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High mortality of juvenile gilthead sea bream (*Sparus aurata*) from photobacteriosis is associated with alternative macrophage activation and anti-inflammatory response: Results of gene expression profiling of early responses in the head kidney

Caterina Pellizzari [a], Aleksei Krasnov [b], Sergey Afanasyev [b,c], Nicola Vitulo [d], Rafaella Franch [a], Sara Pegolo [a], Tomaso Patarnello [a], Luca Bargelloni [a,*]

[a] Department of Comparative Biomedicine and Food Science, University of Padova, viale dell'Università 16, 35020 Legnaro, Italy
[b] Nofima, Norwegian Institute of Food, Fisheries and Aquaculture Research, P.O. Box 5010, Ås No-1430, Norway
[c] Sechenov Institute of Evolutionary Physiology and Biochemistry, St Petersburg 194223, Russia
[d] CRIBI, University of Padova, Complesso Biologico Vallisneri, Via Ugo Bassi 58/B, 35131 Padova, Italy

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ABSTRACT

The halophilic bacterium *Photobacterium damselae* subsp. *piscicida* (*Phdp*) represents a substantial health problem for several fish species in aquaculture. Bacteria that reside free and inside phagocytes cause acute and chronic forms of photobacteriosis. Infections of juveniles rapidly kill up to 90–100% fish. Factors underlying failure of the immune protection against bacteria remain largely unknown. The reported study used a transcriptomic approach to address this issue. Juvenile sea breams (0.5 g) were challenged by immersion in salt water containing 2.89×10^8 CFU of a virulent *Phdp* and the head kidney was sampled after 24- and 48-h. Analyses were performed using the second version of a 44 k oligonucleotide DNA microarray that represents 19,734 sea bream unique transcripts and covers diverse immune pathways. Expression changes of selected immune genes were validated with qPCR. Results suggested rapid recognition of the pathogen, as testified by up-regulation of lectins and antibacterial proteins (bactericidal permeability-increasing protein lectins, lysozyme, intracellular and extracellular proteases), chemokines and chemokine receptors. Increased expression of proteins involved in iron and heme metabolism also could be a response against bacteria that are dependent on iron. However, negative regulators of immune/inflammatory response were preponderant among the up-regulated genes. A remarkable finding was the increased expression of IL-10 in concert with up-regulation of arginase I and II and proteins of the polyamine biosynthesis pathway that diverts the arginine flux from the production of reactive nitrogen species. Such expression changes are characteristic for alternatively activated macrophages that do not develop acute inflammatory responses. Immune suppression can be induced by the host to reduce tissue damages or by the pathogen to evade host response.

1. Introduction

Photobacteriosis, described also as fish pasteurellosis, is a septicaemia caused by the gram negative halophilic bacterium *Photobacterium damselae* subsp. *piscicida* (*Phdp*), which was originally isolated in 1963 from mortalities occurring in natural populations of white perch (*Morone americanus*) and striped bass in Chesapeake Bay. Since 1969, this has been one of the most important fish diseases in Japan, affecting mainly yellowtail (*Seriola quinqueradiata*), and since 1990 it has caused economic losses in the marine culture of gilthead sea bream, sea bass, and sole species (*Solea senegalensis* and *Solea solea*) in the Mediterranean countries of Europe, and of hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) in the USA [1]. This bacterial septicaemia occurs mainly during summer months and has been associated with high temperatures, low salinity, and poor water quality. Larvae and juveniles are highly susceptible to the disease, whereas animals larger than 50 g are comparatively more resistant [2]. Bacteria that reside in different tissues and inside phagocytes (mainly macrophages) cause chronic and acute forms of disease. In the former

* Corresponding author. Tel.: +39 (0)49 8272506; fax: +39 (0)49 8272604.
E-mail address: luca.bargelloni@unipd.it (L. Bargelloni).

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case, gross external signs of photobacteriosis are usually inconspicuous and there are generally no surface lesions. Only some affected fish may exhibit a darkening of body colour and/or slight haemorrhagic areas in the head and gills. Chronic lesions in internal organs are characterized by the presence of white tubercles of about 0.5–3.5 mm in diameter [3]. In the acute form, liver, kidney and spleen show a multifocal necrosis and the presence of bacterial accumulations, free and within phagocytes, in the capillaries and in the interstitial spaces. Acute infections of juvenile sea bream induce rapid mortality that can reach 90–100% of infected populations with large economic losses for affected fish farms [4].

Until present, studies aiming at explaining the high pathogenicity of *Phdp* have focused on the virulence mechanisms. Adherence and invasive capacities are essential in the first stage of infection [5]. Although the pathogen shows a moderate binding capacity to fish cell lines, it is able to adhere in high numbers to gilthead sea bream intestine [6]. The presence of a capsular material of polysaccharide nature does not affect bacterial adhesion to cell receptors but confers resistance to serum killing, and increases fish mortality [7]. The weak invasiveness is compensated by the *Phdp* ability to survive for at least five days in phagocytic and non-phagocytic cells [8,9]. The intracellular survival of *Phdp* is likely to provide effective protection against specific and non-specific host defences and exogenous antimicrobial agents including antibiotics. In addition, intracellular bacteria damage infected cells, resulting in the release of microorganisms and subsequent invasion of adjacent cells. A fundamental virulence factor of *Phdp* is a high-affinity siderophore-mediated iron sequestering system. The ability to acquire iron from hemin and haemoglobin is essential for the growth of pathogenic bacteria within the host and essential, therefore, to cause infection. Iron also plays a regulatory role in the synthesis of some proteolytic enzymes such as gelatinase and caseinase [10]. Other virulence factors with an important role in the pathogenesis of photobacteriosis are extracellular products with strong phospoliphase, cytotoxic, and haemolytic activities [11] as well as a plasmid-encoded pro-apoptotic protein, targeting peritoneal neutrophils and macrophages, which leads to secondary necrosis [12–14].

At present, little is known about the fish responses to *Phdp*. Occurrence of acute and chronic forms suggests failure of both innate and acquired immunity. This can be due to absence or low level of immune responses. An alternative possibility is the direct suppression of immunity by the host or by the pathogen that can contribute to persistent disease [15]. Given the limited knowledge on interactions between sea bream and bacteria and a large number of possible scenarios, multiple gene expression profiling appears an appropriate research strategy. A hypotheses-free gene expression profiling was carried out to seek for an answer to the question: why juvenile sea breams are unable to resist acute photobacteriosis? A new version of the sea bream high-density oligo-DNA microarray developed by Ferraresso et al. [16] was designed and used to analyse rapid changes at the transcriptome level upon experimental infection in the head kidney.

2. Material and methods

2.1. Disease challenge, sampling and RNA extraction

Fish material for this experiment was provided by the fish farm "Valle Ca' Zuliani" (Monfalcone, Italy). Fertilized eggs originating from a single broodstock in a mass-spawning event with over 60 potential parents were collected and kept in a dedicated water tank. After hatching, larvae were raised following standard procedures. Juvenile sea breams were maintained in a dedicated aquarium until reaching an average weight of 0.4–0.6 g (110 days old). All animals were then transferred to the Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Italy) for the experimental infections. A total of 78 fish were divided into two aerated tanks each with 200 l of re-circulating seawater (salinity 35‰). Water temperature was maintained constantly at 19 °C. After one week of acclimation the challenge experiment was carried out. This study was performed in strict accordance with the requirements of Italian Law n. 116 of 27 January 1992 (O.J.I.R. 18 February 1992, n. 40, O.S.) and further amendments on the protection of animals used in experiments. The protocol fulfilled the requirements as presented in Annex 4 to Circular n. 8 of 22 April 1994 of the Ministry of Health. All animal manipulations were performed under MS-222 anaesthesia and all efforts were made to minimize suffering. At the end of the experiment the animals were euthanatized with an excess of anaesthetic.

The day before exposure to the pathogen (*Phdp*) 8 individuals (4 for each tank) were randomly sampled among experimental population to check for the presence of the pathogen. Histological evaluation of head kidney, spleen, and liver was carried out. Moreover, total DNA was extracted from spleen following standard procedures. All DNA samples were subjected to PCR-RFLP test for the presence of *Phdp* [17].

A total of 35 fish were exposed by immersion in 50 l of aerated sea water containing 2.89×10^8 CFU of a virulent *Phdp* strain (249/ittio99) for 30 min [17]. After pathogen exposure, all fish were returned to the original tank. A control was treated with the same experimental procedures in the tank without pathogen. At 24 h and 48 h post infection, 5 fish were collected from control and treatment aquaria and euthanized by over anaesthetization, for a total of 20 individuals. Samples of head kidney were taken and stored in RNA later (Ambion, Austin, Texas) at −20 °C until RNA extraction. Spleen and liver were divided into two aliquots, one was used for DNA extraction and Phdp specific RFLP-PCR test, the other was fixed in buffered formalin and processed for histological examination in all challenged animals.

Total RNA was extracted from tissue samples using the RNAeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA concentration was determined using a UV–Vis spectrophotometer, NanoDrop® ND-1000 (NanoDrop Technologies, Wilmington, USA). RNA integrity and quality was estimated on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA Integrity Number (RIN) index was calculated for each sample using Agilent 2100 Expert software. RIN provides a numerical assessment of the integrity of RNA that facilitates the standardization of the quality interpretation. In order to reduce experimental biases in microarray analysis due to poor RNA quality, only RNA samples with RIN number >8 were further processed. On the basis of RNA concentration and quality, 4 controls and 4 challenged individuals for both 24 h and 48 h post infection time points for a total of 16 samples were selected for labelling and hybridization.

2.2. Microarray design and annotation

Sea bream high-density oligonucleotide DNA microarray used in this study represents an updated version of the platform developed by Ferraresso et al. [16]. Selection of sequences for designing probes began with clusterization of 67,670 ESTs, all from directional cDNA libraries sequenced using Sanger technology and 307 mRNA sequences from GenBank (http://www.ncbi.nlm.nih.gov/genbank). The sequences were masked for vector and repetitive sequences using RepeatMasker software (http://www.repeatmasker.org) and fish repetitive element database. MEGABLAST ver 2.2.15 [18] identified sequences (e-value = e^{-10}) to be included in clusters and Cap3 [19] assembled the members of clusters and produced the consensus sequences. ESTs were assigned to the same cluster if