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Characterisation of *arginase* paralogues in salmonids and their modulation by immune stimulation/ infection

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ABSTRACT

In this study we show that four arginase isoforms (*arg1a*, *arg1b*, *arg2a*, *arg2b*) exist in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). We have characterised these molecules in terms of a) sequence analysis, b) constitutive expression in different tissues, and modulated expression following c) stimulation of head kidney macrophages *in vitro*, or d) vaccination/infection with *Yersinia ruckeri* and e) parasite infection (AGD caused by *Paramoeba perurans* and PKD caused by *Tetracapsuloides bryosalmonae*). Synteny analysis suggested that these arginase genes are paralogues likely from the Ss4R duplication event, and amino acid identity/similarity analyses showed that the proteins are relatively well conserved across species. In rainbow trout constitutive expression of one or both paralogues was seen in most tissues but different constitutive expression patterns were observed for the different isoforms. Stimulation of rainbow trout head kidney macrophages with PAMPs and cytokines also revealed isoform specific responses and kinetics, with *arg1a* being particularly highly modulated by the PAMPs and pro-inflammatory cytokines. In contrast the type II arginase paralogues were induced by rIl-4/13, albeit to a lesser degree. Vaccination and infection with *Y. ruckeri* also revealed isoform specific responses, with variation in tissue expression level and kinetics. Lastly, the impact of parasite infection was studied, where down regulation of *arg1a* and *arg1b* was seen in two different models (AGD in salmon and PKD in trout) and of *arg2a* in AGD. The differential responses seen are discussed in the context of markers of type II responses in fish and paralogue subfunctionalization.

1. Introduction

Arginase (amidinohydrolase, EC3.5.3.1) is an ureohydrolase enzyme widely distributed in living organisms, from bacteria and yeast to plants and animals [1,2]. It catalyses the conversion of L-arginine into L-ornithine plus urea in the Krebs-Henselheit urea cycle. Most studied microorganisms and invertebrates have only one type of arginase, localized in the mitochondria [2,3]. Arginase gene duplication occurred after the separation of vertebrates and invertebrates with the appearance of a cytosolic arginase in ureotelic animals [1–3]. These two isoforms have been studied extensively in mammals and are termed arginase type I and type II. Type I is the cytoplasmic form and is expressed in liver as part of the urea cycle whilst type II is the mitochondrial associated enzyme which is expressed in several peripheral tissues but primarily in the kidney, prostate, small intestine and lactating glands [4]. Hence the two enzymes catalyse the same biochemical reaction but differ in cellular expression, regulation and subcellular localization [1,4].

Within the immune system arginase is known to be a marker of type II responses that are broadly anti-inflammatory and associated with tissue healing, as seen in parasite infections. In the classical polarization model, activated macrophages can either 1) convert L-arginine to L-citrulline and produce nitric oxide (NO)/reactive nitrogen species by the action of inducible nitric oxide synthase (iNOS) after stimulation by T helper 1 (Th1) cytokines such as interferon (IFN)-γ, or 2) they can express arginase after activation with Th2 cytokines including interleukin (IL)-4, IL-10 and IL-13 [4]

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thereby generating the "repair" molecule ornithine that is involved in polyamine and collagen biosynthesis, the latter an important extracellular matrix component that promotes tissue remodelling/fibrosis during healing. These polarized macrophage populations are referred to as classically activated (M1) or alternatively activated (M2) cells respectively. More recently it has become apparent that M1 and M2 may represent extremes of a large array of activation states and that polarization of macrophages first during an innate immune response likely directs T cells to produce Th1 or Th2 adaptive responses, where their secreted cytokines serve to amplify the macrophage dichotomy [5].

M2 macrophages with elevated levels of arginase activity have also been found in fish [5–8]. Common carp (*Cyprinus carpio*) infected with *Trypanosoma carassii* show elevated levels of arginase enzyme activity during the later phase of infection and lack a prominent NO response. Moreover, stimulation of head kidney leukocytes from *T. carassii* infected carp with dibutyryl cyclic adenosine mono phosphate (cAMP, 0.5 mg/ml) increases arginase activity 3–4 fold but these same cells do not increase nitrite production after lipopolysaccharide (LPS, 50 mg/ml) stimulation [7]. Similar findings were obtained with macrophage cultures from uninfected fish stimulated with cAMP but now LPS induced iNOS expression and nitrite production [9,10]. Modulation of arginase gene expression has also been shown in salmonids during parasite infection. Arginase type I is up regulated in skin of Atlantic salmon (*Salmo salar*) infected with sea lice (*Lepeophtheirus salmonis*) [6] but is down regulated in posterior kidney after *Tetracapsuloides bryosalmonae* infection in rainbow trout (*Oncorhynchus mykiss*) and in gills of salmon after *Paramoeba perurans* infection [11,12]. Functional divergence of arginase type I and arginase type II in fish vs mammals has been recently hypothesized in common carp, where it is suggested that arginase type II is a better marker for alternatively activated macrophages in teleost fish rather than arginase type I [5].

A whole-genome duplication (WGD) event occurred at the base of the teleost fish during evolution, and a further WGD (the Ss4R salmonid-specific autotetraploidization event) occurred in the common ancestor of salmonids about 80 Mya after their divergence from Esociformes [13,14]. Following genome duplication events, duplicated genes can either be lost by pseudogenization or retained as two copies that can diverge by the partitioning of the ancestral gene functions (i.e. subfunctionalization) or by the acquisition of a novel function (i.e. neofunctionalization) [15]. In rainbow trout and Atlantic salmon four different isoforms of arginase (*arg1a*, *arg1b*, *arg2a*, *arg2b*) have been found in this study, which has characterised these molecules in terms of sequence analysis, constitutive expression in different tissues, and modulated expression following stimulation of cultured head kidney macrophages *in vitro* or after vaccination and/or infection *in vivo*.

2. Materials and methods

2.1. Cloning of arginase isoforms and sequence analysis

The rainbow trout whole genome shotgun (WGS) sequence was searched with tBLASTn [16], using fish Arginase I and II protein sequences. Candidate WGS contigs (contigs 25562, *arginase 1a*; 44798, *arginase 1b*; 119897, *arginase 2a*; and 23604, *arginase 2b*) were identified and exons predicted as described previously [17,18]. Primers (Table 1) were designed to the 5'- and 3'- untranslated regions (UTR) and used for PCR amplification and cloning of the complete coding region using a mixed tissue cDNA sample. The cloning, DNA and protein sequence analysis was as described previously [19,20]. Briefly, the nucleotide sequences generated were assembled and analysed using the AlignIR™ Software (LI-COR, Inc.). The translated trout protein sequences were used to find the four different isoforms in Atlantic salmon in SalmoBase (http://salmobase.org) using BLAST search (tBLASTn). The Atlantic salmon amplified products obtained using newly designed primers were confirmed by cloning and sequence analysis. Briefly, the PCR products were cloned into pGEM®-T Easy Cloning Vector (Promega) and transformed into competent *Escherichia coli* cells (RapidTrans™ TAM1; Active Motif). The competent cells were grown on MacConkey agar plates (Sigma-Aldrich, UK) with ampicillin (100 μg/ml) at 37 °C for 45 min and colonies with the correct insert size were grown overnight in 4 ml of Luria Bertani (LB) broth (Melford Laboratories Ltd., UK) with ampicillin (100 μg/ml) in a shaking incubator at 37 °C. Plasmid DNA purification was performed using a QIAprep®spin DNA miniprep kit (QIAGEN, UK) according to the manufacturer's instructions and purified plasmids were then sent to be sequenced by Eurofins MWG Operon. Protein sequences were then aligned with MAFFT v7 [21].

For the phylogenetic tree, protein sequences from other species were predicted from the ENSEMBL (http://www.ensembl.org/index.html) or NCBI websites (http://www.ncbi.nlm.nih.gov/) and verified in UniProt (http://www.uniprot.org/blast/). Agmatine ureohydrolase or agmatinase was used as the outgroup for the phylogenetic tree as it is an important evolutionary related enzyme also involved in arginine and proline metabolism [2]. The software BioEdit [22] was used to align all protein sequences from different species and generate a file for the Guidance2 server [23–25], where sequence alignment was performed using Fast Fourier Transform, MAFFT v7 [21], as an algorithm for Multiple Sequence Alignment (MSA) with a final score of 0.92 from the original alignment and a statistical confidence cut-off score of 0.93 after the removal of unreliable columns [26]. A final resulting alignment of 239 amino acids was uploaded to MEGA v6 software [27] to predict the best-fitting amino acid substitution model which was the LG [28] and Gamma site heterogeneity model for arginase with the smallest Akaike information criterion (AIC) of 5177.901 and the lowest Bayesian information criterion (BIC) of 5591.264. Bayesian phylogenetic analysis was performed using Bayesian Evolutionary Analysis, by means of the Sampling Trees (BEAST) software package v1.7 [29] employing the best-fitting substitution model (LG + G), an uncorrelated lognormal relaxed clock model [30], a Yule speciation process, and a Unweighted Pair Group Method with Arithmetic Mean (UPGMA) starting tree. Two runs of BEAST were performed, each with a Markov chain Monte Carlo (MCMC) algorithm of 10,000,000 generations for Bayesian phylogenetic inference. Bayesian Evolutionary Analysis Utility (BEAUti), Tracer v1.6 (http://beast.bio.ed.ac.uk/tracer) [31], LogCombiner (http://beast.bio.ed.ac.uk/logcombiner) with 10% of burn-in, TreeAnnotator (http://beast.bio.ed.ac.uk/treeannotator) and FigTree (http://beast.bio.ed.ac.uk/figtree) were used to construct the phylogenetic tree. The software MatGAT (Matrix Global Alignment Tool) was used to predict amino acid identity/similarity between sequences using the BLOcks amino acid SUbstitution 62 (BLOSUM62) Matrix [32]. The exon-intron structures of arginase genes were determined in Salmobase for *S. salar*, ENSEMBL for *Danio rerio* and *Homo sapiens*, and by aligning the mRNAs with the corresponding genomic DNA using Splign (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi) for *Esox lucius* and *O. mykiss* [33]. The exception was for *arg2b* in *O. mykiss* where the exons had to be inferred using the genomic sequence for *O. mykiss arg2a* (accession number CCAF010119897.1) as it had the highest query cover (99%) allowed for alignment in Splign (58% identity) and hence only exon phase could be determined and not intron size. 100% identity of *arg2b* in *O. mykiss* matched the accession number CCAF010123604.1, supporting the presence of this gene in contig 23604. The synteny of the arginase loci was analysed in Salmobase using BLAST search (tBLASTn).