Evaluation of vectors for gene expression in *Pseudovibrio* bacteria and their application in *Aplysina* marine sponge studies

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Abstract

The filter feeding capacity of marine sponges contributes to biogeochemical cycling and they are also involved in habitat formation, properties that are critical to marine ecology. Sponge-associated microbes are crucial to the functional roles provided by sponges. α-Proteobacteria belonging to the *Pseudovibrio* genus have been isolated from many different marine sponge genera and have been proposed to contribute to sponge health. We recently reported specialized metabolites we named pseudovibriamides from *Pseudovibrio brasiliensis* Ab134. The pseudovibriamide encoding *ppp* gene cluster is found in two thirds of *Pseudovibrio* genomes. Pseudovibriamides coordinate motility and biofilm formation, behaviors that are known to be important for host colonization. Although reverse genetics methods to delete genes via homologous recombination have been established, no self-replicative vectors have been reported for *Pseudovibrio*. We show that plasmid vectors containing three different broad-host-range replicons, RSF1010, RK2, and pBBR1, can be used in *P. brasiliensis* for fluorescent protein expression and consequent labeling. We then applied GFP and mCherry expressing strains to answer the question of whether pseudovibriamides affect the uptake of *P. brasiliensis* by *Aplysina aerophoba* sponges. *P. brasiliensis* cell counts decreased in the sponge aquaria at an equivalent rate for wild-type and pseudovibriamide-defective Δ*pppA* mutant strains, indicating that the sponge filters each strain indiscriminately under the conditions tested. Yet, the filtering capacity varied for each sponge individual tested, stressing the importance of performing experiments with wild-type and mutant bacterial strains in the same aquarium to allow for rigorous conclusions, which is now enabled with the methods established here.

36 ## Importance

37 Marine sponges are ecosystem engineers. They transform nutrients into a bioavailable form for
38 other marine organisms. Microbes are critical to the functional roles provided by sponges because
39 they expand the metabolic capabilities of the sponge host. Yet, most of our knowledge on sponge
40 microbes comes from genomic studies, since cultivability and the ability to perform genetics with
41 sponge bacterial isolates is limited. The genus *Pseudovibrio* of α-Proteobacteria has consistently
42 been isolated from marine sponges and it has been hypothesized to contribute to marine sponge
43 health. Moreover, *Pseudovibrio* bacteria are a source of antibiotics and other secondary
44 metabolites with the potential to be developed into pharmaceuticals. Here we established vectors
45 for the expression of fluorescent proteins in *Pseudovibrio* bacteria and demonstrated their utility
46 in *in vivo* studies with marine sponges. The availability of genetic tools is important to enable us
47 to explore the emerging ecological and biotechnological potential of *Pseudovibrio* bacteria.

48

49 **Keywords:** plasmid vectors, *Pseudovibrio*, marine sponges, *Aplisyna aerophoba*, flow cytometry

Introduction

The term 'holobiont' has been coined to express the crucial association between animals and their microbiota (1, 2). Host-associated microbiota have nutritional and protective roles, contributing to animal health (3). Marine sponges are ancient animals that are vital to the ecology of the oceans. They provide habitat to other organisms, and they affect the biogeochemical cycling in the ecosystem. Sponge microbes support these functional roles thanks to their unique metabolism. For example, sponge microbes produce secondary metabolites that support the persistence of sponges against predators or competitors (4, 5). Moreover, sponge microbes help the holobiont assimilate inorganic nutrients such as nitrogen, facilitating nutrient transfer through the ocean's food webs (6).

Marine sponges harbor diverse, specific, and stable microbiomes with Pseudomonadota being the most dominant phylum, particularly α-and γ-Proteobacteria (7, 8). Although a certain level of resilience has been observed, environmental perturbations such as climate change and pollution that go beyond a certain threshold can challenge the stability of sponge microbiomes leading to dysbiosis, that is, the disruption of community diversity (1). The level of disease affecting marine animals such as sponges is unprecedented (9). Dysbiosis is hypothesized to increase the susceptibility of animals to disease, as dysbiosis is followed by opportunistic infections (10). Conversely, a more diverse microbiome is correlated with resilience to stressors such as elevated seawater temperature and ocean acidification (11). A mechanistic understanding of sponge-microbiota interactions is important from a basic science perspective but also to help counteract negative effects of climate change and anthropogenic influences. For example, how do specialized metabolites produced by sponge microbes contribute to holobiont homeostasis?

Sponge-microbiota model systems would help address mechanistic questions related to holobiont homeostasis. Desired characteristics of experimental sponge-microbiota models

include ease of collection, maintenance in aquaculture, availability of omics data, cultivability of symbionts, and amenability to genetic manipulation of both the host and its microbiota (12). Although no sponge model has yet been established, several sponges are candidates, including *Aplysina aerophoba*, a Mediterranean/East Atlantic sponge that can be kept in aquarium conditions and for which microbial metagenome data and symbiont genomes are available (13, 14). Moreover, the genus *Aplysina* has nearly global distribution in temperate and tropical regions (12). Due to limitations in *in vitro* reproduction thus far, lab experimentation depends on field collections. Cultivability and the ability to perform reverse genetics with sponge symbionts is also limited.

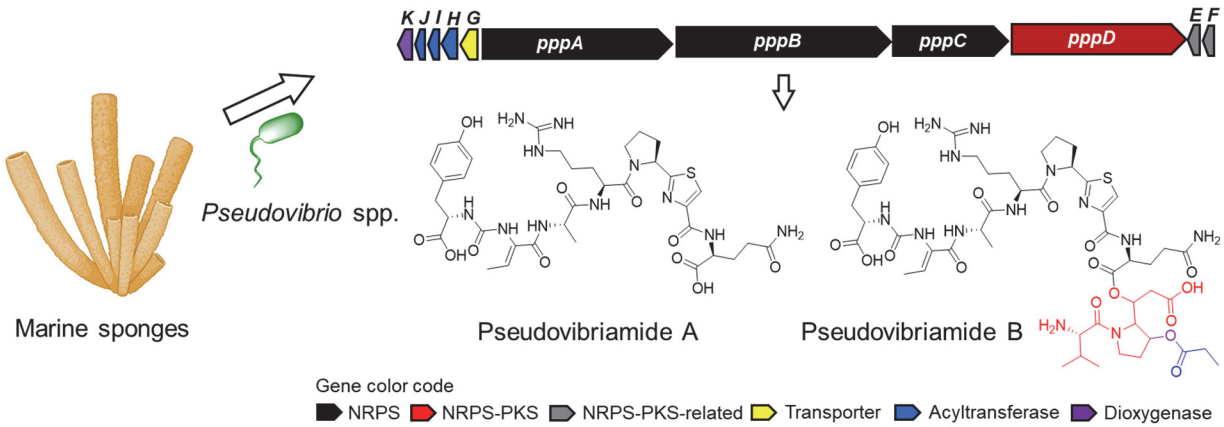
The genus *Pseudovibrio* of α-Proteobacteria has consistently been isolated from marine sponges, in addition to corals and algae, among other marine organisms (15). *Pseudovibrio* has been hypothesized to contribute to marine sponge health because it has been isolated only from healthy sponges, it produces antibiotics that prevent the growth of sponge pathogens, and evidence for vertical transmission has been provided (15). Beyond sponges, *Pseudovibrio* has also been shown to induce coral larvae settlement behavior (16).

We recently discovered specialized metabolites we named pseudovibriamides (**Figure 1**) from *Pseudovibrio brasiliensis* Ab134 (17). The pseudovibriamide encoding gene cluster (termed *ppp*) is present in approximately two thirds of *Pseudovibrio* genomes (17). Reverse genetics methods we established (gene knockout via homologous recombination) helped us discover a link between pseudovibriamides and *Pseudovibrio* collective behaviors, that is, flagella-mediated motility and biofilm formation (17). Motility and biofilm formation are known to be important for bacterial survival and for host colonization (18).

No self-replicative vectors that could aid with gene expression have been reported for *Pseudovibrio*. Here we compared broad-host range self-replicative vectors and identified those

98 that can be used with *P. brasiliensis* to express fluorescent proteins. We then used the vectors to

99 test whether pseudovibriamides affect uptake by *A. aerophoba* sponges.



100

101 **Figure 1**. **Pseudovibriamides from *Pseudovibrio* bacteria isolated from marine sponges.** Structures

102 and encoding gene cluster. Pseudovibriamides were isolated from *Pseudovibrio brasiliensis* Ab134 (17).

103 The *ppp* gene cluster is present in 2/3 of *Pseudovibrio* genomes isolated from various marine sponges

104 including *Arenosclera brasiliensis*, *Axinella dissimilis*, *Spongia officinalis*, *Polymastia penicillus*, (17) and

105 *Aplysina aerophoba* (19). The sponge icon is from BioRender.com.

106

107 ## Results

108 **Establishing broad-host range vectors for *P. brasiliensis* Ab134.** We started by obtaining and
109 testing plasmid vectors containing three different broad-host-range replicons, RSF1010, RK2, and
110 pBBR1. The corresponding plasmids pAM4891 (20), pSEVA227M, pSEVA234M, and
111 pSEVA237R_Pem7 (21) were transferred into *P. brasiliensis* Ab134 by conjugation from *E. coli*
112 and obtained clones were confirmed by plasmid extraction, restriction digestion and gel
113 electrophoresis (**SI Figure S1**). We then used short-read Illumina sequencing of total DNA to
114 estimate relative plasmid copy number by mapping the obtained Illumina reads to the Ab134
115 reference genome (17). We have previously shown that Illumina sequencing is comparable to
116 quantitative PCR for relative plasmid copy number determination (22). Others have also used
117 sequencing instead of quantitative PCR for this purpose (23). The vector coverage normalized to
118 the chromosome coverage indicated that RSF1010 and RK2 replicons are of medium copy
119 number (49 and 47, respectively) and that pBBR1 is of high copy number (148 to 210) in *P.*
120 *brasiliensis* Ab134 (**Table 1**).

121 **Table 1**. **Normalized plasmid vector copy number in *P. brasiliensis* Ab134.** Data obtained from Illumina
122 sequencing of total DNA.

Plasmid vector name	pAM4891	pSEVA227M	pSEVA234M	pSEVA237R_Pem7
Replicon	RSF1010	RK2	pBBR1	pBBR1
Fluorescent protein	GFP	GFP	GFP	mCherry
Promoter	*Ptac*, constitutive	none	*lacI*^{q}*-Ptrc*, IPTG-inducible	*Pem7*, constitutive
Average Chromosome Coverage	112	72	66	94
Average Vector Coverage	5,526	3,389	13,838	13,957
Vector to Chromosome Ratio	49	47	210	148

123

124 Growth comparison in liquid cultures showed that *P. brasiliensis* Ab134 carrying vectors
125 with RSF1010 or RK2 replicons have comparable growth to each other and to the wild type

126 (**Figure 2**). In contrast, Ab134 carrying pSEVA234M and pSEVA237R_Pem7, both with the same
127 pBBR1 replicon, showed distinct growth phenotypes, with pSEVA234M displaying a growth defect
128 (**Figure 2**). The marked growth defect in Ab134 carrying pSEVA234M was consistent across
129 independent clones (**SI Figure S2**). Although it has been shown that high plasmid copy number
130 can result in growth defects (24, 25), Ab134 carrying pSEVA237R_Pem7 with the same pBBR1
131 replicon did not exhibit this defect (**Figure 2** and **SI Figure S2**). This discrepancy could be due to
132 the higher plasmid copy number of 210 for pSEVA234M compared to 148 for pSEVA237R_Pem7
133 (**Table 1**). Another possibility is that other components within the pSEVA234M vector, such as
134 repressor-promoter *lacI*^{q}-*Ptrc*, may be responsible. The mutation in the promoter of the *lacI*
135 repressor gene (*lacI*^{q}) results in higher levels of LacI within cells (26). The overexpression of
136 foreign genes can lead to decreased growth rate as demonstrated with *E. coli* (27). Thus, the
137 combination of high plasmid copy number and high expression of the *lacI* repressor gene from
138 pSEVA234M could be the underlying cause of the observed growth defect.

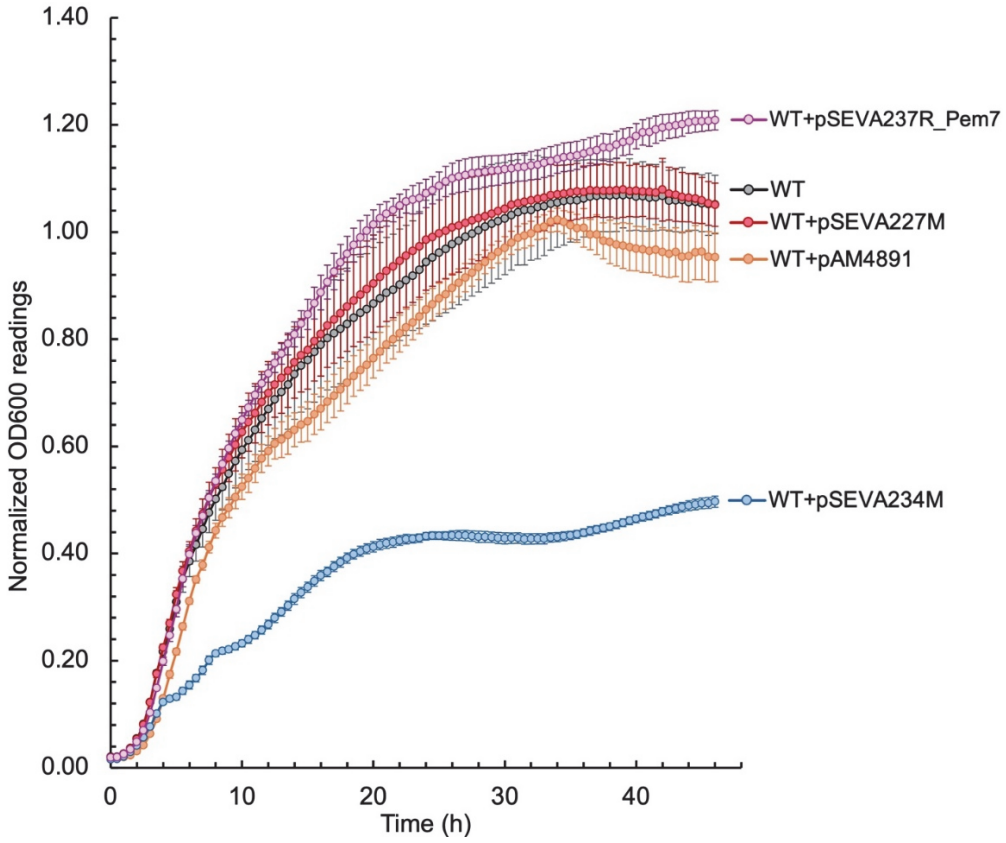


Figure 2. **Growth of *P. brasiliensis* Ab134 containing different vectors.** Growth in liquid cultures was measured by optical density at 600 nm (OD_{600}). *N*=10. Error bars indicate standard deviation. WT, *P. brasiliensis* Ab134 wild type. See Table 1 for vector details.

The self-replicative vectors delay flagellar motility but it is still possible to distinguish wild type and pseudovibriamide-defective Δ*pppA* mutant strains. We have previously shown that a pseudovibriamide-defective mutant strain (Δ*pppA*) displays reduced swarming motility compared to the wild type (17). In preparation for marine sponge studies, we next investigated whether carrying a self-replicative vector would affect the flagellar motility of wild type and Δ*pppA* strains, and whether fluorescent proteins could be detected without selection. We chose to use pAM4891 containing GFP and pSEVA237R_Pem7 containing mCherry since each vector carries a constitutive promoter (**Table 1**) and results in growth comparable to the wild type (**Figure 2**). The swarming motility of strains carrying the pAM4891 vector was comparable to the parent

strains, whereas strains carrying the pSEVA237R_Pem7 vector exhibited a delayed swarming phenotype (**Figure 3A** and **SI Figure S3**). Nevertheless, as for the parent strains, the swarming motility of Δ*pppA* strains carrying either vector was still reduced compared to the wild type carrying the same vectors (**Figure 3A** and **SI Figure S3**). Finally, we were able to detect fluorescent protein expression in these strains after three days even without antibiotic selection, indicating that the vectors are stably maintained over the course of the experiment (**Figure 3B**).

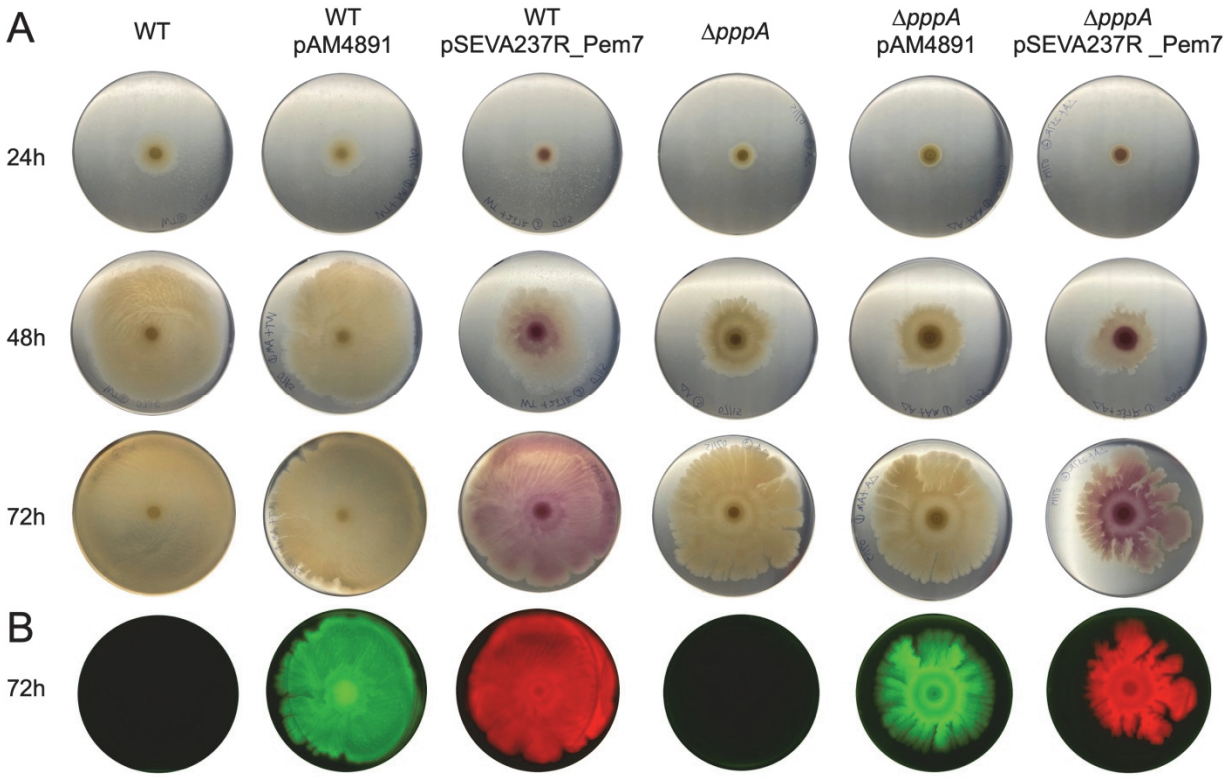


Figure 3. **Swarming motility of *P. brasiliensis* Ab134 wild type or Δ*pppA* mutant parent strains compared to strains carrying self-replicative vectors.** (**A**) Swarming assays performed on marine broth with 0.5 % Eiken agar. Pictures shown were taken at 24, 48, and 72 hours after inoculation, respectively. The assay was performed in triplicates (**SI Figure S3**) and representative images are shown. (**B**) Fluorescence imaging of swarming plates at 72 hours to show the expression of GFP and mCherry in strains carrying pAM4891 and pSEVA237R_Pem7, respectively.

To test the vectors further, we next set out to answer the question of whether pseudovibriamide production affects the uptake of *P. brasiliensis* by marine sponges by comparing the uptake of the wild-type and of the pseudovibriamide-defective Δ*pppA* mutant strain.

Tools for *P. brasiliensis* bacteria-sponge studies. *A. aerophoba* marine sponge specimens from the Mediterranean Sea, a sponge species from which *Pseudovibrio* containing the *ppp* gene cluster have been previously isolated (19), were collected by SCUBA. The field-collected sponges were kept in aquaria with unfiltered, circulating seawater where they can be maintained for several months (**Figure 4A-3C** and **SI Figure S4A**). *Pseudovibrio* wild type and Δ*pppA* mutant expressing either green (GFP) or red (mCherry) fluorescent protein encoded in plasmids pAM4891 and pSEVA237R_Pem7, respectively, were obtained as described above (**Figure 4D**).

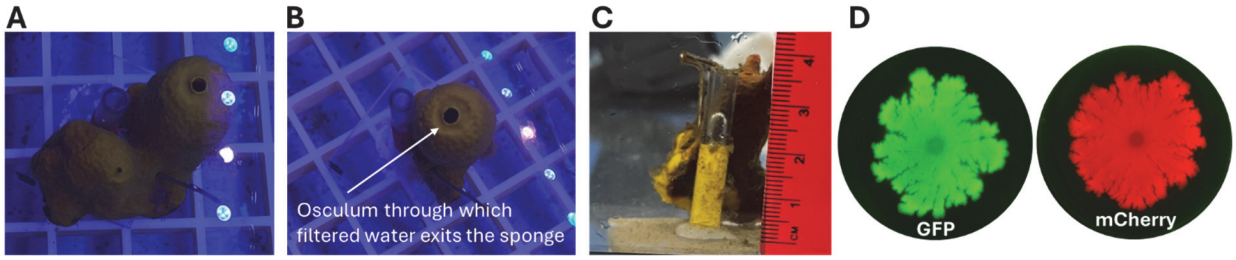
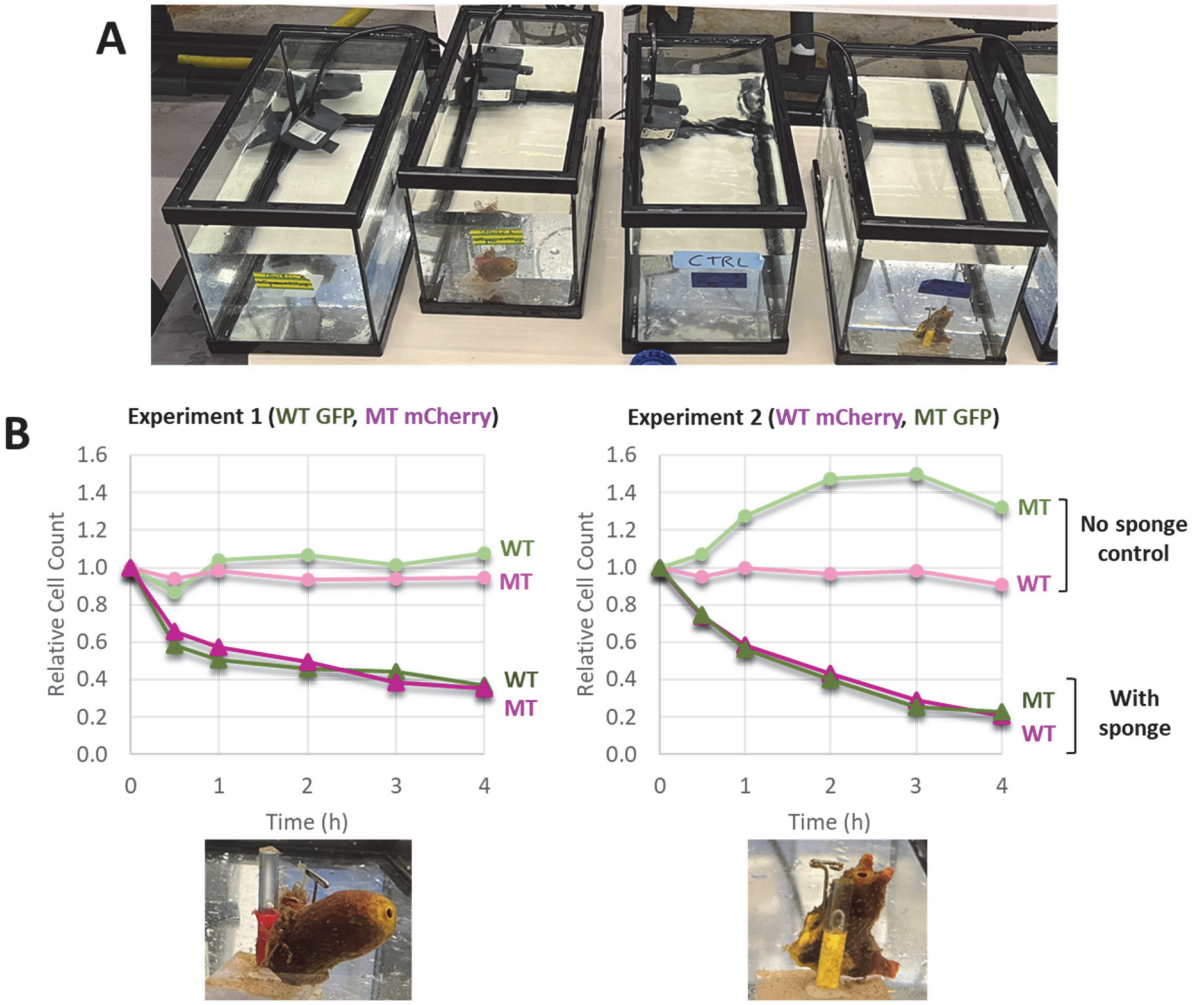


Figure 4. Tools for marine sponge-bacteria studies. (**A**) *A. aerophoba* marine sponge specimens were collected by SCUBA and kept in aquaria with unfiltered, circulating seawater until use. Sponges can be kept as such for several months. (**B**) Single individuals containing one osculum were sectioned using a scalpel and attached to a plastic support. These single-osculum individuals were used in sponge-bacteria experiments. (**C**) Individuals used were about 3 cm long but can vary in shape and filtering capacity. (**D**) Fluorescence imaging of strains of *P. brasiliensis* Ab134 that express green or red fluorescent proteins (GFP and mCherry) from pAM4891 and pSEVA237R_Pem7, respectively (select swarming pictures of the Δ*pppA* mutant at 72 hours are shown). The two-color labels allow competition experiments to be performed in the same aquarium which is important to account for sponge individual variability.

187 ***A. aerophoba* sponges take up *P. brasiliensis* wild-type and Δ*pppA* mutant strains**
188 **indiscriminately**. For the bacteria-sponge studies, *A. aerophoba* sponge specimens containing
189 one osculum (**Figure 4B**) were taken from the long-term aquarium and transferred into individual
190 aquaria with 4-L filtered seawater each (**Figure 5A** and **SI Figure S4**). An aquarium pump was
191 used for water circulation. Sponges were challenged with a mixture of wild-type and Δ*pppA* mutant
192 strains expressing either GFP or mCherry (and vice versa to account for any potential fluorescent
193 protein or vector effects). Control aquaria contained no sponge (**Figure 5A**).



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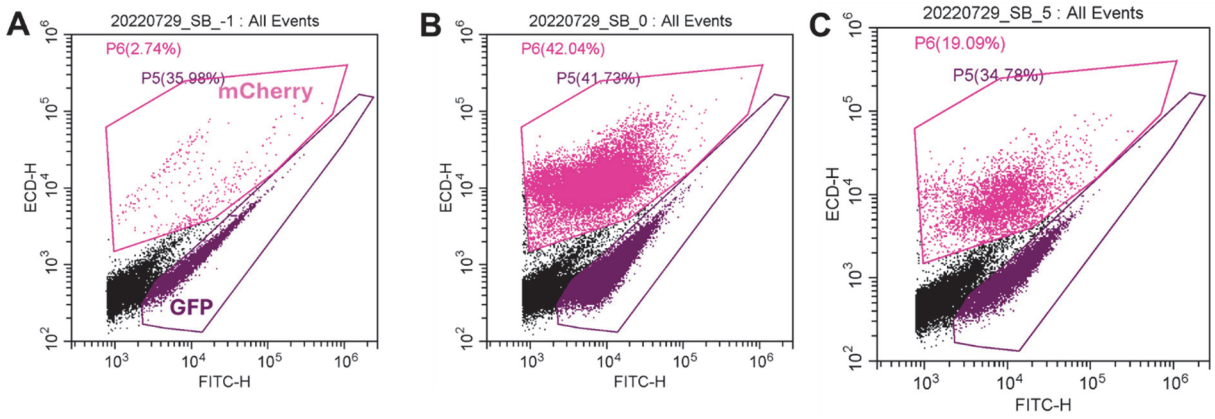
195 **Figure 5**. ***A. aerophoba* marine sponge-*P. brasiliensis* bacteria experiments.** (**A**) Four aquaria were
196 set up with 50-µm filtered seawater, two of which contained sponge individuals, and two of which served
197 as no-sponge control. A mixture of wild-type (WT) and Δ*pppA* mutant (MT) expressing either green (GFP)

198 or red (mCherry) fluorescent protein were added to each aquarium at a final concentration of 10^6 cells/ml
199 as measured by flow cytometry. In Experiment 1, aquaria contained WT with GFP and MT with mCherry,
200 and the labels were inverted for Experiment 2. (**B**) Relative cell count over time for each experiment as
201 measured by flow cytometry (see **Table S2** for raw data). The sponge individuals used in each experiment
202 are shown at the bottom. The increase in green fluorescence signal in aquaria without sponges (particularly
203 marked for experiment 2) is likely due to seawater contamination as the sponge specimen was transferred
204 from the long-term aquarium. Note that the filtering capacity varies for each individual sponge specimen,
205 highlighting the need to perform WT and MT experiments in the same aquarium to derive rigorous
206 conclusions.

207 Our first observation was that it is important to use a bacterial concentration of 10^6 cells
208 per ml seawater as measured by flow cytometry. More (10^7) overwhelms the sponge leading to
209 osculum closure and filtering cessation (**SI Figure S5**). Less (10^5) prevents accurate cell
210 quantification over the course of the experiment. We used flow cytometry both to determine
211 bacterial cell count before starting the experiments and for time course quantification (**Figure 6**).



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213 **Figure 6**. **Example of flow cytometry data.** Flow cytometry was used for relative cell count of *P.*
214 *brasiliensis* bacteria expressing either GFP or mCherry as presented in Figure 5. (**A**) Aquarium seawater
215 before inoculation. (**B**) Time 0 directly after inoculation. (**C**) End point. The pink and purple gates represent
216 mCherry and GFP, respectively.

217

218 The second observation was that there was more interference in the GFP window (**Figure 6A**)
219 due to background green fluorescence. Note the sponges are wild specimens and not axenic, no
220 axenic sponge model has been reported. The green fluorescence interference is more apparent
221 in the no-sponge aquaria, likely due to the growth of contaminating bacteria, which can be filtered
222 out by the sponges in the sponge aquaria (**Figure 5B**). It was still possible to accurately compare
223 wild-type and mutant using GFP in the sponge aquaria as we corrected for the water background
224 (**Figure 5B**).

225 Third, the bacteria cell counts either stay the same or increase in the no-sponge aquaria over
226 the course of 4 hours (**Figure 5B**). The increase is associated with GFP and likely due to
227 background contamination of bacteria with natural green fluorescence. The cell counts decrease
228 in the sponge aquaria at an equivalent rate for wild-type and Δ*pppA* mutant strains indicating that
229 the sponge filters each strain indiscriminately under the conditions tested.

230 Forth, note that the filtering capacity (**Figure 5B**) varied for each sponge individual (e.g., a
231 lower cell count was reached for experiment 2 compared to experiment 1), stressing the
232 importance of performing experiments with wild-type and mutant bacterial strains in the same
233 aquarium to allow for rigorous conclusions, which is now enabled with the methods established
234 here.

235

236

237 ## Discussion

238 The genus *Pseudovibrio* was first described in 2004 (28). Since then, *Pseudovibrio* bacteria have
239 been isolated or detected predominantly from marine sponges and corals worldwide but also from
240 tunicates, flatworms, algae and other sources (15). In addition to beneficial roles such as
241 supporting biogeochemical cycling and providing nutrients, *Pseudovibrio* has also been proposed
242 to contribute to marine sponge health, and to promote coral larvae settlement (15, 16). The
243 availability of genetic tools is important to enable us to explore the emerging ecological and
244 biotechnological potential of *Pseudovibrio* bacteria.

245 Although we have previously established reverse genetics methods for *P. brasiliensis*
246 using a suicide vector (17, 29), no self-replicative vectors for gene expression have been reported
247 for *Pseudovibrio*. Here we showed that vectors containing three distinct, broad-host range
248 replicons can be used in *P. brasiliensis* to express fluorescent proteins GFP and mCherry.

249 The RSF1010 replicon belongs to the IncQ group of plasmids. The largest of the replicons
250 tested here (5.4 kb) is composed of *oriV*, three replication genes (*repA*, *repB*, and *repC*) and three
251 mobilization proteins (*mobA*, *mobB* and *mobC*). Its broad host range expands to
252 Pseudomonadota, Bacillota, Actinomycetota, and Cyanobacteriota (30). The reported low copy
253 number of 10 to 12 for wild type RSF1010 in *E. coli* (31) may be explained due to self-mobilization
254 which results in DNA nicking and low yields of extracted DNA. The pAM4891 vector tested here
255 contains a mutation in the RSF1010 *mobA* gene (Y25F) that results in plasmid preparations
256 containing mostly supercoiled DNA in contrast to the mostly nicked wild-type DNA (20), which
257 may explain the higher copy number of 49 we observed (**Table 1**).

258 The RK2 replicon belongs to the IncPα group and it replicates in many Gram-negative
259 bacteria (32). The minimal replicon (2.2 kb) included in the pSEVA plasmids contains *oriV* and the
260 replication protein gene *trfA* (21). The parental RK2 plasmid (56.4 kb) displays a low copy number

261 of 4-7 in *E. coli* whereas deletions leading to a mini replicon increase the copy number to 35-40
262 (33), in accordance with the relative copy number of 47 we observed here (**Table 1**).

263 The pBBR1 replicon does not belong to IncP, IncQ or IncW groups and may represent a new
264 incompatibility group (34). It has a broad-host range in Gram-negative bacteria including
265 Pseudomonadota (34) and Thermodesulfobacteriota (35), and it has also been used in
266 *Microbacterium* (Actinomycetota) (36). The replicon is composed of *oriV*, one *rep* and one *mob*
267 gene, and as such, it is relatively small (34). The pBBR1 replicon (1.52 kb) in the SEVA plasmids
268 is the smallest of the broad-host range replicons we tested (21). It contains *oriV* and *rep*. Instead
269 of the *mob* gene present in the original replicon (34), all SEVA plasmids use *oriT* from RK2 (21).
270 A medium copy number of 30-40 was reported for *E. coli* and *Bordetella pertussis* (34), whereas
271 a growth defect was reported with some bacteria, including *Pseudomonas putida* (24), *Geobacter*
272 *sulfurreducens* (35) and *Burkholderia* sp. (22) corresponding to the higher copy number of pBBR1
273 in these strains. Both vectors we tested that contain the pBBR1 replicon resulted in high copy
274 number of 148-210 in *P. brasiliensis* (**Table 1**) but only pSEVA234M led to a growth defect (**Figure**
275 **2**). The observed growth defect with pSEVA234M may be due to a combination of higher plasmid
276 copy number of 210 and high expression of the *lacI*^{q} repressor gene from pSEVA234M (26, 27).

277 Although *P. brasiliensis* carrying pSEVA237R_Pem7 and pAM4891 showed comparable
278 growth rate to the wild type, their swarming motilities were affected to different extents.
279 Specifically, *P. brasiliensis* carrying pSEVA237R_Pem7, with a higher plasmid copy number
280 compared to pAM4891, exhibited a more noticeable reduction in swarming motility (**Figure 3A**).
281 Previous studies have shown that the production of unnecessary proteins, including fluorescent
282 proteins, imposes a significant metabolic burden on the host, slowing native protein synthesis (37-
283 40). Thus, the production of mCherry from the high copy number pSEVA237R_Pem7 in *P.*
284 *brasiliensis* might induce a burden on cellular processes, reducing the availability of resources to
285 produce essential proteins for swarming motility.

286 We next applied pAM4891 and pSEVA237R_Pem7 vectors in studies with marine sponges.
287 Specifically, we aimed to answer the question of whether pseudovibriamides (**Figure 1**) affect the
288 uptake of *P. brasiliensis* by *A. aerophoba* sponges. We observed no difference in uptake between
289 wild type and pseudovibriamide-defective Δ*pppA* mutants. Future studies should vary
290 experimental conditions such as using no pump to allow for comparing the difference in motility
291 of each strain that may affect sponge uptake. Long-term experiments in which sponges are
292 evaluated after the filtering experiment to determine whether strains can be established in the
293 host would also be helpful, although this may first require the development of axenic sponge
294 models which has been attempted but remains unresolved (41, 42). Alternatively, assays to study
295 how sponges differentiate between food and symbionts, for example, may prove insightful (43).
296 Finally, it is noteworthy that the filtering capacity varied for each sponge individual (**Figure 4B**),
297 stressing the importance of performing experiments with wild-type and mutant bacterial strains in
298 the same aquarium to allow for rigorous conclusions, as demonstrated here using the evaluated
299 vectors.

300 In conclusion, given the emerging roles of *Pseudovibrio* as part of sponge, coral and other
301 marine holobionts, and their production of secondary metabolites with biomedical and
302 biotechnological potential (15), the availability of genetic tools is important for biotechnological
303 applications but also to facilitate probing mechanistic questions regarding *Pseudovibrio* biology,
304 microorganism-microorganism and microorganism-host interactions.

305

306 ## Materials and Methods

307 **General experimental procedures.** Chemicals and enzymes were acquired from Sigma-Aldrich,
308 VWR, Becton Dickinson (BD), and Thermo Fisher Scientific, unless otherwise noted. Restriction
309 enzymes were purchased from New England Biolabs (NEB). Genomic DNA was isolated using
310 GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich). ZymoPURE Plasmid Miniprep kits were
311 used to extract plasmid DNA. Whole plasmid sequencing by Primordium labs was performed to
312 confirm the accuracy of plasmid vectors.

313 **Bacterial cultivation.** *P. brasiliensis* Ab134 wild type and Δ*pppA* mutant strains were cultivated
314 at 30˚C on BD Difco™ Marine Agar 2216 (MA) or in BD Difco™ Marine Broth 2216 (MB) for 18-
315 24 hours unless otherwise noted. Kanamycin (200 µg/mL) was used for mutant selection as
316 appropriate. *E. coli* strains were cultured in BD Difco™ Luria Broth (LB) or on LB agar for 18-20
317 hours. Kanamycin (50 µg/mL) was used for mutant selection as appropriate. *E. coli* DH5α was
318 used for plasmid vector propagation and *E. coli* S17-1 for conjugation with Ab134. All strains were
319 cryo-preserved in 20% glycerol (*v/v*) at -80 ˚C.

320 **Plasmid transfer into *P. brasiliensis* Ab134.** Plasmid vectors were transferred into Ab134 by
321 conjugation from *E. coli* S17-1, using a similar protocol as previously described (17). Vectors were
322 first electroporated into *E. coli* S17-1, then introduced into the Ab134 wild-type or the Δ*pppA*
323 mutant. Selection of exconjugants was carried out on MA (20 mL) containing kanamycin (200
324 µg/mL) for the incoming plasmid and carbenicillin (50 µg/mL) to selectively kill *E. coli*. Positive
325 clones were inoculated into MB (5 mL) containing kanamycin (200 µg/mL) for plasmid extraction
326 after overnight growth at 30°C. Plasmids were confirmed using restriction digestions (**SI Figure**
327 **S1**).

328 **Relative plasmid copy number determination.** Total DNA was isolated from *P. brasiliensis*
329 carrying one of four plasmids (pAM4891, pSEVA227M, pSEVA234M or pSEVA237R_Pem7)

330 using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich) and following the
331 manufacturer's instructions. The quality of the total DNA was evaluated by gel electrophoresis
332 and Nanodrop measurements. DNA samples were sequenced using Illumina paired-end whole
333 genome sequencing (400 Mbp, 2.67 M reads package) by the SeqCenter. Sample libraries were
334 prepared using the Illumina DNA Prep kit along with IDT 10bp UDI indices. Sequencing was
335 conducted on an Illumina NextSeq 2000, generating 2×151bp paired-end reads. Demultiplexing,
336 quality control and adapter trimming was performed using bcl-convert (v3.9.3). The obtained
337 sequence reads (**SI Table S1**) were mapped to the reference sequences – Ab134 genome
338 (GenBank: GCA_018282095.1) (17) plus each of the plasmids introduced using the in-built "Map
339 to References" function with medium sensitivity and five iterations in Geneious. In the alignment
340 file, the coverage statistics, which indicates the number of reads mapped at each position, was
341 used to calculate the plasmid copy number as shown below:

342 $$Plasmid\ Copy\ Number = \frac{Coverage\ of\ Introduced\ Plasmid}{Coverage\ of\ Chromosome}$$

343 **Growth curves.** The growth of each bacterial strain was tracked for 48 h using a microtiter plate
344 method. Seed cultures were prepared by inoculating 50 µL of each cryo-preserved culture into 5
345 mL of MB. All cultures were incubated at 30°C overnight (18-20 h). Seed cultures were then diluted
346 in MB to OD_{600} of 0.05. OD-standardized cultures were dispensed (200 µL into each well, 10
347 replicates for each strain) into Falcon™ 96-well, non-treated, flat-bottom microplate using a multi-
348 channel pipettor. Border wells were filled with MB to maintain humidity. Plates were incubated at
349 30°C and shaken linearly at a speed of 887 revolutions per minute (amplitude 1 mm) using an
350 Infinite M Plex plate reader (Tecan). OD_{600} readings of each well were taken every 30 min over 48
351 h.

352 **Swarming motility assays.** Swarming assays were performed as previously described (17).
353 Details are provided here for convenience. Briefly, swarming agar with reduced surface tension

354 was prepared by combining MB and 0.5% (*w/v*) of Eiken agar (Eiken Chemical CO., Japan). The
355 autoclaved agar medium equilibrated to 55 °C for 2 hours was aliquoted onto Petri dishes (exactly
356 20 mL), which were air dried for 20 min in a laminar flow hood. Fresh cultures of bacterial strains
357 were normalized to OD_{600} of 1.0, and 5 µL of OD-normalized cultures were dropped on the center
358 of each plate. Plates were air dried until the droplet of culture was absorbed and incubated at
359 30°C. Visible light pictures were taken using a cell phone camera. Fluorescence imaging was
360 obtained using a ChemiDoc™ MP Imaging System. Multi-channel (auto-exposure) with Cy2 (532
361 nm / 28 mm) and Alexa 546 (602 nm / 50 mm) was used to visualize green and red fluorescence.
362 Pictures were taken daily for 3 days.

363 **Sponge collection.** *Aplysina aerophoba* sponge specimens were collected by SCUBA in June
364 2022 off the coast of Girona, Spain, and kept in an aquarium with circulating, unfiltered seawater
365 at the facilities in ICM-CSIC (**SI Figure S4A**). Using a scalpel, sponge specimens were divided to
366 contain only one osculum (**Figure 4B**) while maintaining them submerged in seawater. Each
367 specimen was affixed to a plastic support using a metal pin (**Figure 4C** and **SI Figure S4B**) and
368 kept in the aquarium with circulating, unfiltered seawater until use. We used the sponge
369 specimens within three weeks of collection, but they can be kept in this way for a few months.

370 **Preparation of *P. brasiliensis* cells for sponge-bacteria studies.** *P. brasiliensis* Ab134 wild
371 type and Δ*pppA* mutant cultures containing either pSEVA237R_Pem7 (mCherry) (21) or
372 pAM4891 (GFP) (20) were initiated by inoculating 0.2 ml cryopreserved cultures onto 20 ml of
373 marine broth with kanamycin (200 µg/ml final concentration). The cultures contained in 250-ml
374 Erlenmeyer flasks were incubated at 27 °C, 200 rpm, for 23 h. Cells were harvested by
375 centrifugation (10 min at 25 °C and 3,000 rpm = 1,730 ×g). After discarding the supernatant, cells
376 pellets were washed twice, each time with 20 ml seawater (same centrifugation conditions as
377 above). After the last wash, the cell pellets were resuspended in 20 ml fresh seawater. An aliquot
378 of each cell suspension was diluted 1:1,000 with seawater and cell count was performed using

379 flow cytometry (CytoFLEX S, Beckman Coulter) set at medium flow (30 µl/min), with data
380 acquisition for 2 min per sample. A seawater sample was used as “blank” to correct the total cell
381 count. Wild type and mutant strains expressing either GFP or mCherry, respectively (or vice versa)
382 were mixed at 1:1 ratio (5×10^8 cells of each per ml) to yield a total of 1×10^9 cells per ml. The
383 mixed cell suspensions were kept on ice until use.

384 **Sponge-bacteria studies.** Four experimental aquaria were prepared, each containing 4 L of
385 filtered (50 µm) seawater and an aquarium pump (**Figure 5A** and **SI Figure S4B**). *A. aerophoba*
386 sponge specimens containing one osculum were transferred to two of the experimental aquaria,
387 whereas the other two aquaria remained as no sponge controls. The sponge specimens were
388 transferred using a plastic bag, taking care that they were always submerged in seawater. Aliquots
389 (1.62 ml) were taken from each aquarium before inoculation (time -1). 4 ml of each bacterial cell
390 mixture (wild type expressing GFP and Δ*ppp*A mutant expressing mCherry or wild type expressing
391 mCherry and Δ*ppp*A mutant expressing GFP) was then added to two aquaria (to make up 1×10^6
392 cells per ml final concentration), one of which contained a sponge specimen. Water samples (1.62
393 ml) were taken from each aquarium at 0, 0.5, 1, 2, 3, and 4 hours, transferred to 2-ml Eppendorf
394 tubes and mixed with 0.18 ml fixative (10% paraformaldehyde, 0.5% glutaraldehyde). After 10 min
395 incubation at room temperature, the fixed samples were stored at -20 °C. Flow cytometry was
396 performed on all samples as described above to determine cell count originating from cells
397 expressing mCherry and GFP (**Figure 5**). The mCherry and GFP gates (**Figure 6**) were set based
398 on pure cultures. The relative cell count for each aquarium was calculated after correcting for the
399 corresponding -1 (before inoculation) sample (**SI Table S2**) to yield the results shown in **Figure**
400 **5**.

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