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Bacterial composition and activity determines host gene-expression responses in gnotobiotic Atlantic cod (*Gadus morhua*) larvae

Torunn Forberg [a,*], Ragnhild Inderberg Vestrum [a], Augustine Arukwe [b], Olav Vadstein [a]

[a] Department of Biotechnology, Norwegian University of Science and Technology, N7491 Trondheim, Norway
[b] Department of Biology, Norwegian University of Science and Technology, N7491 Trondheim, Norway

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ABSTRACT

In this study the effects of different live versus dead bacteria on the survival and gene-expression patterns in Atlantic cod (*Gadus morhua*) larvae were examined. Seventeen different gnotobiotic conditions were tested in a 5-day experiment, and the effects of these conditions on the expression of selected genes involved in immune response, xenobiotic breakdown and nutrition (*C3*, *Gpx*, *Cyp1a1* and *Fdps*) were investigated. For some of the conditions where dead bacteria were added, we observed significantly higher survival compared to the axenic control. Microbial specificity of host-responses was observed for all four genes, for both live and dead bacteria, although expression of *Gpx* and *Fdps* was more influenced by active bacteria. Live bacteria generally resulted in higher transcript levels of all the genes investigated, indicating that these host-responses are partly dependent on microbial activity, and not just due to interactions with bacteria as particles.

The observed microbial specificity toward dead bacteria highlights the need to take into account the role of bacterial components when investigating host–microbe relationships.

1. Introduction

The vertebrate gut is host to numerous bacteria, many of which are essential for normal development and function (Hooper and Gordon, 2001; McFall-Ngai, 2002). In aquatic systems, where microbes have easy access to host surfaces, the intimate host–microbe relationship must be highly regulated (Olafsen, 2001; Vadstein et al., 2004). Previously we have investigated gene-expression patterns in bacteria-free Atlantic cod (*Gadus morhua* L.) larvae and larvae raised in seawater containing undefined microbial communities (Forberg et al., 2011a,b). Significant variation in larval survival and gene expression was observed between biological replicates exposed to different bacterial communities. Gnotobiotic studies (where the microflora present is known and pre-determined) on mice and zebrafish have revealed microbial specificity of host gene-expression responses (Hooper et al., 2001; Rawls et al., 2004). However, it is not known to what extent type and magnitude of a specific host-response is due to interaction with the bacteria as a particle or dependent on bacterial activity. Bacterial components such as lipopolysaccharide (LPS), lipoprotein and DNA have potent immunostimulatory effects on the host (Jijon et al., 2004; Nya and Austin, 2010; Rakoff-Nahoum et al., 2004; Vadstein, 1997). Bacterial activity in the gut has been shown to drive host-responses in mice and zebrafish, bacterial colonization of germ-free animals led to decreased expression of genes involved in xenobiotic breakdown, probably due to degradation of xenobiotics by the gut microbiota (Gill et al., 2006; Hooper and Gordon, 2001; Rawls et al., 2004).

The aims of this study were to (1) investigate whether expression of genes previously found to be regulated by bacteria in zebrafish; *C3*, *Cyp*1a1, *Fdps* and *Gpx* (Rawls et al., 2004), show microbial specificity in cod larvae, and (2) evaluate the dependency of these host-responses on microbial activity, as opposed to interactions with

* Corresponding author at: Sem Sealandsveg 6/8, 7491 Trondheim, Norway. Tel.: +47 73591685.
E-mail address: torunn.forberg@biotech.ntnu.no (T. Forberg).

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bacterial components. Eight combinations of either live or dead bacteria were used as gnotobiotic conditions for cod larvae.

2. Materials and methods

2.1. Biological material, rearing and sampling

All experiments were performed in a laminar flow hood, using pre-sterilized equipment.

Bacterial strains were cultured for 2 days at 22 °C in liquid Marine Broth (Difco™), harvested by centrifugation, and re-suspended in filtered (0.22 μm Micropore) autoclaved seawater (FASW) before addition to the rearing bottles. The bacterial density was determined by measuring the absorbance (660 nm) of the suspension, and converting it to cells/mL by strain specific calibrations. To obtain dead bacteria, FASW solutions with known bacterial concentrations were autoclaved (121 °C, 20 min). Atlantic cod eggs were supplied by Nofima Marin (Tromsø, Norway). Bacteria-free eggs were obtained as described in Forberg et al. (2011a), using two rounds of glutaraldehyde disinfection and incubation in rifampicin and ampicillin until hatching.

Sterile Easyflasks™ (Nunclon™) were filled with 50 mL FASW (approximately 6 °C), and stocked with 30 larvae per bottle. The bottles were placed horizontally to maximize the water surface area. After stocking, the temperature was increased by 1 °C per day until 12 °C was achieved. Continuous light was used from day 3 post-hatch, and no feed was added. Dead larvae were counted from day 1 after stocking, without disturbing the bottles. At the end of the experiment (day 5 post-hatch), larvae were sacrificed using MS-222 (0.5 g/L) and placed in RNA*later*® (Ambion) for storage at −20 °C until RNA isolation.

2.2. Design of gnotobiotic experiments

Three probiotic candidates (*Vibrio* RD 5–30, *Pseudoalteromonas* RA 7–14 and *Microbacterium* ND 2–7) isolated by Fjellheim et al. (2010), as well as one pathogen (*Vibrio anguillarum* HI 610 obtained from Institute of Marine Research, Bergen, Norway, courtesy of Professor Øivind Bergh) were used in gnotobiotic conditions. The 17 conditions tested contained live or dead bacteria, with all seven possible combinations of the three probiotic candidates, as well as the pathogenic bacteria alone and an axenic control (each condition with three biological replicates). Bacteria were added to a final density of 10^6 CFU/mL, immediately after stocking larvae into rearing bottles (day 0 post-hatch). For the live and dead conditions with all three probiotic candidates together, the final density was 10^9 CFU/mL. The gnotobiotic cod larvae were observed until day 5 post-hatch, with the exception of the live *V. anguillarum* condition, which had to be terminated at day 3 post-hatch as a result of high mortality.

2.3. Verification of gnotobiotic conditions

Samples from the cod rearing bottles were taken at the end of the experiment. Liquid and solid M65 media (0.5 g peptone, 0.5 g tryptone and 0.5 g yeast extract, dissolved in 800 mL FASW and 200 mL MilliQ water) and Marine Broth were used to check for bacterial contamination. Serial dilution plating was used to estimate the density of bacteria in the bottles containing mono-associations of bacteria.

2.4. RNA isolation and gene expression analysis by quantitative PCR

To isolate total RNA, the cod larvae were homogenized in TRK lysis buffer and β-mercaptoethanol, using a rotor-stator. Larvae were pooled (10 individuals per replicate) to reduce the effect of inter-individual variation on gene-expression. Three biological replicates were included per treatment. RNA purity and integrity was confirmed by spectrophotometric analysis and inspection of intact ribosomal 28S and 18S bands after denaturing gel electrophoresis. Complementary DNA (cDNA) for qPCR was generated from 1 μg total RNA for all samples, using a mixture of random and poly-T primers from iScript cDNA synthesis kit (BioRad), according to the manufacturer's protocol. A control lacking reverse transcriptase enzyme was included in each run. The synthesized cDNA was diluted 1:6 prior to qPCR.

Quantitative polymerase chain reaction (qPCR) was performed using the Mx3000p REAL-TIME PCR SYSTEM (Stratagene). Each 25 μL reaction contained 12.5 μL iTAQ™SYBR® Green Supermix with ROX (Bio-Rad), 5 μL diluted cDNA, 6.5 μL dH_2O and 200 nM of both the forward and the reverse primers. The PCR program consisted of an initial step at 95 °C for 3 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 20 s at 72 °C. All reactions were run in triplicate. A non-template control, as well as the control sample from the reverse transcription was included for each gene. Standard curves for individual genes were generated using 10-fold dilutions of known concentrations of plasmids containing the specific amplicon. Messenger RNA (mRNA) copy numbers were estimated based on the standard curves and the Ct values obtained. Data from the triplicate runs were averaged, and results expressed as copies/μg initial RNA.

2.5. Statistical analysis

The survival data were arcsin transformed before analysis by ANOVA, and the contrast to the control-treatment (axenic) was calculated to determine if survival was higher or lower than for the control larvae. Gene expression data were log transformed before analysis by ANOVA. To evaluate the effect of additions of bacteria, the contrast to the axenic treatment was calculated as for the survival data. Tukey's honestly significant-difference test was performed in order to detect statistically significant differences in gene expression within the different live and dead gnotobiotic conditions. Student's *t* test was used to evaluate differences in gene expression between live and dead treatments with the same bacterial composition. The statistical analysis was done with Systat V.12, and a *p*-value <0.05 was considered a statistically significant difference.