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.

Importance

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

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

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

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

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

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

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

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

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

Results

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

Table 1. **Normalized plasmid vector copy number in *P. brasiliensis* Ab134.** Data obtained from Illumina 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

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

(**Figure 2**). In contrast, Ab134 carrying pSEVA234M and pSEVA237R_Pem7, both with the same

pBBR1 replicon, showed distinct growth phenotypes, with pSEVA234M displaying a growth defect

(**Figure 2**). The marked growth defect in Ab134 carrying pSEVA234M was consistent across

independent clones (**SI Figure S2**). Although it has been shown that high plasmid copy number

can result in growth defects (24, 25), Ab134 carrying pSEVA237R_Pem7 with the same pBBR1

replicon did not exhibit this defect (**Figure 2** and **SI Figure S2**). This discrepancy could be due to

the higher plasmid copy number of 210 for pSEVA234M compared to 148 for pSEVA237R_Pem7

(**Table 1**). Another possibility is that other components within the pSEVA234M vector, such as

repressor-promoter *lacI^q-Ptrc*, may be responsible. The mutation in the promoter of the *lacI*

repressor gene (*lacI^q*) results in higher levels of LacI within cells (26). The overexpression of

foreign genes can lead to decreased growth rate as demonstrated with *E. coli* (27). Thus, the

combination of high plasmid copy number and high expression of the *lacI* repressor gene from

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.

***A. aerophoba* sponges take up *P. brasiliensis* wild-type and Δ*pppA* mutant strains**

indiscriminately. For the bacteria-sponge studies, *A. aerophoba* sponge specimens containing

one osculum (**Figure 4B**) were taken from the long-term aquarium and transferred into individual

aquaria with 4-L filtered seawater each (**Figure 5A** and **SI Figure S4**). An aquarium pump was

used for water circulation. Sponges were challenged with a mixture of wild-type and Δ*pppA* mutant

strains expressing either GFP or mCherry (and vice versa to account for any potential fluorescent

protein or vector effects). Control aquaria contained no sponge (**Figure 5A**).

Figure 5. ***A. aerophoba* marine sponge-*P. brasiliensis* bacteria experiments.** (**A**) Four aquaria were

set up with 50-μm filtered seawater, two of which contained sponge individuals, and two of which served

as no-sponge control. A mixture of wild-type (WT) and Δ*pppA* mutant (MT) expressing either green (GFP)

or red (mCherry) fluorescent protein were added to each aquarium at a final concentration of 10^6 cells/ml

as measured by flow cytometry. In Experiment 1, aquaria contained WT with GFP and MT with mCherry,

and the labels were inverted for Experiment 2. (**B**) Relative cell count over time for each experiment as

measured by flow cytometry (see **Table S2** for raw data). The sponge individuals used in each experiment

are shown at the bottom. The increase in green fluorescence signal in aquaria without sponges (particularly

marked for experiment 2) is likely due to seawater contamination as the sponge specimen was transferred

from the long-term aquarium. Note that the filtering capacity varies for each individual sponge specimen,

highlighting the need to perform WT and MT experiments in the same aquarium to derive rigorous

conclusions.

Our first observation was that it is important to use a bacterial concentration of 10^6 cells

per ml seawater as measured by flow cytometry. More (10^7) overwhelms the sponge leading to

osculum closure and filtering cessation (**SI Figure S5**). Less (10^5) prevents accurate cell

quantification over the course of the experiment. We used flow cytometry both to determine

bacterial cell count before starting the experiments and for time course quantification (**Figure 6**).

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Figure 6. **Example of flow cytometry data.** Flow cytometry was used for relative cell count of *P.*

brasiliensis bacteria expressing either GFP or mCherry as presented in Figure 5. (**A**) Aquarium seawater

before inoculation. (**B**) Time 0 directly after inoculation. (**C**) End point. The pink and purple gates represent

mCherry and GFP, respectively.

The second observation was that there was more interference in the GFP window (**Figure 6A**)

due to background green fluorescence. Note the sponges are wild specimens and not axenic, no

axenic sponge model has been reported. The green fluorescence interference is more apparent

in the no-sponge aquaria, likely due to the growth of contaminating bacteria, which can be filtered

out by the sponges in the sponge aquaria (**Figure 5B**). It was still possible to accurately compare

wild-type and mutant using GFP in the sponge aquaria as we corrected for the water background

(**Figure 5B**).

Third, the bacteria cell counts either stay the same or increase in the no-sponge aquaria over

the course of 4 hours (**Figure 5B**). The increase is associated with GFP and likely due to

background contamination of bacteria with natural green fluorescence. The cell counts decrease

in the sponge aquaria at an equivalent rate for wild-type and Δ*pppA* mutant strains indicating that

the sponge filters each strain indiscriminately under the conditions tested.

Forth, note that the filtering capacity (**Figure 5B**) varied for each sponge individual (e.g., a

lower cell count was reached for experiment 2 compared to experiment 1), 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.

Discussion

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

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

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

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

of 4-7 in *E. coli* whereas deletions leading to a mini replicon increase the copy number to 35-40

(33), in accordance with the relative copy number of 47 we observed here (**Table 1**).

The pBBR1 replicon does not belong to IncP, IncQ or IncW groups and may represent a new

incompatibility group (34). It has a broad-host range in Gram-negative bacteria including

Pseudomonadota (34) and Thermodesulfobacteriota (35), and it has also been used in

Microbacterium (Actinomycetota) (36). The replicon is composed of *oriV*, one *rep* and one *mob*

gene, and as such, it is relatively small (34). The pBBR1 replicon (1.52 kb) in the SEVA plasmids

is the smallest of the broad-host range replicons we tested (21). It contains *oriV* and *rep*. Instead

of the *mob* gene present in the original replicon (34), all SEVA plasmids use *oriT* from RK2 (21).

A medium copy number of 30-40 was reported for *E. coli* and *Bordetella pertussis* (34), whereas

a growth defect was reported with some bacteria, including *Pseudomonas putida* (24), *Geobacter*

sulfurreducens (35) and *Burkholderia* sp. (22) corresponding to the higher copy number of pBBR1

in these strains. Both vectors we tested that contain the pBBR1 replicon resulted in high copy

number of 148-210 in *P. brasiliensis* (**Table 1**) but only pSEVA234M led to a growth defect (**Figure**

2). The observed growth defect with pSEVA234M may be due to a combination of higher plasmid

copy number of 210 and high expression of the *lacI^q* repressor gene from pSEVA234M (26, 27).

Although *P. brasiliensis* carrying pSEVA237R_Pem7 and pAM4891 showed comparable

growth rate to the wild type, their swarming motilies were affected to different extents.

Specifically, *P. brasiliensis* carrying pSEVA237R_Pem7, with a higher plasmid copy number

compared to pAM4891, exhibited a more noticeable reduction in swarming motility (**Figure 3A**).

Previous studies have shown that the production of unnecessary proteins, including fluorescent

proteins, imposes a significant metabolic burden on the host, slowing native protein synthesis (37-

40). Thus, the production of mCherry from the high copy number pSEVA237R_Pem7 in *P.*

brasiliensis might induce a burden on cellular processes, reducing the availability of resources to

produce essential proteins for swarming motility.

We next applied pAM4891 and pSEVA237R_Pem7 vectors in studies with marine sponges.

Specifically, we aimed to answer the question of whether pseudovibriamides (**Figure 1**) affect the

uptake of *P. brasiliensis* by *A. aerophoba* sponges. We observed no difference in uptake between

wild type and pseudovibriamide-defective Δ*pppA* mutants. Future studies should vary

experimental conditions such as using no pump to allow for comparing the difference in motility

of each strain that may affect sponge uptake. Long-term experiments in which sponges are

evaluated after the filtering experiment to determine whether strains can be established in the

host would also be helpful, although this may first require the development of axenic sponge

models which has been attempted but remains unresolved (41, 42). Alternatively, assays to study

how sponges differentiate between food and symbionts, for example, may prove insightful (43).

Finally, it is noteworthy that the filtering capacity varied for each sponge individual (**Figure 4B**),

stressing the importance of performing experiments with wild-type and mutant bacterial strains in

the same aquarium to allow for rigorous conclusions, as demonstrated here using the evaluated

vectors.

In conclusion, given the emerging roles of *Pseudovibrio* as part of sponge, coral and other

marine holobionts, and their production of secondary metabolites with biomedical and

biotechnological potential (15), the availability of genetic tools is important for biotechnological

applications but also to facilitate probing mechanistic questions regarding *Pseudovibrio* biology,

microorganism-microorganism and microorganism-host interactions.

Materials and Methods

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

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

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

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

using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich) and following the

manufacturer's instructions. The quality of the total DNA was evaluated by gel electrophoresis

and Nanodrop measurements. DNA samples were sequenced using Illumina paired-end whole

genome sequencing (400 Mbp, 2.67 M reads package) by the SeqCenter. Sample libraries were

prepared using the Illumina DNA Prep kit along with IDT 10bp UDI indices. Sequencing was

conducted on an Illumina NextSeq 2000, generating 2×151bp paired-end reads. Demultiplexing,

quality control and adapter trimming was performed using bcl-convert (v3.9.3). The obtained

sequence reads (**SI Table S1**) were mapped to the reference sequences – Ab134 genome

(GenBank: GCA_018282095.1) (17) plus each of the plasmids introduced using the in-built "Map

to References" function with medium sensitivity and five iterations in Geneious. In the alignment

file, the coverage statistics, which indicates the number of reads mapped at each position, was

used to calculate the plasmid copy number as shown below:

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

Growth curves. The growth of each bacterial strain was tracked for 48 h using a microtiter plate

method. Seed cultures were prepared by inoculating 50 µL of each cryo-preserved culture into 5

mL of MB. All cultures were incubated at 30°C overnight (18-20 h). Seed cultures were then diluted

in MB to OD_{600} of 0.05. OD-standardized cultures were dispensed (200 µL into each well, 10

replicates for each strain) into Falcon™ 96-well, non-treated, flat-bottom microplate using a multi-

channel pipettor. Border wells were filled with MB to maintain humidity. Plates were incubated at

30°C and shaken linearly at a speed of 887 revolutions per minute (amplitude 1 mm) using an

Infinite M Plex plate reader (Tecan). OD_{600} readings of each well were taken every 30 min over 48

h.

Swarming motility assays. Swarming assays were performed as previously described (17).

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

flow cytometry (CytoFLEX S, Beckman Coulter) set at medium flow (30 µl/min), with data

acquisition for 2 min per sample. A seawater sample was used as "blank" to correct the total cell

count. Wild type and mutant strains expressing either GFP or mCherry, respectively (or vice versa)

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

mixed cell suspensions were kept on ice until use.

Sponge-bacteria studies. Four experimental aquaria were prepared, each containing 4 L of

filtered (50 µm) seawater and an aquarium pump (**Figure 5A** and **SI Figure S4B**). *A. aerophoba*

sponge specimens containing one osculum were transferred to two of the experimental aquaria,

whereas the other two aquaria remained as no sponge controls. The sponge specimens were

transferred using a plastic bag, taking care that they were always submerged in seawater. Aliquots

(1.62 ml) were taken from each aquarium before inoculation (time -1). 4 ml of each bacterial cell

mixture (wild type expressing GFP and Δ*ppp*A mutant expressing mCherry or wild type expressing

mCherry and Δ*ppp*A mutant expressing GFP) was then added to two aquaria (to make up 1×10^6

cells per ml final concentration), one of which contained a sponge specimen. Water samples (1.62

ml) were taken from each aquarium at 0, 0.5, 1, 2, 3, and 4 hours, transferred to 2-ml Eppendorf

tubes and mixed with 0.18 ml fixative (10% paraformaldehyde, 0.5% glutaraldehyde). After 10 min

incubation at room temperature, the fixed samples were stored at -20 °C. Flow cytometry was

performed on all samples as described above to determine cell count originating from cells

expressing mCherry and GFP (**Figure 5**). The mCherry and GFP gates (**Figure 6**) were set based

on pure cultures. The relative cell count for each aquarium was calculated after correcting for the

corresponding -1 (before inoculation) sample (**SI Table S2**) to yield the results shown in **Figure**

5.

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