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Solea senegalensis genes responding to lipopolysaccharide and copper sulphate challenges: Large-scale identification by suppression subtractive hybridization and absolute quantification of transcriptional profiles by real-time RT-PCR

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ABSTRACT

Solea senegalensis is a commercially relevant aquaculture species that remains largely unexplored at the genomic level. The aim of this study was to identify novel genomic responses to lipopolysaccharide and copper sulphate challenges using suppression subtractive hybridization (SSH) and real-time RT-PCR. Forward- and reverse-subtractive libraries were generated for the identification of genes whose transcription is altered in response to lipopolysaccharide (LPS) (immunomodulator) in head kidney (immunologically important organ) and to $CuSO_4$ (common algacide) in liver (central metabolic organ and important source of immune transcripts). A total of 156 genes involved in major physiological functions were identified by SSH, the identified sequences representing a significant increase in the number of sole ESTs in public databases. Fifteen genes represented in the subtracted libraries were selected for further tissue, temporal and inducible transcriptional profiling by real-time RT-PCR. A rigorous quantification of transcript copy numbers was performed for this purpose in both pooled and individual samples from two independent experiments. More than half of the investigated mRNAs encode proteins that deal with different aspects of the immune response, like NCCRP1 (non-specific cytotoxic cell receptor), C3 and C7 (complement components), and ferritin M, HP and TF (iron homeostasis), or play a crucial role in its regulation, like TRAF3. Other mRNAs studied encode proteins involved in metabolism, like TKT and NDUFA4, the response to stimulus, like CEBPB (transcription factor) and CIRBP (RNA-binding protein), and other cell processes. Highly abundant (>500 molecules/pg total RNA) and rare ($\leq$1 molecules/pg) mRNA species were quantified in each sole organ examined, and outstanding differences were also recorded in the comparison between the two organs, e.g. C3 and TF mRNAs were largely overexpressed in liver (>5000 molecules/pg) as compared to head kidney (<5 molecules/pg). Most investigated mRNAs displayed significant alterations in their steady-state copy number following LPS and/or $CuSO_4$ stimulation, i.e. they were (i) up-regulated in response to both treatments in at least one of the two organs (NCCRP1, CEBPB, SQSTM1, NDUFA4, C7 and HP), (ii) up-regulated (TF, CIRBP, TRFA, C3) or down-regulated (TKT) by LPS, their levels remaining essentially unchanged upon $CuSO_4$ challenge, or (iii) down-regulated by LPS, though up-regulated by $CuSO_4$ (ferritin M). Quantifications in individual fish were consistent with those in pooled samples with respect to both the direction and the absolute changes in transcript abundance.

1. Introduction

Solea senegalensis is an important aquaculture species. A serious problem in Senegalese sole culture is the development of infectious diseases. Immunosuppression under aquaculture conditions has been indicated as being the cause of pathological problems (Magnadottir, 2006). Increasing resistance to pathogens and stressors is thus a main priority to keep up a sustainable growth pattern and avoid economic losses (Dinis et al., 1999). The immune and inflammatory systems are activated in response to both infectious and non-infectious stimuli. Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria. In mammals, LPS has been extensively used to mimic bacterial infection and to activate host defences. In fish, LPS has been found to be responsible for the pathogenicity of several bacterial diseases (Swain et al., 2008; Zelikoff, 1993).

In fish, the innate immune system is of primary importance in combating infections due to the constraints placed on the adaptive

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immune response by their poikilothermic nature, plus the limited antibody repertoires, affinity maturation and memory and relatively slow lymphocyte proliferation. (Magnadottir, 2006; Whyte, 2007). The head kidney is an important haematopoietic organ in teleosts, and exhibits morphological similarities with the bone marrow of higher vertebrates. The head kidney also serves as a secondary lymphoid organ in the induction and elaboration of immune responses, and in the clearance of soluble and particulate antigens from the circulation. Furthermore, teleost head kidney is the major site for antibody production and melanomacrophage accumulation of the parenchyma retains antigens for long periods (Whyte, 2007). Thus, the head kidney provides a good model for studying the effects of LPS on the immune systems of Senegalese sole.

Although copper is an essential trace element, its overload is potentially toxic to most organisms. Copper sulphate ($CuSO_4$) is used in aquaculture, as an algacide and to control the outbreaks of infectious diseases in fish ponds and hatcheries (Griffin and Mitchell, 2007). Due to its central role in metabolism, particular attention has been paid to liver in toxicological investigations of both organic and inorganic chemicals in different fish species. Hepatic histological changes and copper accumulation have been reported in Senegalese sole after sublethal $CuSO_4$ exposure, in agreement with fish liver being one of the major target organs for this metal (Arellano et al., 1999).

Suppression subtractive hybridization (SSH) is a simple and efficient PCR-based technique for generating cDNAs enriched for differentially expressed genes (Diatchenko et al., 1996). Since there are relatively few sequenced and defined genes in Senegalese sole, we performed SSH to identify differentially expressed genes in LPS- or $CuSO_4$-stimulated head kidney and liver, respectively. Nowadays, real-time RT-PCR is the most powerful tool for mRNA quantification. Most studies using RT-PCR are taken semiquantitatively (fold-variation) and assume that the mRNAs of reference genes are stably expressed or that any changes that might occur are balanced (e.g. see Manchado et al., 2007). We and others (Monje-Casas et al., 2004; Prieto-Álamo et al., 2003; van de Peppel et al., 2003) have demonstrated that such assumptions can bias the result interpretation. Actually, we have recently emphasized the need for quantification of the real copy number of transcripts in whole-animal studies (Jurado et al., 2003, 2007; Prieto-Álamo et al., 2003; Ruiz-Laguna et al., 2006). Here, the differential mRNA expression of 15 selected genes was further investigated by obtaining their absolute transcript expression signatures.

2. Materials and methods

2.1. Fish treatments

Fish (65 ± 13 g) came from the "Centros IFAPA *Agua del Pino* and *El Toruño*" (Andalusia, Spain). Animals were dosed via a single intraperitoneal (IP) injection with 25 mg kg^{-1} of *Escherichia coli* LPS (serotype 0111:B4, Sigma) or 2 mg kg^{-1} $CuSO_4$. The mRNA expression of fish immune-relevant genes is routinely investigated following IP injection with pathogenic bacteria or LPS. Therefore, it has recently been shown that IP-injected LPS at similar dose (30 mg kg^{-1}) to that used in the current study produces a significant transcriptional response of the c-type lysozyme gene in Senegalese sole (Fernández-Trujillo et al., 2008). To simplify the exposure experiments and to reduce the number of animals to be studied, copper contamination was also performed by IP injection. The copper dose (2 mg kg^{-1}) was chosen as it has been seen to induce oxidative stress when IP injected into the sea bass (*Dicentrarchus labrax*) at the times used in this experiment (Roméo et al., 2000). Individuals injected with phosphate-buffered saline (PBS) served as vehicle controls of all LPS- and $CuSO_4$-treated fish. Neither mortality nor phenotypic effects were observed in any of the fish. Sampling was performed at different times after injection. Fish were sacrificed by immersion in tricaine methanesulfonate (MS-222). The head kidney and the liver were then rapidly dissected, frozen in liquid nitrogen and stored at −80 °C.

2.2. Construction of SSH libraries

Fish were sampled at 6 and 24 h post-injection. Total RNA was extracted from pooled head kidneys and livers (≥10 individuals/condition), using TRIZOL reagent (Invitrogen) according to manufacturer's instructions. Genomic DNA contamination was avoided by a subsequent cleanup with RNeasy reagents (Qiagen Inc.). RNA quality was checked electrophoretically, and quantification was done spectrophotometrically. Five hundred micrograms of head kidney total RNA from each of the 6 and 24 h pools from the LPS-treated and PBS-control fish were combined for poly(A+) mRNA purification (Oligotex mRNA kit by Qiagen Inc.). Similarly, liver total RNA from each of the two pools (6 and 24 h) of the $CuSO_4$-treated and PBS-control fish were combined for poly(A^+) mRNA isolation. In both cases, forward (F) and reverse (R) libraries were designed to obtain clones of genes that had up-regulated or down-regulated mRNA level in the LPS- or $CuSO_4$-treated fish relative to the PBS control.

SSH libraries were produced using the PCR-Select™ cDNA subtraction kit (Clontech) following the manufacturer's protocol. Briefly, double stranded cDNA was digested with RsaI and ligated to specialized adapters. Two successive hybridisations were carried out, resulting in equalisation and enrichment of differentially expressed, adaptor ligated sequences. These were amplified by suppressive PCR, ligated to the TOPO 2.1 vector (Invitrogen) and transformed into *E. coli*. Clones were randomly selected and analyzed for inserts by PCR using vector-based primers. PCR products were purified by the Rapid PCR Purification System (Marligen Bioscience, Inc.). DNA was sequenced on an ABI PRISM™ 3130XL sequencer (Applied Biosystems) using M13 primers.

Sequence output was edited to remove vector sequences, PCR primers, and terminal ambiguities. Trimmed sequences were uploaded to the BLAST server at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to search for homologous nucleotide and protein sequences. BLAST searches were performed using the tBLASTx algorithm and default search conditions. Clones with similar homology were aligned using the SeqMan module of the DNASTAR Lasergene software, to determine the presence of multiple clone copies and of separate parts of the same gene. Classification of the annotated sequences was searched by Gene Ontology (http://www.geneontology.org). Sequences were deposited in the expressed sequence tag database dbEST of GenBank under the accession numbers FF682216 to FF682663 inclusive.

2.3. Absolute transcript quantification by real-time RT-PCR

Primers (supplementary data 1) directed against 15 selected genes were designed with the Oligo 6.1 software (Molecular Biology Insights), as detailed in (Pueyo et al., 2002). To obtain high specificity and performance, primers were required to have high Tm (≥80 °C), optimal 3′-ΔG (≥−3 kcal/mol) value, and to be hairpin and duplex free. All primer pairs produced amplicons of the predicted size. All PCR products were further verified by nucleotide sequencing.

Total RNA was extracted as described for the construction of SSH libraries. cDNA was generated from 2 μg of total RNA from each sample, using the M-MLV reverse transcriptase (Life Technologies) and random hexamers (Invitrogen). Real-time PCR reactions were performed in quadruplicate by using 50 ng of cDNA template,