Bacterial host interaction of GFP-labelled *Listonella anguillarum* HI-610 with gnotobiotic sea bass (*Dicentrarchus labrax* L.) larvae

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Running title: bacterial interaction in gnotobiotic larval gut

Abstract

The location and cell damage caused by *Vibrio anguillarum*, the causative agent of classical vibriosis, within the developing gut of the newly hatched sea bass (*Dicentrarchus labrax* L.) is unknown. A gnotobiotic sea bass model was used to investigate the early interactions of *V. anguillarum* with sea bass larvae. In the present study, germ-free sea bass larvae were orally exposed to a *V. anguillarum* HI-610 pathogen labelled with the Green Fluorescent Protein (GFP-HI-610) and sampled at regular intervals. Pathogenic colonisation of gut enterocytes was observed 2 hrs post exposure (p.e.) and onwards, whereas bacteria within the swim bladder were visualized 48 hrs p.e and onwards. Ultrastructural findings demonstrated direct bacterial contact with the host cell in the oesophageal mucosa and putative attachment to microvilli of mid- and hindgut enterocytes. The present findings form a starting point for studies assessing the impact of potential candidates (probiotics, prebiotics, antimicrobial peptides) to mitigate bacterial virulence.

Keywords: larviculture, gnotobiotic model system, GFP-labelled pathogen, microscopy, ultrastructure

Introduction

Knowledge of the location of adhesion and/or translocation of pathogenic bacteria to and within the enterocytes of various gut segments in fish larvae is of utmost importance in the design of mitigation strategies. However, the diversity of micro-organisms colonising the gut in the early larval stages is hindering the study of the cause-effect relationship in microbial larval interactions. Using a gnotobiotic model (Dierckens, Rekecki, Laureau, Sorgeloos, Boon, Van den Broeck & Bossier 2009) starting with germ-free larvae, and hence eliminating indigenous microbiota, may provide an excellent tool to unravel the modes-of-action of host microbe interactions (Gunasekara, Rekecki, Baruah, Bossier & Van den Broeck 2010). Recently we described the zootechnical aspects of a standardised challenge test with the pathogen *Vibrio anguillarum* HI-610 (Dierckens *et al.* 2009). However, the invasion strategy of this pathogen is unknown in the developing gut of sea bass larvae as the location of and cell damage caused by *V. anguillarum* has not been investigated yet. In the larviculture industry, exogenous feeding of sea bass larvae with *Artemia* start at day 8-9 after hatching (AH) (Chatain 1997). This initial period without food is considered as a stress inducing factor. Furthermore, bacteria are taken up in the gut before fish larvae switch from endogenous to exogenous feeding regime (Ringø & Birckbeck 1999). By using the gnotobiotic model, sea bass larvae are feed-deprived until day 7 AH. During the first two weeks AH, germ-free sea bass larvae do not possess mucus producing goblet cells (Rekecki, Dierckens, Laureau, Boon, Bossier & Van den Broeck 2009). As a consequence, the mucus layer, normally acting as a physical barrier in the gut is lacking, except for in the pharyngeal and oesophageal regions (Rekecki *et al.* 2009) whereby the mid- and hindgut are exposed and may become more vulnerable to bacteria at this stage. It is suggested that the absence of indigenous bacteria by shedding of epithelial cells in stressed fish and the lack of protective mucus might have a significant role in pathogenesis as the pathogenic bacteria can then attach to cell surfaces,

translocate through enterocytes and cause disease (Ringø, Myklebust, Mayhew & Olsen 2007).

Vibrio appears to interact differently with different host species as described by Sandlund, Rødseth, Knappskog, Fiksdal & Bergh (2010). *V. anguillarum* serotype O2α (HI-610) was detected in the GI tract, abdominal cavity, gallbladder and kidney of turbot (*Scophthalmus maximus*) larvae. In Atlantic cod (*Gadus morhua*) larvae, this bacterium was visualized only in the GI tract, whereas necrosis of dermal cells and the presence of these bacteria in sensory cells of the head region were observed in Atlantic halibut (*Hippoglossus hippoglossus*) larvae. In another study, a high mortality but little histopathology was observed in cod larvae challenged with the HI-610 strain; therefore it is supposed that bacterial toxins may have a vital role in pathogenesis (Engelsen, Sandlund, Fiksdal & Bergh 2008).

The aim of the present study was to define the presence and location of pathogenic *V. anguillarum* strain HI-610 in gnotobiotic sea bass larvae at different time intervals after experimental infection using fluorescence, confocal, light and electron microscopy.

Materials and methods

Bacterial strains and media

Insertion of the Green Fluorescent Protein (GFP) gene into GFP-HI-610 transconjugants was obtained by conjugation between rifampicin resistant *V. anguillarum* HI-610, originally isolated from cod (Samuelsen, Bergh & Ervik 2003) and *Escherichia coli* DH5α carrying the suicide plasmide pJBA120 containing a mini-Tn5 transposon with the *gfp* gene (Andersen, Sternberg, Poulsen, Bjørn, Givskov & Molin 1998) and subsequent selection on Luria Broth agar containing rifampicin and kanamycin as described by Boon, Goris, De Vos, Verstraete & Top (2001). *V. anguillarum* GFP-HI-610 and wild type *V. anguillarum* WT-HI-610 were grown as described by Dierckens *et al*. (2009). To determine the growth curve of strain GFP-

HI-610 and strain WT-HI-610, batch cultures were made by inoculating 2 day-old cultures into 10 ml of liquid media (MB) in 20 ml plastic centrifugation tubes and incubating at 16°C with agitation. Every 3 hrs, bacterial growth was checked by aseptically removing 1 ml of the aliquot and measuring the absorbance at a wavelength of 550 nm with a spectrophotometer (Genesys 20, Thermospectronic, Aalst, Belgium).

Experimental set-up

Eggs of *Dicentrarchus labrax* were obtained from natural spawning at the hatchery of Ecloserie Marine de Gravelines (Gravelines, France). To obtain germ-free eggs, a standard disinfection protocol was used previously described by Dierckens *et al.* (2009). After hatching (60 hrs), a transparent sterile screw cap vial (total volume: 15 ml) was filled with 10 ml filtered, autoclaved sea water (FASW) containing 10 mg rifampicin l^{-1}. Twelve larvae were transferred to each vial. A total amount of 100 vials was placed on a rotor turning at 4 rpm with an axis tangential to the axis of the vials. Throughout the experiment, eggs and larvae were kept in a temperature controlled room at 16 ± 0.5°C in constant dim light (10 candela steradian m^{-2}) at a salinity of 36 g l^{-1}. From day 7 AH until day 13, thirty freshly hatched germ-free *Artemia franciscana* nauplii (Marques, François, Dhont, Bossier & Sorgeloos 2004) were added to each vial (± 2.5 nauplii/larva), every second day. The experiment was approved by the ethical committee of Ghent University under the number EC2005/95.

Exposure study

Two trials were carried out in this study: trial 1 was performed according to the study of Dierckens *et al.* (2009). A standard suspension of 10^5 cfu *V. anguillarum* ml^{-1} was administered via immersion to day 3 AH larvae in order to test the virulence of WT-HI-610 and GFP-HI-610 *Vibrio anguillarum*. On day 7, 9, 11 and 13 AH (4 days to 10 days post

exposure; p.e.), 5 replicate vials (60 larvae) were used for survival counts, and for fluorescence, confocal and light microscopy. In trial 2, day 4 AH larvae were exposed to a density of 10^8 cfu ml^{-1} GFP-HI-610 in order to track early pathogenic invasion of the gut**.** Whole larval bodies were sampled for fluorescence, confocal, light microscopy and TEM at 2-6-12-24-48-72-120-168 hrs p.e. (2 hrs to 7 days p.e. until day 11 AH). Day 4 AH larvae were used to ensure possible uptake of bacteria by drinking as their mouth is supposed to be already open from day 3 AH, as the bacteria were tracked already after 2 hrs p.e. For both trials, control germ-free larvae were kept under similar conditions and sampled at the same time points as challenged larvae. All larvae were euthanized by 0.1% benzocain immersion before sampling except when mentioned otherwise.

Testing germ-free status and quantification of bacterial density inside the fish larvae

After 24 hrs of incubation, 30 sea bass eggs were aseptically removed from the incubation bottles and processed as described by Rekecki *et al*. (2009). In addition, 1 ml water from each incubation bottle and from all sampled vials was added to a tube containing 9 ml of 10% sterile MB from the first time point until the end of the experiment. No colony forming bacteria and no turbidity were detected after 72 hours incubation of eggs and larvae during the course of the trial.

In order to determine the density of gut microbiota in sea bass larvae in trial 1, live challenged and control germ-free larvae were anesthetized with 0.1% benzocain for 10 s. After surface disinfection with 0.1% benzalconium chloride for 10 s the larvae were washed 3 times in sterile NSS for 10 s, before being homogenized and plated on 10% Marine Agar (MA) with 10 mg l^{-1} rifampicin. Agar plates and the marine broth tubes were incubated at an increasing temperature from 20°C to 28°C over a 72 hrs period and were checked for bacterial growth.

Sampling procedure and microscopy

Fluorescence and confocal microscopy*:* for every time point, 5 challenged larvae were anesthetized with 0.1% benzocain for 10 s, the external body surface was disinfected with 0.1% benzalconium chloride for 10 s, and washed in FASW for 10 s, before fixation in 4% paraformaldehyde for 2 hrs. The whole larval body was individually mounted on glass slides with a solution of glycerine and 1,4-diaza-bicyclo[2,2,2]-octane (DABCO) (ACROS Organics, Geel, Belgium). To examine bacterial colonization of the skin, 3 additional larvae were sampled and treated at every time point in the same way but without the disinfection step. Serial optical sections of larvae were acquired using a LEICA TCS SP2 confocal microscope using the Argon 488 nm excitation laser line (Leica Microsystems Belgium BVBA, Groot-Bijgaarden, Belgium) and Olympus BX61 fluorescence microscope (Olympus Belgium N.V., Aartselaar, Belgium) using the filter cube U-MWIB2.

Light microscopy: five sea bass larvae were fixed for 3 hours in Carnoy's fixative (6 parts absolute alcohol, 3 parts chloroform, 1 part glacial acetic acid 100%) for Giemsa staining and processed and cut as described by Rekecki *et al*. (2009).

Transmission electron microscopy (TEM): whole larvae were fixed in Karnovsky fixative (2% formaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.4) overnight at 4°C, washed in sodium cacodylate buffer (pH 7.4), post-fixed overnight with 1% osmium tetroxide, dehydrated in a graded series of alcohol and embedded in SPURR's resin. Serial ultrathin sections of 60 nm ranging from the ventral border of the yolk sac up to the swim bladder of the larval GI tract were made at intervals of 7 µm, using the Leica EM UC6 ultramicrotome (Leica Microsystems Belgium BVBA), mounted on formvar coated single slot copper grids (Laborimpex N.V., Brussels, Belgium) and post-stained with uranyl acetate and lead citrate (Leica EM stain, Leica Microsystems Belgium BVBA). Specimens were

viewed on a Jeol 1200 EXII transmission electron microscope (JEOL Europe BV, Zaventem, Belgium) at 80kV accelerating voltage. Different segments of the gut, namely oesophagus, gastric region, mid- and hindgut of sea bass larvae were investigated by TEM.

Statistics

Statistical analysis was performed by means of SPSS 15.0 software. Survival was reported as mean ± standard error of the mean (S.E.M.). Data were tested for normality and in case of normal distribution, they were subjected to one-way analysis of variance (ANOVA). Bonferroni and Tukey test was used for multiple comparisons among means in case of non-homogeneity. For non-parametric tests, Kruskal Wallis was used to compare response variables from three treatment groups. Mann-Whitney U test was used for pair-wise comparisons. Significance was accepted at $p< 0.05$.

RESULTS

Mortality and bacterial density - trial 1

In vitro, the growth curves of both strains GFP-HI-610 and WT-HI-610 were similar (data not shown). A significantly higher mortality was demonstrated in both wild-type and germ-free challenged groups (76 ± 19 % and 44 ± 6 %) as compared to the control group (4 ± 2 %) from day 11 AH onwards. This difference in mortality became more apparent by the end of the experiment on day 13 AH, being 100% , 89 ± 9 % and 11 ± 2 %, respectively. On day 7 AH, the bacterial density within the sea bass larvae exposed to WT-HI-610 and GFP-HI-610 strains was 6.0×10^4 cfu larva^{-1} and 1.8×10^3 cfu larva^{-1}, respectively. On day 9 AH, the strain WT-HI-610 density (7.1×10^4 cfu larva^{-1}) had not increased compared to day 7 AH, whereas the strain GFP-HI-610 density had increased by 1 log (1.0×10^4 cfu larva^{-1}). By day 11 AH,

194 the density of both WT-HI-610 and GFP-HI-610 strains became similar (1.8 x 10^5 cfu larva^{-1}
195 and 3.4 x 10^5 cfu larva^{-1}), respectively.

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197 Microscopy

198 Fluorescence, confocal and light microscopic findings

199 In trial 1, five larvae sampled per time point were examined for the presence of green
200 fluorescent bacteria. The first indication of bacterial adhesion in the gut and the localization of
201 bacteria within the swim bladder occurred both from day 9 AH (6 days p.e.) onwards .

202 During trial 2, samples were taken at 2-6-12-24-48-72-120-168 hrs p.e. After 2 hrs p.e.,
203 bacteria were localized in the oesophagus, mid- and hindgut of day 4 AH larvae. Light
204 microscopic study revealed bacterial adhesion to gut enterocytes already at 2 hrs p.e. as
205 confirmed by Giemsa staining, while at 48 hrs p.e. bacteria, single or grouped, were clearly
206 seen in close contact to the apical brush border of midgut and hindgut enterocytes visualized
207 by fluorescence and confocal microscopy (Figs. 1.1-1.2). At 12 hrs p.e., a few bacteria could
208 be detected in the lumen of the ureter. Starting from 48 hrs p.e. onwards (day 6 AH larvae),
209 bacteria were visible within the swim bladder (Figs. 1.3-1.4). In total, bacteria were present in
210 the swim bladders of 10% (6 out of 60 larvae) of the investigated sea bass larvae. Bacterial
211 presence on the skin was observed sporadically at the yolk sac and the cranial regions in 13%
212 of larvae. During the whole experiment, the number of bacteria present on the skin never
213 reached the initial levels of bacteria observed in the GI tract at 2 hrs p.e.. Control germ-free
214 larvae did not contain any GFP-labelled bacteria.

215 Ultrastructural findings

216 Ciliated cells were observed in fore-, mid- or hindguts in both uninfected and challenged
217 larvae. At 48 hrs p.e., numerous intraluminal bacteria were visualized in close contact with
218 ciliated cells of the oesophageal mucosa (Fig. 2.1a). Bacterial adhesion to the oesophageal

epithelium characterised by electron dense area between the bacterial outer membrane and the host cell membrane was observed (Fig. 2.1b). Shedding of epithelial cells into the gut lumen was regularly seen. Bacterial-like structures within the gut lumen and close contact (interspace of 100-200 nm) of bacteria to the microvilli of midgut and hindgut enterocytes were visualized during the course of the experiment (Figs. 2.2-2.3). Bacterial clusters within the midgut lumen were surrounded by and in direct physical contact with membrane-like structures at 120 hrs p.e. (Fig. 2.4). A putative dendritic cell with phagosomes protruding with cytoplasmic extensions towards the midgut lumen was visualized at 168 hrs p.e. (Fig. 2.5). In both challenged and control animals, intraepithelial macrophages situated at the basal portions of midgut and hindgut enterocytes were visualized at 48 hrs p.e. (Fig. 2.6).

DISCUSSION

Although *in vitro* the growth curves of both bacterial strains were similar, *in vivo* their growth seemed to be delayed. The sea bass larvae rely only upon endogenous yolk reserves during the first days of development and hence the larval gut is a nutrient-poor environment for bacteria. In this respect, the GFP production may lead to slower bacterial growth and slightly delayed larval mortality. Incorporation of GFP can be a metabolic burden for the bacterial cell, as more energy is required for this extra protein (Allison & Sattenstall 2007). However, at day 11 AH, when the larvae were fed already for several days the bacterial density in both groups became similar. As an alternative we put forward the controversial hypothesis that the growth of strain GFP-HI-610 in the larval GI tract may be delayed due to down regulation and/or inactivation of a virulence factor due to chromosomal GFP insertion.

Engelsen and colleagues (2008) did not detect bacteria associated with the skin of cod larvae during immersion challenge with *V. anguillarum* HI-610. O'Toole, von Hofsten, Rosqvist, Olsson & Wolf-Watz (2004) localised GFP-labelled *V. anguillarum* firstly in the gut of

zebrafish larvae, followed by the fish surface. In our study, only a few bacteria were noted on the skin in 13% of larvae, thus it can be concluded that no significant colonisation of the skin was observed. An increasing number of GFP-labelled *V. anguillarum* was localised in the GI tract lumen in the present study. Already from 2 hrs p.e. onwards, putative adhesion to and colonisation of gut enterocytes was observed. At 48 hrs p.e. the presence of bacteria in the swimbladder was demonstrated by fluorescence and confocal microscopy. Sea bass larvae belong to the transient physostomous group using a functional pneumatic duct for a limited time in order to inflate their swim bladder (Chatain 1986; Kitajima, Watanabe, Tsukashima & Fujita 1994). Inflation occurs when sea bass larvae switch from endogenous to exogenous feeding, whereafter a gas gland, located ventrally in the swim bladder, will be responsible for buoyancy control (Chatain 1986; Battaglene & Talbot 1990; Trotter, Pankhurst & Battaglene 2005). Immunosuppressed larvae may become vulnerable to bacterial aerocystitis, an inflammation of the swim bladder epithelium that causes dysfunction of the gas gland and collapse of a partially inflated swim bladder (Summerfelt 1996). Similar findings were reported in walleye (*Stizostedion vitreum*) larvae (Kindschi & MacConnell 1989; Marty, Hinton & Summerfelt 1995) and in zebrafish (*Danio rerio*) larvae (Kent, Matthews, Laver & Schech 2007). Inflation failure is considered to be irreversible, forming one of the major bottlenecks in larviculture as buoyancy control, swimming ability and feeding success become jeopardized (Peruzzi, Westgaard & Chatain 2007). It needs to be taken into account that in commercial hatcheries, both allochthonous and autochthonous gut bacteria, can cause disease under certain conditions (Ringø *et al.* 2007) and can also enter and cause collapse and noninflation of the swim bladder, eventually leading to starvation and death. Therefore, application of probiotics and their putative beneficial effect as ecological competitor in the gut may be questioned when larval survival is endangered by bacterial aerocystitis.

Ciliated cells were observed in the different segments of the GI tract in both challenged and control germ-free larvae of different ages. In the midgut of two weeks old herring (*Clupea harengus*) larvae, ciliated cells have been described which are supposed to propel bacteria towards the hindgut where antigen uptake occurs (Hansen, Strøm & Olafsen 1992). The function of ciliated cells could be a cleansing mechanism, preventing organisms to make contact with binding sites on mucosal surfaces. Bacteria must adhere first to intestinal mucosa in order to withstand this action (Lu & Walker 2001). In our study, bacterial adhesion to the oesophageal mucosa was demonstrated at 48 hrs p.e.. Electron dense contact between the bacterial outer membrane and an invaginated host cell membrane is considered to be an attachment site (Neutra 1984). Next to avoiding cleansing mechanisms, adhesion is also important for infection resulting in other benefits for the bacteria such as growth advantages, enhanced toxicity to the host, and increased resistance against antimicrobial or antitoxin activities (Ofek & Doyle 1994). Our study additionally demonstrated scattered bacteria in the lumen of mid- and hindgut enterocytes in close contact with the brush border layer (Fig. 2.4). Whether this implies that bacteria attached to the brush border effectively enter the fish by this intestinal route, is not certain as suggested by Engelsen *et al.* (2008).

In general, the hindgut is considered to be mainly involved in phagocytotic activity and less involved in nutrient absorption (Buddington & Diamond 1987). In both the challenged and germ-free groups, engulfment of particles, but not of bacteria was observed in both mid- and hindgut intraepithelial macrophages. The exact nature of these particles hitherto is not clear.

In *in vivo* studies with *V. anguillarum*, the pathogen did not penetrate turbot juvenile epithelial cells nor was it taken up intracellularly (Olsson, Jöborn, Westerdahl, Blomberg, Kjelleberg & Conway 1996). In contrast, immunohistochemical studies evidenced a release of free *V. anguillarum* from an enterocyte endosome into the lamina propria in turbot larvae (Grisez, Chair, Sorgeloos, & Ollevier 1996). In epithelial cell lines, *V. anguillarum* strains can

survive intracellularly as a result of uptake by phagocytosis or endocytosis induced by bacteria (Wang, Oon, Ho, Wong, Lim & Leung 1998). In a preliminary TEM study on conventional sea bass juveniles, internalized bacterial colonies within the gut enterocytes were described (Rekecki, unpublished results). However, in the current study, no intra- or intercellular bacterial translocation from the GI tract was observed.We observed expulsion of enterocytes into the gut lumen which can be either due to natural shedding of gut enterocytes needed for tissue homeostasis, or a major host defense mechanism of the gut epithelium. The latter involves rapid exfoliation of damaged cells infected by bacterial pathogens in order to prevent bacterial colonisation (Kim, Ashida, Ogawa, Yoshikawa, Mimuro & Sasakawa 2010). Whether these cells in the gut lumen are part of the hosts defense mechanism, is unknown.

In conclusion, by using the established sea bass model and by labelling the pathogenic bacterium with GFP, we managed to study bacteria *in situ* during early colonisation of the host. The present findings provide a baseline for investigating the effects of probiotica (e.g. quroum sensing inhibitors), antimicrobial peptides, prebiotica, immunostimulants and other substances affecting virulence factors, hence colonisation and infection. Finally, whether probiotics can exert a protective role in fish larvae once their swim bladder is infected, is a topic for further research.

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