Functional analysis of cyclic diguanylate-modulating proteins in *Vibrio fischeri*

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ABSTRACT

As bacterial symbionts transition from a motile free-living state to a sessile biofilm state, they must coordinate behavior changes suitable to each lifestyle. Cyclic diguanylate (c-di-GMP) is an intracellular signaling molecule that can regulate this transition, and it is synthesized by diguanylate cyclase (DGC) enzymes and degraded by phosphodiesterase (PDE) enzymes. Generally, c-di-GMP inhibits motility and promotes biofilm formation. While c-di-GMP and the enzymes that contribute to its metabolism have been well-studied in pathogens, considerably less focus has been placed on c-di-GMP regulation in beneficial symbionts. *Vibrio fischeri* is the sole beneficial symbiont of the Hawaiian bobtail squid (*Euprymna scolopes*) light organ, and the bacterium requires both motility and biofilm formation to efficiently colonize. C-di-GMP regulates swimming motility and cellulose exopolysaccharide production in *V. fischeri*. The genome encodes 50 DGCs and PDEs, and while a few of these proteins have been characterized, the majority have not undergone comprehensive characterization. In this study, we use protein overexpression to systematically characterize the functional potential of all 50 *V. fischeri* proteins. All 28 predicted DGCs and 14 predicted PDEs displayed at least one phenotype consistent with their predicted function, and a majority of each displayed multiple phenotypes. Finally, active site mutant analysis of proteins with the potential for both DGC and PDE activities revealed potential activities for these proteins. This work presents a systems-level functional analysis of a family of signaling proteins in a tractable animal symbiont and will inform future efforts to characterize the roles of individual proteins during lifestyle transitions.

IMPORTANCE

C-di-GMP is a critical second messenger that mediates bacterial behaviors, and *V. fischeri* colonization of its Hawaiian bobtail squid host presents a tractable model in which to interrogate

56 the role of c-di-GMP during animal colonization. This work provides systems-level
57 characterization of the 50 proteins predicted to modulate c-di-GMP levels. By combining
58 multiple assays, we generated a rich understanding of which proteins have the capacity to
59 influence c-di-GMP levels and behaviors. Our functional approach yielded insights into how
60 proteins with domains to both synthesize and degrade c-di-GMP may impact bacterial
61 behaviors. Finally, we integrated published data to provide a broader picture of each of the 50
62 proteins analyzed. This study will inform future work to define specific pathways by which c-di-
63 GMP regulates symbiotic behaviors and transitions.
64
65 **INTRODUCTION**
66
67 Many bacteria exist in the environment in a free-living state, and upon encountering an animal
68 host undergo dramatic developmental transitions. These adjustments enable symbiotic
69 microbes–including mutualists, commensals, and pathogens–to acclimate to the physical,
70 chemical, and nutritional milieu in the host; to resist immune responses; and to engage in
71 behaviors required for survival and growth within the distinct host environment (1–5). To
72 manage such transitions successfully, bacteria often inversely regulate motility and adhesion
73 (6–11). In the motile state, it would be counterproductive to be adherent, and environmental
74 bacteria use motility and chemotaxis to colonize novel niches, seek nutrition, and avoid
75 predation (12–14). In contrast, adherent bacteria, especially those that have formed a
76 multicellular biofilm, do not have a need for swimming motility. As a result there are multiple
77 mechanisms that bacteria use to coordinately and inversely regulate these two broad behaviors
78 (15–18). Alteration of the levels of the intracellular second messenger cyclic diguanylate (c-di-
79 GMP) is a common mechanism used by bacteria to accomplish this purpose (19). In general, c-
80 di-GMP promotes biofilm formation and inhibits motility (20). Enzymes that regulate c-di-GMP
81 levels are diguanylate cyclases (DGCs), which synthesize c-di-GMP, and phosphodiesterases

(PDEs), which degrade the molecule. DGCs contain GGDEF domains with conserved GG(D/E)EF active site residues, while PDEs contain EAL domains with conserved ExLxR active site residues or HD-GYP domains with conserved HD and GYP active site residues (21–26). Although many proteins have both GGDEF and EAL domains, only one domain is usually active, even when the amino acid motif for the other domain should function based on sequence conservation (27–30). However, environmental conditions may influence whether some dual-function proteins exhibit primarily DGC or PDE activity (31).

Much of what is known about c-di-GMP regulation of biofilm, motility, and host colonization is from studies on pathogenic species (32–34). Activation of cellulose production was the first role defined for c-di-GMP, in *Acetobacter xylinum* (now *Komagataeibacter xylinus*), and has remained one of most well characterized c-di-GMP-regulated phenotypes across taxa (20, 21). C-di-GMP-mediated cellulose production has been implicated in host colonization defects by pathogens *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (33). Although much focus has been placed on defining roles for c-di-GMP in pathogenic associations, recent studies have focused on the impacts of c-di-GMP in bacteria during beneficial host associations. Host-derived ligands inactivate *Aeromonas veronii* DGC SpeD, which promotes host colonization (35). Additionally, high levels of c-di-GMP negatively impact the establishment of the symbiosis between the bioluminescent marine bacterium *Vibrio fischeri* and its host the Hawaiian bobtail squid (*Euprymna scolopes*) (36). *V. fischeri* is the sole, beneficial light organ symbiont of the Hawaiian bobtail squid (*Euprymna scolopes*), and the bacterium requires both swimming motility and biofilm formation to successfully colonize the host light organ (37–40). *V. fischeri* express polar flagella in seawater and form biofilm aggregates in the host mucus before migrating into the light organ (37, 38, 41–44). *V. fischeri* produce cellulose, which is activated by c-di-GMP, and genetic manipulation of c-di-GMP levels through deletion or overexpression of DGCs and PDEs modulates cellulose production (39, 45–48). Although cellulose is not required for

symbiotic biofilm or squid colonization, an *in vivo* regulatory interaction exists between cellulose and the symbiosis polysaccharide (Syp) (36, 38, 39, 42, 49, 50).

We previously examined how global c-di-GMP levels impact bacterial behaviors in *V. fischeri*, including cellulose production, cellulose synthase and *syp* transcriptional reporter activities, and flagellar motility (36). For that work, we took advantage of a strain lacking seven DGCs and another strain lacking six PDEs to adjust the global c-di-GMP pool. That effort sparked our interest in the potential redundancy of the dozens of predicted c-di-GMP modulating enzymes and the capacity of the encoded proteins to impact bacterial behavior. *V. fischeri* strain ES114 encodes 50 genes predicted to modulate c-di-GMP levels (**FIG. 1**) (51, 52). Of these possible c-di-GMP-modulating proteins, only a few have been characterized in depth for their roles in biofilm formation and/or swimming motility. DGCs MifA and MifB regulate magnesium-dependent motility by contributing to flagellar biogenesis (45). CasA is a DGC that is activated by calcium to inhibit motility and promote cellulose biofilm formation (48). Reduction of c-di-GMP levels by PDE BinA reduces cellulose synthesis (46). PdeV is a PDE that activates biofilm dispersal by decreasing the c-di-GMP pool that LapD, a protein with predicted degenerate GGDEF and EAL domains, uses to promote biofilm formation (47). In this model, the degenerate GGDEF or EAL domains of LapD may act as c-di-GMP binding sites (47). In *V. fischeri* strain KB2B1, the ortholog of DGC VF_1200 was shown to inhibit swimming motility (53). The other 44 *V. fischeri* predicted c-di-GMP-modulating proteins have not undergone comprehensive phenotypic characterization, although a recent study was published where motility of c-di-GMP-modulating gene mutants were assessed (52). Additionally, none of the 50 c-di-GMP-modulating proteins have been shown to impact host colonization individually, although putative PDE VF_A0879 was predicted to be required for host colonization in a transposon sequencing study (54). In this report, we systematically dissected the capability of *V. fischeri* predicted c-di-GMP-modulating enzymes to impact various biofilm and motility

phenotypes through an approach combining systems-level overexpression analysis and targeted active site mutations.

RESULTS

V. fischeri encodes 50 proteins predicted to modulate c-di-GMP levels

The *V. fischeri* genome (strain ES114) encodes 50 proteins containing DGC and/or PDE domains (49–52). Twenty-eight are predicted DGCs with GGDEF domains, 14 are predicted PDEs (12 with EAL domains, 2 with HD-GYP domains), 5 are predicted dual-function proteins with both GGDEF and EAL domains, and 3 are predicted to be nonfunctional due to degenerate active sites. The genes encoding these proteins are spread across both *V. fischeri* chromosomes, with most (31 out of 50) of the genes located on the smaller second chromosome (**FIG. 1**). To further characterize the functions of all 50 proteins, we took an overexpression approach to examine the function of each protein when individually overexpressed in *V. fischeri*. We sought this approach to be resilient against the redundancy we expect to exist within the large gene families. A similar overexpression approach has been effective to evaluate phenotypes of DGCs and PDEs in *V. cholerae* (55–59) and in other bacteria (28, 60–62). We used an IPTG-inducible vector to overexpress each protein in *V. fischeri* and performed assays to quantify cellulose production, swimming motility, and c-di-GMP levels. We also included control strains overexpressing VC1086 (a *V. cholerae* PDE) and QrgB (a *Vibrio campbellii* BB120 DGC), which have served as effective controls in multiple organisms (57, 59, 62–67). We note that the vector backbone used is the same in as many of the *V. cholerae* studies. Assays were conducted in a 96-well format to facilitate simultaneous, reproducible assays of the complete set of 54 test and control strains.

160 **Predicted *V. fischeri* DGCs and PDEs impact cellulose polysaccharide production**

161

162 C-di-GMP promotes cellulose synthesis in many bacteria including *V. fischeri* (46, 48). To
163 assess cellulose production across the set of proteins, we performed Congo red binding assays
164 (46) of strains overexpressing each protein. Most DGCs (17/28 *V. fischeri* proteins) increased
165 cellulose production, consistent with their predicted function, including characterized DGCs MifA
166 and CasA (**FIG. 2A**). Known *V. campbellii* DGC QrgB also increased cellulose production (**FIG.**
167 **2A**). The increase in cellulose production upon overexpression of known DGC MifA is consistent
168 with published results (45), and the CasA overexpression result is consistent with data showing
169 decreased cellulose production in a Δ*casA* strain (48). Only DGC VF_A0796 showed the
170 unexpected result of diminished cellulose production upon overexpression, though the effect
171 was modest (**FIG. 2A**). While overexpression of the remaining predicted DGCs did not
172 significantly alter Congo red binding levels, most had a trend in the positive direction as
173 expected and consistent with an increase in c-di-GMP levels (**FIG. 2A**).

174

175 Overexpression of 12/14 predicted PDEs significantly decreased cellulose production upon
176 overexpression, consistent with their predicted function, including characterized PDEs BinA and
177 PdeV. Known *V. cholerae* PDE VC1086 also decreased cellulose production (**FIG. 2A**). Our
178 results are consistent with published results showing effects on cellulose production for PDEs
179 BinA and PdeV (46, 47).

180

181 *V. fischeri* encodes five proteins with both GGDEF and EAL domains. Proteins that contain both
182 GGDEF and EAL domains typically only have one domain exhibit activity, even if the amino acid
183 motif for the other domain is conserved (27–30), though there is evidence of a dual-function
184 protein capable of exhibiting either DGC or PDE activity depending on conditions (31). We
185 therefore expected these dual-function proteins to behave predominantly as DGCs or PDEs. Of

186 the five predicted dual-function proteins, overexpression of VF_0985 and VF_A0244 increased
187 cellulose production while VF_0094, VF_0494, and VF_A0475 decreased cellulose production
188 compared to the empty vector control (**FIG. 2A**).
189
190 Finally, we examined the three predicted “degenerate” c-di-GMP modulating enzymes; i.e.,
191 proteins that encode GGDEF and/or EAL domains but with degenerate active sites that are not
192 predicted to function. Overexpression of one such protein, LapD (with a degenerate GGDEF
193 and EAL domains), slightly decreased cellulose production, but to a level comparable to
194 overexpression of GFP from the pEVS143 control plasmid (**FIG. 2A**). Therefore the small effect
195 of LapD on cellulose production may not be a catalytic effect. The other two degenerate
196 proteins, VF_0355 and VF_A0216 did not significantly impact cellulose production (**FIG. 2A**).
197
198 **Predicted *V. fischeri* DGCs and PDEs influence flagellar motility**
199
200 As a second behavioral output of c-di-GMP levels, we proceeded to assay swimming motility in
201 the same set of strains. We conducted swimming motility assays of strains overexpressing each
202 protein in TBS soft agar and in TBS soft agar with the addition of either magnesium or calcium,
203 which are known to influence swimming behavior by *V. fischeri (52, 68)*. We observed that most
204 DGCs (20/28) and PDEs (10/14) showed the expected overexpression results of inhibiting or
205 promoting motility, respectively, including known *V. fischeri* DGCs MifA, MifB, and CasA and
206 known PDE BinA (**FIG. 2B**). *V. campbellii* DGC QrgB and *V. cholerae* PDE VC1086 also
207 showed the expected results (**FIG. 2B**). Overexpression of either DGC MifA, MifB, or CasA
208 diminished motility under all conditions tested despite having known cation-specific motility
209 phenotypes (**FIG. 2B; FIG. S1**). MifA and MifB inhibit motility in the presence of magnesium
210 (45), while CasA inhibits motility in the presence of calcium (48), and we suspect that
211 overexpression of the proteins likely amplified their respective DGC activities and bypassed the

212 requirement for the respective cations. One example where we observed a media-specific effect
213 is for VF_0985, which strongly inhibited motility upon overexpression in TBS and TBS-Ca,
214 relative to the empty vector control, but not in TBS-Mg (**FIG. 2B; FIG. S1**).
215
216 One predicted DGC and three PDEs had significant effects on motility in the opposite direction
217 as expected under at least tested condition, but all were fairly weak in magnitude and not
218 observed under all three motility conditions, except for CdgG (**FIG. 2B; FIG. S1**). The most
219 consistent response was for an increase in motility upon overexpression of predicted DGC
220 CdgG, which is consistent with a report demonstrating that a Δ*cdgG* strain exhibits diminished
221 motility (52). Overexpression of predicted degenerate proteins VF_0355 and VF_A0216 did not
222 significantly impact motility (**FIG. 2B; FIG. S1**). Overexpression of LapD decreased motility in all
223 three motility media (**FIG. 2B; FIG. S1**), but this is likely independent of c-di-GMP modulation
224 and is consistent with the function of LapD in inhibiting biofilm dispersal (47), which is
225 associated with decreased motility.
226
227 **Predicted *V. fischeri* DGCs and PDEs modulate c-di-GMP reporter levels**
228
229 Congo red and motility assays revealed that most of the *V. fischeri* DGCs and PDEs influence
230 phenotypes associated with c-di-GMP. We next asked to what capacity these proteins are
231 capable of modulating c-di-GMP levels using the pFY4535 fluorescent c-di-GMP reporter
232 plasmid (69), which we used previously to quantify c-di-GMP levels in *V. fischeri (36)*. We
233 expected overexpression of DGCs to increase c-di-GMP levels and overexpression of PDEs to
234 decrease c-di-GMP levels relative to the empty vector control. We also expected proteins with
235 the strongest cellulose and/or motility phenotypes to have the strongest effects of c-di-GMP
236 reporter activity. Consistent with their predicted function, overexpression of 27/28 predicted
237 DGCs increased c-di-GMP reporter activity, including characterized *V. fischeri* DGCs MifA and

238 MifB (**FIG. S2A**). The results for overexpression of MifA and MifB are consistent with published
239 results (45). The one exception among *V. fischeri* DGCs was VF_1561, which did exhibit
240 elevated c-di-GMP reporter activity but not to statistical significance (**FIG. S2A**). Given that the
241 construct increased cellulose production and decreased motility (**FIG. 2**), we suspect this result
242 is a limitation to the sensitivity of the reporter. Similar results were observed for known *V.*
243 *campbellii* DGC QrgB (**FIG. 2; FIG. S2A**). In contrast to the results with DGCs, upon
244 overexpression only 2/14 predicted PDEs, VF_0087 and VF_A0506, had the expected effect of
245 significantly decreasing c-di-GMP reporter activity (**FIG. S2A**). These results are also in contrast
246 to the cellulose and motility results, where overexpression of the majority of PDEs (12/14 for
247 cellulose, 10/14 for motility) elicited the expected phenotypes, and no PDEs had significant
248 opposite cellulose or motility phenotypes (**FIG. 2**). BinA is a characterized PDE that negatively
249 influences c-di-GMP levels (absence of BinA resulted in increased c-di-GMP compared to WT)
250 (46). Our results show that BinA overexpression diminished c-di-GMP reporter activity by 57%,
251 but this result was not statistically significant (**FIG. S2A**). However, the cellulose and motility
252 results for BinA are among the strongest of the PDEs (**FIG. 2**). Similarly, overexpression of
253 known *V. cholerae* PDE VC1086 also did not significantly alter c-di-GMP reporter activity (**FIG.**
254 **S2A**), but did significantly alter cellulose production and motility in the expected directions (**FIG.**
255 **2**). Surprisingly, four predicted PDEs, VF_A1076, VF_0091, VF_A0344, and VF_A0879,
256 significantly increased c-di-GMP reporter activity upon overexpression (**FIG S2A**). The same
257 results were observed when the same overexpression strains were assayed individually rather
258 than in the arrayed 96-well format (**FIG. S2B**), suggesting perhaps there is some feedback
259 regulation that is providing this counterintuitive result. Of these four PDEs, VF_0091,
260 VF_A0879, and VF_A1076 significantly decreased cellulose production and VF_0091
261 significantly increased motility, but these phenotypes do not correspond with increased c-di-
262 GMP reporter activity (**FIG. 2; FIG. S2A**). We previously reported that in a high c-di-GMP strain
263 of *V. fischeri*, overexpression of PDE VC1086 reduced c-di-GMP reporter activity 1.6-fold when

264 measured with the same reporter (36). However, overexpression with the same vector in wild-
265 type *V. fischeri* had no effect on the already-low c-di-GMP reporter activity. Therefore, while the
266 c-di-GMP reporter levels were well-correlated with c-di-GMP-responsive phenotypes for the
267 DGCs, information from the reporter was not informative for predicting phenotypes from the low
268 basal wild-type levels. To test whether the pFY4535 c-di-GMP biosensor could better report
269 decreased c-di-GMP in a high c-di-GMP background, we overexpressed select PDEs in a strain
270 of *V. fischeri* deleted for six PDEs (Δ6PDE) and has been demonstrated to have high basal c-di-
271 GMP levels (36). Every PDE diminished c-di-GMP reporter activity in the high c-di-GMP
272 background, including PdeV, VF_0091, VF_A0706, VF_A1076, and VF_1367, all of which had
273 high reporter activity in the wild-type background (**FIG. S2C**). Dual-function protein VF_0494
274 diminished reporter activity comparable to the strongest PDE in the assay, while dual-function
275 protein VF_A0244 did not significantly impact reporter levels (**FIG. S2C**). These results are
276 consistent with the cellulose and motility results for these proteins in the wild-type background
277 (**FIG. 2**). Further, DGC VF_A0342 lowered c-di-GMP reporter activity in the High cdG
278 background (**FIG. S2C**). In the wild-type background, VF_A0342 did not significantly alter
279 cellulose production or motility (**FIG. 2**) and increased reporter activity at a level comparable to
280 the PDEs with elevated activity (**FIG. S2A**), suggesting other DGCs that did not have the
281 expected cellulose production and motility phenotypes may also have unreliable c-di-GMP
282 reporter results in the wild-type background. Given these questions about the utility of the
283 reporter activity in the wild-type background when c-di-GMP levels are diminished, for our
284 overall question of the catalytic potential of the proteins assayed we are focusing on the
285 phenotypic assays of the cellulose production and swimming motility.
286
287 **Select *V. fischeri* DGCs and PDEs impact motility and biofilm phenotypes through their c-**
288 **di-GMP catalytic sites**
289

290 Most of the DGCs and PDEs exhibited the expected cellulose and motility phenotypes upon
291 overexpression (**FIG. 2**). If the results we observed were due to catalytic function, then mutation
292 of the active sites should impact these phenotypes. Changing the second amino acid in the
293 GGDEF motif to A (60, 70, 71) drastically reduced the effects of MifA, VF_A0057, VF_A0152,
294 and VF_A0398 on cellulose production and motility (**FIG. 3**). These results confirm that intact
295 GGDEF domains are required for the regulatory functions of these proteins.
296
297 VF_A0057 is one of the more interesting predicted DGCs because it has a non-consensus
298 GGDEF domain with a serine instead of a glycine in the first position of the motif (i.e., SGDEF).
299 The altered motif is not functional in *V. cholerae* CdgG, which led us to predict it to be similarly
300 nonfunctional in *V. fischeri* VF_A0057 (70). Surprisingly, though, mutation of the second amino
301 acid of the motif in VF_A0057, resulting in the amino acid sequence SADEF, substantially
302 mitigated the effects of the protein on cellulose production and motility, suggesting that the non-
303 canonical SDGEF motif in this protein is functional and that VF_A0057 likely has DGC activity
304 (**FIG. 3**). CdgG, a homolog of the nonfunctional *V. cholerae* protein CdgG, is another predicted
305 DGC with a non-consensus active site where the first amino acid is a serine: SGEEF. However,
306 overexpression of CdgG or the variant (SAEEF) did not promote cellulose production or inhibit
307 motility (**FIG. 3**).
308
309 To examine the catalytic site of the PDEs, we similarly replaced the first residue of the ExLxR
310 motif with an alanine, a mutation that has been used previously to disrupt EAL domain activity
311 (46, 57, 72, 73). AxLxR mutants of BinA and VF_0087 attenuated the effects of these proteins
312 on cellulose production and motility (**FIG. 3**). These results confirm previous work (46, 52, 53),
313 and are the first demonstration of the requirement for the active site residues for VF_0087
314 function.
315

316 VF_1367 is one of two predicted HD-GYP domain PDEs encoded by *V. fischeri*. The motif
317 encoded is HD-GYL, but the presumed HD catalytic dyad for HD superfamily proteins is still
318 intact (23). Here, we changed the second amino acid to an alanine, which has been used to
319 disrupt HD-GYP domain activity (25, 58). Overexpression of an HA-GYL variant of VF_1367
320 obviated the impact on cellulose production, which was the major phenotype observed on
321 overexpression of the wild-type protein (**FIG. 3**). Overexpression of active site mutants of
322 predicted and characterized DGCs and PDEs thus supports the predicted biochemical activities
323 of individual proteins and validates our overall approach of overexpressing predicted c-di-GMP-
324 modulating proteins to understand the genome-wide functional landscape.
325
326 **Challenges in assigning functions for dual-domain proteins**
327
328 Three of the five *V. fischeri* dual-function proteins had clear phenotypes in both cellulose
329 production and motility assays, suggesting they may primarily function as DGCs or PDEs (**FIG.**
330 **2**). However, overexpression of proteins with both GGDEF and EAL domains can only hint at
331 whether a protein primarily has DGC or PDE activity and does not discern which domains are
332 active/inactive. To assess whether one, both, or neither of the DGC and PDE domains
333 contribute to the effects of these predicted dual-domain protein, we assayed strains
334 overexpressing GGDEF mutants, EAL mutants, or GGDEF/EAL double mutants of each protein.
335 As one example, phenotypic assays strongly suggested that VF_0985 behaved as a DGC given
336 its impact on cellulose and motility (**FIG. 2**). A VF_0985 AAL mutant behaved like the wild-type
337 protein in all assays when overexpressed, while a GADEF mutant drastically reduced the effects
338 of VF_0985 on cellulose production and motility (**FIG. 4**). Interestingly, mutation of the GGDEF
339 domain seemed to reverse the effects of VF_0985: the GGDEF mutant behaved similar to a
340 PDE in both assays (**FIG. 4**), suggesting the EAL domain may be active. When the VF_0985
341 double GADEF/AAL mutant was overexpressed, cellulose production resembled the single

342 GGDEF mutant, while little effect on motility was observed (**FIG. 4**). These results suggest that,
343 under the conditions tested, VF_0985 likely functions predominantly as a DGC, but that we may
344 be detecting cryptic PDE activity in the variant in which DGC activity is absent.
345
346 Active site mutant analysis of the remaining four dual-function proteins similarly suggest a more
347 complicated picture than one dominant domain. For example, cellulose production upon
348 overexpression of VF_A0244 was dependent on the GGDEF motif in the wild-type background,
349 but was insensitive to mutation of this motif in a mutant PDE domain background (**FIG. 4**). We
350 also observed differences in phenotypes across assays in ways that were rarely observed for
351 wild-type strains (e.g., VF_0494 cellulose compared to motility response). Further in-depth
352 studies of these proteins will be required to dissect their roles in cellulose production and
353 motility, and the mechanisms by which these proteins perform those roles.
354
355 **DISCUSSION**
356
357 *V. fischeri* is an emerging model for studies of c-di-GMP regulation, and especially for how c-di-
358 GMP impacts animal host colonization. We previously demonstrated that high global levels of c-
359 di-GMP impair colonization, though it is unknown how levels remain low to facilitate a productive
360 host-microbe symbiosis. An ongoing goal is to elucidate relevant signaling that enables proper
361 c-di-GMP in the host and during transitions to and from the host-associated state. Therefore, the
362 goal of the current study was to identify which of the predicted 50 c-di-GMP modulating
363 proteins–DGCs, PDEs, dual-function DGC/PDEs, and likely degenerate enzymes–are able to
364 elicit c-di-GMP-responsive phenotypes in *V. fischeri*. In **Figure 5** we assembled the results from
365 our analyses to demonstrate cellulose production and swimming motility results for all 50 *V.*
366 *fischeri* proteins and the two control proteins. Furthermore, we integrated data from a recent
367 study (52) that examined swimming motility of single-gene mutants in the same *V. fischeri*

368 proteins. The resulting table (**FIG. 5**) presents a powerful visualization of current knowledge of
369 the catalytic potential of these gene families. Conducted in different labs, there is remarkable
370 consistency among the results. There are more proteins with significant effects observed upon
371 overexpression (37/50 in at least one motility assay) compared to gene deletion (20/50), which
372 was expected, and supports our approach to use the overexpression approach to reveal
373 function in a family where redundancy is expected. The two cases in which discrepancies were
374 noted were for DGC VF_A0368, where we had consistent results across all conditions and the
375 deletion approach yielded significant results only on TBS swim plates; and for PDE VF_A0526,
376 in which the deletion approach yielded consistent results and we observed different results
377 swimming on TBS-Mg compared to all other conditions. The only case in which a phenotype
378 was observed solely in the deletion study was for DGC VF_A0476, where a Δ*VF_A0476* strain
379 yielded the unexpected phenotype of elevated motility and we did not observe any significant
380 cellulose or motility phenotypes.
381
382 The proteins we observed to exhibit the strongest phenotypes were DGCs VF_1200,
383 VF_A0057, VF_A0152, VF_A0155, VF_A0276, VF_A0368, VF_A0381, VF_A0567, and
384 VF_A0692; PDEs VF_1603, VF_2480, VF_A0506, and VF_A0551; and dual-function protein
385 VF_0985 (**FIG. 2 and 5**). We also identified interesting phenotypes in DGC VF_0596, which has
386 strong phenotypes in the opposite direction from expected, and in VF_0985, which inhibits
387 motility only when magnesium is absent. For all of these proteins, little work has been
388 conducted on a mechanistic basis, and our study presents candidates to pursue that may
389 mediate relevant signaling in the host or during key lifestyle transitions. A benefit of the
390 overexpression approach is that it can reveal phenotypes that are not apparent during single
391 deletions. For example, a protein that is expressed during host colonization or in a specific
392 environmental condition, but not during culture growth, may be assayed for function in this
393 manner. Conversely, a limitation of our method is that the likely higher levels of each examined

394 protein obscures natural regulation that will certainly be relevant to understand signaling *in vivo*.
395 Therefore, this work pares down a complex family of 50 proteins to a narrower set for more
396 focused individual studies.
397
398 While the data in **Figure 5** are remarkably consistent, it is clear that there are situations in which
399 protein overexpression impacts motility and not cellulose, or motility in some media and not
400 others. In fact, on a fine scale there are 18 different patterns to the overexpression data among
401 *V. fischeri* proteins in **Figure 5**. This result is even more remarkable given that there was no
402 difference in transcriptional regulation in our experimental setup. In addition to the possibility of
403 posttranscriptional differences in expression, our results suggest that local signaling effects—
404 e.g., localization, protein-protein interactions—may play a major role in how these factors
405 mediate host interactions. There is growing evidence for local signaling and regulated activity of
406 distinct DGCs and PDEs (74–76), and unique patterns in our data provide hints as to where
407 such regulation could occur in *V. fischeri*.
408
409 We previously demonstrated that global *V. fischeri* c-di-GMP levels need to be kept sufficiently
410 low in order to successfully colonize squid (36), but the specific signals and pathways involved
411 are yet to be determined. During colonization initiation, *V. fischeri* responds to squid-derived
412 signals to help guide them to the light organ (37, 77–80). Therefore it is likely that one or more
413 squid-specific signals may be sensed by c-di-GMP-modulating proteins to elicit effects on
414 certain biofilm and/or motility pathways. Our study presents a set of DGCs and PDEs that are
415 demonstrably functional in multiple assays in *V. fischeri*, and provide strong candidates as
416 proteins to regulate c-di-GMP levels to facilitate functional and reproducible squid host
417 colonization.
418

419 **MATERIALS AND METHODS**

420

421 **Bacterial strains, plasmids, and media**

422 *V. fischeri* and *E. coli* strains used in this study are listed in Table 1. Plasmids used in this study
423 are listed in Table 2. *V. fischeri* strains were grown at 25°C in Luria-Bertani salt (LBS) medium
424 (per liter: 25 g Difco LB broth [BD], 10 g NaCl, 50 mM Tris buffer [pH 7.5]) or tryptone broth salt
425 (TBS) medium (per liter: 10 g Bacto Tryptone [Gibco], 20 g NaCl, 35 mM $MgSO_4$ [where noted],
426 10 mM $CaCl_2$ [where noted], 50 mM Tris buffer [pH 7.5]) where noted. *E. coli* strains used for
427 cloning and conjugation were grown at 37°C in Luria-Bertani (LB) medium (per liter: 25 g Difco
428 LB broth [BD]). When needed, antibiotics were added to the media at the following
429 concentrations: kanamycin, 100 µg/mL for *V. fischeri* and 50 µg/mL for *E. coli*; gentamicin, 2.5
430 µg/mL for *V. fischeri* and 5 µg/mL for *E. coli*. When needed, 100 µM isopropyl ß-D-1-
431 thiogalactopyranoside (IPTG) was added to the media. For Congo red media, 40 µg/mL Congo
432 red and 15 µg/mL Coomassie blue were added to LBS. When needed, growth media was
433 solidified using 1.5% agar. Plasmids were introduced from *E. coli* strains into *V. fischeri* strains
434 using standard techniques (81, 82).

435

436 **DNA synthesis and sequencing**

437 Primers used in this study are listed in Table 3 and were synthesized by Integrated DNA
438 Technologies (Coralville, IA). Gibson primers for all plasmids except pRYI039 and pEVS143-
439 VF_A0506 were designed using the NEBuilder online tool. Site-directed mutagenesis primers
440 were designed using the NEBaseChanger online tool. Full inserts for cloned constructs and
441 active site mutant constructs were confirmed by Sanger Sequencing at Northwestern University
442 Center for Genetic Medicine, ACGT, Inc. (Wheeling, IL), Functional Biosciences (Madison, WI),
443 and/or whole plasmid sequencing by Plasmidsaurus (Eugene, OR). Sequence data were
444 analyzed using DNASTAR or Benchling. PCR to amplify constructs for cloning and sequencing

445 was performed using Pfx50 DNA Polymerase (Invitrogen) or Q5 High-Fidelity DNA polymerase
446 (NEB). Diagnostic PCR was performed using GoTaq polymerase (Promega).
447
448 **Construction of pRYI039**
449 pEVS143 was amplified using primers RYI354 and RYI355. pRYI039 was assembled by Gibson
450 assembly using the NEBuilder HiFi DNA Assembly Master Mix (NEB). The assembly reaction
451 was transformed into chemically competent NEB5α cells and candidate transformants were
452 selected using kanamycin. The plasmid was screened by PCR using primers M13 -48 rev and
453 MRH049. The plasmid was confirmed by Sanger sequencing using primers M13 -48 rev and
454 MRH049.
455
456 **Construction of the overexpression plasmids**
457 pEVS143 was amplified using pEVS143_expF and pEVS143_expR primers. Gene ORFs were
458 amplified from MJM1100 genomic DNA using gene-specific forward and reverse primers.
459 Amplified gene inserts were assembled with amplified pEVS143 by Gibson assembly using NEB
460 Gibson Assembly Cloning Kit (NEB) or NEBuilder HiFi DNA Assembly Master Mix (NEB). The
461 assembly reactions were transformed into chemically competent NEB5α or DH5α λpir cells and
462 candidate transformants were selected using kanamycin. Each plasmid was screened by PCR
463 using primers MJM-738F and MJM739R; M13 -48 rev and MJM-739R; or MJM-738F and the
464 respective assembly reverse primer, MJM-739R and the assembly forward primer, and/or MJM-
465 738F and MJM739R. To construct pEVS143-VF_A1014, *VF_A1014* was synthesized with
466 flanking AvrII and BamHI restriction sites and cloned into pEVS143 by GenScript (Piscataway,
467 NJ) and plasmid was confirmed by whole plasmid sequencing. pEVS143-VF_A0506 was
468 confirmed by Sanger sequencing using primers M13 -48 rev, RYI225, RYI226, RYI227, RYI228,
469 RYI229, RYI230, and MRH049. Remaining plasmids were confirmed by Sanger sequencing
470 using primers MJM-738F and MJM-739R; MJM-738F and the respective assembly reverse

471 primer and/or MJM-739R and the assembly forward primer; or pEVS143_seqF and
472 pEVS143_seqR.
473
474 **Construction of the DGC and PDE active site mutant overexpression plasmids**
475 Plasmids were amplified using site-directed mutagenesis primers to introduce active site
476 mutations. Active site mutant plasmids were assembled using the Q5 Site-Directed Mutagenesis
477 Kit (NEB). Site-directed mutagenesis reactions were transformed into chemically competent
478 NEB5α or DH5α λpir cells and candidate transformants were selected using kanamycin. Each
479 plasmid was screened by PCR using primers M13 -48 rev and MJM739R; or MJM-738F and the
480 respective assembly reverse primer, MJM-739R and the assembly forward primer, and MJM-
481 738F and MJM739R. pEVS143-VF_0989(GAEEF) was confirmed by Sanger sequencing using
482 primers RYI370 and RYI372. pEVS143-VF_A0057(SADEF) was confirmed by Sanger
483 sequencing using primers RYI365 and RYI366. pEVS143-VF_A1038 was confirmed by Sanger
484 sequencing using primers RYI520, RYI521, RYI522, RYI523, MJM-738F, and MJM-739R.
485 Remaining active site mutant plasmids were confirmed by whole plasmid sequencing or Sanger
486 sequencing using primers MJM-738F and MJM-739R; MJM-738F and the respective template
487 plasmid Gibson assembly reverse primer and/or MJM-739R and the template plasmid Gibson
488 assembly forward primer; or pEVS143_seqF and pEVS143_seqR.
489
490 **Assembly of arrayed libraries**
491 Strains were streaked on LBS agar and incubated overnight at 25°C. Liquid LBS was inoculated
492 with single colonies of each strain in a deep-well 96-well plate and grown overnight at 23-25°C
493 on a shaker. Overnight cultures were saved in glycerol stocks in 96-well plates in triplicate.
494

495 **C-di-GMP quantification**

496 Liquid LBS was inoculated with 5 µL of glycerol stock of each strain from an arrayed library and
497 grown at room temperature (23-25°C) overnight on a shaker. 2 or 4 µL of liquid culture were
498 spotted onto LBS agar and incubated at 25°C for 24 h. For assay of strains not in 96-well format
499 (**FIG. S2B and C**), strains were streaked on LBS agar and single colonies were inoculated into
500 liquid LBS and grown at room temperature (23-25°C) overnight on a shaker. 4 or 8 µL of liquid
501 culture were spotted onto LBS agar and incubated at 25°C for 24 h. For both versions of the
502 assay, spots were resuspended in 500 µL 70% Instant Ocean (IO), then OD_{600}, TurboRFP (555
503 nm excitation/585 nm emission), and AmCyan (453 nm excitation/486 nm emission) for each
504 resuspended spot were measured in triplicate using BioTek Synergy Neo2 plate reader. To
505 calculate c-di-GMP reporter activity, TurboRFP values (reports on c-di-GMP) were normalized
506 to AmCyan values (constitutively expressed).

507

508 **Congo red biofilm assay**

509 Liquid LBS was inoculated with 2 µL of glycerol stock of each strain from an arrayed library and
510 grown at room temperature (23-25°C) overnight on a shaker. 2 µL spots of liquid culture were
511 spotted on LBS Congo red agar and incubated 24 h at 25°C. For VF_A0152 in **FIG. 2A**, 2 or 8
512 µL spots of liquid cultures of *V. fischeri* overexpressing VF_A0152 and *V. fischeri* with the
513 pRYI039 empty vector control were individually spotted onto LBS Congo red agar and incubated
514 24 h at 25°C. Spots were transferred onto white printer paper (83) and images were scanned as
515 TIFF files. Congo red binding was quantified using ImageJ software (version 2.0.0-rc-69/1.52p)
516 as described below.

517

518 **Motility assay**

519 Liquid LBS was inoculated 2 µL of glycerol stock of each strain from an arrayed library and
520 grown at room temperature (23-25°C) overnight on a shaker. 2 µL spots of liquid culture were

521 spotted on TBS, TBS-Mg^{2+} (35 mM $MgSO_4$), and TBS-Ca^{2+} (10 mM $CaCl_2$) agar and incubated
522 overnight at 25°C. The spotted strains were inoculated into TBS, TBS-Mg^{2+}, and TBS-Ca^{2+} soft
523 (0.3%) agar, respectively, using a 96-pin replicator and incubated at 25°C for 3 h for TBS plates
524 and 2.5 h for TBS-Mg^{2+} and TBS-Ca^{2+} plates. Images of plates were taken using a Nikon D810
525 digital camera and diameter of migration was measured using ImageJ software (version 2.0.0-
526 rc-69/1.52p).
527
528 **Data analysis**
529 For all assays, statistical significance was assigned when the 95% confidence interval of the
530 test strain was outside the 95% confidence interval of the empty vector control (pRYI039).
531 Congo red binding was quantified using ImageJ (version 2.0.0-rc-69/1.52p) by subtracting the
532 WT gray value from the mutant gray value and multiplying the value by -1 (36). Fluorescence of
533 strains in liquid culture were measured using a BioTek Synergy Neo2 plate reader. GraphPad
534 Prism was used to generate graphs and conduct statistical analyses. Graphs were further
535 refined in Adobe Illustrator.
536
537 **ACKNOWLEDGMENTS**
538
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540 Kotla for active site mutant construction; and Chris Waters for the pEVS143-QrgB and
541 pEVS143-VC1086 plasmids. This study was funded by NIGMS grant R35 GM148385 to M.J.M.,
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543
544 **Table 1. Strains**
545

Strain	Genotype	Source or Reference(s)

V. fischeri		
MJM1100 = ES114	Natural isolate, squid light-organ (Mandel Lab Stock)	(50, 84)
MJM2775	MJM1100 / pEVS143	(63)
MJM4094	MJM1100 / pRYI039	This study
MJM3822	MJM1100 / pEVS143-QrgB	This study (plasmid from Chris Waters)
MJM3823	MJM1100 / pEVS143-VC1086	This study (plasmid from Chris Waters)
MJM3091	MJM1100 / pEVS143-VF_0087	This study
MJM3092	MJM1100 / pEVS143-VF_0091	This study
MJM3093	MJM1100 / pEVS143-VF_0094	This study
MJM3094	MJM1100 / pEVS143-VF_0355	This study
MJM2766	MJM1100 / pEVS143-VF_0494	This study
MJM2772	MJM1100 / pEVS143-VF_0596	This study
MJM3815	MJM1100 / pEVS143-VF_0985	This study
MJM2773	MJM1100 / pEVS143-VF_0989	This study
MJM3095	MJM1100 / pEVS143-VF_1200	This study
MJM3096	MJM1100 / pEVS143-VF_1245	This study
MJM3097	MJM1100 / pEVS143-VF_1350	This study
MJM3098	MJM1100 / pEVS143-VF_1367	This study
MJM3099	MJM1100 / pEVS143-VF_1515	This study
MJM2774	MJM1100 / pEVS143-VF_1561	This study
MJM3820	MJM1100 / pEVS143-VF_1603	This study
MJM3100	MJM1100 / pEVS143-VF_1639	This study
MJM3932	MJM1100 / pEVS143-VF_2261	This study
MJM3101	MJM1100 / pEVS143-VF_2362	This study
MJM3821	MJM1100 / pEVS143-VF_2480	This study
MJM2767	MJM1100 / pEVS143-VF_A0056	This study
MJM3102	MJM1100 / pEVS143-VF_A0057	This study

MJM2769	MJM1100 / pEVS143-VF_A0152	This study
MJM3103	MJM1100 / pEVS143-VF_A0155	This study
MJM3816	MJM1100 / pEVS143-VF_A0216	This study
MJM3818	MJM1100 / pEVS143-VF_A0244	This study
MJM2770	MJM1100 / pEVS143-VF_A0276	This study
MJM3104	MJM1100 / pEVS143-VF_A0323	This study
MJM3105	MJM1100 / pEVS143-VF_A0342	This study
MJM3106	MJM1100 / pEVS143-VF_A0343	This study
MJM3814	MJM1100 / pEVS143-VF_A0344	This study
MJM3824	MJM1100 / pEVS143-VF_A0344	This study
MJM3933	MJM1100 / pEVS143-VF_A0368	This study
MJM2768	MJM1100 / pEVS143-VF_A0368	This study
MJM3107	MJM1100 / pEVS143-VF_A0381	This study
MJM3108	MJM1100 / pEVS143-VF_A0398	This study
MJM3109	MJM1100 / pEVS143-VF_A0475	This study
MJM3817	MJM1100 / pEVS143-VF_A0476	This study
MJM3110	MJM1100 / pEVS143-VF_A0526	This study
MJM3111	MJM1100 / pEVS143-VF_A0551	This study
MJM3112	MJM1100 / pEVS143-VF_A0567	This study
MJM3113	MJM1100 / pEVS143-VF_A0692	This study
MJM3114	MJM1100 / pEVS143-VF_A0706	This study
MJM3115	MJM1100 / pEVS143-VF_A0796	This study
MJM3116	MJM1100 / pEVS143-VF_A0879	This study
MJM3117	MJM1100 / pEVS143-VF_A0959	This study
MJM3819	MJM1100 / pEVS143-VF_A0976	This study
MJM2771	MJM1100 / pEVS143-VF_A1012	This study
MJM2765	MJM1100 / pEVS143-VF_A1038	This study
MJM3118	MJM1100 / pEVS143-VF_A1076	This study

MJM3119	MJM1100 / pEVS143-VF_A1166	This study
MJM5574	MJM1100 / pEVS143-VF_0091(GAAEF, AAL)	This study
MJM5573	MJM1100 / pEVS143-VF_0091(GAAEF)	This study
MJM5263	MJM1100 / pEVS143-VF_0094(AAL, GADEF)	This study
MJM5095	MJM1100 / pEVS143-VF_0094(AAL)	This study
MJM5096	MJM1100 / pEVS143-VF_0094(GADEF)	This study
MJM5099	MJM1100 / pEVS143-VF_0596(SAEEF)	This study
MJM4092	MJM1100 / pEVS143-VF_0989(GAEEF)	This study
MJM4084	MJM1100 / pEVS143-VF_A0057(SADEF)	This study
MJM5572	MJM1100 / pEVS143-VF_A0152(GADEF)	This study
MJM5256	MJM1100 / pEVS143-VF_A0244(AAL, GADEF)	This study
MJM5097	MJM1100 / pEVS143-VF_A0244(AAL)	This study
MJM5254	MJM1100 / pEVS143-VF_A0244(GADEF)	This study
MJM5255	MJM1100 / pEVS143-VF_A0475(AAL)	This study
MJM5098	MJM1100 / pEVS143-VF_A0475(GADEF)	This study
MJM5100	MJM1100 / pEVS143-VF_A0796(GAEEF)	This study
MJM5354	MJM1100 / pEVS143-VF_A0976(GADEF)	This study
MJM4296	MJM1100 / pEVS143-VF_A1038(AAL)	This study
MJM4010	MJM1100 / pEVS143;pFY4535	This study
MJM4821	MJM1100 / pEVS143-QrgB;pFY4535	This study
MJM4971	MJM1100 / pEVS143-VC1086;pFY4535	This study
MJM4767	MJM1100 / pEVS143-VF_0087;pFY4535	This study
MJM4793	MJM1100 / pEVS143-VF_0091;pFY4535	This study
MJM4794	MJM1100 / pEVS143-VF_0094;pFY4535	This study
MJM4795	MJM1100 / pEVS143-VF_0355;pFY4536	This study
MJM4686	MJM1100 / pEVS143-VF_0494; pFY4535	This study
MJM4791	MJM1100 / pEVS143-VF_0596;pFY4535	This study
MJM4689	MJM1100 / pEVS143-VF_0985; pFY4535	This study

MJM4024	MJM1100 / pEVS143-VF_0989;pFY4535	This study
MJM4796	MJM1100 / pEVS143-VF_1200;pFY4535	This study
MJM4797	MJM1100 / pEVS143-VF_1245;pFY4535	This study
MJM4798	MJM1100 / pEVS143-VF_1350;pFY4535	This study
MJM4687	MJM1100 / pEVS143-VF_1367; pFY4535	This study
MJM4799	MJM1100 / pEVS143-VF_1515;pFY4535	This study
MJM4792	MJM1100 / pEVS143-VF_1561;pFY4535	This study
MJM4769	MJM1100 / pEVS143-VF_1603;pFY4535	This study
MJM4768	MJM1100 / pEVS143-VF_1639;pFY4535	This study
MJM4770	MJM1100 / pEVS143-VF_2261;pFY4535	This study
MJM4800	MJM1100 / pEVS143-VF_2362;pFY4535	This study
MJM4820	MJM1100 / pEVS143-VF_2480;pFY4535	This study
MJM4787	MJM1100 / pEVS143-VF_A0056;pFY4535	This study
MJM4108	MJM1100 / pEVS143-VF_A0057;pFY4535	This study
MJM4788	MJM1100 / pEVS143-VF_A0152;pFY4535	This study
MJM4110	MJM1100 / pEVS143-VF_A0155;pFY4535	This study
MJM4816	MJM1100 / pEVS143-VF_A0216;pFY4535	This study
MJM4818	MJM1100 / pEVS143-VF_A0244;pFY4535	This study
MJM4789	MJM1100 / pEVS143-VF_A0276;pFY4535	This study
MJM4801	MJM1100 / pEVS143-VF_A0323;pFY4535	This study
MJM4802	MJM1100 / pEVS143-VF_A0342;pFY4535	This study
MJM4803	MJM1100 / pEVS143-VF_A0343;pFY4536	This study
MJM4815	MJM1100 / pEVS143-VF_A0344;pFY4535	This study
MJM4822	MJM1100 / pEVS143-VF_A0368;pFY4535	This study
MJM4804	MJM1100 / pEVS143-VF_A0381;pFY4535	This study
MJM4688	MJM1100 / pEVS143-VF_A0398; pFY4535	This study
MJM4805	MJM1100 / pEVS143-VF_A0475;pFY4535	This study
MJM4817	MJM1100 / pEVS143-VF_A0476;pFY4535	This study

MJM4823	MJM1100 / pEVS143-VF_A0506;pFY4535	This study
MJM4806	MJM1100 / pEVS143-VF_A0526;pFY4535	This study
MJM4807	MJM1100 / pEVS143-VF_A0551;pFY4535	This study
MJM4808	MJM1100 / pEVS143-VF_A0567;pFY4535	This study
MJM4809	MJM1100 / pEVS143-VF_A0692;pFY4535	This study
MJM4203	MJM1100 / pEVS143-VF_A0706; pFY4535	This study
MJM4810	MJM1100 / pEVS143-VF_A0796;pFY4535	This study
MJM4811	MJM1100 / pEVS143-VF_A0879;pFY4535	This study
MJM4812	MJM1100 / pEVS143-VF_A0959;pFY4535	This study
MJM4819	MJM1100 / pEVS143-VF_A0976;pFY4535	This study
MJM4790	MJM1100 / pEVS143-VF_A1012;pFY4535	This study
MJM4023	MJM1100 / pEVS143-VF_A1038;pFY4535	This study
MJM4813	MJM1100 / pEVS143-VF_A1076;pFY4535	This study
MJM4814	MJM1100 / pEVS143-VF_A1166;pFY4535	This study
MJM5576	MJM1100 / pFY4535;pEVS143-VF_0091(GAAEF)	This study
MJM5659	MJM1100 / pFY4535;pEVS143-VF_0091(AAL)	This study
MJM5637	MJM1100 / pFY4535;pEVS143-VF_0091(GAAEF,AAL)	This study
MJM5638	MJM1100 / pFY4535;pEVS143-VF_0094(GADEF)	This study
MJM5639	MJM1100 / pFY4535;pEVS143-VF_0094(AAL)	This study
MJM5640	MJM1100 / pFY4535;pEVS143-VF_0094(GADEF,AAL)	This study
MJM5226	MJM1100 / pEVS143-VF_0094(AAL);pFY4535	This study
MJM5227	MJM1100 / pEVS143-VF_0094(GADEF);pFY4535	This study
MJM4969	MJM1100 / pEVS143-0494(AAL);pFY4535	This study
MJM4970	MJM1100 / pEVS143-0494(GADEF);pFY4535	This study
MJM5645	MJM1100 / pFY4535;pEVS143-VF_0494(AAL,GADEF)	This study
MJM5641	MJM1100 / pFY4535;pEVS143-VF_0596(SAEEF)	This study
MJM5642	MJM1100 / pFY4535;pEVS143-VF_0985(GADEF)	This study
MJM5643	MJM1100 / pFY4535;pEVS143-VF_0985(AVL)	This study

MJM5644	MJM1100 / pFY4535;pEVS143-VF_0985(GADEF,AVL)	This study
MJM4107	MJM1100 / pEVS143-VF_0989(GAEEF);pFY4535	This study
MJM5648	MJM1100 / pFY4535;pEVS143-VF_1639(GAEEF)	This study
MJM4109	MJM1100 / pEVS143-VF_A0057(SADEF);pFY4535	This study
MJM5575	MJM1100 / pFY4535;pEVS143-VF_A0152(GADEF)	This study
MJM5655	MJM1100 / pFY4535;pEVS143-VF_A0244(AAL)	This study
MJM5646	MJM1100 / pFY4535;pEVS143-VF_A0244(GADEF)	This study
MJM5656	MJM1100 / pFY4535;pEVS143-VF_A0244(AAL, GADEF)	This study
MJM5647	MJM1100 / pFY4535;pEVS143-VF_A0344(AVL)	This study
MJM5650	MJM1100 / pFY4535;pEVS143-VF_A0475(AAL)	This study
MJM5649	MJM1100 / pFY4535;pEVS143-VF_A0475(GADEF)	This study
MJM5651	MJM1100 / pFY4535;pEVS143-VF_A0475(AAL,GADEF)	This study
MJM5654	MJM1100 / pFY4535;pEVS143-VF_A0796(GAEEF)	This study
MJM5652	MJM1100 / pFY4535;pEVS143-VF_A0976(GADEF)	This study
MJM5653	MJM1100 / pFY4535;pEVS143-VF_A1038(AAL)	This study
MJM4106	Δ6PDE / pRYI039;pFY4535	This study
MJM5861	Δ6PDE / pVF_0087;pFY4535	This study
MJM5884	Δ6PDE / pVF_0091;pFY4535	This study
MJM5862	Δ6PDE / pVF_0494;pFY4535	This study
MJM4399	Δ6PDE / pVF_1367;pFY4535	This study
MJM4400	Δ6PDE / pVF_1603;pFY4535	This study
MJM4401	Δ6PDE / pVF_2480;pFY4535	This study
MJM5863	Δ6PDE / pVF_A0216;pFY4535	This study
MJM5864	Δ6PDE / pVF_A0244;pFY4535	This study
MJM4202	Δ6PDE / pVF_A0506;pFY4535	This study
MJM4204	Δ6PDE / pVF_A0706;pFY4535	This study
MJM5885	Δ6PDE / pVF_A1014;pFY4535	This study
MJM4205	Δ6PDE / pVF_A1038;pFY4535	This study

MJM5866	Δ6PDE / pVF_A1076;pFY4535	This study
MJM5867	Δ6PDE / pVF_A1166;pFY4535	This study
MJM4403	Δ6PDE / pVC1086;pFY4535	This study
E. coli		
MJM534	CC118 λpir / pEVS104	*(81)*
MJM2466	DH5α λpir / pEVS143	*(63)*
MJM3999	NEB5α / pFY4535	*(69)*
MJM4064	NEB5α / pEVS143-ΔGFP-CmR	This study
MJM2470	DH5α λpir / pEVS143-VC1086	This study (plasmid from Chris Waters)
MJM2468	DH5α λpir / pEVS143-QrgB	This study (plasmid from Chris Waters)
MJM2524	NEB5α / pEVS143-VF_0087	This study
MJM2521	NEB5α / pEVS143-VF_0091	This study
MJM2811	NEB5α / pEVS143-VF_0094	This study
MJM2516	NEB5α / pEVS143-VF_0355	This study
MJM2500	NEB5α / pEVS143-VF_0494	This study
MJM2506	NEB5α / pEVS143-VF_0596	This study
MJM2525	NEB5α / pEVS143-VF_0985	This study
MJM2507	NEB5α / pEVS143-VF_0989	This study
MJM2526	NEB5α / pEVS143-VF_1200	This study
MJM2807	NEB5α / pEVS143-VF_1245	This study
MJM2522	NEB5α / pEVS143-VF_1350	This study
MJM2518	NEB5α / pEVS143-VF_1367	This study
MJM2527	NEB5α / pEVS143-VF_1515	This study
MJM2508	NEB5α / pEVS143-VF_1561	This study
MJM3089	NEB5α / pEVS143-VF_1603	This study
MJM2509	NEB5α / pEVS143-VF_1639	This study
MJM2528	NEB5α / pEVS143-VF_2261	This study
MJM2515	NEB5α / pEVS143-VF_2362	This study

MJM2815	NEB5α / pEVS143-VF_2432	This study
MJM3090	NEB5α / pEVS143-VF_2480	This study
MJM2501	NEB5α / pEVS143-VF_A0056	This study
MJM2519	NEB5α / pEVS143-VF_A0057	This study
MJM2503	NEB5α / pEVS143-VF_A0152	This study
MJM2802	NEB5α / pEVS143-VF_A0155	This study
MJM2803	NEB5α / pEVS143-VF_A0216	This study
MJM2806	NEB5α / pEVS143-VF_A0244	This study
MJM2504	NEB5α / pEVS143-VF_A0276	This study
MJM2809	NEB5α / pEVS143-VF_A0323	This study
MJM2833	NEB5α / pEVS143-VF_A0342	This study
MJM2512	NEB5α / pEVS143-VF_A0343	This study
MJM2520	NEB5α / pEVS143-VF_A0344	This study
MJM2502	NEB5α / pEVS143-VF_A0368	This study
MJM2513	NEB5α / pEVS143-VF_A0381	This study
MJM2805	NEB5α / pEVS143-VF_A0398	This study
MJM2523	NEB5α / pEVS143-VF_A0475	This study
MJM2804	NEB5α / pEVS143-VF_A0476	This study
MJM3982	DH5α λpir / pEVS143-VF_A0506	This study
MJM2810	NEB5α / pEVS143-VF_A0526	This study
MJM2514	NEB5α / pEVS143-VF_A0551	This study
MJM2510	NEB5α / pEVS143-VF_A0567	This study
MJM2517	NEB5α / pEVS143-VF_A0692	This study
MJM2529	NEB5α / pEVS143-VF_A0706	This study
MJM2511	NEB5α / pEVS143-VF_A0796	This study
MJM2813	NEB5α / pEVS143-VF_A0879	This study
MJM2808	NEB5α / pEVS143-VF_A0959	This study
MJM2812	NEB5α / pEVS143-VF_A0976	This study

MJM2505	NEB5α / pEVS143-VF_A1012	This study
MJM4907	DH5α λpir / pEVS143-VF_A1014	This study
MJM2499	NEB5α / pEVS143-VF_A1038	This study
MJM2814	NEB5α / pEVS143-VF_A1076	This study
MJM2801	NEB5α / pEVS143-VF_A1166	This study
MJM4921	DH5α λpir / pEVS143-VF_0087(ACL)	This study
MJM5307	NEB5α / pEVS143-VF_0091(AAL)	This study
MJM5551	NEB5α / pEVS143-VF_0091(GAAEF, AAL)	This study
MJM5550	NEB5α / pEVS143-VF_0091(GAAEF)	This study
MJM5248	DH5α λpir / pEVS143-VF_0094(AAL, GADEF)	This study
MJM5038	DH5α λpir / pEVS143-VF_0094(AAL)	This study
MJM5039	DH5α λpir / pEVS143-VF_0094(GADEF)	This study
MJM4922	DH5α λpir / pEVS143-VF_0494(AAL)	This study
MJM4923	DH5α λpir / pEVS143-VF_0494(GADEF)	This study
MJM5042	DH5α λpir / pEVS143-VF_0596(SAEEF)	This study
MJM4935	DH5α λpir / pEVS143-VF_0985(AVL)	This study
MJM5499	NEB5α / pEVS143-VF_0985(GADEF, AVL)	This study
MJM4974	DH5α λpir / pEVS143-VF_0985(GADEF)	This study
MJM4065	NEB5α / pEVS143-VF_0989(GAEEF)	This study
MJM4287	NEB5α / pEVS143-VF_1038(AAL)	This study
MJM4936	DH5α λpir / pEVS143-VF_1367(HAIGK)	This study
MJM3179	NEB5α / pEVS143-VF_1639(GAEEF)	This study
MJM4070	NEB5α / pEVS143-VF_A0057(SADEF)	This study
MJM3180	NEB5α / pEVS143-VF_A0152(GADEF)	This study
MJM5249	DH5α λpir / pEVS143-VF_A0244(AAL, GADEF)	This study
MJM5040	DH5α λpir / pEVS143-VF_A0244(AAL)	This study
MJM5093	DH5α λpir / pEVS143-VF_A0244(GADEF)	This study
MJM5301	NEB5α / pEVS143-VF_A0344(AVL)	This study

MJM4924	DH5α λpir / pEVS143-VF_A0398(GADEF)	This study
MJM5232	DH5α λpir / pEVS143-VF_A0475(AAL, GADEF)	This study
MJM5094	DH5α λpir / pEVS143-VF_A0475(AAL)	This study
MJM5041	DH5α λpir / pEVS143-VF_A0475(GADEF)	This study
MJM5043	DH5α λpir / pEVS143-VF_A0796(GAEEF)	This study

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547 **Table 2. Plasmids**
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Plasmid	**Description**	**Source or Reference(s)**
pEVS104	Conjugal helper plasmid (Kan^R)	(81)
pEVS143	IPTG-inducible overexpression vector (Kan^R)	(63)
pRYI039	Empty vector control for pEVS143 constructs; pEVS143-Δ*gfp-cmR* (Kan^R)	This study
pEVS143-VC1086	pEVS143 carrying *VC1086* (Kan^R)	(57)
pEVS143-QrgB	pEVS143 carrying *QrgB* (Kan^R)	(57)
pEVS143-VF_0087	pEVS143 carrying *VF_0087* (Kan^R)	This study
pEVS143-VF_0091	pEVS143 carrying *VF_0091* (Kan^R)	This study
pEVS143-VF_0094	pEVS143 carrying *VF_0094* (Kan^R)	This study
pEVS143-VF_0355	pEVS143 carrying *VF_0355* (Kan^R)	This study
pEVS143-VF_0494	pEVS143 carrying *VF_0494* (Kan^R)	This study
pEVS143-VF_0596	pEVS143 carrying *VF_0596* (Kan^R)	This study
pEVS143-VF_0985	pEVS143 carrying *VF_0985* (Kan^R)	This study

pEVS143-VF_0989	pEVS143 carrying *VF_0989* (*mifA*) (Kan^R)	This study
pEVS143-VF_1200	pEVS143 carrying *VF_1200* (Kan^R)	This study
pEVS143-VF_1245	pEVS143 carrying *VF_1245* (Kan^R)	This study
pEVS143-VF_1350	pEVS143 carrying *VF_1350* (Kan^R)	This study
pEVS143-VF_1367	pEVS143 carrying *VF_1367* (Kan^R)	This study
pEVS143-VF_1515	pEVS143 carrying *VF_1515* (Kan^R)	This study
pEVS143-VF_1561	pEVS143 carrying *VF_1561* (Kan^R)	This study
pEVS143-VF_1603	pEVS143 carrying *VF_1603* (Kan^R)	This study
pEVS143-VF_1639	pEVS143 carrying *VF_1639* (*casA*) (Kan^R)	This study
pEVS143-VF_2261	pEVS143 carrying *VF_2261* (Kan^R)	This study
pEVS143-VF_2362	pEVS143 carrying *VF_2362* (Kan^R)	This study
pEVS143-VF_2432	pEVS143 carrying *VF_2432* (Kan^R)	This study
pEVS143-VF_2480	pEVS143 carrying *VF_2480* (Kan^R)	This study
pEVS143-VF_A0056	pEVS143 carrying *VF_A0056* (Kan^R)	This study
pEVS143-VF_A0057	pEVS143 carrying *VF_A0057* (Kan^R)	This study
pEVS143-VF_A0152	pEVS143 carrying *VF_A0152* (Kan^R)	This study
pEVS143-VF_A0155	pEVS143 carrying *VF_A0155* (Kan^R)	This study

pEVS143-VF_A0216	pEVS143 carrying *VF_A0216* (Kan^R)	This study
pEVS143-VF_A0244	pEVS143 carrying *VF_A0244* (Kan^R)	This study
pEVS143-VF_A0276	pEVS143 carrying *VF_A0276* (Kan^R)	This study
pEVS143-VF_A0323	pEVS143 carrying *VF_A0323* (Kan^R)	This study
pEVS143-VF_A0342	pEVS143 carrying *VF_A0342* (Kan^R)	This study
pEVS143-VF_A0343	pEVS143 carrying *VF_A0343* (Kan^R)	This study
pEVS143-VF_A0344	pEVS143 carrying *VF_A0344* (Kan^R)	This study
pEVS143-VF_A0368	pEVS143 carrying *VF_A0368* (Kan^R)	This study
pEVS143-VF_A0381	pEVS143 carrying *VF_A0381* (Kan^R)	This study
pEVS143-VF_A0398	pEVS143 carrying *VF_A0398* (Kan^R)	This study
pEVS143-VF_A0475	pEVS143 carrying *VF_A0475* (Kan^R)	This study
pEVS143-VF_A0476	pEVS143 carrying *VF_A0476* (Kan^R)	This study
pEVS143-VF_A0506	pEVS143 carrying *VF_A0506* (Kan^R)	This study
pEVS143-VF_A0526	pEVS143 carrying *VF_A0526* (Kan^R)	This study
pEVS143-VF_A0551	pEVS143 carrying *VF_A0551* (Kan^R)	This study
pEVS143-VF_A0567	pEVS143 carrying *VF_A0567* (Kan^R)	This study
pEVS143-VF_A0692	pEVS143 carrying *VF_A0692* (Kan^R)	This study

pEVS143-VF_A0706	pEVS143 carrying *VF_A0706* (Kan^R)	This study
pEVS143-VF_A0796	pEVS143 carrying *VF_0796* (Kan^R)	This study
pEVS143-VF_A0879	pEVS143 carrying *VF_A0879* (Kan^R)	This study
pEVS143-VF_A0959	pEVS143 carrying *VF_A0959* (*mifB*) (Kan^R)	This study
pEVS143-VF_A0976	pEVS143 carrying *VF_A0976* (Kan^R)	This study
pEVS143-VF_A1012	pEVS143 carrying *VF_A1012* (Kan^R)	This study
pEVS143-A1014	pEVS143 carrying *VF_A1014* (*pdeV*) (Kan^R)	This study
pEVS143-VF_A1038	pEVS143 carrying *VF_A1038* (*binA*) (Kan^R)	This study
pEVS143-VF_A1076	pEVS143 carrying *VF_A1076* (Kan^R)	This study
pEVS143-VF_A1166	pEVS143 carrying *VF_A1166* (*lapD*) (Kan^R)	This study
pEVS143-VF_0087(ACL)	pEVS143 carrying *VF_0087* with ACL active site mutation (Kan^R)	This study
pEVS143-VF_0091(AAL)	pEVS143 carrying *VF_0091* with AAL active site mutation (Kan^R)	This study
pEVS143-VF_0091(GAAEF, AAL)	pEVS143 carrying *VF_0091* with GAAEF and AAL active site mutations (Kan^R)	This study
pEVS143-VF_0091(GAAEF)	pEVS143 carrying *VF_0091* with GAAEF active site mutation (Kan^R)	This study
pEVS143-VF_0094(AAL, GADEF)	pEVS143 carrying *VF_0094* with AAL and GADEF active site mutations (Kan^R)	This study
pEVS143-VF_0094(AAL)	pEVS143 carrying *VF_0094* with AAL active site mutation (Kan^R)	This study
pEVS143-VF_0094(GADEF)	pEVS143 carrying *VF_0094* with GADEF active site mutation (Kan^R)	This study

pEVS143-VF_0494(AAL)	pEVS143 carrying *VF_0494* with AAL active site mutation (Kan^R)	This study
pEVS143-VF_0494(GADEF)	pEVS143 carrying *VF_0494* with GADEF active site mutation (Kan^R)	This study
pEVS143-VF_0596(SAEEF)	pEVS143 carrying *VF_0596* with SAEEF active site mutation (Kan^R)	This study
pEVS143-VF_0985(AVL)	pEVS143 carrying *VF_0985* with AVL active site mutation (Kan^R)	This study
pEVS143-VF_0985(GADEF, AVL)	pEVS143 carrying *VF_0985* with GADEF and AVL active site mutations (Kan^R)	This study
pEVS143-VF_0985(GADEF)	pEVS143 carrying *VF_0985* with GADEF active site mutation (Kan^R)	This study
pEVS143-MifA(GAEEF)	pEVS143 carrying *VF_0989* (*mifA*) with GAEEF active site mutation (Kan^R)	This study
pEVS143-VF_1367(HAIGK)	pEVS143 carrying *VF_1367* with HAIGK active site mutation (Kan^R)	This study
pEVS143-VF_CasA(GAEEF)	pEVS143 carrying *VF_1639* (*casA*) with GAEEF active site mutation (Kan^R)	This study
pEVS143-VF_A0057(SADEF)	pEVS143 carrying *VF_A0057* with SADEF active site mutation (Kan^R)	This study
pEVS143-VF_A0152(GADEF)	pEVS143 carrying *VF_A0152* with GADEF active site mutation (Kan^R)	This study
pEVS143-VF_A0244(AAL, GADEF)	pEVS143 carrying *VF_A0244* with AAL and GADEF active site mutations (Kan^R)	This study
pEVS143-VF_A0244(AAL)	pEVS143 carrying *VF_A0244* with AAL active site mutation (Kan^R)	This study
pEVS143-VF_A0244(GADEF)	pEVS143 carrying *VF_A0244* with GADEF active site mutation (Kan^R)	This study
pEVS143-VF_A0344(AVL)	pEVS143 carrying *VF_A0344* with AVL active site mutation (Kan^R)	This study
pEVS143-VF_A0398(GADEF)	pEVS143 carrying *VF_A0398* with GADEF active site mutation (Kan^R)	This study
pEVS143-VF_A0475(AAL, GADEF)	pEVS143 carrying *VF_A0475* with AAL and GADEF active site mutations (Kan^R)	This study

pEVS143-VF_A0475(AAL)	pEVS143 carrying *VF_A0475* with AAL active site mutation (Kan^R)	This study
pEVS143-VF_A0475(GADEF)	pEVS143 carrying *VF_A0475* with GADEF active site mutation (Kan^R)	This study
pEVS143-VF_A0796(GAEEF)	pEVS143 carrying *VF_A0796* with GAEEF active site mutation (Kan^R)	This study
pEVS143-BinA(AAL)	pEVS143 carrying *VF_A1038* (*binA*) with AAL active site mutation (Kan^R)	This study
pFY4535	c-di-GMP reporter plasmid (Gent^R)	(69)

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550 **Table 3. Primers**
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Primer name	**Sequence (5'-3')**	**Notes**
RYI354	GGAAGCTAAAGATCCGGTGAttgattgagcaa	Forward primer to amplify pEVS143 without *gfp* and *cmR* cassettes for Gibson assembly of pRYI039
RYI355	TCACCGGATCTTTAGCTTCCttagctccctgaattc	Reverse primer to amplify pEVS143 without *gfp* and *cmR* cassettes for Gibson assembly of pRYI039
pEVS143_expF	GATCCGGTGATTGATTGAGCAA	Forward primer to amplify pEVS143 excluding *gfp* and *cmR* for Gibson assembly to insert ORF of gene of interest
pEVS143_expR	TTTAGCTTCCTTAGCTCCTGAATTC	Reverse primer to amplify pEVS143 excluding *gfp* and *cmR* for Gibson assembly to insert ORF of gene of interest
0087_F	gagctaaggaagctaaaATGCAAACTCTCTCATTTAGTG	Forward primer to amplify *VF_0087* for Gibson assembly into pEVS143
0087_R	caatcaatcaccggatcTTACGTCTTAATGTGTAACGATTTAG	Reverse primer to amplify *VF_0087* for Gibson assembly into pEVS143
0091_F	gagctaaggaagctaaaATGACAGTGGTACTTAACAGCC	Forward primer to amplify *VF_0091* for Gibson assembly into pEVS143
0091_R	caatcaatcaccggatcTCACGCCTGAGAACGATGAATC	Reverse primer to amplify *VF_0091* for Gibson assembly into pEVS143
0094_F	gagctaaggaagctaaaATGACAGAACCAACACATAAAAAAC	Forward primer to amplify *VF_0094* for Gibson assembly into pEVS143
0094_R	caatcaatcaccggatcTTATTCTGTTGGCCAATCTTCTAAAG	Reverse primer to amplify *VF_0094* for Gibson assembly into pEVS143
0355_F	gagctaaggaagctaaaATGAGAAAAACGCCTAGTTTAC	Forward primer to amplify *VF_0355* for Gibson assembly into pEVS143

0355_R	caatcaatcaccggatcTTATTTACCCCAACGGCTTCTTC	Reverse primer to amplify *VF_0355* for Gibson assembly into pEVS143
0494_F	gagctaaggaagctaaaATGTCTCTTTCTCAAATACAACATTG	Forward primer to amplify *VF_0494* for Gibson assembly into pEVS143
0494_R	caatcaatcaccggatcTTATCTAGCGCGTTTGTTTTGTAC	Reverse primer to amplify *VF_0494* for Gibson assembly into pEVS143
0596_F	gagctaaggaagctaaaATGATTGAAGTATCCATTGTTGCC	Forward primer to amplify *VF_0596* for Gibson assembly into pEVS143
0596_R	caatcaatcaccggatcCTACGCACTAATGAGTTGCTCAATG	Reverse primer to amplify *VF_0596* for Gibson assembly into pEVS143
0985_F	gagctaaggaagctaaaATGCTGCATAAGTCTGATAAAAG	Forward primer to amplify *VF_0985* for Gibson assembly into pEVS143
0985_R	caatcaatcaccggatcTTATGCATATTTTGCTTTTAATTCAC	Reverse primer to amplify *VF_0985* for Gibson assembly into pEVS143
0989_F	gagctaaggaagctaaaATGAATCTCAAGCAAATAAAATATTTTATC	Forward primer to amplify *VF_0989* (*mifA*) for Gibson assembly into pEVS143
0989_R	caatcaatcaccggatcTCATGCGATTTGATCCATTTCAC	Reverse primer to amplify *VF_0989* (*mifA*) for Gibson assembly into pEVS143
1200_F	gagctaaggaagctaaaATGGCTAAGAATCAATGGAATG	Forward primer to amplify *VF_1200* for Gibson assembly into pEVS143
1200_R	caatcaatcaccggatcTTAATACATGGTTTCTTTGATATGC	Reverse primer to amplify *VF_1200* for Gibson assembly into pEVS143
1245_F	gagctaaggaagctaaaATGCCTCAATCTCGTCTTCAGC	Forward primer to amplify *VF_1245* for Gibson assembly into pEVS143
1245_R	caatcaatcaccggatcTTATATATCTTGATAGTGAGTGATTTTATTACGC	Reverse primer to amplify *VF_1245* for Gibson assembly into pEVS143
1350_F	gagctaaggaagctaaaATGAACCTTAGGCTTTTAACAG	Forward primer to amplify *VF_1350* for Gibson assembly into pEVS143
1350_R	caatcaatcaccggatcTTAAATATCATAAAAGTGATTGCTCTG	Reverse primer to amplify *VF_1350* for Gibson assembly into pEVS143
1367_F	gagctaaggaagctaaaATGTGTGTTTTTTCTTCATTTGTTTC	Forward primer to amplify *VF_1367* for Gibson assembly into pEVS143
1367_R	caatcaatcaccggatcTTACTGATTATTCTCAAAAATAGCTC	Reverse primer to amplify *VF_1367* for Gibson assembly into pEVS143
1515_F	gagctaaggaagctaaaATGAGGTTCGTTTATCTATCCG	Forward primer to amplify *VF_1515* for Gibson assembly into pEVS143
1515_R	caatcaatcaccggatcTCAGCCTCGAATGGTAACTTGA	Reverse primer to amplify *VF_1515* for Gibson assembly into pEVS143
1561_F	gagctaaggaagctaaaATGAGCATTTTTCAAAAAATTGC	Forward primer to amplify *VF_1561* for Gibson assembly into pEVS143
1561_R	caatcaatcaccggatcTTAATTCATATAATGTATATTTGAACTTGAG	Reverse primer to amplify *VF_1561* for Gibson assembly into pEVS143

1603_F	gagctaaggaagctaaaATGTACACATATGTTGCTCGTC	Forward primer to amplify *VF_1603* for Gibson assembly into pEVS143
1603_R	aatcaatcaccggatcCTATTTTGCTATCTCATCTACCC	Reverse primer to amplify *VF_1603* for Gibson assembly into pEVS143
1639_F	gagctaaggaagctaaaATGCCGAAATTTAATTTAAAACATATC	Forward primer to amplify *VF_1639* (*casA*) for Gibson assembly into pEVS143
1639_R	caatcaatcaccggatcTTATGAAAAGTAAACTCGGTTTTTAC	Reverse primer to amplify *VF_1639* (*casA*) for Gibson assembly into pEVS143
2261_F	gagctaaggaagctaaaATGCATTATAAAAAAACGAAAGC	Forward primer to amplify *VF_2261* for Gibson assembly into pEVS143
2261_R	caatcaatcaccggatcCTATGCTAAGTCCTCTAAACTAG	Reverse primer to amplify *VF_2261* for Gibson assembly into pEVS143
2326_F	gagctaaggaagctaaaATGATCTACATGGATGTTTATATG	Forward primer to amplify *VF_2326* for Gibson assembly into pEVS143
2326_R	caatcaatcaccggatcTTATGATTTATCAATTTTCTCTTGGTAC	Reverse primer to amplify *VF_2326* for Gibson assembly into pEVS143
2480_F	gagctaaggaagctaaaATGTATGCATACGTTGCCAGAC	Forward primer to amplify *VF_2480* for Gibson assembly into pEVS143
2480_R	aatcaatcaccggatcCTATTTAGACAAAATACTCTGATACCAC	Reverse primer to amplify *VF_2480* for Gibson assembly into pEVS143
A0056_F	gagctaaggaagctaaaATGTTTCGGTGTTGTATGAGTG	Forward primer to amplify *VF_A0056* for Gibson assembly into pEVS143
A0056_R	caatcaatcaccggatcTCAATCCGTTTCTCGTTTAAGC	Reverse primer to amplify *VF_A0056* for Gibson assembly into pEVS143
A0057_F	gagctaaggaagctaaaATGATACTTCAATGGTTCAGTG	Forward primer to amplify *VF_A0057* for Gibson assembly into pEVS143
A0057_R	caatcaatcaccggatcTTAAACGAGAAACGGATTGATTTC	Reverse primer to amplify *VF_A0057* for Gibson assembly into pEVS143
A0152_F	gagctaaggaagctaaaATGAGGGTAAAAAATATCGTTG	Forward primer to amplify *VF_A0152* for Gibson assembly into pEVS143
A0152_R	caatcaatcaccggatcTTACTCAGATATAAAAACTTGGTTTC	Reverse primer to amplify *VF_A0152* for Gibson assembly into pEVS143
A0155_F	gagctaaggaagctaaaATGAAAAATCGAAGTGCTTTTTTTTATAG	Forward primer to amplify *VF_A0155* for Gibson assembly into pEVS143
A0155_R	caatcaatcaccggatcTTACAATTCAAACCTAACACAG	Reverse primer to amplify *VF_A0155* for Gibson assembly into pEVS143
A0216_F	gagctaaggaagctaaaATGAAAAGTAAAATAAATATAATATTAGTAGATGATATTG	Forward primer to amplify *VF_A0216* for Gibson assembly into pEVS143
A0216_R	caatcaataccggatcTTAACATTGAAGGTTTTCAGTTTC	Reverse primer to amplify *VF_A0216* for Gibson assembly into pEVS143
A0244_F	gagctaaggaagctaaaATGATTATGAATAAATACTTCATTTATCAGCC	Forward primer to amplify *VF_A0244* for Gibson assembly into pEVS143

A0244_R	caatcaatcaccggatcTCATGCTGGGTGAAGCTTATTTTTC	Reverse primer to amplify *VF_A0244* for Gibson assembly into pEVS143
A0276_F	gagctaaggaagctaaaATGAAACCAATCCATATAGACTC	Forward primer to amplify *VF_A0276* for Gibson assembly into pEVS143
A0276_R	caatcaatcaccggatcTTAATCCTCAGGTGAAACAATTTG	Reverse primer to amplify *VF_A0276* for Gibson assembly into pEVS143
A0323_F	gagctaaggaagctaaaATGATCATGACAAATAAGAAAATGC	Forward primer to amplify *VF_A0323* for Gibson assembly into pEVS143
A0323_R	caatcaatcaccggatcTTATATAGAATCAGAGCACTTTTTTG	Reverse primer to amplify *VF_A0323* for Gibson assembly into pEVS143
A0342_F	gagctaaggaagctaaaATGTTTAATGTTAAATTATTTGGATTAGATAAG	Forward primer to amplify *VF_A0342* for Gibson assembly into pEVS143
A0342_R	caatcaatcaccggatcCTAAAAATCATAATTGTTCTTATCTAATGTAAC	Reverse primer to amplify *VF_A0342* for Gibson assembly into pEVS143
A0343_F	gagctaaggaagctaaaATGATTTTTAGCAATGTTGATAATAATAATATG	Forward primer to amplify *VF_A0343* for Gibson assembly into pEVS143
A0343_R	caatcaatcaccggatcTTATCCTTCAAATACCGTTACTTTG	Reverse primer to amplify *VF_A0343* for Gibson assembly into pEVS143
A0344_F	gagctaaggaagctaaaATGAAATTTATTACTAATAAATATATTGTTTATTTGTTG	Forward primer to amplify *VF_A0344* for Gibson assembly into pEVS143
A0344_R	caatcaatcaccggatcTTAATCACGCATTATGGAATGAAAATTATC	Reverse primer to amplify *VF_A0344* for Gibson assembly into pEVS143
A0368_F	gagctaaggaagctaaaATGAAAAAATTAATCTTGCTGCTTG	Forward primer to amplify *VF_A0368* for Gibson assembly into pEVS143
A0368_R	caatcaatcaccggatcTTAATAGATACTGTCGTTCAAAATAAC	Reverse primer to amplify *VF_A0368* for Gibson assembly into pEVS143
A0381_F	gagctaaggaagctaaaATGGATGGCATAATCCAACTCTC	Forward primer to amplify *VF_A0381* for Gibson assembly into pEVS143
A0381_R	caatcaatcaccggatcTTATTCGCTGACGCAGTTACGT	Reverse primer to amplify *VF_A0381* for Gibson assembly into pEVS143
A0398_F	gagctaaggaagctaaaATGGACAGTCTTTTAAATCGAATAG	Forward primer to amplify *VF_A0398* for Gibson assembly into pEVS143
A0398_R	caatcaatcaccggatcTTAATAAGAACATACTTTATTCTTTCCG	Reverse primer to amplify *VF_A0398* for Gibson assembly into pEVS143
A0475_F	gagctaaggaagctaaaTTGAATTCAACAATTTCTTTCTTATATC	Forward primer to amplify *VF_A0475* for Gibson assembly into pEVS143
A0475_R	caatcaatcaccggatcTCACTGTAATTTTCTGCTTTTG	Reverse primer to amplify *VF_A0475* for Gibson assembly into pEVS143
A0476_F	gagctaaggaagctaaaATGCTATCGTTTATTTATATGAGTG	Forward primer to amplify *VF_A0476* for Gibson assembly into pEVS143
A0476_R	caatcaatcaccggatcTTATACTTTATTTCTATTTTTATATTGAAACTTTG	Reverse primer to amplify *VF_A0476* for Gibson assembly into pEVS143

A0506_F	caggagctaaggaagctaaaATGCGTCATTACCTATCTCTAG	Forward primer to amplify *VF_A0506* for Gibson assembly into pEVS143
A0506_R	gctcaatcaatcaccggatcTTAATCGGGATATTCAAGTCGAATATC	Reverse primer to amplify *VF_A0506* for Gibson assembly into pEVS143
A0526_F	gagctaaggaagctaaaATGAGATTAATTGAAGCAAAACTG	Forward primer to amplify *VF_A0526* for Gibson assembly into pEVS143
A0526_R	caatcaatcaccggatcTCAATAATAATTCTTTTTAAAATTTGAAATTGTATTTTTATAATTAAC	Reverse primer to amplify *VF_A0526* for Gibson assembly into pEVS143
A0551_F	gagctaaggaagctaaaATGCTATTGGCAACACAGGACG	Forward primer to amplify *VF_A0551* for Gibson assembly into pEVS143
A0551_R	caatcaatcaccggatcTTAAGCCGCTTGATGATTTTGTTC	Reverse primer to amplify *VF_A0551* for Gibson assembly into pEVS143
A0567_F	gagctaaggaagctaaaATGAAGTGGATAGAAAATGCATC	Forward primer to amplify *VF_A0567* for Gibson assembly into pEVS143
A0567_R	caatcaatcaccggatcTCAAATTTTGTGAAACATATACTTTC	Reverse primer to amplify *VF_A0567* for Gibson assembly into pEVS143
A0692_F	gagctaaggaagctaaaATGACGATGCTAAGACGATTAATG	Forward primer to amplify *VF_A0692* for Gibson assembly into pEVS143
A0692R	caatcaatcaccggatcTCAATTTGCCATAGTGACACGG	Reverse primer to amplify *VF_A0692* for Gibson assembly into pEVS143
A0706_F	gagctaaggaagctaaaGTGTTTAAACGTAAGAATTCGC	Forward primer to amplify *VF_A0706* for Gibson assembly into pEVS143
A0706_R	caatcaatcaccggatcTTATTTATTACGATTGGTATCAATCTG	Reverse primer to amplify *VF_A0706* for Gibson assembly into pEVS143
A0796_F	gagctaaggaagctaaaATGAATTCTGATATGAGTGATTTTCATTG	Forward primer to amplify *VF_A0796* for Gibson assembly into pEVS143
A0796_R	caatcaatcaccggatcTTATGGCATTAAGGTGGTGCAA	Reverse primer to amplify *VF_A0796* for Gibson assembly into pEVS143
A0879_F	gagctaaggaagctaaaATGCCTACTTATACGTTCAAAAAC	Forward primer to amplify *VF_A0879* for Gibson assembly into pEVS143
A0879_R	caatcaatcaccggatcTCAGAACGCTGATAATGCATCAC	Reverse primer to amplify *VF_A0879* for Gibson assembly into pEVS143
A0959_F	gagctaaggaagctaaaATGATTTCTCGCCCATATGTGAG	Forward primer to amplify *VF_A0959* (*mifB*) for Gibson assembly into pEVS143
A0959_R	caatcaatcaccggatcTTACTGCTGATAATAAGCTTTTTTCTC	Reverse primer to amplify *VF_A0959* (*mifB*) for Gibson assembly into pEVS143
A0976_F	gagctaaggaagctaaaGTGATTACATTTGGTAAGTCCAATAAATTATTTTTTTG	Forward primer to amplify *VF_A0976* for Gibson assembly into pEVS143
A0976_R	caatcaatcaccggatcTCATAGCTTTTCAAATACTTTAAATCC	Reverse primer to amplify *VF_A0976* for Gibson assembly into pEVS143

A1012_F	gagctaaggaagctaaaATGTTAACTGACCAAAAAATTTTAATTG	Forward primer to amplify *VF_A1012* for Gibson assembly into pEVS143
A1012_R	caatcaatcaccggatcTCAAATGGTTATTGTTGATACAC	Reverse primer to amplify *VF_A1012* for Gibson assembly into pEVS143
A1038_F	gagctaaggaagctaaaATGCAAAAAACGTTAACGTCTG	Forward primer to amplify *VF_A1038* (*binA*) for Gibson assembly into pEVS143
A1038_R	caatcaatcaccggatcTTACACAAAGTGAAAGTAGGGG	Reverse primer to amplify *VF_A1038* (*binA*) for Gibson assembly into pEVS143
A1076_F	gagctaaggaagctaaaATGTTCTCAATTAAGAAATTGGTTAATTTTATG	Forward primer to amplify *VF_A1076* for Gibson assembly into pEVS143
A1076_R	caatcaatcaccggatcTTAAGGAATAAGTAGCGGTCTTC	Reverse primer to amplify *VF_A1076* for Gibson assembly into pEVS143
A1166_F	gagctaaggaagctaaaATGACATTATATAAACAACTAGTAGC	Forward primer to amplify *VF_A1166* (*lapD*) for Gibson assembly into pEVS143
A1166_R	caatcaatcaccggatcTTAAATGCCTTCCACTTTTTCATTAATAAAG	Reverse primer to amplify *VF_A1166* (*lapD*) for Gibson assembly into pEVS143
A0152_mutF	TCGCTTGGGCGCTGACGAGTTTG	Forward primer to amplify pEVS143-A0152 for site-directed mutagenesis
A0152_mutR	GCGAAATGATCAGATTCACAG	Reverse primer to amplify pEVS143-A0152 for site-directed mutagenesis
0087(ACL)_F_V2	agcatcaacaGCAtgtctaatgcg	Forward primer to amplify pEVS143-0087 for site-directed mutagenesis to introduce ACL active site change
0087(ACL)_R_V2	agctcatgagtatgcgctttataaataggt	Reverse primer to amplify pEVS143-0087 for site-directed mutagenesis to introduce ACL active site change
0091(GAAEF)_F	CGCATTGGTGcaGCTGAGTTTG	Forward primer to amplify pEVS143-0091 for site-directed mutagenesis to introduce GAAEF active site change
0091(GAAEF)_R	GGCCATTTCTATATTTTGATC	Reverse primer to amplify pEVS143-0091 for site-directed mutagenesis to introduce GAAEF active site change
0091(AAL)_F	CATGGTGCAGcAGCGTTAATTC	Forward primer to amplify pEVS143-0091 for site-directed mutagenesis to introduce AAL active site change
0091(AAL)_R	AATTTTACCGTCTTCAAAATTC	Reverse primer to amplify pEVS143-0091 for site-directed mutagenesis to introduce AAL active site change
0094(AAL)_F	ATCGGTGCGGcAGCATTAATTC	Forward primer to amplify pEVS143-0094 for site-directed mutagenesis to introduce AAL active site change
0094(AAL)_R	GGTTTTTCCACTCTTTATATCAATTATTG	Reverse primer to amplify pEVS143-0094 for site-directed mutagenesis to introduce AAL active site change

0094(GADEF)_F	CGAGTTGGTGcaGATGAATTCGC	Forward primer to amplify pEVS143-0094 for site-directed mutagenesis to introduce GADEF active site change
0094(GADEF)_R	AGCAATGGCAATGTTGTC	Reverse primer to amplify pEVS143-0094 for site-directed mutagenesis to introduce GADEF active site change
0494(AAL)_F	TAATGGCGCAgcaGCCCTTGTTC	Forward primer to amplify pEVS143-0494 for site-directed mutagenesis to introduce AAL active site change
0494(AAL)_R_V2	attctaaaatcacgagcacttacttttggttggtac	Reverse primer to amplify pEVS143-0494 for site-directed mutagenesis to introduce AAL active site change
0494(GADEF)_F	CCATTTAGGTgcaGATGAATTTGGA ATACTATTTC	Forward primer to amplify pEVS143-0494 for site-directed mutagenesis to introduce GADEF active site change
0494(GADEF)_R	CCAACAACAGCGTGTTTTG	Reverse primer to amplify pEVS143-0494 for site-directed mutagenesis to introduce GADEF active site change
0596(SAEEF)_F	CGTTTTAGTGcaGAAGAATTTTTAAT TTTATTTAC	Forward primer to amplify pEVS143-0596 for site-directed mutagenesis to introduce SAEEF active site change
0596(SAEEF)_R	AGCAACAAAATTAGTATCTG	Reverse primer to amplify pEVS143-0596 for site-directed mutagenesis to introduce SAEEF active site change
0985(AVL)_F_V2	TTATGGTGTAgcaGTGCTTTCTCG	Forward primer to amplify pEVS143-0985 for site-directed mutagenesis to introduce AVL active site change
0985(AVL)_R_V2	ATCTCACCTGTTTTAGTATC	Reverse primer to amplify pEVS143-0985 for site-directed mutagenesis to introduce AVL active site change
0985(GADEF)_F	ACGTTTTGGCgcaGATGAATTTG	Forward primer to amplify pEVS143-0985 for site-directed mutagenesis to introduce GADEF active site change
0985(GADEF)_R	GCAACTATTTCATCTTTTTCAATAAA G	Reverse primer to amplify pEVS143-0985 for site-directed mutagenesis to introduce GADEF active site change
1367(HAIGK)_F	TTCGCTGCATgcaATTGGAAAGATT G	Forward primer to amplify pEVS143-1367 for site-directed mutagenesis to introduce HAIGK active site change
1367(HAIGK)_R	GCAATCATACGAATTTCTTC	Reverse primer to amplify pEVS143-1367 for site-directed mutagenesis to introduce HAIGK active site change
A0057(SADEF)_F	TCGTCTATCTgcaGATGAGTTTTTAC TTGG	Forward primer to amplify pEVS143-A0057 for site-directed mutagenesis to introduce SADEF active site change

A0057(SADEF)_R	GCGACAAGATCGTTTTCAAAAAC	Reverse primer to amplify pEVS143-A0057 for site-directed mutagenesis to introduce SADEF active site change
A0244(GADEF)_F	CGTTTTGGTGcAGATGAGTTTATTTTATG	Forward primer to amplify pEVS143-A0244 for site-directed mutagenesis to introduce GADEF active site change
A0244(GADEF)_R	AATTAATAAATCTTCTTTTCTTAAGTATTTATTAATTAAAC	Reverse primer to amplify pEVS143-A0244 for site-directed mutagenesis to introduce GADEF active site change
A0244(AAL)_F	GTTTCTTACGcAGCATTAATTAGATTTAAAG	Forward primer to amplify pEVS143-A0244 for site-directed mutagenesis to introduce AAL active site change
A0244(AAL)_R	AATAGAACCTTCAAAGAAGTTAG	Reverse primer to amplify pEVS143-A0244 for site-directed mutagenesis to introduce AAL active site change
A0344(AVL)_F	ATTGGAGGGGcaGTATTAGCTC	Forward primer to amplify pEVS143-A0344 for site-directed mutagenesis to introduce AVL active site change
A0344(AVL)_R	GATATTTTCATTAGCATCAACAATAG	Reverse primer to amplify pEVS143-A0344 for site-directed mutagenesis to introduce AVL active site change
A0398(GAEEF)_F	ACGTTATGGAgcaGAAGAGTTTC	Forward primer to amplify pEVS143-A0398 for site-directed mutagenesis to introduce GAEEF active site change
A0398(GAEEF)_R	AGTGTATAGTGAGAAGGTG	Reverse primer to amplify pEVS143-A0398 for site-directed mutagenesis to introduce GAEEF active site change
A0475(AAL)_F	ATCAGTGTTGcAGCTTTACTAAG	Forward primer to amplify pEVS143-A0475 for site-directed mutagenesis to introduce AAL active site change
A0475(AAL)_R	AATACTTCCATTCTGTACTTTTG	Reverse primer to amplify pEVS143-A0475 for site-directed mutagenesis to introduce AAL active site change
A0475(GADEF)_F	AGATACGGTGcaGATGAATTTTTGATTTTTAC	Forward primer to amplify pEVS143-A0475 for site-directed mutagenesis to introduce GADEF active site change
A0475(GADEF)_R	TATGATAAGGTCTTCCTTTC	Reverse primer to amplify pEVS143-A0475 for site-directed mutagenesis to introduce GADEF active site change
A0796(GAEEF)_F	CGATATGGTGcaGAAGAGTTTACC	Forward primer to amplify pEVS143-A0796 for site-directed mutagenesis to introduce GAEEF active site change
A0796(GAEEF)_R	CCCACAAATATCTGTGTTAC	Reverse primer to amplify pEVS143-A0796 for site-directed mutagenesis to introduce GAEEF active site change

A0976(GADEF)_F	CGATTAGGTGcaGATGAATTTGCC	Forward primer to amplify pEVS143-A0976 for site-directed mutagenesis to introduce GADEF active site change
A0976(GADEF)_R	AGCGACGTAACTATTTTC	Reverse primer to amplify pEVS143-A0976 for site-directed mutagenesis to introduce GADEF active site change
BinA(AAL)_F	attggttgtGCAgcgctattac	Forward primer to amplify pEVS143-BinA for site-directed mutagenesis to introduce AAL active site change
BinA(AAL)_R	CCATTTTTTATTTACTGGGC	Reverse primer to amplify pEVS143-BinA for site-directed mutagenesis to introduce AAL active site change
MifA(GAEEF)_F	AAGAATTGGCgcaGAAGAGTTTG	Forward primer to amplify pEVS143-MifA for site-directed mutagenesis to introduce GAEEF active site change
MifA(GAEEF)_R	GCTACAAAATCAATACTTCG	Reverse primer to amplify pEVS143-MifA for site-directed mutagenesis to introduce GAEEF active site change
pEVS143_seqF	gcactcccgttctggata	Forward primer to amplify gene inserts in pEVS143
pEVS143_seqR	gtatgagtcagcaacacc	Reverse primer to amplify gene inserts in pEVS143
MJM-738_F	acaatttcacacaggaaacagctc	Forward primer to amplify gene inserts in pEVS143
MJM-739_R	agccagtaatcgaattggctagta	Reverse primer to amplify gene inserts in pEVS143
M13 -48 rev	agcggataacaatttcacacagg	Forward primer to amplify gene inserts in pEVS143
MRH049	aggaaagtctacacgaaccct	Reverse primer to amplify gene inserts in pEVS143
RYI231	atgcgtcattacctatctctagtttgtg	Sequencing primer for *VF_A0506*
RYI232	tgatacctacagcaacgataggtagc	Sequencing primer for *VF_A0506*
RYI233	gctacctatcgttgctgtaggtatca	Sequencing primer for *VF_A0506*
RYI234	cgatagcttcagtgttatccaaggaaag	Sequencing primer for *VF_A0506*
RYI235	gggatcatcgttactcagtacattgc	Sequencing primer for *VF_A0506*
RYI236	ggcaagttaacaccagaagaaagaact	Sequencing primer for *VF_A0506*
RYI365	gatgggttaacacaattagctaaccgt	Sequencing primer for *VF_A0057*
RYI366	ttaaacgagaaacggattgatttcttttgc	Sequencing primer for *VF_A0057*
RYI370	cagagttgggaaggtgaagttgtt	Sequencing primer for *mifA*

RYI372	tcatgcgatttgatccatttcactgg	Sequencing primer for *mifA*
RYI520	tgatcgcctattgtgggttacctc	Sequencing primer for *binA*
RYI521	acttttgcatatacattttgctaatagccgt	Sequencing primer for *binA*
RYI522	agtcgtcattatctgaacacaaaattgaacg	Sequencing primer for *binA*
RYI523	gtggcttaatttctgatgaaccgct	Sequencing primer for *binA*

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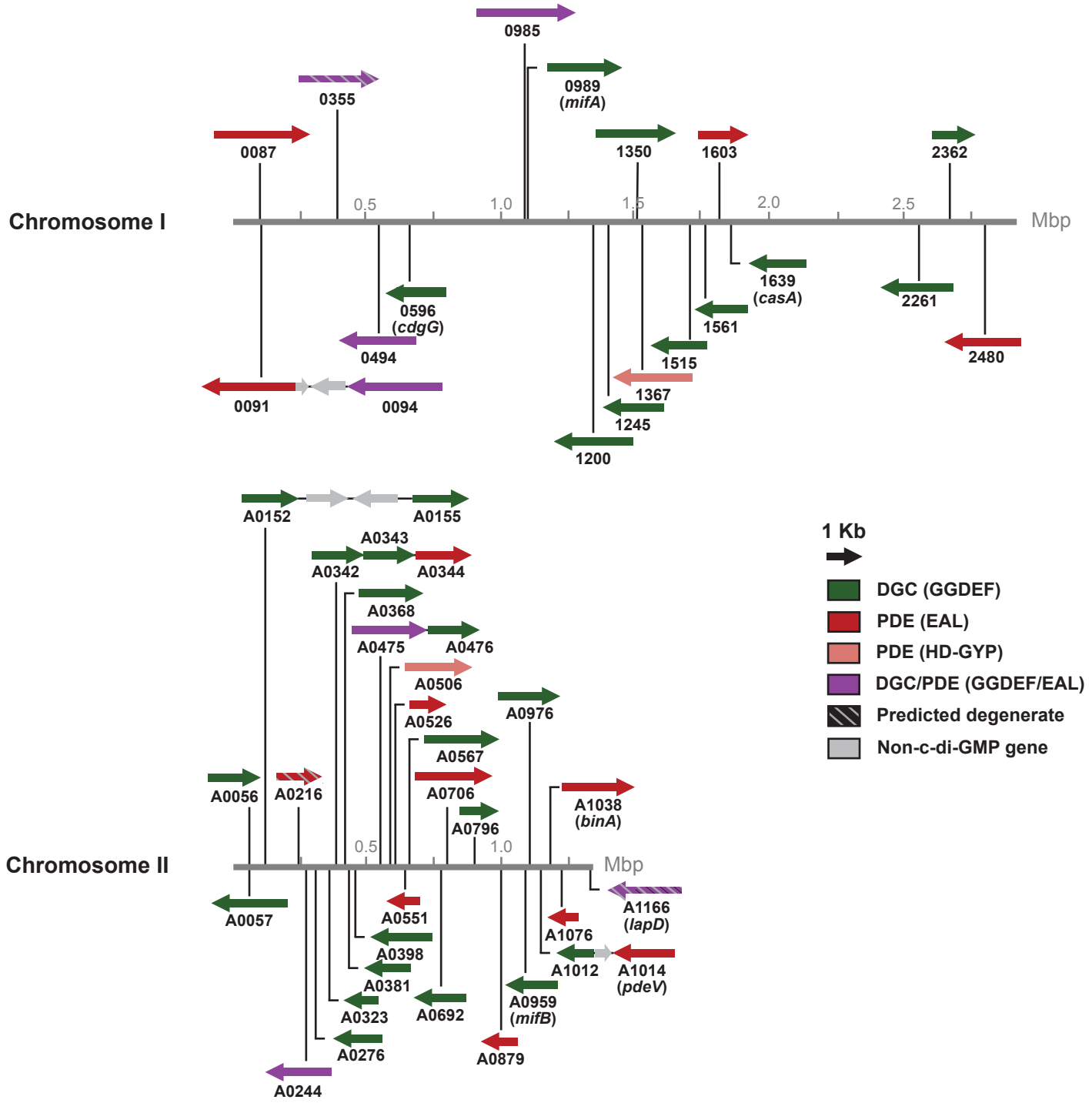

FIG 1. *V. fischeri* encodes 50 proteins across both chromosomes predicted to modulate c-di-GMP levels. The circular chromosomes are represented in a linear fashion for this representation. Numbers represent VF_ locus tags (e.g., VF_0087, VF_A0056, etc.).

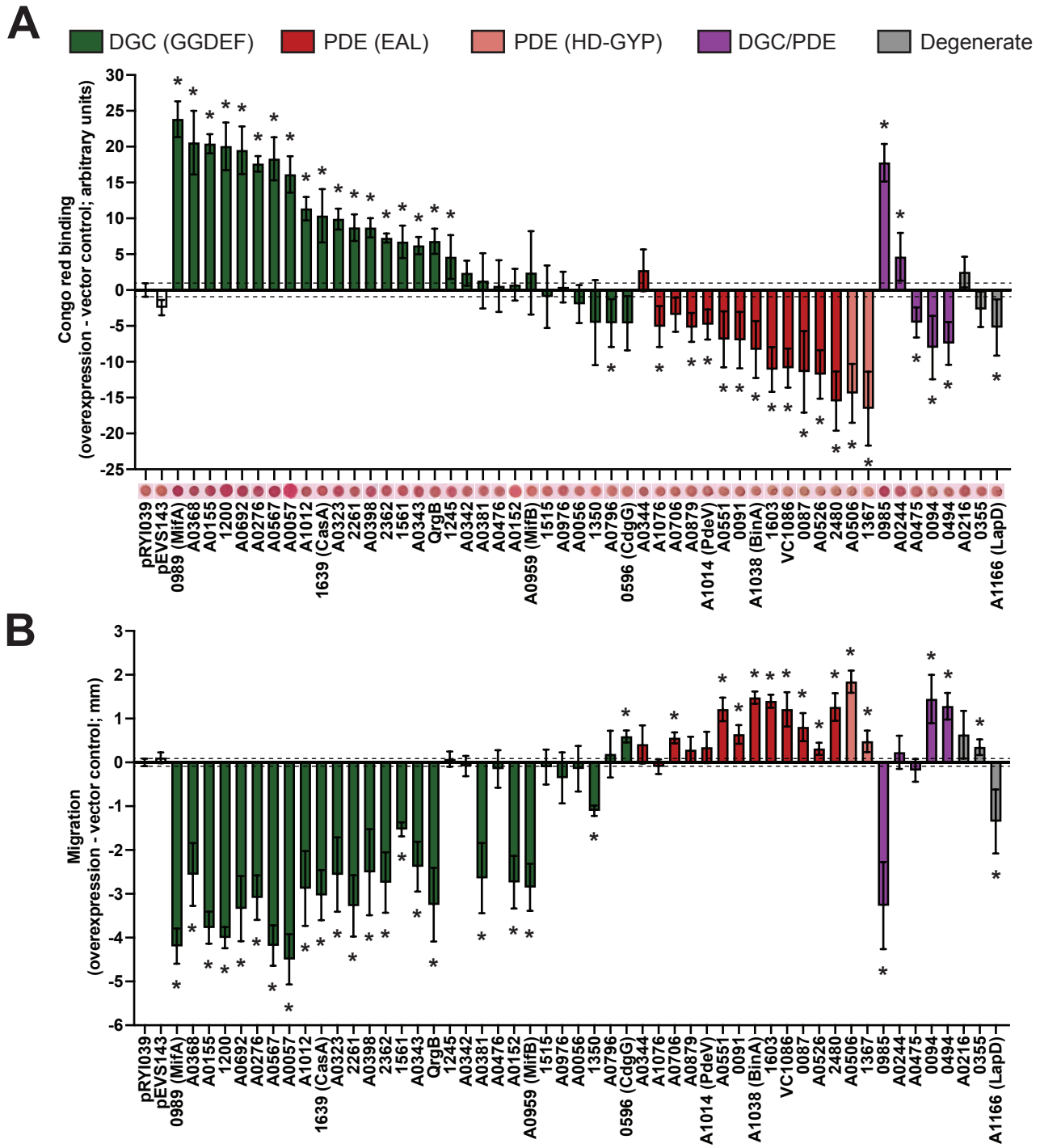

FIG 2. Many predicted *V. fischeri* DGCs and PDEs impact biofilm formation and swimming motility when overexpressed. A. Quantification of Congo red binding for *V. fischeri* strains overexpressing the indicated proteins relative to the pRYI039 empty vector control. For each strain, n = 5 biological (15 for controls) and n = 3-5 technical replicates per biological replicate. Congo red images are representative. **B.** Quantification of migration through soft (0.3%) agar for *V. fischeri* strains overexpressing the indicated proteins relative to the pRYI039 empty vector control. For each strain, n = 4 biological (12 for controls) and n = 3-5 technical replicates per biological replicate. For A and B, each bar represents the means of biological replicates, error bars represent 95% confidence interval, dashed lines denote the upper and lower limits of the 95% confidence interval of a strain overexpressing the empty vector control (pRYI039), * denotes statistical significance (95% confidence interval of the test strain was outside the 95% confidence interval of the empty vector control pRYI039), numbers represent VF_ locus tags (e.g., VF_0087, VF_A0056, etc.); non-*V. fischeri* controls QrgB and VC1086 are also listed.

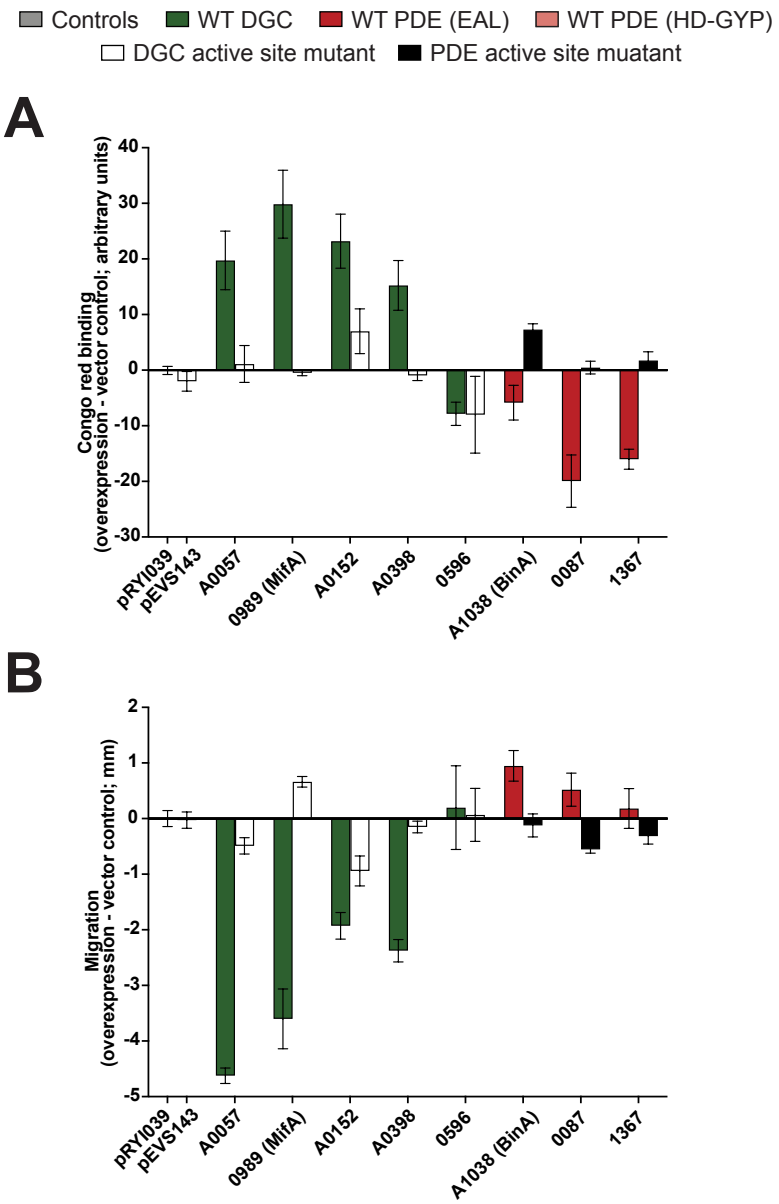

FIG 3. Active sites are required to modulate cellulose production and motility for selected DGCs and PDEs. A. Quantification of Congo red binding for *V. fischeri* strains overexpressing the indicated proteins relative to the pRYI039 empty vector control. For each strain, n = 4-5 biological (10 for controls) and n = 4-5 technical replicates per biological replicate. **B.** Quantification of migration through soft (0.3%) agar for *V. fischeri* strains overexpressing the indicated proteins relative to the pRYI039 empty vector control. For each strain, n = 5 biological (10 for controls) and n = 3-5 technical replicates per biological replicate. For A and B, each bar represents the means of biological replicates, error bars represent 95% confidence interval, dashed lines denote the upper and lower limits of the 95% confidence interval of a strain overexpressing the empty vector control (pRYI039), numbers represent VF_ locus tags (e.g., VF_0087, VF_A0056, etc.); non-*V. fischeri* controls QrgB and VC1086 are also listed.

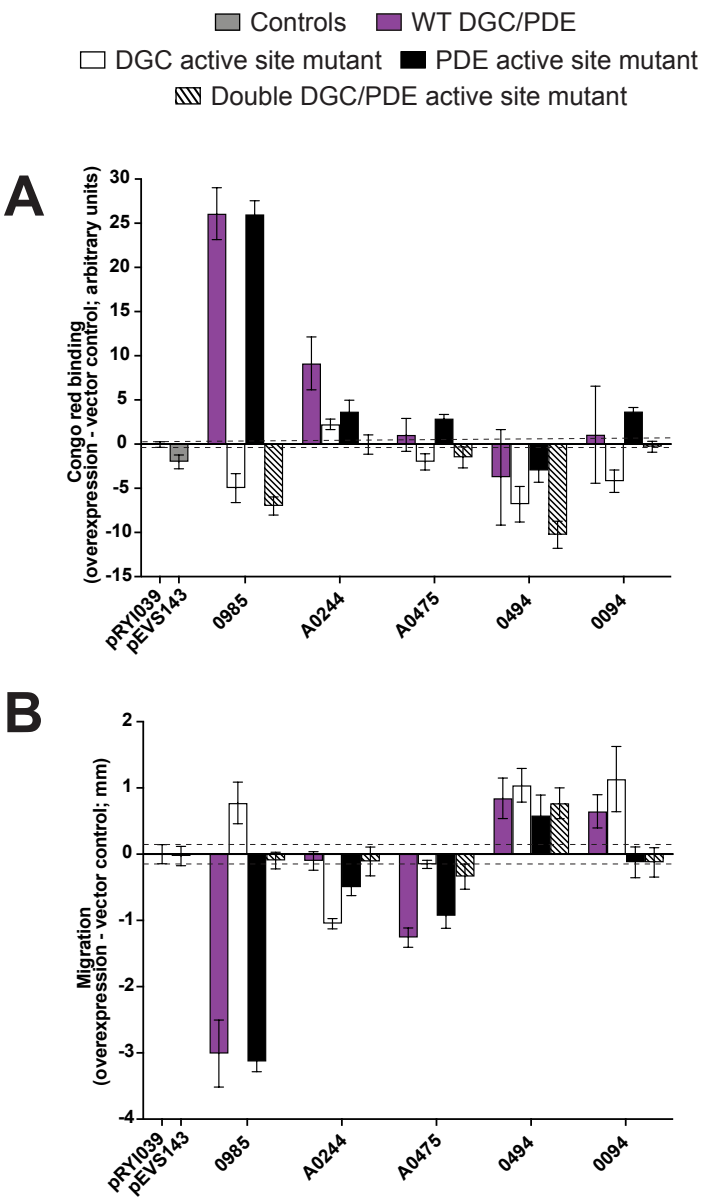

FIG 4. Dual-function protein VF_0985 predominantly functions as a DGC. A. Quantification of Congo red binding for *V. fischeri* strains overexpressing the indicated proteins relative to the pRYI039 empty vector control. For each strain, n = 4-5 biological (10 for controls) and n = 4-5 technical replicates per biological replicate.
B. Quantification of migration through soft (0.3%) agar for *V. fischeri* strains overexpressing the indicated proteins relative to the pRYI039 empty vector control. For each strain, n = 5 biological (10 for controls) and n = 3-5 technical replicates per biological replicate. For A and B, each bar represents the means of biological replicates, error bars represent 95% confidence interval, dashed lines denote the upper and lower limits of the 95% confidence interval of a strain overexpressing the empty vector control (pRYI039), numbers represent VF_ locus tags (e.g., VF_0087, VF_A0056, etc.); non-*V. fischeri* controls QrgB and VC1086 are also listed.

		Overexpression[a]				Deletion[b]		
			Motility			Motility		
	Protein	CR	TBS	Mg	Ca	TBS	Mg	Ca
DGCs (GGDEF)	0596 (CdgG)		+	+	+	-	-	-
	0989 (MifA)	+	-	-	-	+		+
	1200	+	-	-	-	+		+
	1245	+						
	1350		-	-				
	1515							
	1561	+	-	-	-			
	1639 (CasA)	+	-	-	-			+
	2261	+	-	-	-	+		
	2362	+	-	-	-			
	A0056							
	A0057	+	-	-	-			
	A0152		-	-	-			
	A0155	+	-	-	-			+
	A0276	+	-	-	-			+
	A0323	+	-	-	-			
	A0342							
	A0343	+	-	-	-			
	A0368	+	-	-	-	-		
	A0381		-	-	-	+		+
	A0398	+	-	-	-			
	A0476							-
	A0567	+	-	-	-			
	A0692	+	-	-	-			
	A0796	-						
	A0959 (MifB)		-	-	-	+	+	+
	A0976				+			-
	A1012	+	-	-	-			
	QrgB	+	-	-	-	N/A		
PDEs (EAL)	0087	-	+		+			
	0091	-	+					
	1603	-	+		+	-	-	-
	2480	-	+		+	-	-	-
	A0344			-	+			
	A0526	-	+	-	+	-	-	-
	A0551	-	+		+			
	A0706		+					
	A0879	-						
	A1014 (PdeV)	-						
	A1038 (BinA)	-	+		+			
	A1076	-			-			
	VC1086	-	+	+	+	N/A		
PDEs (HD-GYP)	1367	-	+	+				
	A0506	-	+	+	+			
DGC/PDEs	0094	-	+		+			-
	0494	-	+		+	-	-	-
	0985	+	-		-	+		
	A0244	+						
	A0475	-						
Degenerate	0355		+					-
	A0216							
	A1166 (LapD)	-	-	-	-			

FIG 5. Integration of phenotypic data for the V. fischeri DGCs and PDEs. Numbers represent VF_ locus tags (e.g., VF_0087, VF_A0056, etc.); non-*V. fischeri* controls QrgB and VC1086 are also listed. Overexpression data are from this study; deletion data are integrated from Shrestha *et al*., 2022. Olive coloring indicates phenotypes expected from elevated c-di-GMP, whereas orange indicates phenotypes expected from reduced c-di-GMP. White indicates no significant change. [a]This study, [b]Shrestha *et al*. 2022.

A

	Protein	Motility TBS	Mg	Ca
	0596 (CdgG)	+	+	+
	0989 (MifA)	-	-	-
	1200	-	-	-
	1245			
	1350	-	-	
	1515			
	1561	-	-	-
	1639 (CasA)	-	-	-
	2261	-	-	-
	2362	-	-	-
	A0056			
	A0057	-	-	-
	A0152	-	-	-
DGCs (GGDEF)	A0155	-	-	-
	A0276	-	-	-
	A0323	-	-	-
	A0342			
	A0343	-	-	-
	A0368	-	-	-
	A0381	-	-	-
	A0398	-	-	-
	A0476			
	A0567	-	-	-
	A0692	-	-	-
	A0796			
	A0959 (MifB)	-	-	-
	A0976			+
	A1012	-	-	-
	QrgB	-	-	-
	0087	+		+
	0091	+		
	1603	+		+
	2480	+		+
	A0344		-	+
	A0526	+	-	+
PDEs (EAL)	A0551	+		+
	A0706	+		
	A0879			
	A1014 (PdeV)			
	A1038 (BinA)	+		+
	A1076			-
	VC1086	+	+	+
PDEs (HD-GYP)	1367	+	+	
	A0506	+	+	+
	0094	+		+
	0494	+		+
DGC/PDEs	0985	-		-
	A0244			
	A0475			
	0355	+		
Degenerate	A0216			
	A1166 (LapD)	-	-	-

B

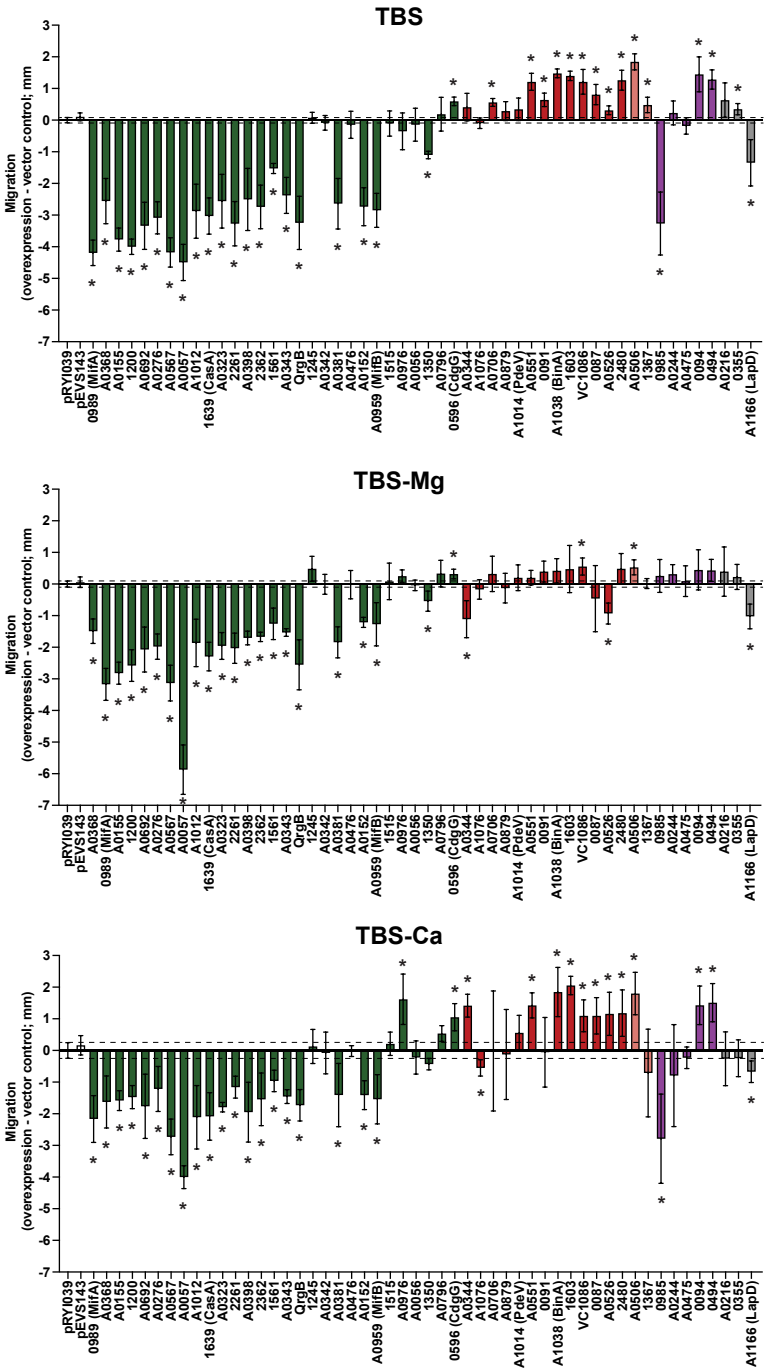

FIG S1. Many predicted *V. fischeri* DGCs and PDEs swimming motility when overexpressed. A. Summary of motility results for overexpression of indicated proteins in TBS, TBS-Mg, and TBS-Mg soft (0.3%) agar. Olive coloring indicates phenotypes expected from elevated c-di-GMP, whereas orange indicates phenotypes expected from reduced c-di-GMP. White indicates no significant change. **B.** Quantification of migration through TBS, TBS-Mg, and TBS-Ca soft (0.3%) agar for *V. fischeri* strains overexpressing the indicated proteins relative to the pRYI039 empty vector control. For each strain, n = 4-5 biological (15 for controls) and n = 3-5 technical replicates per biological replicate, bars represent the means of biological replicates, error bars represent 95% confidence interval, dashed lines denote the upper and lower limits of the 95% confidence interval of a strain overexpressing the empty vector control (pRYI039), * denotes statistical significance (95% confidence interval of the test strain was outside the 95% confidence interval of the empty vector control pRYI039). Data for TBS graph is the same as represented in Fig. 2B. For A and B, numbers represent VF_ locus tags (e.g., VF_0087, VF_A0056, etc.); non-*V. fischeri* controls QrgB and VC1086 are also listed.

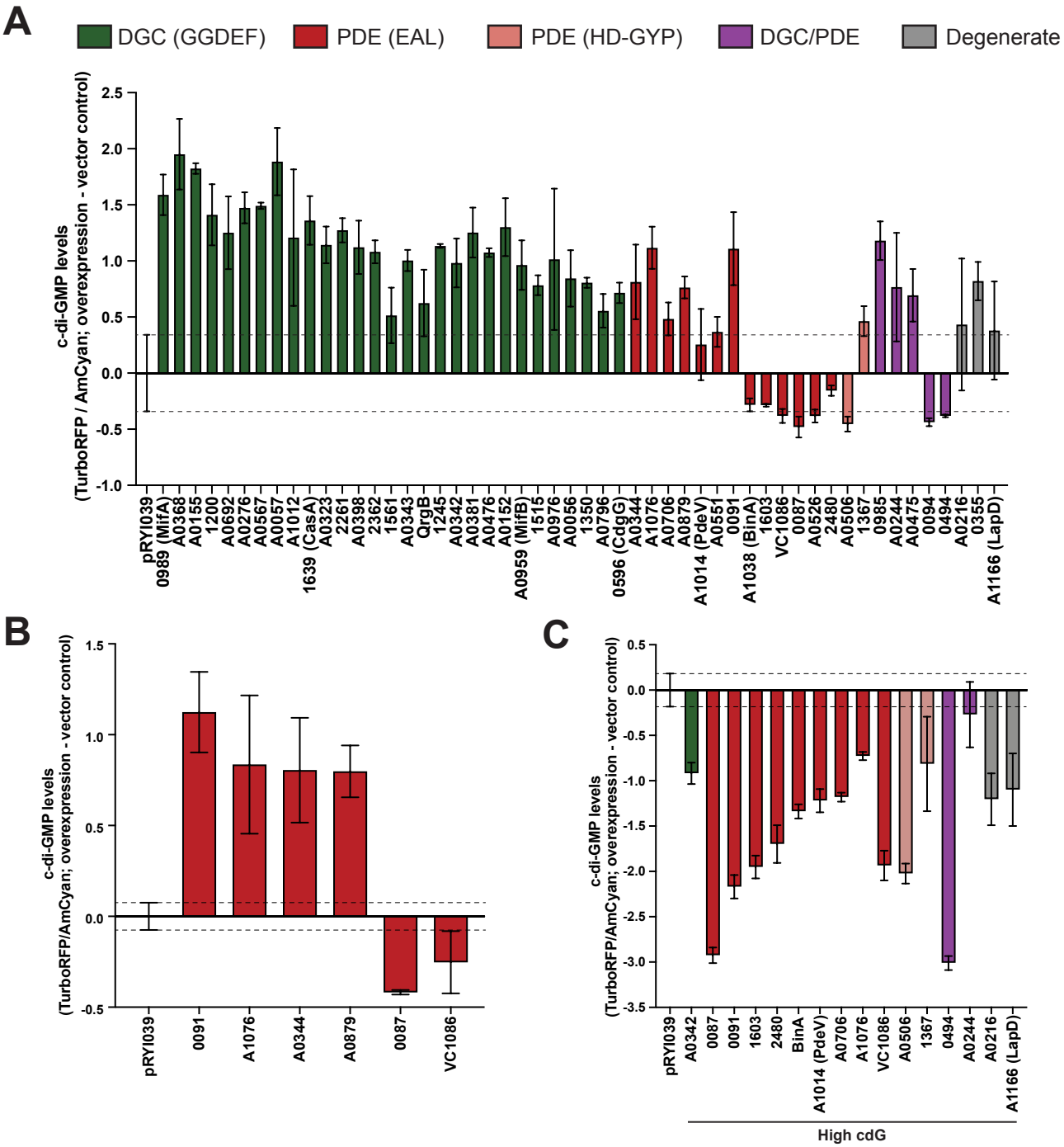

FIG S2. C-di-GMP biosensor activity. Quantification of c-di-GMP concentration for *V. fischeri* strains overexpressing the indicated proteins using the pFY4535 c-di-GMP reporter plasmid. **A.** Assays performed in 96-well format. Values are relative to the pRYI039 empty vector control. For each strain, n = 3 (9 for controls) biological and n = 3 technical replicates per biological replicate, each bar represents the means of biological replicates, error bars represent the 95% confidence interval. Dashed lines denote the upper and lower limits of the 95% confidence interval of a strain overexpressing the empty vector control (pRYI039). **B.** Assays performed in non-96-well format. Values are relative to the pRYI039 empty vector control. For each strain, n = 3 biological and n = 3 technical replicates per biological replicate, each bar represents the means of biological replicates, error bars represent the 95% confidence interval. **C.** Assays of indicated proteins overexpressed in a high c-di-GMP background. For each strain, n = 3-9 biological and n = 3 technical replicates per biological replicate, each bar represents the means of biological replicates, error bars represent the 95% confidence interval. For A to C, constitutive AmCyan was used to normalize TurboRFP to cell density and numbers represent VF_ locus tags (e.g., VF_0087, VF_A0056, etc.); non-*V. fischeri* controls QrgB and VC1086 are also listed.