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Disruption of Cell-to-Cell Signaling Does Not Abolish the Antagonism of *Phaeobacter gallaeciensis* toward the Fish Pathogen *Vibrio anguillarum* in Algal Systems

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Quorum sensing (QS) regulates *Phaeobacter gallaeciensis* antagonism in broth systems; however, we demonstrate here that QS is not important for antagonism in algal cultures. QS mutants reduced *Vibrio anguillarum* to the same extent as the wild type. Consequently, a combination of probiotic *Phaeobacter* and QS inhibitors is a feasible strategy for aquaculture disease control.

Bacteria predominantly exist in mixed populations, and the behavior of the complete population is often affected by highly structured and sophisticated ways of communication. Many bacteria secrete small pheromone-like substances that allow the complete population to sense its density and subsequently alter, in a coordinated manner, gene expression. This type of communication between bacteria is known as quorum sensing (QS). QS systems play an important role in the regulation of genes related to virulence and, also, in genes involved in the production of bioactive compounds, such as antibiotics and enzymes (1–4).

Roseobacter clade bacteria are some of the most common prokaryotes in oceanic and aquaculture environments (5–7). Some *Roseobacter* clade species, such as *Phaeobacter gallaeciensis*, *Phaeobacter inhibens*, and *Ruegeria mobilis*, produce a broad-spectrum antibacterial compound known as tropodithietic acid (TDA) (7–9). TDA does not induce resistance in the target organisms (10) and is the key compound for antagonism in laboratory broth cultures (11), in algae and rotifers, and in fish larval systems (12, 13). The ecological role of TDA is not known, but as TDA-producing bacteria can inhibit fish and shellfish pathogens, they have interesting prospects for application as probiotics in aquaculture (13, 14).

QS compounds of the acylated homoserine lactone (AHL) class are produced by some *Roseobacter* clade species (15–17), and TDA production is influenced by AHLs in *Phaeobacter gallaeciensis*. Genes homologous to the classical *luxI* (*pgaI*) and *luxR* (*pgaR*) genes have been identified, and a 24-h culture supernatant of a *pgaI*-negative mutant that is unable to produce the AHL compound did not antagonize other bacteria as the wild type did (18). *Ruegeria mobilis* does not produce AHLs, but in this bacterium, TDA itself can act as an autoinducer, controlling the expression of key genes for TDA production (19).

Roseobacter bacteria are frequently associated with algae, both in oceanic waters and aquaculture systems (5, 7). In aquaculture, algae are used as a direct addition to fish larva-rearing tanks and to enrich live prey organisms, such as rotifers and *Artemia*, with fatty acids. Both algae and live feed have been suggested as vehicles for the introduction of probiotics into rearing systems (20, 21), and a strategy of adding *Phaeobacter* strains to reduce pathogenic *Vibrio* in live feed and larval cultures has been proposed (12). As *P. gallaeciensis* antagonism is mainly caused by TDA and since TDA production is regulated by QS, we hypothesized that *P. gallaeciensis* antagonism in aquaculture settings (e.g., algal cultures) would also be affected by QS and, hence, could potentially be enhanced by the addition of synthetic QS compounds.

New approaches to control pathogenic bacteria in aquaculture systems include the use of quorum-sensing inhibition (QSI) compounds (22, 23, 24), and given the link between QS and TDA production, the use of QSI compounds could potentially limit the effectiveness of TDA-producing bacteria. Therefore, to develop effective strategies for the application of TDA-producing bacteria for disease control in aquaculture, we need to understand the effect of QS on TDA production in aquaculture systems. In the present study, we compared the antagonistic effects of *P. gallaeciensis* wild type and two QS mutants (*pgaI* and *pgaR*) (18) against the fish pathogen *Vibrio anguillarum* NB10 in cultures of the green alga *Tetraselmis suecica*, which is often used as live feed in aquaculture. We also determined whether the addition of the AHL compound *N*-3-hydroxydecanoylhomoserine lactone ($3OHC_{10}$-HSL), which is naturally produced by *P. gallaeciensis* strain DSM17395 had any effect on the antagonism of the *pgaI* mutant.

Organisms and culture media. *P. gallaeciensis* strain DSM 17395 was originally isolated from scallop-rearing systems (25). *P. gallaeciensis* insertion mutants with mutations of *pgaI* (unable to produce the AHL compound $3OHC_{10}$-HSL) and *pgaR* (devoid of the AHL receptor) (18) were kindly provided by Thorsten Brinkhoff, University of Oldenburg, Germany, and grown on half-strength marine agar (MA) (27.55 g MA 2216, Difco, 7.5 g agar, 1 liter deionized water) supplemented with 25 mg/liter gentamicin. The TDA-negative mutant, *P. gallaeciensis* MJG-G6, was created by transposon mutagenesis using the EZ-Tn*5* <R6Kγ*ori*/KAN>Tnp Transposome kit (Epicentre, Madison, WI) as described by Geng et al. (38) and was cultured in 1/2 YTSS (yeast extract, tryptone, sea salts) (25) supplemented with 75 mg/liter kanamycin. *Vibrio anguillarum* serotype O1 strain NB10 (26, 27), which was tagged by insertion of the plasmid pNQFlaC4-gfp27 (*cat gfp*) into an intergenic region, was kindly provided by Debra Milton, University of Umeå, Sweden (28), and was cultured

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on tryptone-soy agar (TSA CM0131; Oxoid) containing 6 mg/liter chloramphenicol. Bacterial precultures were grown in 5 ml 1/2 YTSS at 25°C and 200 rpm. For serial 10-fold dilutions, autoclaved 3% Instant Ocean (IO; Aquarium Systems, Inc.) was used.

Tetraselmis suecica CCAP 66/4 (*Prasinophyceae*) was obtained as an axenic culture from the Culture Collection of Algae and Protozoa (Oban, United Kingdom) and cultured in B medium, prepared as follows. Solution 1 was composed of 45 g Na_2EDTA, 100 g $NaNO_3$, 33.6 g H_3BO_3, 20 g H_2PO_4, 0.36 g $MnCl_2 \cdot 4H_2O$, 1.3 g $FeCl_3 \cdot 6H_2O$, and 1 liter demineralized H_2O; solution 2 contained 2.1 g $ZnCl_2$, 2.0 g $CoCl_2 \cdot 6H_2O$, 0.9 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2.0 g $CuSO_4 \cdot 5H_2O$, and 1 liter demineralized H_2O, with the addition of drops of 0.1 N HCl until the solution was clear; and vitamin stock solution consisted of 200 mg thiamine, 1 mg biotin, 1 mg cyanocobalamin, and 1 liter demineralized H_2O. The three solutions were autoclaved separately, and a mineral stock solution was made of 1 liter solution 1 and 1 ml solution 2. One milliliter of this stock solution and 1 ml of the vitamin stock solution were added to 1 liter 3% Sigma artificial seawater (29). Algal concentrations and axenicity were checked according to the method of D'Alvise et al. (12).

Effect of QS deficiency on *P. gallaeciensis* antagonism against *V. anguillarum* in an algal (*Tetraselmis suecica*) system. *T. suecica* cultures (6.6×10^4 cells/ml) containing 10^2 CFU/ml of the appropriate *P. gallaeciensis* strain/mutant were prepared in B medium (150 ml). After 24 h of incubation at 25°C, the AHL compound $3OHC_{10}$-HSL (University of Nottingham, United Kingdom) was added to a concentration of 1 μM in *P. gallaeciensis* *pgaI* mutant cultures and, after 48 h, *V. anguillarum* was added at a level of 10^3 CFU/ml. *T. suecica* cultures inoculated with the TDA-negative mutant *P. gallaeciensis* MJG-G6 or without any bacteria added were used as negative controls. The growth of *V. anguillarum* in *T. suecica* cultures was checked in the absence and presence of $3OHC_{10}$-HSL. All cultures were run in duplicate at 25°C, with continuous aeration and under a light intensity of 13,000 lx. Two independent experiments were performed. Two-milliliter samples of each culture were collected in a sterile tube daily for 5 days (120 h) after *V. anguillarum* inoculation and used for quantification of algae and bacteria. The algal concentration was determined by optical density at 665 nm after calibration with counts of axenic reference cultures in a Neubauer counting chamber. Formaldehyde (0.5% final concentration) was used as the fixation agent. Quantification of *P. gallaeciensis* wild type and AHL mutants was done by plating 10-fold dilutions on MA plates and incubating them for 72 h at 25°C. The numbers of *V. anguillarum* organisms were determined on TSA plates supplemented with 6 mg/liter chloramphenicol and incubated at 25°C for 48 h. A one-way analysis of variance (ANOVA) and Tukey test (level of significance, 0.05) were used to analyze the differences between *V. anguillarum* concentrations alone or in coculture with the different *P. gallaeciensis* strain/mutants in algal cultures.

After 48 h in algal coculture, *P. gallaeciensis* wild type and all mutants had grown to 10^6 CFU/ml. *V. anguillarum* grew to 10^4 CFU/ml in control cultures. After 120 h in coculture with the *P. gallaeciensis* wild type, the *V. anguillarum* concentration was significantly reduced, to 10 CFU/ml ($P = 0.002$), whereas in the presence of the *tdaB* mutant MJG-G6, which is unable to produce TDA, *V. anguillarum* growth was only slightly affected and reached 10^4 CFU/ml after 120 h (Fig. 1) ($P > 0.05$). Both the *pgaI* and *pgaR* mutants significantly reduced the counts of *V. anguillarum* (*pgaI*, $P = 0.002$; *pgaR*, $P = 0.006$), similarly to the *P. gallaeciensis* wild type. The addition of $3OHC_{10}$-HSL had no significant effect on the growth or antagonism of the *pgaI* mutant ($P > 0.05$), nor did the addition of the AHL compound have any effect on *V. anguillarum* growth ($P > 0.05$) (Fig. 1). None of the introduced bacteria affected the growth of the algae, which grew to a concentration of 10^6 to 10^7 cells/ml at the end of the experiment.

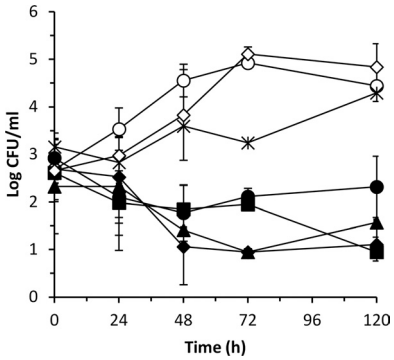

FIG 1 Growth of *V. anguillarum* in *T. suecica* cultures alone (○) or supplemented with 1 μM $3OHC_{10}$-HSL (◇) or in coculture with *P. gallaeciensis* wild type (◆), *tdaB* mutant (✱), *pgaI* mutant (■), *pgaI* mutant supplemented with 1 μM $3OHC_{10}$-HSL (●), or *pgaR* mutant (▲). Data are means and standard deviations of two independent experiments.

Antagonism of *P. gallaeciensis* QS mutants in broth systems. Since the QS mutants, unexpectedly, were as inhibitory as the wild type, we studied the kinetics of TDA production in *P. gallaeciensis* wild type and QS mutants in detail, by means of bioactivity and pigment production. The strains/mutants were grown in marine broth (MB 2216, Difco) for 3 days at 25°C. Aeration of *P. gallaeciensis* cultures influences TDA production in MB, and the most-pronounced TDA production occurs under stagnant conditions (15, 30). Therefore, TDA production was analyzed under both stagnant and agitated (200 rpm) conditions. Different cultures were used for each sampling point. Spent culture supernatants of *P. gallaeciensis* wild type and QS mutants were sampled daily for pigment measurement (optical density at 398 nm [OD_{398}]) and analysis of antagonism against *V. anguillarum* (22). The bioactivities of the different strains/mutants were checked by using a previously described bioassay (6). Briefly, *V. anguillarum* was cultured in MB for 24 h at 25°C and infused in 3% IO agar with 0.4% glucose and 0.3% Casamino Acids at 43 to 44°C. Wells (diameter, 6 mm) were punched in the solidified agar, and sterile filtered supernatants of *P. gallaeciensis* wild type and QS mutants were added to the wells. The plates were incubated for 24 h at 25°C and checked for the presence of clearing zones. Pigment was measured, as its occurrence in MB cultures coincides with TDA production (15). The significance of the differences in pigment production and bioactivity between the *P. gallaeciensis* wild type and QS mutants grown in MB was ascertained by using a one-way ANOVA and Tukey test (level of significance, 0.05).

The supernatant from a wild-type, 24-h shaken culture was inhibitory against *V. anguillarum*, whereas supernatants from the *pgaI* and *pgaR* mutants were not ($P < 0.05$) (Fig. 2). However, when sampled after 48 and 72 h, the QS mutants were inhibitory and no significant differences in inhibitory activities were observed ($P > 0.05$). Under stagnant conditions, all three strains inhibited the target organism at the 48- and 72-h sampling points,

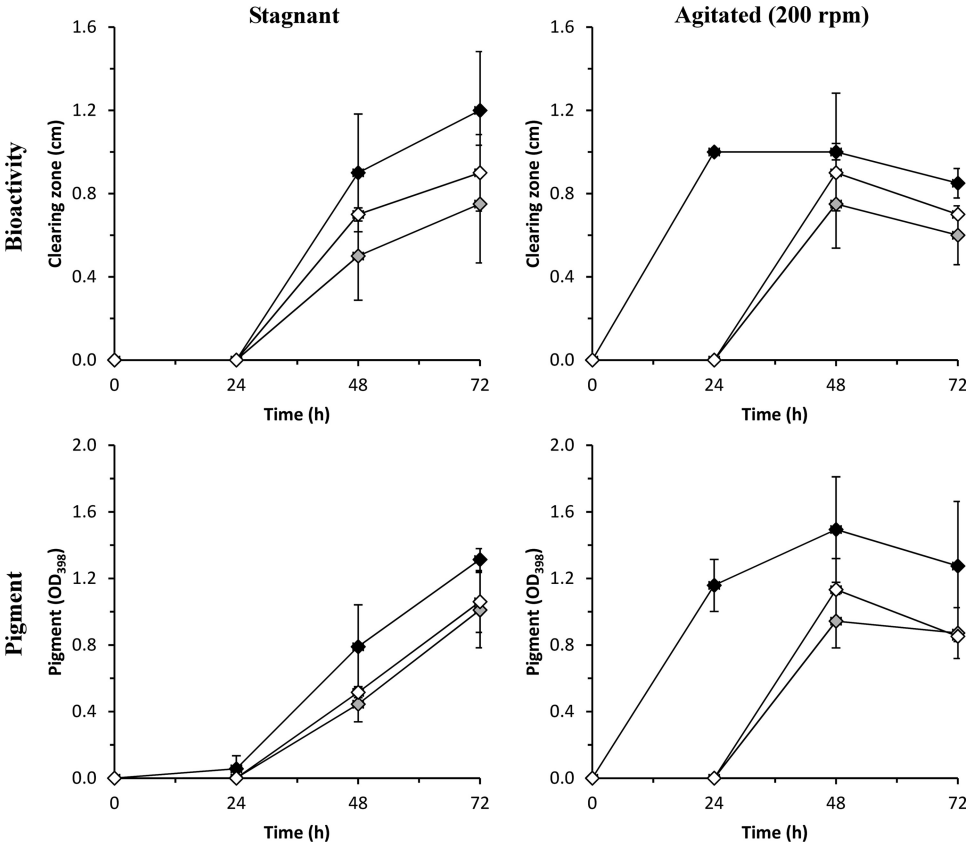

FIG 2 Bioactivity (inhibition zone diameter) and pigment production (OD_{398}) of *P. gallaeciensis* wild type (black) and *pgaI* (white) and *pgaR* (gray) knockout mutants in marine broth. Data are means and standard deviations of two independent assays.

and no significant differences were detected ($P > 0.05$). The inhibitory activity correlated with the production of pigment in all strains (Fig. 2). Pigment production in QS mutants was significantly different from that in the wild type only after 24 h of incubation under aerated conditions ($P = 0.001$), and the stagnant cultures of *pgaI* and *pgaR* mutants did not show pigmentation significantly different from that of the wild type ($P > 0.05$).

The production of secondary metabolites, such as antifungal and antibacterial compounds, can be regulated by QS, and this regulation can be absolute (31, 32) or just a modulation (33, 34). Therefore, a mutation in the QS system can lead to the complete loss of antibiotic production (31, 32) or just cause a reduction in the production of the antagonistic compound (33, 34). Berger et al. (18) showed that TDA production in *P. gallaeciensis* is QS regulated. However, by extending the growth period from 24 to 48 and 72 h, we found that the production of TDA, as determined by bioactivity and pigment production, was delayed but not completely abolished. Similarly, mutations in QS genes reduced but did not totally suppress the production of biofilm-inhibiting cyclic lipopeptides in *Pseudomonas putida* (33) or proteases in *Pseudomonas chlororaphis* (34). Our results show that TDA production in *P. gallaeciensis* is only modulated by the PgaI-PgaR QS system, and this indicates that QS is just one of several regulatory systems involved. This is in agreement with previous observations by Berger et al. (35), who showed that *pgaI* and *pgaR* mutants are able to produce TDA in a minimal medium with phenylalanine as the sole carbon source. In some bacteria, the *luxI-luxR* QS system forms part of a hierarchically organized network where other regulators are integrated for the control of secondary-metabolite production (34, 36), and this type of organization has been suggested previously for TDA production in *P. gallaeciensis* (35). Our group has recently demonstrated that the expression of the *tdaC* gene, key in the synthesis of TDA in *Ruegeria mobilis*, coincides with high levels of the second messenger signal cyclic dimeric guanosin-monophosphate (c-di-GMP), and we have hypothesized that c-di-GMP could be involved in the regulation of TDA synthesis in *Roseobacter* bacteria (37). Since the genes responsible for the synthesis and degradation of c-di-GMP are present in *P. gallaeciensis* (37) and since TDA production is modulated by QS, one could speculate that this modulation could occur through c-di-GMP.

We can conclude that an AHL-mediated QS system modulates *P. gallaeciensis* antagonism in a broth system but that this regulation does not seem to be important in algal systems mimicking an aquaculture environment. Several studies have used QSI compounds to target aquaculture pathogens (22–24), and since TDA production can be regulated through QS, one could be concerned that such QSI compounds would limit the effectiveness of beneficial bacteria, such as TDA-producing *Phaeobacter*. However, in this study, we demonstrate that the inhibitory activity of *P. gallaeciensis* is independent of the QS system, and therefore, this inhibition will likely not be hampered by the possible use of QSI compounds. Hence, one can envision a dual disease control strategy, combining probiotic *Phaeobacter* strains with pathogen-targeted QSI compounds.

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