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Immune gene expression in trout cell lines infected with the fish pathogenic oomycete *Saprolegnia parasitica*

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ABSTRACT

The oomycete *Saprolegnia parasitica* causes significant losses in the aquaculture industry, mainly affecting salmon, trout and catfish. Since the ban of malachite green, effective control measures are currently not available prompting a re-evaluation of the potential for immunological intervention. In this study, the immune response of salmonid cells is investigated at the transcript level, by analysis of a large set of immune response genes in four different rainbow trout cell lines (RTG-2, RTGill, RTL and RTS11) upon infection with *S. parasitica*. Proinflammatory cytokine transcripts were induced in all four cell lines, including IL-1β1, IL-8, IL-11, TNF-α2, as well as other components of the innate defences, including COX-2, the acute phase protein serum amyloid A and C-type lectin CD209a and CD209b. However, differences between the four cell lines were found. For example, the fold change of induction was much higher in the epithelial RTL and macrophage-like RTS11 cell lines compared to the fibroblast cell lines RTG-2 and RTGill. Several antimicrobial peptides (AMPs) were also up-regulated in response to *Saprolegnia* infection, including hepcidin and cathelicidin 1 (rtCATH1) and 2 (rtCATH2). An rtCATH2 peptide was synthesised and tested for activity and whilst it showed no killing activity for zoospores, it was able to delay sporulation of *S. parasitica*. These results demonstrate that particular immune genes are up-regulated in response to *S. parasitica* infection and that AMPs may play a crucial role in the first line of defence against oomycetes in fish.

1. Introduction

The class Oomycota contain both animal and plant pathogenic species that infect humans, fish, and plants, as exemplified by the human pathogen *Pythium insidiosum*, the fish pathogen *Saprolegnia parasitica* and the late blight pathogen of potato *Phytophthora infestans* (Phillips et al., 2008). Even though their morphology looks quite similar to fungi, oomycetes are more closely related to brown algae than to the true fungi (Baldauf et al., 2000). The world-wide growing fish and shellfish industry is heavily affected by oomycetes belonging to the Saprolegniales, i.e. *Saprolegnia* spp. which cause million dollar losses in salmon and catfish aquaculture (van West, 2006). Not only farmed fish are affected by *Saprolegnia* spp. but also natural populations of salmon and fresh water fish, and even amphibians (van West, 2006). Since the ban on the use of malachite green only formalin and Pyceze remain as a means to stop *Saprolegnia* from infecting fish (Phillips et al., 2008). *S. parasitica* causes Saprolegniosis or Saprolegniasis, characterised by white patches of mycelium on the skin, gills and fins of the fish. *S. parasitica* has clearly defined life stages, including motile zoospores that are thought to spread the disease and which encyst and germinate upon attachment to fish tissue, subsequently forming mycelial mats (van West, 2006). However, the mechanism by which *S. parasitica* infects and penetrates fish tissues is not known. We have shown recently that *S. parasitica* produces a protein that translocates into rainbow trout fibroblast (RTG-2) cells, indicating that this protein may be involved in the host-pathogen interaction and putatively in the infection process (van West et al., 2010; Wawra et al., 2012).

Macrophages play an important role in the defence of fish against oomycetes. For example, macrophages of silver barbs, gourami and tilapia can inhibit germination of cysts of the extremely destructive oomycete *Aphanomyces invadans* (Miles et al., 2001), although macrophages from snakeheads cannot (Miles et al., 2001). In addition, the rainbow trout monocyte/macrophage cell line RTS11 has been shown to move and aggregate around the mycelium and spores of both *Achlya* and *Saprolegnia* (Kales et al., 2007). In response to *Achlya* infection, gene expression analysis showed strong up-regulation of the proinflammatory genes COX-2, IL-1β and TNFα in the RTS11

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cells (Kales et al., 2007). IL-8, a chemoattractant for neutrophils, was also moderately up-regulated in these studies. Microarray analysis of juvenile Atlantic salmon (whole fish homogenates) showed that several immune genes were induced upon *Saprolegnia* infection, including components of the complement system, C type lectin receptor, a CD209-like protein and TAP2, amongst others (Roberge et al., 2007).

As part of the innate immune system, plants, mammals and fish produce a range of cationic antimicrobial peptides (AMPs) as a first line of defence. AMPs are amphipathic molecules with both hydrophobic and hydrophilic moieties and target the microbial membrane (Zasloff, 2002). One well-studied AMP is cathelicidin, with peptides identified in mammals, birds and fish (Radek and Gallo, 2007; Lu et al., 2011). Cathelicidins are produced as inactive precursors, or pre-pro-proteins, which have a N-terminal signal peptide, a conserved cathelin domain and a variable C-terminal mature peptide. After the signal peptide is cleaved off, proteolytic processing by elastase or other proteases generates the active C-terminal peptide (Shinnar et al., 2003; Broekman et al., 2011). In several fish species, genes encoding for cathelicidins have been identified and show the conserved four cysteine residues in the cathelin domain and a variable C-terminal peptide (Uzzell et al., 2003; Chang et al., 2005, 2006; Maier et al., 2008; Broekman et al., 2011). In both rainbow trout and Atlantic salmon two cathelicidin genes, cathelicidin 1 and cathelicidin 2, have been identified to date (Chang et al., 2005, 2006).

Whilst previous gene expression studies on whole salmon infected with *S. parasitica* or RTS11 cells infected with *Achlya* have been undertaken by RT-PCR and microarray analysis, respectively, a real-time quantitative PCR (Q-PCR) analysis of targeted immune genes has not yet been performed. Since *Saprolegnia* infection is usually initiated in the skin and gills, the response towards *Saprolegnia* infection of four rainbow trout cell lines derived from different tissues was investigated in this study. Two fibroblast cell lines (RTG-2 and RTGill), an epithelial cell line (RTL) and a macrophage cell line (RTS11) were challenged with *S. parasitica* cysts and at various times post-infection approximately 186 immune genes were initially screened for their transcriptional response in these cells, with 40 genes selected for in depth study on the basis that they appeared to be modulated by infection. Besides genes encoding for proinflammatory cytokines, genes encoding for antimicrobial peptides, including hepcidin and cathelicidin 1 and 2, were highly up-regulated. A peptide containing the first 36 amino acids of the mature cathelicidin molecule was synthesised ($rtCATH2_{1-36}$), as this peptide has been shown to be antibacterial in our previous studies, and was tested for activity against *S. parasitica*. Whilst the peptide did not kill the *S. parasitica* zoospores it was found to delay sporulation.

2. Materials and methods

2.1. Microbe culture conditions

S. parasitica isolate CBS223.65 (C65) was obtained from the Centraal Bureau voor Schimmelcultures (CBS), the Netherlands. Culture conditions and collection of zoospores, cysts and germinating cysts of *S. parasitica* were as described in Van West et al. (2010). Zoospores/cysts were collected by pouring the culture filtrate through a 40–70 μm cell strainer and concentrated by centrifugation for 5 min at 1500*g*. Zoospore density was calculated using a haemocytometer.

2.2. Maintenance and challenge of the fish cell lines

The RTG-2 cell line is a continuous cell line derived from rainbow trout (*Oncorhynchus mykiss*) gonadal tissue and was obtained from ATCC (ATCC CCL-55) (Wolf and Quimby, 1962). It was maintained at 24 °C in 25- or 75-cm^2 tissue culture flasks (Nunc) in Leibovitz (L-15) medium (Gibco) supplemented with 10% foetal calf serum (FCS, Biosera), 200 U ml^{-1} penicillin and 200 μg ml^{-1} streptomycin (Fisher). The fibroblast RTGill-W1 cell line derived from *O. mykiss* gill cells (American Type Tissue Collection (CRL2523) and epithelial RTL-W1 derived from *O. mykiss* liver cells (Lee et al., 1993), were maintained as for the RTG-2 cells. The *O. mykiss* macrophage RTS11 cell line derived from spleen tissue (Ganassin and Bols, 1998) was maintained at 24 °C in 25- or 75-cm^2 tissue culture flasks in L-15 medium supplemented with 200 U ml^{-1} penicillin, 200 μg ml^{-1} streptomycin and 15–30% FCS. Confluent flasks of cells were split weekly into three flasks containing 2/3 fresh media. For challenge experiments, cells were washed once with fresh medium, centrifuged at 500*g* for 5 min (with slow brake) and adjusted to approximately 10^6 cells ml^{-1}. Five millilitres cells was added to 25-cm^2 flasks or 1 ml to 24 well plates.

2.3. Time-course experiment and gene expression analysis

RTG-2, RTGill and RTL cells were grown as confluent monolayers in 25- or 75-cm^2 cell culture flasks (Nunc) and challenged with 1.7×10^4 or 5×10^4 cysts, respectively, and incubated at 24 °C for up to 24 h. At the time of sampling the media was discarded and 1 ml (25-cm^2 flask) or 5 ml (75-cm^2 flask) of Trizol reagent (Invitrogen) was added to each flask. For time point 0 h, cysts were added after addition of Trizol. Cells were loosened with a cell scraper (Fisher) and the suspension was aliquoted as 1 ml portions into 2-ml screw-cap tubes containing 10–35 glass beads of 1 mm diameter (Biospec). Samples were frozen immediately in liquid nitrogen, and frozen cells placed in a Fastprepmachine (ThermoSavant) and shaken several times at a speed of 5.0 for 45 s until defrosted and homogenised. RNA was then isolated according to the manufacturer's protocol, modified with an extra 1:1 (v/v) phenol:chloroform extraction after the first chloroform addition. RNA was treated with Turbo DNA-free DNase (Ambion) according to the manufacturer's protocol and checked for quality and genomic DNA on a 1% agarose gel. The concentration and purity of RNA were determined spectrophotometrically with a Nanodrop at 260 nm and by calculating the 260/230 and 260/280 nm ratios, respectively. Samples with a 260/280 nm ratio lower than 1.7 were discarded. Subsequent cDNA synthesis was performed using a First strand cDNA synthesis kit (GE Healthcare) with 5–7 μg of RNA per 33 μl sample, using $pd(N)_6$ random hexamers according to the manufacturer's protocol. Transcript levels of the immune genes were analysed with a LightCycler 480 (Roche), using the LightCyclers 480 SYBR Green I Master mix (Roche), with 0.5 μl of cDNA in a total of 10 μl and according to the manufacturer's protocol. The reaction was performed with an initial incubation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60–65 °C (Table S1) for 30 s, 72 °C for 30 s, 80 or 84 °C for 5 s, respectively. A dissociation curve, as described in the LightCyclers 480 SYBR Green I Master mix (Roche), was performed to check the specificity of the primers. An initial screen of 186 trout immune genes (see Table S1) was performed using pooled cDNA from replicate samples of RTG-2 cells challenged with *S. parasitica* at two time points, 8 h and 24 h. Subsequently, 40 genes that appeared to be modulated by infection were selected for in depth analysis. The primers of the 40 selected immune genes were tested on cDNA of mycelium of *S. parasitica* and none of the selected primers amplified a specific amplicon, confirming their specificity for host genes only. The primer sequences of the selected genes are described in Table S2. To correct for differences in the template concentration EF-1α was used as a reference gene. The immune genes showed differential transcript levels when compared to the reference genes in uninfected cDNA samples (Table S3). For analysis, a standard curve