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Effect of TDA-producing *Phaeobacter inhibens* on the fish pathogen *Vibrio anguillarum* in non-axenic algae and copepod systems

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Summary

The expanding aquaculture industry plays an important role in feeding the growing human population and with the expansion, sustainable bacterial disease control, such as probiotics, becomes increasingly important. Tropodithietic acid (TDA)-producing *Phaeobacter* spp. can protect live feed, for example rotifers and *Artemia* as well as larvae of turbot and cod against pathogenic vibrios. Here, we show that the emerging live feed, copepods, is unaffected by colonization of the fish pathogen *Vibrio anguillarum*, making them potential infection vectors. However, TDA-producing *Phaeobacter inhibens* was able to significantly inhibit *V. anguillarum* in non-axenic cultures of copepod *Acartia tonsa* and the copepod feed *Rhodomonas salina*. *Vibrio* grew to $10^6$ CFU ml$^{-1}$ and $10^7$ CFU ml$^{-1}$ in copepod and *R. salina* cultures, respectively. However, vibrio counts remained at the inoculum level ($10^4$ CFU ml$^{-1}$) when *P. inhibens* was also added. We further developed a semi-strain-specific qPCR for *V. anguillarum* to detect and quantify the pathogen in non-axenic systems. In conclusion, *P. inhibens* efficiently inhibits the fish larval pathogen *V. anguillarum* in the emerging live feed, copepods, supporting its use as a probiotic in aquaculture. Furthermore, qPCR provides an effective method for detecting vibrio pathogens in complex non-axenic live feed systems.

Introduction

Aquaculture provides high-quality protein for approximately three billion people worldwide and with the estimated increase in the human population combined with overfishing, this number is likely to increase (WWF and ZSL, 2015; FAO, 2016). Larvae of several commercially important species including cod, halibut and turbot are fed using live feed due to the lack of suited artificial feed formulations. Traditionally, marine fish larvae have been fed using rotifers and *Artemia*, although copepods are more representative of the natural diet (Turner, 2004). Copepods have several advantages over traditional aquaculture live feed including desirable amino acid profiles, a high fatty acid content and swimming patterns that promote feeding in fish larvae (Støttrup et al., 1986; Støttrup and Norsker, 1997; Bell et al., 2003; Turingan et al., 2007). Furthermore, substituting rotifers with copepods (*Acartia tonsa*) in the initial larval feeding stage has a long-term positive effect on survival, growth and viability of Atlantic cod and ballan wrasse fish larvae (Oie et al., 2017). So far, the use of copepods as live feed in larval rearing has not been cost-effective; however, new approaches to copepod breeding are improving the cost balance (Abate et al., 2016), thus making copepods a relevant live feed in commercial aquaculture.

A major bottleneck in aquaculture is disease outbreaks where bacterial infections account for approx. 55% (Kibenge et al., 2012). One of the most prominent bacterial genera causing disease in aquaculture is *Vibrio* including *Vibrio anguillarum*, *V. harveyi*, *V. vulnificus* and *V. splendidus* (Thompson et al., 2004; Toranzo et al., 2005). Live feed used in aquaculture is a potential point of entry and vector for pathogenic bacteria (Olafsen, 2001), and several *Vibrio* species, including the human and fish pathogens *V. cholerae*, *V. vulnificus* and *V. splendidus*, are found in association with zooplankton, for example copepods, which in the marine environment seem to serve as natural *Vibrio* reservoirs (Sochard et al., 1979; Heidelberg et al., 2002; Colwell et al., 2003; Vezzulli et al., 2015). The role of copepods as hosts for *V. cholerae* has especially been studied extensively, and
V. cholerae has repeatedly been isolated from the surface, gut and exuviae of these small crustaceans (Huq et al., 1983; Tamplin et al., 1990; Gugliandolo et al., 2008). Thus, although vibrios such as Vibrio para-haemolyticus, V. alginolyticus and V. mimicus have been reported to be unable to colonize the copepod species Temora stylifera, Acartia clausi, Centropages typicus and Paracalanus parvus (Dumontet et al., 1996), it is a valid concern that Acartia tonsa may function as infection vectors for other Vibrio species including the fish pathogenic V. anguillarum in aquaculture systems.

Historically, antibiotics have been used to control bacterial infections in aquaculture. However, risk of antibiotic resistance development and the associated health risk have led to a search for sustainable alternatives (Skjermo and Vadstein, 1999; Sommerset et al., 2005; Defoirdt et al., 2011). The deployment of vaccines has limited the use of antibiotics especially in Europe and North America (Defoirdt et al., 2011; Ringø et al., 2014). However, antibiotics are still used in many countries and fish larviculture as their undeveloped immune system does not allow for vaccination (Sommerset et al., 2005; Defoirdt et al., 2011). An alternative to antibiotics in such systems is probiotics, that is live microorganisms which exert beneficial effects on the host health (FAO and WHO, 2001), for example by inhibiting growth of pathogenic bacteria (Gatesoupe, 1999; Skjermo and Vadstein, 1999; Kesarcdodi-Watson et al., 2008). Members of the genera Phaeobacter and Ruegeria which produce the broad-spectrum antimicrobial compound tropodithietic acid (TDA) have proven probiotic properties (Ruiz-Ponte et al., 1999; Hjelm et al., 2004; Planas et al., 2006; D'Alvise et al., 2012). Phaeobacter spp. inhibit the growth of pathogenic vibrios and protect live feed such as rotifers and Artemia (D'Alvise et al., 2012; Grotkjaer et al., 2016a, b). Furthermore, Phaeobacter spp. can protect larvae of turbot and cod in challenge trials (Planas et al., 2006; D'Alvise et al., 2012, 2013). Phaeobacter spp. have repeatedly been isolated from different aquaculture facilities (Ruiz-Ponte et al., 1998; Hjelm et al., 2004; Porsby et al., 2008; Gram et al., 2015; Grotkjaer et al., 2016b), which indicate that they are part of the natural microbiota in these aquaculture units. Most studies on the probiotic effect of Phaeobacter spp. and Ruegeria spp. have been carried out in axenic live feed or fish larvae systems (Planas et al., 2006; D'Alvise et al., 2012, 2013; Grotkjaer et al., 2016b). Although non-axenic algae and Artemia systems have been tested (Grotkjaer et al., 2016a), the probiotic effect of Phaeobacter spp. and Ruegeria spp. in these more ‘natural’ systems requires further studies.

The purpose of this study was to investigate whether the copepod species Acartia tonsa would potentially function as an infection vector for fish pathogenic vibrio, and whether the TDA-producing P. inhibens could antagonize V. anguillarum in non-axenic copepod systems as well as in algae used as copepod feed. Furthermore, we aimed at developing a strain-specific quantitative PCR (qPCR) protocol for detection and quantification of pathogens in complex systems without the dependency on selection markers, such as antibiotic resistance that potentially could affect pathogen behaviour.

**Results**

**Invasion of copepods by GFP-tagged V. anguillarum**

To test whether V. anguillarum could invade the copepod Acartia tonsa and make the crustacean a potential infection vector in larviculture, a GFP-tagged V. anguillarum strain NB10 was added to non-axenic A. tonsa feed, that is Rhodomonas salina. After 4 days of incubation, the GFP signal of NB10 was detected on the surface and inside the copepods (Fig. 1). Specifically, the vibrios appeared to be concentrated within the intestinal system of the crustacean. No GFP signal was detected in non-inoculated copepods. Throughout the experiment, Vibrio abundances in the surrounding medium were stable around the inoculum level of 10⁵ CFU ml⁻¹ (data not shown). Furthermore, A. tonsa seemed unaffected by the addition of vibrios, exhibiting mortalities after 4 days of 1.47 ± 0.72%.

**Co-culture in non-axenic Rhodomonas salina**

As A. tonsa was found to be a potential vector for V. anguillarum, we investigated whether P. inhibens could antagonize vibrios in cultures of copepod and in the copepod live feed R. salina. We conducted a co-culture experiment in non-axenic R. salina cultures. Counts of R. salina were stable during the co-culture experiment, and no significant difference was seen between the different treated cultures (data not shown). Phaeobacter inhibens established itself and remained at approx. 10⁷ CFU ml⁻¹ in all set-ups, independently of addition of V. anguillarum strain 90-11-286 (Fig. 2). TSA counts showed that V. anguillarum was significantly reduced when grown in the presence of P. inhibens. Without P. inhibens, V. anguillarum grew to approx. 10⁷ CFU ml⁻¹, while in the presence of P. inhibens, V. anguillarum numbers were significantly reduced with approximately three orders of magnitude (P < 0.0001) and never exceeded the inoculum level of 10⁴ CFU ml⁻¹.

**Co-culture in non-axenic A. tonsa, and detection and quantification of V. anguillarum with qPCR**

Non-axenic A. tonsa were not affected by the presence of either the probiont or the pathogen resulting in
mortalities after 3 days of 5.7 ± 2.52%, 7.3 ± 1.15%, 5.7 ± 2.08% and 6.3 ± 3.51% in cultures with P. inhibens, V. anguillarum, a combination of P. inhibens and V. anguillarum, or no addition (control), respectively. As for the R. salina co-culture experiment, no differences were observed between P. inhibens abundances in the presence or absence of V. anguillarum (Fig. 3) with counts being stable around the inoculum level of 10^6–10^7 CFU ml^{-1}. The abundance of V. anguillarum was significantly reduced by P. inhibens (P = 0.0004). With the addition of P. inhibens, Vibrio counts remained at the inoculum level around 10^4 CFU ml^{-1}, approx. two orders of magnitude below the abundances observed in cultures without P. inhibens. Numbers of V. anguillarum were estimated both by counts on TSA plates and by qPCR resulting in similar abundances (P > 0.05; Table 1).

Verification of primer specificity and standard curve

Primer-BLAST was used to design and test the specificity of the V. anguillarum 90-11-286 primers in silico, and this indicated that the primers were strain-specific. The primers were subsequently tested in vitro against 11 V. anguillarum strains and one Vibrio harveyi strain (Table 2). The primers amplified the DNA of three strains resulting in cycle threshold (C_t) values within the limits of detection (C_t ≤ 30). Furthermore, one strain, NB10, was close to the limit of detection with a C_t value of 30.29 ± 0.13. Strains 90-11-286 and PF7 showed similar C_t values of 19.56 ± 0.05 and 19.16 ± 0.11, respectively, and a higher C_t value of 23.53 ± 0.08 was seen for PF4. For quantification, a standard curve was made with strain 90-11-286 overnight culture that was 10-fold serial diluted from approx. 10^7 to 10^3 (Fig. 4). The

standard curve was used to compare $C_t$ values to bacterial density (CFU ml$^{-1}$) for *Vibrio* samples from the non-axenic *A. tonsa* co-culture.

**Discussion**

Live feed is essential at the larval stage in several commercially important species of finfish, and controlling pathogenic bacteria in the live feed is important as these may act as vectors for the pathogens. Here, we demonstrate that *P. inhibens* can inhibit the highly virulent *V. anguillarum* strain 90-11-286 in different non-axenic live feed systems. Specifically, we demonstrate the probiotic potential in copepods as these are superior as larval feed compared to *Artemia* and rotifers, and future aquaculture is expected to rely increasingly on a copepod-based live feed.

Live feed is known as a point of entry and vector for pathogenic bacteria (Olafsen, 2001). Copepods have previously been shown to host some *Vibrio* spp. (Huq et al., 1983; Tamplin et al., 1990; Heidelberg et al., 2002; Gugliandolo et al., 2008; Vezzulli et al., 2015); however, some *Vibrio* spp. seem to be unable to colonize some copepod species (Dumontet et al., 1996). Using a GFP-tagged *V. anguillarum* NB10, we found that *V. anguillarum* was able to colonize the outside and the gut of the feeding copepods without inducing notable mortality (1.47 ± 0.72% after 4 days) hence making them potential infection vectors in larviculture.

*Vibrio* spp. are part of the established microbiota of live feed used in larviculture (Berland et al., 1970; Austin and Allen, 1982; Salvesen et al., 2000; López-Torres and Lizarraga-Partida, 2001) and when introduced, these interactions in copepods.
opportunistinc pathogens can proliferate rapidly leading to great losses (Reid et al., 2009). In the co-culture experiment, no differences were seen in mortality of the unfed copepods regardless of the presence of P. inhibens or V. anguillarum (5.7 ± 2.52% to 7.3 ± 1.15% mortality). In contrast, V. anguillarum has been shown to cause high mortality (92%) in non-axenic Artemia cultures. Here, the addition of P. inhibens reduced mortality of the infected Artemia to 11% (Grotkjær et al., 2016b). The observed differences in mortality potentially give copepods an advantage over Artemia as live feed, as it has been reported that homogenized Artemia or sea bass larval biomass, simulating the presence of dead Artemia or sea bass larvae, increase the virulence of V. anguillarum, resulting in a significant increase in mortality in challenged sea bass larvae (Li et al., 2014). Thus, copepods with a higher survival rate than Artemia could result in fewer and potentially less virulent vibrios. The differences in mortality between Artemia and copepods can be explained by several factors. A notable difference between the studies is the vibrio abundances observed at the end of the co-culture experiments. Although inoculated at same level (10^4 CFU ml⁻¹), abundances of V. anguillarum in cultures without P. inhibens were 10-fold higher in the Artemia experiment (10⁷ CFU ml⁻¹) (Grotkjær et al., 2016a) as compared to the copepod experiment conducted in the present study (10⁶ CFU ml⁻¹). This suggests that the presence of copepods, or the background microbiota of the copepods, could have an inhibiting effect on vibrio growth, especially as vibrio abundances in the algae cultures of both studies, Tetraselmis suecica and R. salina, respectively, were similar to that of the Artemia cultures, that is 10⁷ CFU ml⁻¹. The immune systems of invertebrates are poorly understood (Loker et al., 2004). However, there are differences between copepods and Artemia, and for instance, the heat shock protein hsp70, which is associated with pathogen resistance, is induced in Artemia when infected with Vibrio (Norouzitallab et al., 2016). In contrast, an analog of this stress responder was not induced in the copepod Eurytemora affinis when exposed to Vibrio spp. (Almada and Tarrant, 2016). Different copepod species are colonized by different bacteria, and some Vibrio spp. are unable to colonize some copepod species (Dumontet et al., 1996). Vibrios colonizing copepods have been reported to trigger upregulation of saposin-like genes and C-type lectins (Almada and Tarrant, 2016) that have antimicrobial properties (Hoeckendorf and Leippe, 2012; Wang et al., 2014). This suggests that copepods have a potential inhibiting effect on the growth of V. anguillarum, which could explain the lower Vibrio abundance and mortality in copepod cultures relative to Artemia cultures. It is also possible that the V. anguillarum strain 90-11-286 is avirulent against A. tonsa. The virulence of strains within the same species can vary immensely as seen for thirty V. anguillarum strains tested in three different hosts (turbot, halibut and cod larvae), causing mortality from 100% to 9.1% with the same infection dose (Rønneseth et al., 2017). Thus, if other V. anguillarum strains had been tested in the Artemia and A. tonsa systems, the outcome might have been different.

Several co-culture studies of P. inhibens and V. anguillarum in axenic systems have shown significant inhibition of vibrios, resulting in lowered vibrio counts relative to the inoculum level (D’Alvise et al., 2010, 2012; Grotkjær et al., 2016b). In contrast, P. inhibens kept V. anguillarum at the inoculum level in both the non-axenic co-culture experiments conducted in the present study and in similar experiments with non-axenic Tetraselmis and Artemia (Grotkjær et al., 2016a). This suggests that the microbiota of the non-axenic systems have a protective effect on V. anguillarum; however, this is not necessarily a problem in an aquaculture context as it is not essential to completely eradicate all vibrios to get a protective effect. Rønneseth et al. (2017) found a significant decrease in virulence for several V. anguillarum strains tested in turbot, halibut and cod larvae challenge trials when added at low density (10^4 CFU ml⁻¹) as compared to high density (10^6 CFU ml⁻¹). Hence, although V. anguillarum was not reduced to below inoculum levels in the presence of P. inhibens, our findings support the use of Phaeobacter spp. as probiotics in copepod systems. In both co-culture experiments, P. inhibens was able to establish itself at a constant level, which is consistent with its reported association with micro- and macroalgae, zooplankton, squids, copepods and fish larvae (Hjelm et al., 2004; Rao et al., 2005; Venmathi Maran et al., 2007; Porsby et al., 2008; Collins et al., 2012; Gram et al., 2015; Grotkjær et al., 2016b). This supports the proposal that one way of utilizing Phaeobacter spp. in aquaculture is by introducing them directly into the live feed systems (Planas et al., 2006; Prol et al., 2009; Grotkjær et al., 2016b).

Previously, non-axenic co-culture experiments have used a chloramphenicol-resistant V. anguillarum as target organism and estimated vibrio abundances on solid substrates supplemented with antibiotics (Grotkjær et al., 2016a). However, using antibiotic as selection marker is not without challenges. The natural microbiome may harbour bacteria resistant to the antibiotic marker, the antibiotic marker may be unstable, and the marker may affect the fitness of the tagged strain, all of which can result in over- or underestimation of the actual abundances (Andersson and Levin, 1999; Allen et al., 2010). Several non-growth dependent methods have been developed for detection and/or quantification of vibrios (Eiler and Bertilsson, 2006; Prol et al., 2009; Saulnier et al., 2009;
Kim and Lee, 2014). However, as previously described, vibrios are part of the established microbiota of live feed, thus genus- or species-specific primers will potentially amplify vibrios from the background microbiota. In this study, we attempted to develop strain-specific primers for quantification of the pathogenic *V. anguillarum* 90-11-286. Three of the 12 tested vibrio strains, including the target strain, were amplified within the limits of detection ($C_T < 30$). The two non-target strains were the closely related *V. anguillarum* PF7 and PF4 (Table 2). All three strains are highly virulent and cluster closely when compared by 163 concatenated virulence factors and by core genome phylogeny (Castillo et al., 2017; Rønneseth et al., 2017). However, the semi-strain-specific primers developed provide an effective method for detecting few specific vibrios in non-axenic systems. As they were made for research and not diagnostic purposes, it is possible to test the applicability of the primer set prior to experimental use.

In conclusion, our study shows that the emerging live feed copepod, *A. tonsa*, potentially can function as an infection vector for pathogenic *V. anguillarum* strains. However, *Vibrio* counts in the copepod cultures were lower than what has previously been seen for *Artemia* and algae cultures, supporting the use of *A. tonsa* as fish larvae feed. *P. inhibens* efficiently inhibited *V. anguillarum* in both non-axenic copepod and algae cultures supporting its use as a probiotic in live feed systems. Lastly, we have described direct qPCR methodology that provides an effective means of detecting vibrios in complex non-axenic live feed systems without the use of selection markers, which potentially could affect pathogen behaviour.

**Experimental procedures**

**Bacterial strain and culture conditions**

Two fish pathogenic *V. anguillarum* strains were used in this study. Strain NB10, used for the adhesion/invasion experiment, was isolated from the Gulf of Bothnia and is pathogenic towards turbot and halibut larvae (Rønneseth et al., 2017). The highly virulent *V. anguillarum* strain 90-11-286 (Rønneseth et al., 2017) was used in the co-culture experiments and was isolated from diseased rainbow trout (*Oncorhynchus mykiss*) from a Danish aquaculture (Pedersen and Larsen, 1993; Skov et al., 1995). *P. inhibens* DSM 17395 was used for the co-culture experiments (Martens et al., 2006; Vandecandelaere et al., 2008; Buddruhs et al., 2013). NB10 and 90-11-286 were grown and counted on TSA (Tryptone Soy Agar, Difco 212185) supplemented with 2.5 mg l$^{-1}$ chloramphenicol for NB10. The vibrios have very distinct colony morphology on TSA. *Phaeobacter inhibens* DSM 17395 is unable to grow on TSA due to its low salinity, thus TSA functions as a semiselective medium. *Phaeobacter inhibens* was grown and counted on MA (Marine Agare, Difco 2216) where it grows as distinct brown colonies due to Fe-TDA complex (D’Alvise et al., 2016). Bacterial stock cultures were stored at $-80^\circ$C in 20% (vol/vol) glycerol. Two to three days prior to use, stock cultures were streaked on agar plates and incubated at 25°C. The purity of the bacteria was checked by colony morphology, and single colonies were used for inoculation of each preculture. All bacterial precultures were grown in 20 ml of $\frac{1}{2}$YTSS (2 g Bacto Yeast extract, 1.25 g Bacto Tryptone, 20 g Sigma Sea Salts, 1 L deionized water) (Sobecky et al., 1997) at 25°C and 200 rpm for 24 h.

**Preparation of non-axenic algae and copepods cultures**

The non-axenic *R. salina* K-1487 originates from Denmark and was provided by Prof. Thomas Kiørboe (National Institute of Aquatic Resources, Technical University of Denmark) (Nielsen and Kiørboe, 2015). A *R. salina* K-1487 stock culture was maintained in f/2 medium (Guillard and Ryther, 1962; Guillard, 1975) without Na$_2$SiO$_3$ but with 5 mM NH$_4$Cl in 3% IO (Instant Ocean salts, Aquarium Systems Inc.) (f/2-Si-NH$_4$) at 18°C and 24 μmol photons m$^{-2}$ s$^{-1}$, photosynthetically active radiation (PAR). The algal density was determined by counting in a Neubauer-improved counting chamber, and the culture was adjusted to approx. 100 000 cell ml$^{-1}$ in f/2-Si-NH$_4$ and 25 ml was distributed into 50-ml falcon tubes. *A. tonsa* eggs were provided by Prof. Benni W. Hansen (Department of Science and Environment, Roskilde University) (Drillet et al., 2006; Hansen et al., 2010) and were kept at 5°C until use. Two days before the experiment, eggs were inoculated in 3% IO and incubated at 18°C and 24 μmol photons m$^{-2}$ s$^{-1}$, photosynthetically active radiation (PAR). For bacterial mono- and co-culture experiments, 50-ml falcon tubes were set up with 25 ml 3% IO with 2–3 *Acartia tonsa* nauplii per millilitre. For the invasion experiment, 50-ml falcon tubes containing 20 ml *R. salina* culture were supplemented with approx. 5 *Acartia tonsa* nauplii per millilitre.

**A. tonsa invasion experiment**

Precultures of *V. anguillarum* NB10 were 10-fold serially diluted in 3% IO, and 250 μl dilutions were used to inoculate non-axenic cultures of *A. tonsa* fed *R. salina*, aiming at an initial concentration of 10$^5$ CFU ml$^{-1}$. © 2018 The Authors. *Microbial Biotechnology* published by John Wiley & Sons Ltd and Society for Applied Microbiology, *Microbial Biotechnology, 11*, 1070–1079
Experiments were carried out over 96 h where bacterial concentrations and copepod mortality were determined every 24 h. At the end of the experiment, A. tonsa from cultures with and without NB10 was investigated for GFP signal using phase-contrast and fluorescence microscopy at 100× magnification using an Olympus BX51 fluorescence microscope (WIB excitation 460–490 nm, emission >515 nm).

Co-culture experiment in non-axenic R. salina and unfed A. tonsa cultures

Precultures of V. anguillarum 90-11-286 were 10-fold serially diluted in 3% IO, and 250 µl diluted culture was used to inoculate non-axenic R. salina and A. tonsa cultures, aiming at an initial concentration of 10^4 CFU ml^-1. Precultures of P. inhabens DSM 17395 were added undiluted to the non-axenic R. salina and A. tonsa cultures, aiming at an initial concentration of 10^7 CFU ml^-1. 250 µl ½YTSS was added to uninoculated controls and cultures where only 90-11-286 had been added. The cultures were incubated at 18°C, lying horizontally on a rotary shaker at 60 rpm in an algae growth cabinet. Experiments were carried out over 72 h where bacterial and algal abundances, and copepod mortality were determined every 24 h. Samples for DNA extraction for qPCR-based quantification of 90-11-286 were taken every 24 h (see below). Bacterial abundances were determined by CFU counts. 90-11-286 counts were determined after the experiment was terminated. All cultures were incubated at 25°C. TSA plate counts were made and DNA was extracted. 

Statistical analysis

CFU ml^-1 values and CFU ml^-1 of the corresponding C_t values for each biological replicate were log10-transformed prior to statistical analysis. Statistical analyses of differences were performed using t-test (alpha = 0.05) in GraphPad Prism 7.04 (GraphPad Software, San Diego CA).

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Conflict of interest

The authors declare no conflict of interest.

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