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Different colonization and residence time of *Listonella anguillarum* and *Vibrio splendidus* in the rotifer *Brachionus plicatilis* determined by real-time PCR and DGGE

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ABSTRACT

Listonella anguillarum 90-11-287 and *Vibrio splendidus* DMC-1 were incorporated in the rotifer *Brachionus plicatilis*, which was subsequently maintained under larval rearing conditions to determine the residence time of both pathogens in rotifers.

Real-time PCR was applied to specifically detect and quantify both pathogens. *L. anguillarum* colonized rotifers more efficiently than *V. splendidus* and both pathogenic strains were released from rotifers to seawater, after infected rotifers were transferred to rearing tanks. *V. splendidus* grew and became predominant in the seawater of tanks. Both pathogens remained in rotifer or seawater enough time to infect fish larvae, but their different behaviour could determine different infection patterns, preferentially by ingestion of prey or by active intake or contact with surrounding seawater.

The effect of *L. anguillarum* and *V. splendidus* on the bacterial community associated with rotifers and seawater of rearing tanks was analysed by DGGE of PCR-amplified 16S rDNA fragments. The bacterial community of rotifers did not present a marked species dominance. The incorporation of *L. anguillarum* or *V. splendidus* did not reduce bacterial diversity and shifts could be explained by bacterial exchange between rotifers and seawater. Main bacterial groups were identified by sequencing the DNA extracted from Marine Agar (MA) bacterial isolates and DGGE excised bands. Only 2 DGGE bands corresponded to bacteria isolated from MA plates, suggesting that bacterial groups present in rotifers may not be easily cultivable. The bacterial community of rotifers was composed by Gram negative bacteria belonging to α-Proteobacteria (*Ruegeria* spp), γ-Proteobacteria (*Alteromonas alvinellae*, *Marinobacter* sp, an *Oceanospirillaceae* bacterium and *Pseudoalteromonas* sp), *Cytophaga–Flexibacter–Bacteroides* group (*Polaribacter dokdonensis*, *Roseivirga spongicola* and *Tenacibaculum soleae*) and a Gram positive bacterium (*Microbacterium* sp).

1. Introduction

Rotifers (*Brachionus plicatilis*) and *Artemia* are essential live prey in larval rearing of marine fish species. Rotifers, commonly used as the first prey in larvae artificial food chain, are major carriers of bacteria (Munro et al., 1993). Most of bacteria in rotifers are not pathogenic but detrimental effects on fish larvae can be caused by the accumulation of bacteria in prey (Dhert, 1996). Bacteria associated with rotifer cultures have been related to unexpected mortalities or to suppressed growth in rotifers (Yu et al., 1990), as well as to low survival and growth in fish larvae (Benavente and Gatesoupe, 1988; Gatesoupe, 1989; Nicolas et al., 1989).

The genus *Vibrio* has been found to be dominant in rotifers (Verdonck et al., 1997) and several *Vibrio* species has been reported to cause high mortality episodes in the culture of some fish species (Thomson et al., 2005; Toranzo et al., 1995). Fish pathogens *Listonella anguillarum* and *Vibrio splendidus* have been found associated with live prey (Thomson et al., 2005; Verdonck et al., 1997), causing mortality to fish larvae (Reid et al., 2009; Sandlund and Bergh, 2008; Thomson et al., 2005). The strains *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 are pathogenic to turbot larvae but the infection patterns for both species seem to be different. *V. splendidus* was detected and isolated from the gut of diseased larvae (Reid et al., 2009; Thomson et al., 2005) whereas *L. anguillarum* was found in the epidermis of experimentally infected turbot larvae (Planas et al., 2005).

Different infection patterns of pathogenic *Vibrionaceae* bacteria could be dependent on the chemotaxis and adhesion capacity to mucosal surfaces (skin, gill, intestine) (Balebona et al., 1995, Bordas et al. 1998) but also on the entry route (prey or seawater) into fish (Grisez et al., 1996; Muroga et al., 1990; Planas et al, 2005). These factors, among others, would determine which organs would be infected (Grisez et al., 1996; Olsson et al., 1996; Villamil et al. 2003).

Transference of pathogens from live prey to target organisms can be performed directly, by ingestion of the prey, and/or indirectly, by release of pathogens from prey to seawater. Therefore, colonization of intestine and adhesion to external epithelium (gill, skin) could be determined by the way pathogens and target organisms come in contact.

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Several models for experimental infection of fish larvae have been developed based on challenge trials with *Vibrionaceae* bacteria, by bioencapsulation of bacteria in rotifers or *Artemia* and administration of the infected live prey to turbot larvae (Grisez et al., 1996; Planas et al., 2005). However, colonization and maintenance of pathogenic strains in live prey or displacement of microbiota associated with prey have not been previously studied. This information would be valuable on the development of experimental infection models or protocols but also on the identification of potential ways of entry (prey, seawater or both) of pathogens into target organisms. All those factors are also determinants to design strategies for infection control in fish larviculture.

Monitoring of introduced pathogens requires the use of specific strain detection techniques. Traditionally, quantification of pathogenic *Vibrionaceae* has been conducted by culture-based methods. These methods may underestimate the concentration of *Vibrionaceae* bacteria (Mizuki et al., 2006; Munro et al., 1993) which can be also biased or hindered by the presence of non-introduced *Vibrionaceae* naturally present in rotifers (Thomson et al., 2005; Verdonck et al., 1997). Furthermore, specificity of TCBS is questionable as it has been demonstrated that other bacteria different to *Vibrionaceae* can grow in this medium (López-Torres and Lizárraga-Partida, 2001). The use of real-time PCR methods, as the recently developed for specific quantification of *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 (Prol et al., 2009), avoids these constraints.

Another important aspect not considered before is the study of displacement or modification of autochthonous bacterial populations due to the introduction of pathogens. Culture dependent techniques are time consuming and underestimate microbial diversity as less than 1% of total bacteria in natural seawater systems are cultivable (Hansen and Olafsen, 1999). The study of autochthonous microbiota modifications in live prey due to introduction of pathogens is more accurate with application of culture independent genetic fingerprinting techniques, such as Denaturing Gradient Gel Electrophoresis (DGGE). DGGE is a reliable and rapid method to study the variation of dominant bacteria and to characterise complex microbial populations (Muyzer et al., 1993). Bacterial community fingerprints have been previously obtained from rotifers (Rombaut et al., 2001; Qi et al., 2009) and several aquaculture systems by using DGGE (Griffiths et al., 2001; McIntosh et al., 2008; Sandaa et al., 2003) being most of studies focussed on seasonal variations. Recently, DGGE has been used to evaluate changes caused by different probiotic strains in rotifers microbial community composition (Qi et al., 2009).

This study is focussed on 2 pathogenic strains, *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1, differing in their infection characteristics. Colonization and residence capabilities of both pathogens in rotifers (*B. plicatilis*) were analysed by specific quantitative real-time PCR. The effect of infection on bacterial community associated with rotifer cultures was analysed by PCR-DGGE.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The pathogens *L. anguillarum* strain 90-11-287 (serotype O1) (Skov et al., 1995) and *V. splendidus* strain DMC-1 (Hjelm et al., 2004a; Thomson et al., 2005) were kindly provided by Prof. Lone Gram (DTU Aqua, Denmark). Both bacteria demonstrated to be pathogenic for turbot larvae (Planas et al., 2005; Thomson et al., 2005) and a challenge model for experimental infection of fish larvae has been successfully developed for *L. anguillarum* 90-11-287 (Planas et al., 2005).

The strains were routinely cultured in Marine Broth (MB, Difco 2219) at 22 °C and 110 rpm. Pre-cultures were incubated for 72 h and 1 ml inoculated in 100 ml of MB, followed by incubation for 24 h and sub-cultured once under the same conditions.

2.2. Infection of rotifers with pathogens

The rotifer *B. plicatilis* was routinely fed on the yeast *Saccharomyces cerevisiae* and enriched with the microalgae *Isochrysis galbana* for 24 h. *I. galbana* was grown in Conway medium (Walne, 1966) at 20 °C and under continuous light.

Colonization of rotifers with *L. anguillarum* 90-11-287 or *V. splendidus* DMC-1 was performed according to the bioencapsulation protocol described by Planas et al. (2005). One million of enriched rotifers were filtered over a nylon mesh of 30 μm pore size and re-suspended in 1 l of 1-μm-filtered seawater. Two-hundred millilitres of a 24 h culture of *L. anguillarum* (10^9 CFU ml^{-1}) or *V. splendidus* ($2 \cdot 10^{10}$ CFU ml^{-1}) were added to rotifer cultures (200 rotifers ml^{-1}) in a volume adjusted to 5 l of 1-μm-filtered seawater. After 3 h, rotifers from each fish pathogen suspension were filtered over a nylon mesh of 50 μm pore size, washed and re-suspended in 1 l of 1-μm-filtered seawater (10^3 rotifers ml^{-1}).

Maintenance of pathogens in rotifers was assayed under conditions similar to those used in turbot larvae rearing and as described by Planas et al. (2006). Rotifers freshly colonized by *L. anguillarum* or *V. splendidus* were transferred (c 2.5 rotifers ml^{-1}) and maintained for 96 h in 60 l larval rearing tanks filled with 1-μm-filtered seawater containing a suspension of *I. galbana* ($2 \cdot 10^5$ cells ml^{-1}). Tanks will be referred as Treatment L and Treatment V depending on whether rotifers were infected with *L. anguillarum* or *V. splendidus*, respectively. Tanks containing rotifers enriched with *I. galbana* and with no added pathogens were used as controls (Treatment Control). All situations were conducted in duplicate and partial renovation (30–40%) of tanks seawater was applied in alternate days, including the proportional addition of microalgae. Rotifer counts were done daily in each tank.

2.3. Preparation of samples for microbiological analysis

After infection of rotifers and transfer to rearing tanks, samples from both rotifers and seawater were taken at 0, 3, 6, 24, 48, 72 and 96 h. All analyses were conducted in duplicate.

Four-hundred (for plate-counting) or 10^6 (for DNA extraction) rotifers were filtered over a nylon mesh of 30 μm pore size, washed with 0.5 ml of autoclaved seawater, collected in an Eppendorf tube and placed in ice for 30 min to facilitate rotifers decantation. Excess seawater was discarded and the final volume was adjusted to 0.1 ml. Rotifers were then homogenized using an Eppendorf micropestle and the final volume adjusted to 0.5 ml with autoclaved seawater.

Five millilitres of seawater from each tank were filtered over a nylon mesh of 30 μm pore size, and the filtrate used for plate-counting. For DNA extraction, 50 ml samples of 30-μm-filtered seawater were centrifuged (5000 ×*g*/10 min/20 °C), the resultant pellet was re-suspended in 1 ml of autoclaved seawater and centrifuged again (5000 ×*g*/10 min/20 °C). The final pellet was stored at −20 °C until DNA extraction.

Serial 10-fold dilutions from rotifer and seawater samples were prepared in autoclaved seawater and then plated on Marine Agar (MA, Difco 2219). Incubation was conducted in the dark at 20 °C for 5 days. Plates containing 30–300 colonies were selected for CFU counts and further isolation of morphologically different colonies with exception of introduced strains. Plates were then replicated on Thiosulfate Citrate Bile Sucrose (TCBS, Cultimed) agar plates (Planas et al., 2005) and incubated for 24–48 h at 20 °C.

2.4. DNA extraction

DNA was extracted from 10^6 rotifers, 50 ml of seawater or 1 colony of the different bacterial morphotypes with a Phenol: Chloroform: Isoamyl Alcohol method (Pintado et al., 2003). Bacteria were lysed by incubating 1 h at 37 °C with 200 μl of lysozyme (Sigma, 50 g·l^{-1}) and 10 μl of mutanolysine (Sigma, $25 \cdot 10^{-5}$ U l^{-1}) and for 50 min at 50 °C