = 5.460 \cdot$
860 10^3 , d.f. = 1, $p < 0.0001$; Vc-mut: $\chi^2 = 2.567 \cdot 10^3$, d.f. = 1, $p < 0.0001$).

861
862 **Figure 3.** Wild-type insertion-deletion mutation (indel) rates per run per generation and
863 frequencies in simple-sequence repeats (SSRs) containing three or more repeats in
864 *Vibrio fischeri*. SSRs were categorized by the length of the repeated unit and indel rates
865 per run per generation were calculated as the number of observed indels in that SSR
866 category, divided by the product of the occurrence of that SSR type in the genome, the
867 number of generations, and the number of MA lineages analyzed. Expected frequencies
868 were calculated based on the target size of each SSR category in the genome.

869
870 **Figure 4.** Mismatch repair deficient conditional base-substitution (bpsm) and insertion-
871 deletion (indel) mutation rates per conditional base-pair per generation for *Vibrio fischeri*
872 $\Delta mutS$ and *Vibrio cholerae* $\Delta mutS$ mutation accumulation lines, estimated by dividing
873 the number of observed mutations by the product of the analyzed sites capable of
874 producing a given mutation and the number of generations of mutation accumulation in
875 each lineage.

876
877 **Figure 5.** Mismatch repair deficient insertion-deletion mutation (indel) rates per run per
878 generation and frequencies in homopolymer repeats containing three or more repeats
879 for *Vibrio fischeri* $\Delta mutS$ (A) and *Vibrio cholerae* $\Delta mutS$ (B). Indel rates per run per
880 generation were calculated as the number of observed indels in each homopolymer
881 length category, divided by the product of the occurrence of homopolymers of that
882 length in the genome, the number of generations, and the number of MA lineages
883 analyzed. Expected frequencies were calculated based on the genome target size for
884 each homopolymer length.

885
886 **Figure 6.** Base-substitution (bpsm) and insertion-deletion (indel) mutation rates per
887 base-pair per generation in 100kb intervals extending bi-directionally from the origin of
888 replication (*OriC*) for all mutation accumulation (MA) experiments in this study. Outer

889 rings on each chromosome represent the mutator MA experiment and inner rings
890 represent the corresponding wild-type MA experiment. A) Bpsm rates of *Vf*-wt and *Vf*-
891 mut; B) Bpsm rates of *Vc*-wt and *Vc*-mut; C) Indel rates of *Vf*-wt and *Vf*-mut; D) Indel
892 rates of *Vc*-wt and *Vc*-mut.

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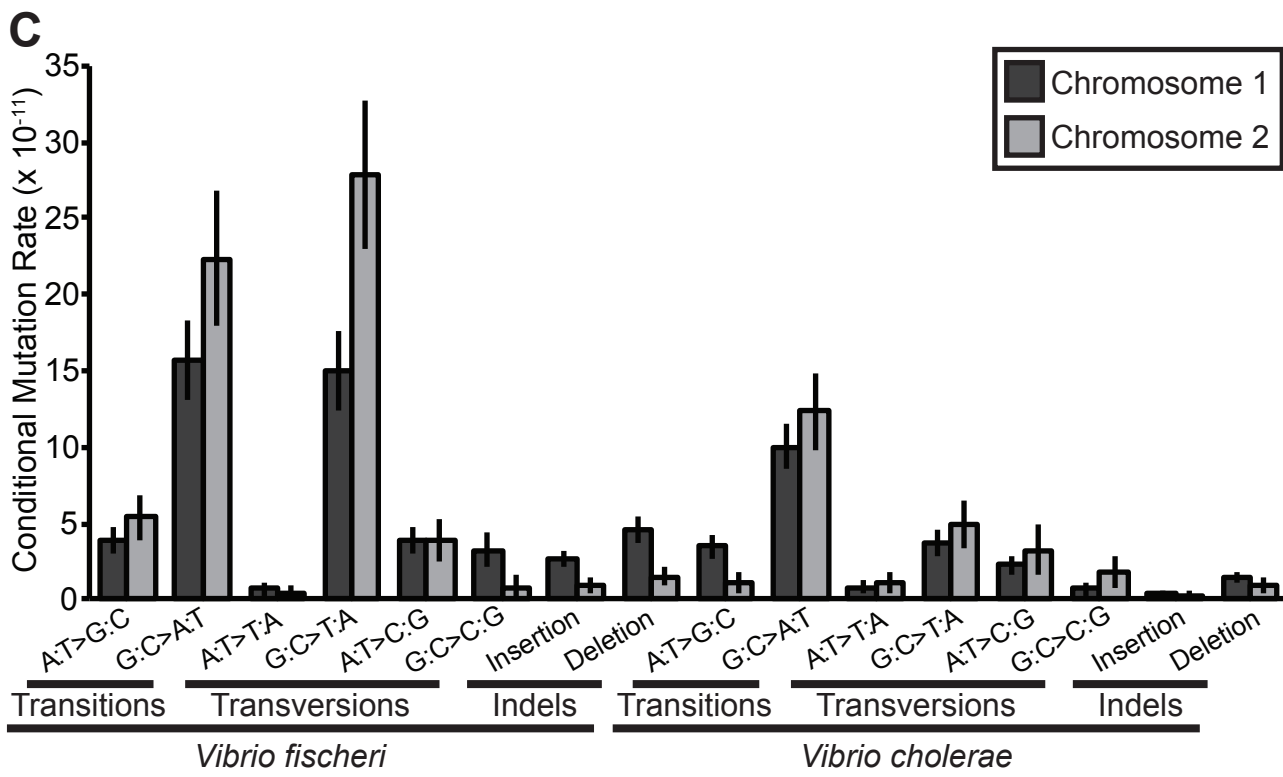
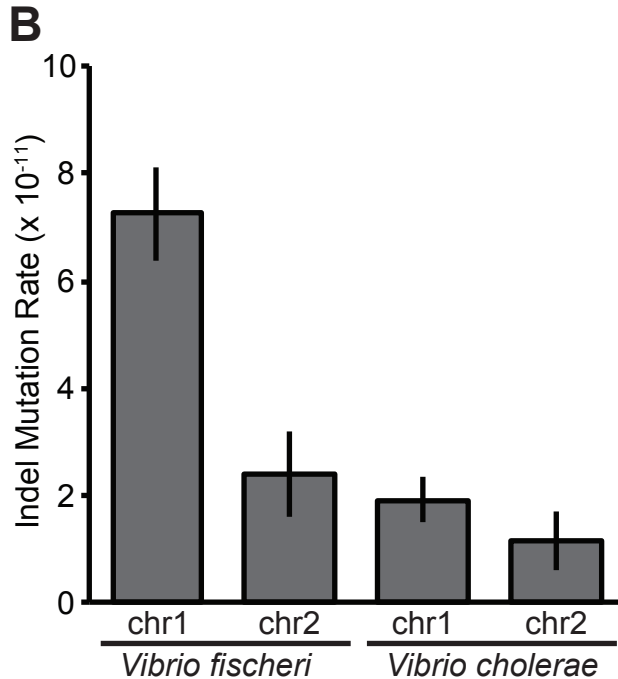
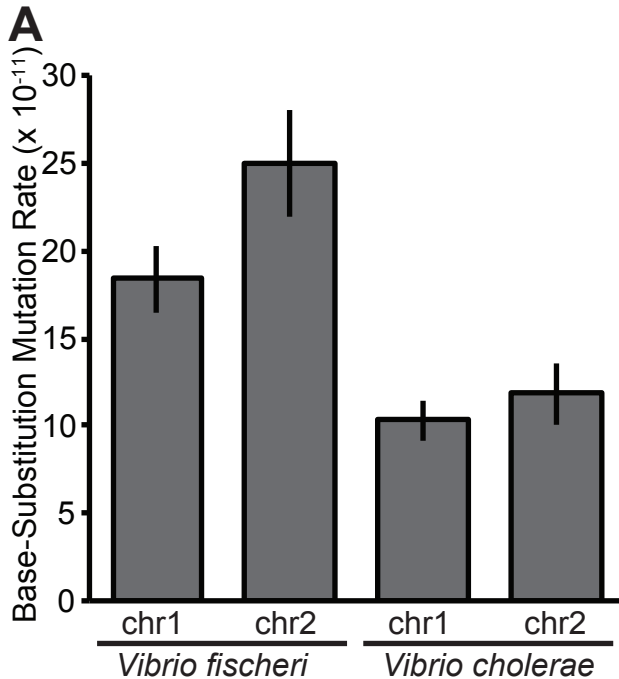
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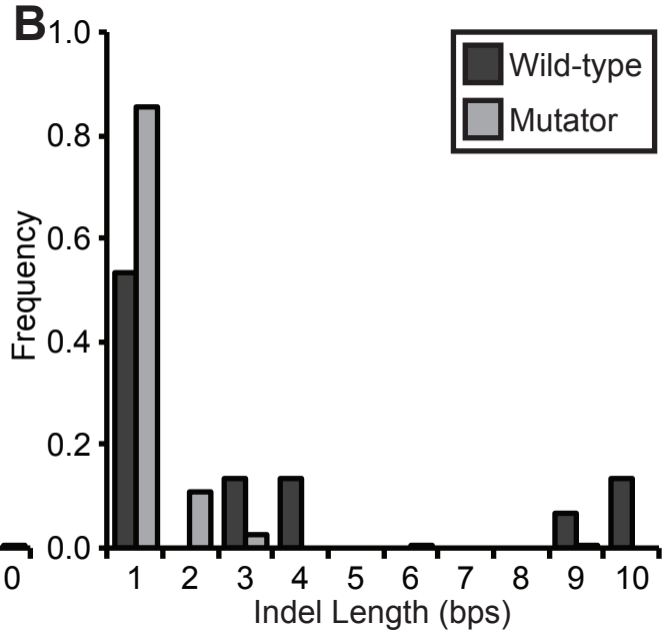
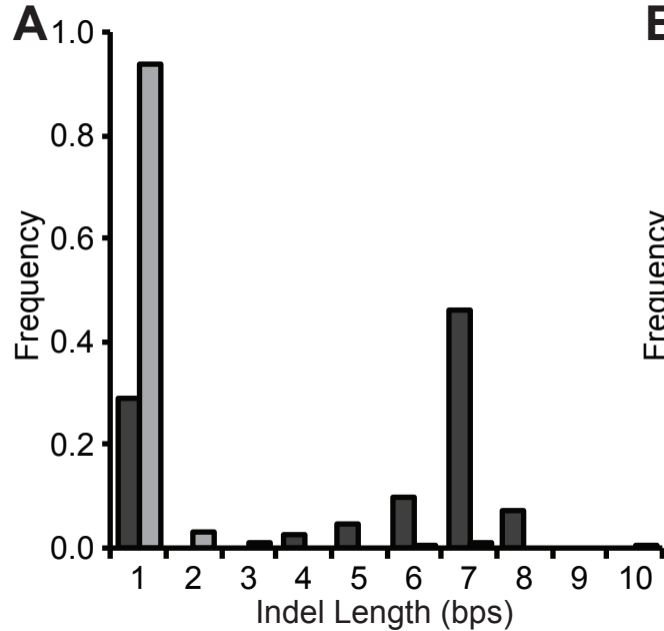
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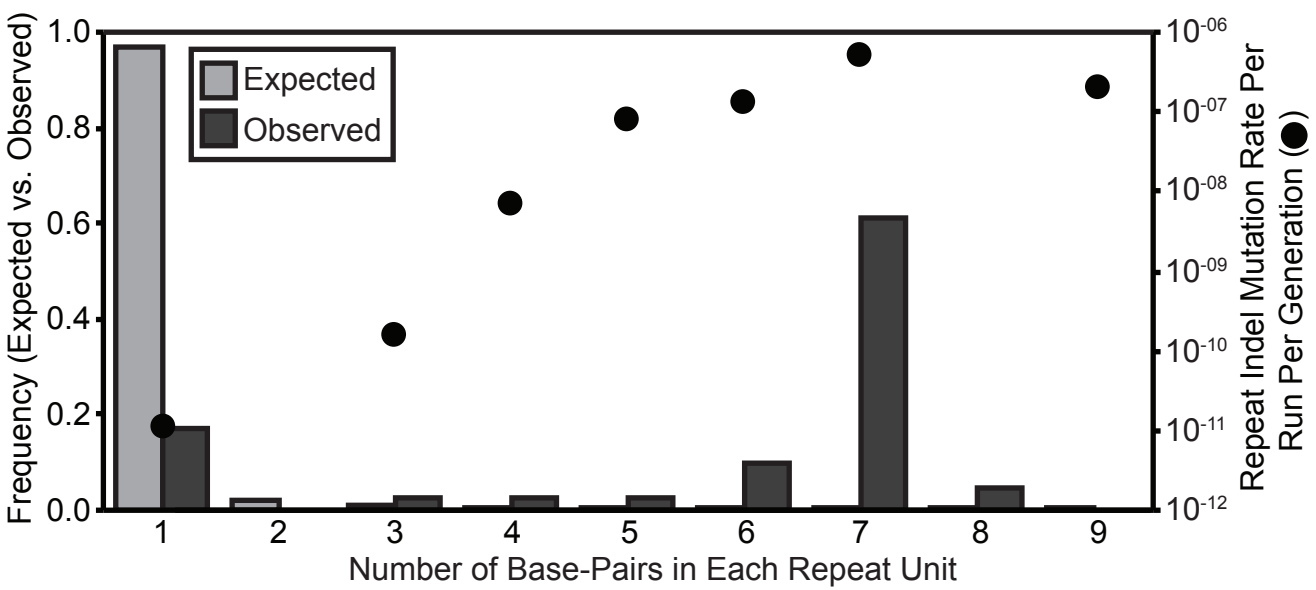
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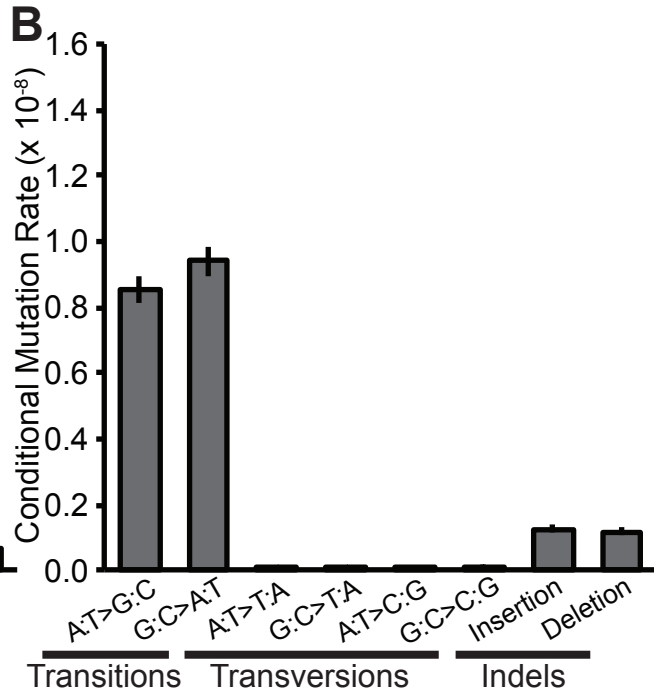
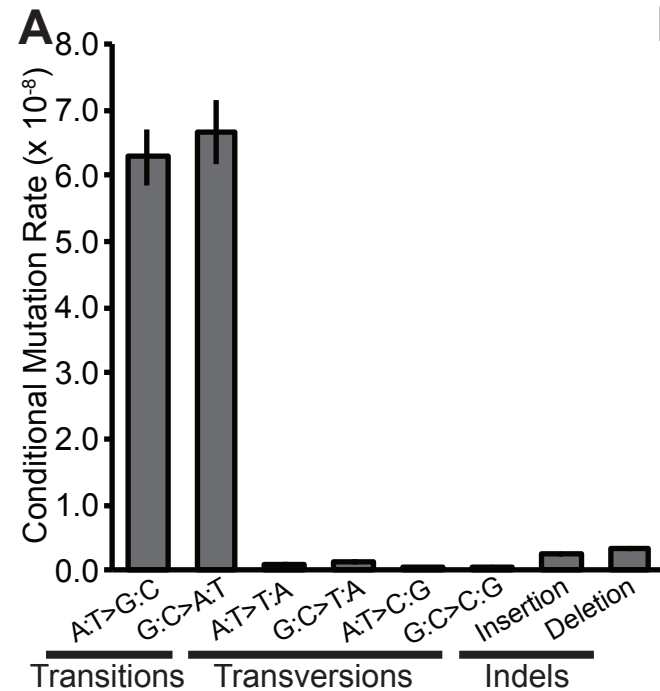
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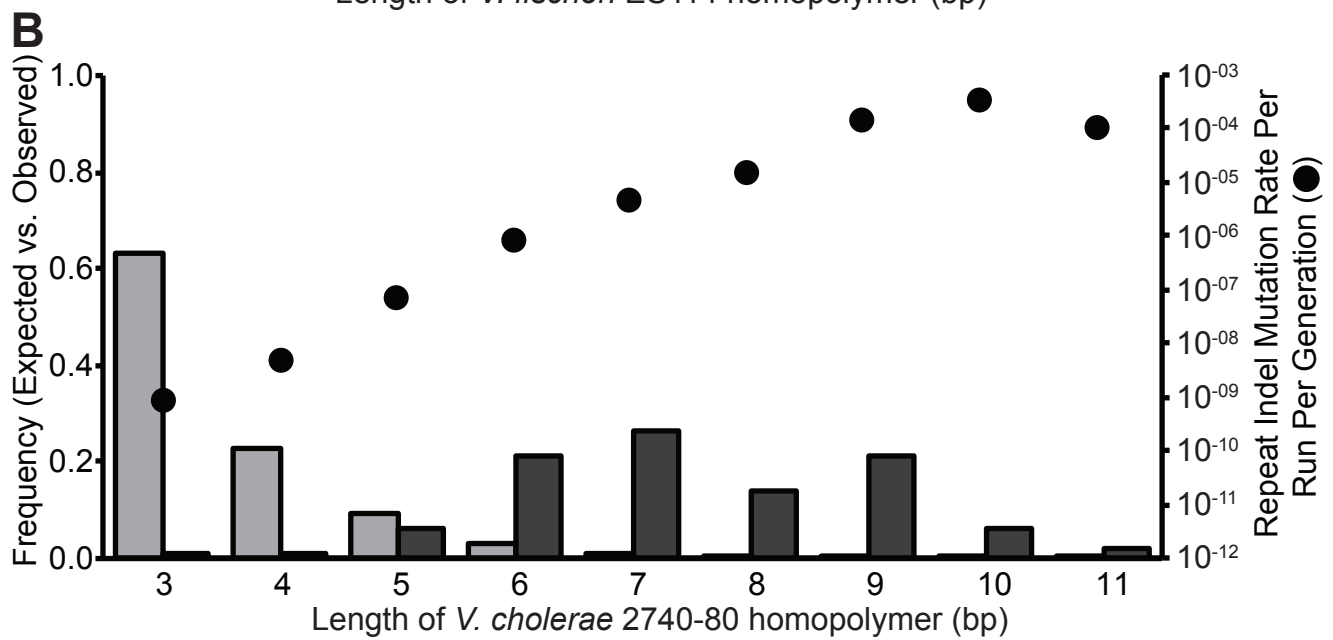
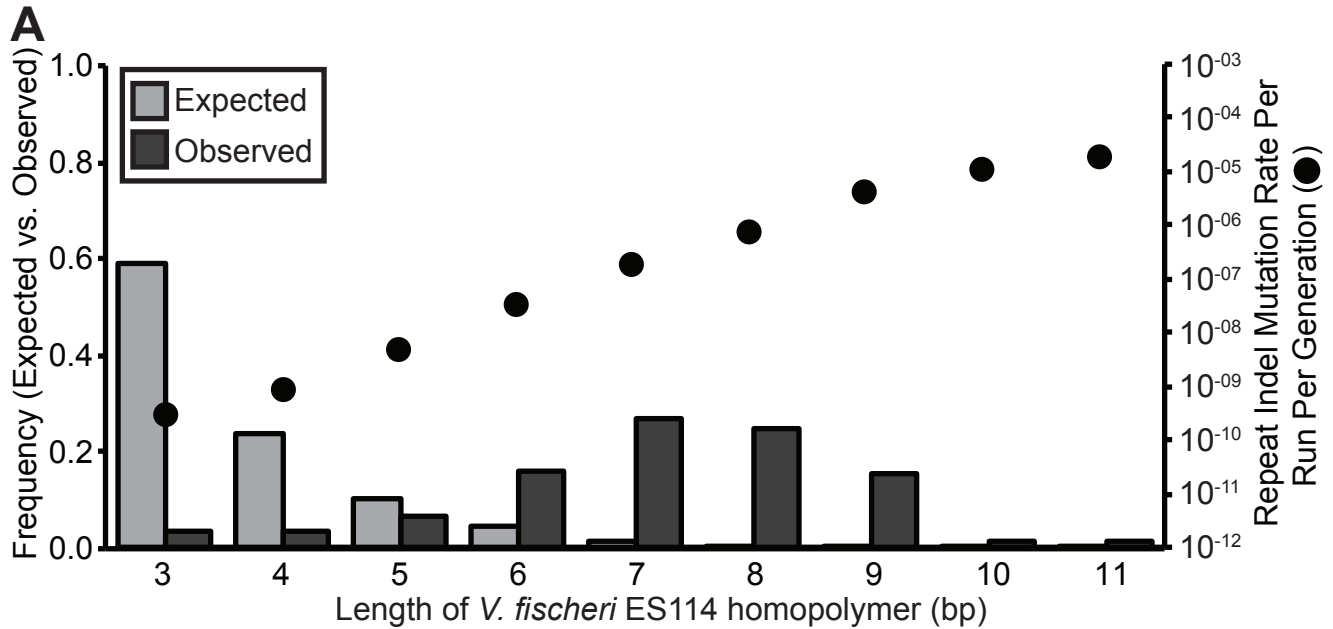
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Vibrio fischeri

Vibrio cholerae

