Phaeobacter gallaeciensis Reduces Vibrio anguillarum in Cultures of Microalgae and Rotifers, and Prevents Vibriosis in Cod Larvae

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Abstract

Phaeobacter gallaeciensis can antagonize fish-pathogenic bacteria in vitro, and the purpose of this study was to evaluate the organism as a probiont for marine fish larvae and their feed cultures. An in vivo mechanism of action of the antagonistic probiotic bacterium is suggested using a non-antagonistic mutant. P. gallaeciensis was readily established in axenic cultures of the two microalgae Tetraselmis suecica and Nannochloropsis oculata, and of the rotifer Brachionus plicatilis. P. gallaeciensis reached densities of 10⁷ cfu/ml and did not adversely affect growth of algae or rotifers. Vibrio anguillarum was significantly reduced by wild-type P. gallaeciensis, when introduced into these cultures. A P. gallaeciensis mutant that did not produce the antibacterial compound tropodithietic acid (TDA) did not reduce V. anguillarum numbers, suggesting that production of the antibacterial compound is important for the antagonistic properties of P. gallaeciensis. The ability of P. gallaeciensis to protect fish larvae from vibriosis was determined in a bath challenge experiment using a multidish system with 1 larva per well. Unchallenged larvae reached 40% accumulated mortality which increased to 100% when infected with V. anguillarum. P. gallaeciensis reduced the mortality of challenged cod larvae (Gadus morhua) to 10%, significantly below the levels of both the challenged and the unchallenged larvae. The TDA mutant reduced mortality of the cod larvae in some of the replicates, although to a much lesser extent than the wild type. It is concluded that P. gallaeciensis is a promising probiont in marine larviculture and that TDA production likely contributes to its probiotic effect.

Introduction

One of the major challenges of marine aquaculture is the continuous and reliable production of juveniles. Severe losses in marine larviculture are caused by infection with opportunistic pathogenic bacteria, including several members of the Vibrionaceae family [1,2], that accounts for approximately 1.5% of the bacterial community in the oceans [3]. Only some Vibrio species are pathogenic to organisms reared in marine aquaculture and one of the most prominent fish and shellfish pathogens is Vibrio (Listonella) anguillarum that causes serious disease and mortalities [2]. The main source of pathogenic bacteria in marine aquaculture is supply water [4], but also brood stock, humans, or starter cultures are possible sources of pathogens [5]. The majority of marine fish larvae are reared intensively in presence of microalgae (green water), which improves feeding, growth, and survival of the larvae [6–9]. The larvae require live feed, and rotifers (Brachionus plicatilis) are typically used as first feed. The rotifers themselves are fed or enriched with live microalgae, such as Tetraselmis suecica, Nannochloropsis oculata, and Isochrysis galbana. Opportunistic pathogens can proliferate in larval feed cultures of phytoplankton and invertebrates due to high concentrations of organic matter. Algae, rotifer and Artemia cultures can therefore harbor high concentrations of pathogenic bacteria [1,5,10,11]. Prophylactic treatment of larvae or their feed cultures with antibiotics can reduce the pathogen load, but has to be avoided, since it leads to emergence of antibiotic-resistant pathogens, and since it impedes the establishment of a normal non-pathogenic microbiota [1,12,13].

The potential use of probiotic bacteria to limit outbreaks or effects of bacterial diseases in fish and invertebrate cultures has been investigated for more than two decades. Most studies have focused on the intestinal microbiota [14–19], although the use of probiotics is not confined to the intestinal tract of the cultured organisms [15,20]. Biotic and abiotic surfaces, algal and fecal particles, and the nutrient-rich water serve as habitat and reservoir of opportunistic pathogenic bacteria in cultures of fish larvae or their food organisms [1,5,10,11,20], and it is hypothesized that competition by non-pathogenic bacteria that are superior in colonizing and persisting in these habitats could reduce the incidence of pathogenic bacteria.

Phaeobacter gallaeciensis (formerly Roseobacter gallaeciensis) is a Gram-negative ß-proteobacterium from the Roseobacter-clade [21]. The bacterium produces the antibacterial compound tropodithietic
acid (TDA) that is an efficient inhibitor of *V. anguillarum* and other fish-pathogenic bacteria [22-25]. *Phaeobacter* spp. are commonly isolated from larval cultures of marine fish and shellfish [26-20], and do not appear to adversely affect fish larvae [22,29]. *Ruegeria mobilis*, a close relative to *Phaeobacter* also producing TDA, is a cosmopolitan marine bacterium that can be isolated from most ocean waters, apart from polar waters [30].

In a previous study, it was demonstrated that *Phaeobacter* and *Ruegeria* isolated from a turf banchy [26] could eliminate *V. anguillarum* isolated from a turbot hatchery [26] could eliminate *V. anguillarum* and *Phaeobacter* strains were grown in MB without shaking at 20°C until stationary phase was reached. All strains were harvested by centrifugation (1,025 x g), washed twice, and resuspended in aerated autoclaved 80% seawater. The bacterial concentrations in these suspensions were determined using a counting chamber for *V. anguillarum*, and for the *P. gallaeciensis* strains by measuring OD600 after centrifugation and dissolving in 0.1M NaOH.

**Generation of a TDA-negative Phaeobacter mutant**

A mutant library of *P. gallaeciensis* BS107 was created by random transposon insertion mutagenesis using the EZ-Tn5 <R6K::Tn5::KAN> Tn5 Transposome Kit (Epigentic, Madison, WI), following the procedure of Geng et al [43]. Ten non-pigmented mutants were selected, and absence of TDA production was confirmed by UHPLC-TOFMS and in an agar-diffusion test against *V. anguillarum* [27]. Growth rates of selected mutants were compared to the wild type in aerated (200 rpm) ½ YTSS cultures at 30°C, and one of the strains with a growth rate comparable to the wild type, BS107-PdAl, was chosen for further experiments. Using rescue cloning as described in the transposome kit manual, the mutated locus was identified as CDS101461, which encodes for a “periplasmic component of a TRAP-type C4-di-carboxylate transport system”, as annotated on the BS107 genome on www.roseobase.org.

**Materials and Methods**

**Bacterial strains and media**

All strains and plasmids are listed in Table 1. *Phaeobacter* (Roseobacter) *gallaeciensis* BS107 (DSM17395) was isolated from seawater in scallop (*Pecten maximus*) cultures [28]. *Vibrio anguillarum* serotype O1 strain NB10 was used in algol and rotifer experiments. It was isolated from the Gulf of Bothnia and has caused disease in rainbow trout [33,34]. The strain has been tagged by insertion of plasmid pNQfHaC1-gfp27 (at a/gfp) into an intergenic region on the chromosome, and was kindly provided by D. Milton, University of Umeå [35]. *V. anguillarum* serotype O2a H1610 was used in challenge trials with cod larvae. The strain was isolated from diseased cod juveniles in the closed seawater basin Lake Parivat in the Institute of Marine Research (IMR), Norway, and has been used in challenge trials with cod [36-39]. It has been selected from a group of *V. anguillarum* strains of different serotypes, being the strain that caused the highest mortality in challenge trials with turbot, halibut and cod larvae [40].

Bacteria from frozen stock cultures (~80°C) were streaked on half-strength Marine Agar (½MA; 27.6 g Difco 212185 Marine Agar, 15 g Instant Ocean Sea Salts, 7.5 g Agar, 1 l deionized water). ½MA was also used for counting *P. gallaeciensis*. *V. anguillarum* was counted on Tryptone-Soy Agar (TSA; Oxoid CM0131) containing 6 mg/l chloramphenicol. The bacterial precultures for the algae and rotifer experiments were grown in 20 ml of ½YTSS (2 g Bacto Yeast extract, 1.25 g Bacto Tryptone, 20 g Sigma Sea Salts, 1 l deionized water) [41] at 25°C with aeration (200 rpm) until OD600 = 1.0. The cells were harvested at 5,000 x g, washed twice, and used as inoculum for algae and rotifer experiments. Bacteria were diluted and washed in artificial seawater (ASW; 2% Sigma Sea Salts). Axenicity of algae and rotifer cultures was controlled by plating 100 ml on ½MA and incubating for 7 days at 25°C.

For the challenge trials, *V. anguillarum* H1610 was grown in tryptone-soy broth with additional 0.5% NaCl at 20°C with shaking at 60 rpm to an OD600 of about 0.5. The *P. gallaeciensis* strains were grown in MB without shaking at 20°C until stationary phase was reached. All strains were harvested by centrifugation (1,025 x g), washed twice, and resuspended in aerated autoclaved 80% seawater. The bacterial concentrations in these suspensions were determined using a counting chamber for *V. anguillarum*, and for the *P. gallaeciensis* strains by measuring OD600 after centrifugation and dissolving in 0.1M NaOH.

**Fluorescence tagging of Phaeobacter**

*P. gallaeciensis* BS107 was tagged chromosomally with a miniTn7(Gm)PA1/04/03:DsRedExpress-a cassette, using a mini-Tn7 tagging system [43,44]. The delivery and helper plasmids were electroporated into *P. gallaeciensis*, followed by selection on ½MA containing 75 µg/ml gentamicin. pPDA11, a transcriptional fusion of the tdaC promoter to a promoterless gfp gene ligated to the broad-host range plasmid pRK415, was constructed in an analogous manner to pHG1011 as described in Geng et al. 2010 [45].

**Phaeobacter antagonism in algae**

*Tetraselmis suecica* CCAP 66/4 (Prasinophyceae) was obtained as axenic culture from the Culture Collection of Algae and Protozoa (Oban, UK). It was cultured in B-medium [46], a mineral algae medium, based on ASW. The 250-ml culture bottles were closed with cotton plugs and slowly aerated through a 0.2 µm syringe filter and a silicone tube, to prevent settling of particles. Light intensity on the bottles was 13,000 lux (daylight spectrum). Algal concentrations were assessed by measuring absorption at 665 nm, and calibrating with counts of axenic reference cultures in a Neubauer-improved counting chamber, using formaldehyde as fixative (0.5% final concentration). For each *V. anguillarum* inoculum level tested, eight bottles of 150 ml of B-medium were inoculated with 6.6 x10⁷ cells/ml axenic *T. suecica*. Two bottles were inoculated with approximately 10⁶ cfu/ml washed *P. gallaeciensis* BS107, two bottles with the same level of washed mutant *P. gallaeciensis* BS107-PdAl cells, and four bottles were left axenic. The cultures were grown for 2 days and axenicity was checked. All cultures, except two axenic negative controls, were inoculated with *V. anguillarum* NB10 to concentrations of 10, 100, 1,000, or 10⁵ cfu/ml. Inoculum levels were verified by plate-counting. Concentrations of algae and both bacterial species were observed until day 5 after inoculation of the pathogen. Two independent experiments were performed for every initial concentration of *V. anguillarum*. *Nannochloropsis oculata* CCMP525 (Eustigmatophyceae) was obtained as axenic culture from the Center for Culture of Marine Phytoplankton (West Boothbay Harbor, ME). Since it did not grow in ASW-based B-medium, it
was cultured in f/2-medium [47] based on Atlantic Seawater obtained from CCMP. *N. oculata* cultures were not aerated. Antagonism experiments were done as in *T. suecica*, but only one initial *V. anguillarum* concentration (10⁵ cfu/ml) was tested. Two independent experiments with two different initial densities of algae (lower density: 4 × 10⁶ cells/ml, higher density: 2 × 10⁷ cells/ml) were carried out.

TDA analysis

Samples of *T. suecica* – *P. gallaeciensis* co-cultures (20 ml) were extracted in 50-ml Falcon tubes with 30 ml ethyl acetate (HPLC grade) containing 1% formic acid (HPLC grade) on a shaking table for 1 h. The samples were centrifuged at 8,000 × g, and 26 ml of the upper phase was transferred to a new Falcon tube and evaporated to dryness at 35°C with nitrogen flow. The samples were reconstituted in 300 µl 85% acetonitrile, vortexed for 5 sec, placed in an ultrasonication bath for 10 min, vortexed again for 5 sec and filtered through a standard 0.22 µm PTFE syringe filter into a HPLC vial. Subsamples of 2 µl were then analyzed by UHPLC-TOFMS on a maXis G3 quadrupole time of flight mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray (ESI) ion source which was connected to an Ultimate 3000 UHPLC system (Dionex, Sunnyvale, CA). Separation was performed at 40°C on a 100 mm × 2.1 mm ID, 2.6 µm Kinetex C₁₈ column (Phenomenex, Torrance, CA) equipped with Kinetex pre-column using a water-acetonitrile gradient (both buffered with 20 mM formic acid) at a flow of 0.4 ml min⁻¹ starting at 10% acetonitrile and increased to 100% in 10 min, keeping this for 3 min. MS was performed in ESI⁺ with a data acquisition range of m/z 100–1000 at a resolution of 40,000 FWHM, the MS was calibrated using 20 mM sodium formate in the presence of [M+H]⁺ ions (± m/z 0.001).

For quantification, *T. suecica* cultures were spiked to a final concentration of 4800, 2400, 1600, 800, 320, 160, and 0 (blank) nM TDA (BioViotica, Dransfeld, Germany) by adding a maximum of 80 µl TDA-acetonitrile solution, and treated as described above. Spiked samples were left at room temperature for at least 1 h prior to extraction. The method was validated on 3 different days using spiked samples as described, and no false positives or negatives were recorded. Relative standard deviation was 30% and the limit of detection was estimated to be <50 nM (signal/noise 5:1), based on the blank samples and lower calibration points. Sensitivity was greatly influenced by the age of the UHPLC column since TDA tailed (although a new precolumn was used) on columns which had been in use for only a few weeks. Samples from two individual biological experiments were analyzed independently.

Absence of TDA in static cultures of the TDA-negative mutant BS107-Pda8 was confirmed by UHPLC-TOFMS analysis.

Phaeobacter antagonism in rotifers, Brachionus plicatilis

A stock of the rotifer *B. plicatilis* (L-type) was obtained from Reed Mariculture (Campbell, CA). Axenic rotifers were attained by disinfecting approximately 50 amictic rotifer eggs in 1 ml of a strong antibiotic solution (150 µg/ml Tetracycline, 300 µg/ml Kanamycin, 60 µg/ml chloramphenicol, 1000 U/ml Penicillin in ASW) for 2 days. The hatched rotifers were filtered onto a sterile 50-µm polyamide mesh, rinsed with ASW and from then on fed with concentrated axenic *T. suecica*. No experiment with *N. oculata* as rotifer feed was conducted, since survival of *V. anguillarum* in presence of *N. oculata* was very variable (see Results section). Rotifer densities were determined by counting in a Sedgewick-Rafter counting chamber. Before counting, the cultures were thoroughly mixed, and 100 µl 5% formaldehyde added to a 1 ml sample. To set up co-culture experiments, axenic rotifer cultures were divided into eight 20-ml batches in 50-ml centrifuge tubes. The initial rotifer concentrations were 94 /ml (first replicate) and 30 /ml (second replicate). For each replicate, duplicate cultures were inoculated with washed wild type and mutant *P. gallaeciensis* at approximately 5 × 10⁵ cfu/ml. On the next day, all cultures except the axenic controls were inoculated with 10⁴ cfu/ml *V. anguillarum*. All rotifer cultures were fed daily with 10-fold concentrated *T. suecica* ([1–2 ml depending on average rotifer density]), so that the algae concentration was at approximately 10⁶ cells/ml after feeding and did not drop below 2 × 10⁵ cells/ml. The rotifer cultures were counted daily, concentrations of *V. anguillarum* and *P. gallaeciensis* were determined, and axenicity of the negative controls was checked. The rotifer culture samples for enumeration of bacteria were homogenized by grinding and repeated pipetting through a 100-µl pipette tip. This was compared to homogenization with an Ultra-Turrax T25 (IKA, Germany) at 16,000 rpm and no significant differences in bacterial counts were found (P = 0.74).

### Table 1. Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant markers</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. gallaeciensis</em> BS107 (DSM17395)</td>
<td>Wild type</td>
<td>Ruiz-Ponte et al. 1998 [28]</td>
</tr>
<tr>
<td><em>P. gallaeciensis</em> BS107-Pda8</td>
<td>CDS104961:αZ-Tn5, Kan⁶</td>
<td>This study</td>
</tr>
<tr>
<td><em>P. gallaeciensis</em> dhsRed</td>
<td>MinTn7(Gm⁶)P₁₀₄₀00DisRedExpress-a</td>
<td>This study</td>
</tr>
<tr>
<td><em>V. anguillarum</em> NB10</td>
<td>Serotype O1, cm⁶, PA1/04/03-RBSII-gfpmut3⁻-T1</td>
<td>Croxatto et al. 2007 [35]</td>
</tr>
<tr>
<td><em>V. anguillarum</em> H610</td>
<td>Serotype O2⁷</td>
<td>Samuelsen &amp; Bergh 2004 [36]</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EZ-Tn5⁶ Transposome</td>
<td>EZ-Tn5&lt;②R6Kyor, Kan⁶&gt;Tnp</td>
<td>Epicentre Biotechnologies</td>
</tr>
<tr>
<td>pAKN132</td>
<td>miniTn7(Gm⁶)P₁₀₄₀₀₀DisRedExpress-a</td>
<td>Lambertsen et al. 2004 [43]</td>
</tr>
<tr>
<td>pUX-BF13</td>
<td>Helper plasmid: Tn7 transposase proteins</td>
<td>Bao et al. 1991 [44]</td>
</tr>
<tr>
<td>pPDA11</td>
<td>tdaCp:&lt;gfp ligated into broad host range vector pRK415</td>
<td>D’Alvise et al. (in preparation)</td>
</tr>
</tbody>
</table>

DOI:10.1371/journal.pone.0043996.t001
Challenge trial
The protocol was adapted from [39,40]. Cod (Gadus morhua) embryos were obtained from the commercial hatchery Havlandet AS, in Flora, Western Norway. Transport of the embryos in polystyrene containers at around 8°C took 4 to 5 hours in total by boat and car. Two independent replicates of the challenge trial were conducted. The embryos used in the first trial were disinfected with Buffolide (Evans Vanodine, Preston, UK), the embryos for the second trial were left untreated. Upon arrival, the embryos were randomly picked and distributed to the wells of 24-well dishes (Nunc, Roskilde, Denmark) filled with 2 ml 80% autoclaved, aerated seawater, placing one embryo in each well. In each trial three dishes for each treatment (72 embryos) were prepared and inoculated immediately. The six treatment groups are listed in Table 2. All inocula were prepared in a volume of 100 µl, and the strains were not mixed before inoculation. Initial bacterial concentrations were 1 × 10⁶ cfu/ml for V. anguillarum HI610 and about 10⁵ cfu/ml for the P. gallaeciensis strains. The plates were incubated in the dark at 7°C. The day when 50% of the larvae had hatched was defined as day 0, which was 6 days after the start of the experiment. Dead larvae were registered daily for 14 days.

Statistics
Differences between concentrations of bacteria or algae were assessed using repeated measures ANOVA after log-transformation. Tukey’s multiple comparison test was used for pairwise comparisons. To address the effects of P. gallaeciensis presence on concentrations of V. anguillarum, initial values (day 0) were omitted in the analysis and the experiments were analyzed separately. Rotifer numbers were not log-transformed before applying ANOVA, and initial values were omitted. Numbers of P. gallaeciensis and homogenization methods were compared using paired t-tests after log-transformation.

The cumulative mortalities in the challenge trials were compared at day 10, prior to the onset of starvation towards the end of the experiment. A chi-square test for 2² contingency tables was implemented, using the software R, version 2.13.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results
Antagonism in algae cultures
Both wild type and mutant P. gallaeciensis colonized the cultures of T. suecica and N. oculata. In T. suecica cultures, P. gallaeciensis reached 10⁷ cfu/ml (Figure 1). In the dense cultures of N. oculata, P. gallaeciensis concentrations were approximately 5 × 10⁶ cfu/ml, and in the less dense cultures approximately 8 × 10⁵ cfu/ml (Figure S1). The wild type Phaeobacter reached slightly higher numbers in T. suecica than the TDA-negative mutant (P = 0.0211). This same slight difference was seen in one of the two Nannochloropsis experiments (P = 0.0335, P = 0.9259). P. gallaeciensis did not affect growth of the algae T. suecica (P = 0.9977) and N. oculata (P = 0.9919). Particles consisting of dead T. suecica and algal cell walls that were shed during cell division served as habitat for rosette forming P. gallaeciensis that formed dense biofilms on the particles (Figure 2 A–D).

V. anguillarum effectively colonized all Tetraselmis cultures that were not inoculated with P. gallaeciensis and numbers increased by up to 2.7 log units within the first day (Figure 3, Figure S2) and reached an average of 3 × 10⁶ cfu/ml after 5 days. V. anguillarum did not colonize particles in the algae cultures, but remained in suspension (Figure 2 E). The numbers of V. anguillarum decreased markedly in presence of wild type P. gallaeciensis (Figure 3, Figure S2). Vibrio reductions were in the order of 3 log units, as compared to the monoxenic controls with only V. anguillarum, and complete elimination of the lowest Vibrio inoculum was achieved in 3 out of 4 replicates (Figure 3A). The effects of wild type P. gallaeciensis on V. anguillarum were highly significant in all Tetraselmis experiments, as compared to the controls (P < 0.001) and to the mutant (P < 0.001). Presence of the TDA-negative mutant did decrease concentrations of the pathogen by about one log unit, although this was only significant (α = 0.05) for two of the initial Vibrio concentrations.

![Figure 1. Concentrations of Tetraselmis suecica and Phaeobacter gallaeciensis in the co-cultures. Means and standard deviations of eight experiments: colony-forming units of P. gallaeciensis wild type (●) and the TDA-negative mutant (●), and concentrations of T. suecica with V. anguillarum (◇), T. suecica with P. gallaeciensis wild type (■), T. suecica with P. gallaeciensis TDA-negative mutant (▲), and axenic T. suecica (□). The P. gallaeciensis strains were inoculated at 10⁷ cfu/ml and remained as a steady population, while the algae went from late log into stationary phase. doi:10.1371/journal.pone.0043996.g001](Image 315x580 to 516x730)

Table 2. Group numbers and treatments in the challenge trial.

<table>
<thead>
<tr>
<th>Group number</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Negative control; no bacteria added</td>
</tr>
<tr>
<td>T2</td>
<td>Positive control; V. anguillarum O²x H1610 10⁵ cfu/ml</td>
</tr>
<tr>
<td>T3</td>
<td>Wild type P. gallaeciensis BS107 (DSM17395) ~10⁷ cfu/ml</td>
</tr>
<tr>
<td>T4</td>
<td>TDA-mutant P. gallaeciensis BS107-Pda8 ~10⁴ cfu/ml</td>
</tr>
<tr>
<td>T5</td>
<td>V. anguillarum O²x H1610 10⁵ cfu/ml and wild type P. gallaeciensis BS107 (DSM17395) ~10⁵ cfu/ml</td>
</tr>
<tr>
<td>T6</td>
<td>V. anguillarum O²x H1610 10⁵ cfu/ml and TDA-mutant P. gallaeciensis BS107-Pda8 ~10⁴ cfu/ml</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0043996.t002
The marked difference in *V. anguillarum* inhibition by the wild type *P. gallaeciensis* and the TDA negative mutant suggested that TDA was a major effector molecule. However, TDA was not detected by chemical analysis of the *Phaeobacter–Tetraselmis* co-cultures, where triplicate cultures were each analyzed in triplicates with a detection limit, 50 nM TDA. The experiment and analysis were repeated with the same result. To determine if the wild type did indeed produce TDA in the algal cultures, a *P. gallaeciensis* carrying pPDA11 (tdaC::gfp) was co-cultured with *T. suecica*. The tdaC promoter, indicative of TDA production, was induced when growing on particles in a *T. suecica* culture, as indicated by GFP fluorescence (Figure 4). Adding pure TDA to *Tetraselmis* cultures inoculated with *V. anguillarum* caused a complete killing of the *Vibrio* population, but also affected survival of algae (50 μM TDA). A 50-fold lower concentration (1 μM) had no effect on the algae, but temporarily reduced *V. anguillarum* below 10 cfu/ml. A concentration of 50 nM TDA, which was the detection limit of the chemical analysis, did not have any effect (data not shown).

*V. anguillarum* was completely eliminated in *Nannochloropsis oculata* cultures by wild type *P. gallaeciensis* within one or two days (Figure S3). However, *V. anguillarum* could only persist in dense cultures of *N. oculata*. In less dense *N. oculata* cultures *V. anguillarum* disappeared from the monoxenic control within 3 days. Consequently, the effect of the wild type *P. gallaeciensis* on *V. anguillarum*, as compared to the control, was significant in the experiment with high algae density (P = 0.001), but not in low density (P = 0.2106).

### Antagonism in rotifer cultures

The concentrations of *P. gallaeciensis* and its mutant in the rotifer cultures were stable at about 10^6–10^7 cfu/ml, and no significant difference between the two strains was observed (P = 0.3689). The rotifers grew faster and reached higher densities in presence of *P. gallaeciensis* than in the axenic or monoxenic (*V. anguillarum*) controls (P<0.05) (Figure 5, Figure S4). Wild type *P. gallaeciensis* reduced *V. anguillarum* concentrations by 3 log units (P<0.01), in average from
6 × 10^5 to 9 × 10^2 cfu/ml (Figure 6). The effect of the TDA-negative mutant on the concentration of the pathogen was not significant (P > 0.05).

**Challenge trial**

Six days after the arrival of the embryos and inoculation, more than 50% of the larvae had hatched. Total cumulative hatching success was 79.2% (first trial 79.6% and second trial 78.7%). The initial mortality was lower in the first trial (16.6%) than in the second (24.8%). In the non-challenged and non-treated control, 34.7% ± 9.8% (average ± standard deviation) of the larvae had died by day 1, yet after the initial mortality only 2.8% ± 0.0% of the larvae died between day 2 and day 10, reaching an accumulated mortality of 37.5% ± 9.8% at day 10. The larvae challenged with *V. anguillarum* HI610 died rapidly and reached 100% ± 0.0%

![Figure 4. Expression of tdaC in co-culture with Tetraselmis suecica. Phase contrast (A) and fluorescence (B) micrographs of *P. gallaeciensis* pPDA11 (tdaCp::gfp) in co-culture with *T. suecica*. The two panels show the same seven algal cells of which some are dividing, and a marine snow-like particle which is colonized by *P. gallaeciensis* carrying the promoter-fusion on a plasmid. The green fluorescence of *P. gallaeciensis* on the particle shows that the gfp gene is expressed from the tdaC promoter, indicating production of TDA. doi:10.1371/journal.pone.0043996.g004](image)

![Figure 5. Influence of bacterial strains on rotifer growth. Rotifer numbers in co-culture with *P. gallaeciensis* wild type (▼), with the TDA-negative mutant of *P. gallaeciensis* (●), with only *V. anguillarum* (▲), and axenic rotifers (■), first experiment. All bacteria were inoculated at day 0. Both *P. gallaeciensis* strains promoted rotifer growth, whereas *V. anguillarum* had no influence. doi:10.1371/journal.pone.0043996.g005](image)

![Figure 6. Reduction of *Vibrio anguillarum* by *Phaeobacter gallaeciensis* in rotifer cultures. Mean values of two duplicate experiments: colony-forming units of *V. anguillarum* in co-culture with *P. gallaeciensis* wild type (▲), with the TDA-negative mutant of *P. gallaeciensis* (▼), and in the monoxenic control (■). doi:10.1371/journal.pone.0043996.g006](image)
accumulated mortality. Treating *Vibrio*-challenged larvae with wild type *P. gallaeciensis* caused a significant reduction in accumulated mortality by day 10 to 12.5% ± 2.0%, which was not only lower than in the challenged larvae but also lower than in the non-challenged (37.5%), 96.1% ± 1.1% of the hatched larvae that had received wild type *P. gallaeciensis* survived until day 10, when starvation set in (Figure 7, Figure S5). The larvae exposed to only *P. gallaeciensis* wild type or mutant had a cumulative mortality of 12.1% ± 3.1% at day 10. The TDA-negative mutant of *P. gallaeciensis* did reduce accumulated mortality of the challenged larvae to 68.8% ± 30.4% (Figure 7, Figure S5), but was not nearly as efficient as the TDA-producing wild type.

**Discussion**

The present study demonstrates that *Phaeobacter gallaeciensis* is harmless and beneficial for the early life stages of cod. Equally important, *P. gallaeciensis* is highly efficient at preventing infections with *V. anguillarum*, and this probiotic effect can be achieved at the low temperature (7°C) used for the cod embryos and yolk sac larvae. It has previously been demonstrated that a *Phaeobacter* sp. can protect turbot larvae against vibriosis at higher temperatures (18°C) [29]. Non-infected larvae showed some level of initial mortality, which may have been due to opportunistic bacteria introduced with the embryos. Both challenged and unchallenged cod larvae exposed to *P. gallaeciensis* had a significantly lower initial mortality, indicating that the inherently occurring microbiota of the chorion may be controlled by the probiont.

A key question in the use of probiotics in aquaculture is how and where the probiont should be introduced to the system. Several studies have emphasized the potential role of feed organisms as a vehicle for probiotic bacteria [29,48–51], or the potential of probiotic bacteria to control pathogenic bacteria in the feed cultures [51–53]. The majority of studies have focused on intestinal probiotic bacteria, and aimed at health-promoting effects within either the reared animal or the feed organism. In contrast, the present study takes a systems approach to preventing bacterial disease in aquaculture organisms, aiming at microbial control throughout the environment of the reared organism and the lower trophic levels of the production. Here it was found that cultures of two aquaculture-relevant algae and of the rotifer *B. phycodunum* can be colonized by *P. gallaeciensis* without compromising their growth, and that *P. gallaeciensis* in these cultures will strongly reduce, or eliminate fish-pathogenic *V. anguillarum*. Introducing *P. gallaeciensis* at this trophic level is very promising, since live feed is a common source of opportunistic pathogens [1,5,10,11]. These findings corroborate the hypothesis from a previous study, that algae and rotifers in aquaculture can be cultured together with probiotic *Roseobacters*, and thus prevent proliferation of pathogens [22]. A reduction of a pathogenic *Vibrio* sp. by 3 log units, as it was achieved in the present study, is very promising in terms of larval health promotion, as only a one log reduction of the bacterial load in rotifers through UV radiation resulted in higher survival of turbot larvae [54]. Using probiotic bacteria, as compared to UV treatment, offers the advantage that nutrients are consumed, niches are occupied, and rapid re-growth of pathogens is prevented. It should be noted that the present study was done using gnotobiotic systems to rule out the influence of the inherent microbiota of algae and rotifer cultures. Thus, it cannot be determined, if or to what extent *P. gallaeciensis* would affect the inherent microbial communities of algae and rotifer cultures.

The inhibition of *V. anguillarum* by a *Phaeobacter* sp. in a model aquaculture setting has been studied once before: Planas et al. [29] demonstrated that mortality in turbot larvae infected with *V. anguillarum* could be reduced by *Phaeobacter* sp. 27-4. A duplicate tank setup was used, and both the pathogen and the probiont were enclosed in rotifers and fed to the larvae. In spite of delivery with the feed, the probiont was only found in the lumen of the larval gut and did not colonize the intestinal epithelium. In contrast to this, the present study did not aim at a probiotic effect in the intestinal tract of the larvae, but assesses the potential of *Phaeobacter* to eliminate the pathogen in the environment of the larvae or embryos. It should be mentioned that, as larvae start to drink shortly after hatching [55], an intestinal presence of pathogens and probionts can occur. *Phaeobacter* sp. 27-4 is a TDA-producer, however, as opposed to *P. gallaeciensis* BS107, it produces TDA only in stagnant culture [26], suggesting that its TDA production may be more delimited and that BS107 could be more antagonistic in vivo.

In the challenge trial the TDA-negative mutant reduced the initial mortality as efficiently as the wild type, but could not prevent infection in the majority of the larvae. The probiotic effect of the mutant could be explained by competition for nutrients, space, or other resources or it could be attributed to a direct immunostimulatory effect on the larvae [56–59]. The mutated gene that renders *P. gallaeciensis* BS107-PdA8 unable to produce TDA belongs to an operon encoding the parts of a transport protein, which has not yet been reported to be involved in TDA production [42]. The role of this transmembrane protein in TDA production has not been investigated. It cannot be excluded that this mutation has pleiotropic phenotypic effects, and other functions than TDA production might be affected and could affect the antagonistic properties of *Phaeobacter gallaeciensis*. Nonetheless, the experiment using pure TDA indicated that this compound indeed has a major inhibitory effect against *V. anguillarum* in the algal system.

The difference in *Vibrio*-antagonism between the TDA-negative mutant and the wild type suggested that TDA production is the trait that enables *P. gallaeciensis* to antagonize *V. anguillarum*. However, TDA was not detectable by chemical analysis of the *Phaeobacter*-Tetraselmis co-cultures. Since a tdaC-promoter fusion (tdaC:gfp), demonstrated that tdaC is expressed by *P. gallaeciensis* in particles in the algae cultures, the reason for the lack of chemical detection could be that the TDA concentration only reaches
inhibitory concentrations in *Phaeobacter*-colonized particles. TDA is likely concentrated within and around the particles, adhering to organic mass of the particle, or being kept within the EPS produced by *P. gallaeciensis*. From an ecological point of view, for a particle-associated marine bacterium the production of an antagonistic compound would be more efficient if the compound was not dispersed, but kept in the close vicinity to fend off possible competitors.

Although TDA-producing *Phaeobacter* and *Ruegeria* spp. are likely to be already present in larviculture systems, their antagonistic properties, which may depend on growth conditions, are probably different from *P. gallaeciensis* BS107 [22,26]. A preliminary experiment to this study showed that only a few of the tested *Phaeobacter* and *Ruegeria* strains were antagonistic in *T. suecica* cultures, whereas all of them did account for large inhibition zones in agar-based assays. Therefore, introduction of *P. gallaeciensis* BS107 in algae and rotifer cultures would likely enhance larval survival even though other *Ruegeria* spp. are already present in the system. Its growth-promoting effect on rotifers may offer an unexpected additional advantage. Whether that is due to the nutritive value of the bacteria or to a potential role in the rotifer gut is not known. In the present study rotifer growth was not adversely affected by *V. anguillarum*. Nevertheless, a *V. anguillarum* strain was reported to cause pronounced growth inhibition of rotifers under suboptimal feeding schemes [60], which could possibly be remediated by *P. gallaeciensis*.

It cannot be predicted, if and how other pathogens in algae and rotifer cultures would be suppressed by *P. gallaeciensis*, however a range of fish pathogens are inhibited in *vitro* by *P. gallaeciensis* [22,25] indicating that it likely could protect against other pathogens than *V. anguillarum*. Forsby et al. [61] have addressed the concern that resistance to TDA could develop, and found, using several experimental approaches, that no resistant mutants or variants could be isolated, neither from short-term selection cultures containing different concentrations of TDA nor from long-term adaptation cultures (>300 generations) containing increasing concentrations of TDA.

A recent study demonstrated that *P. gallaeciensis*, when incubated with p-coumaric acid, produced potent algicides, the roseobactins [62], which are effective against different microalgae, among them *T. suecica* [62]. P-coumaric acid is a degradation product of lignin, which may account for higher local concentrations of p-coumaric acid in algae and rotifer cultures, whereas all of them did account for large inhibition zones in *T. suecica* cultures. Therefore, introduction of *P. gallaeciensis* BS107 in algae and rotifer cultures would likely enhance larval survival even though other *Ruegeria* spp. are already present in the system. Its growth-promoting effect on rotifers may offer an unexpected additional advantage. Whether that is due to the nutritive value of the bacteria or to a potential role in the rotifer gut is not known. In the present study rotifer growth was not adversely affected by *V. anguillarum*. Nevertheless, a *V. anguillarum* strain was reported to cause pronounced growth inhibition of rotifers under suboptimal feeding schemes [60], which could possibly be remediated by *P. gallaeciensis*.

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Based on the present findings, it is hypothesized that *P. gallaeciensis* can be used in marine larviculture, as a means of controlling the ambient, potentially harmful microbiota in cultures of rotifers and microalgae, and as a prophylaxis against vibriosis in fish larvae.

Supporting Information

Figure S1 Concentrations of Nannochloropsis oculata and Phaeobacter gallaeciensis in the co-cultures. Colony-forming units of *P. gallaeciensis* wild type (○) and the TDA-negative mutant (□), and concentrations of *N. oculata* with *V. anguillarum* (▲), *N. oculata* with *P. gallaeciensis* TDA-negative mutant (●), and axenic *N. oculata* (■) in the dense (A) and less dense (B) cultures. (TIF)

Figure S2 Reduction of Vibrio anguillarum by Phaeobacter gallaeciensis in cultures of Tetraselmis suecica. Colony-forming units of *V. anguillarum* inoculated at 10⁷ cfu/ml (A) and at 10⁸ cfu/ml (B) in presence of *P. gallaeciensis* wild type (▲), in presence of the *P. gallaeciensis* TDA-negative mutant (●), and in the monoclonic control (▼). (TIF)

Figure S3 Reduction of Vibrio anguillarum by Phaeobacter gallaeciensis in cultures of Nannochloropsis oculata. Colony-forming units of *V. anguillarum* in presence of *P. gallaeciensis* wild type (▲), in presence of the *P. gallaeciensis* TDA-negative mutant (▼), and in the monoclonic control (■), in dense (3×10⁶ cells/ml; A) and less dense (1–7×10⁶ cells/ml; B) cultures of *N. oculata*. (TIF)

Figure S4 Influence of bacterial strains on rotifer growth. Rotifer numbers in co-culture with *P. gallaeciensis* wild type (▼), with the TDA-negative mutant of *P. gallaeciensis* (●), with only *V. anguillarum* (▲), and axenic rotifers (■), second experiment. All bacteria were inoculated at day 0. Both *P. gallaeciensis* strains promoted rotifer growth, whereas *V. anguillarum* had no influence. (TIF)

Figure S5 Mortality of cod larvae during the challenge trials. Mean values of two independent triplicate experiments with error bars indicating standard deviations. The single-larvae cultures were simultaneously inoculated with *P. gallaeciensis* wild type and *V. anguillarum* (T5, ▼), or with the TDA-negative mutant of *P. gallaeciensis* and *V. anguillarum* (T6, □). Unexposed larvae and larvae exposed to single bacterial strains acted as controls: Negative Control (T1, ▲), only *V. anguillarum* (T2, ●), only *P. gallaeciensis* wild type (T3, ▼), and only *P. gallaeciensis* TDA-negative mutant (T4, △). (TIF)

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Author Contributions

Conceived and designed the experiments: PD SL HWK KFN OB LG. Performed the experiments: PD SL MJP KFN HIW OB. Analyzed the data: PD SL KFN HW OB LG. Wrote the paper: PD LG.

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