Vol. 76: 81–85, 2007 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published June 7

NOTE

Baltic salmon activates immune relevant genes in fin tissue when responding to *Gyrodactylus salaris* infection

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ABSTRACT: Immune mechanisms in 2 strains of *Salmo salar* (Baltic salmon from River Ume Älv in Sweden and East Atlantic salmon from River Skjernå in Denmark) infected with the monogenean ectoparasite *Gyrodactylus salaris* were elucidated by molecular tools (real-time PCR). The gene expression in the fins (the preferred microhabitat of the parasite) of the susceptible but responding Swedish salmon was compared to the expression in the fins of the highly susceptible and non-responding East Atlantic salmon. Experimental infections confirmed that both the Swedish and the Danish salmon allowed initial propagation of the parasite on the fins for a few weeks. Baltic salmon subsequently activated a response from Day 28 and limited the parasite population to a few parasites per host within the following weeks. In contrast, the Danish salmon did not respond and experienced a continuing increase in the parasite load during the same period, which reached several hundreds of parasites per host. RNA was isolated from fins of the 2 salmon strains during the course of infection and subsequent real-time PCR showed an increased expression of *INFγ*, *Mx* and *MHC I* genes in Baltic salmon fins during large segments of the response phase. No upregulation of these genes could be detected in susceptible salmon. No increase in immunoglobulin genes was seen in any of the fish strains, which supports the notion that antibodies are not involved in the response. Further, the work suggests that cellular factors could at least partly contribute to the anti-parasitic response in Baltic salmon.

KEY WORDS: Host response · Real-time PCR · Salmon · *Gyrodactylus salaris*

INTRODUCTION

A series of studies have documented a superior ability of several strains of Baltic salmon to respond to and limit infections of the ectoparasitic monogenean *Gyrodactylus salaris*. In contrast, a range of East Atlantic strains of salmon are highly susceptible and do not activate effective anti-parasitic responses against this particular parasite (Bakke et al. 1990, Bakke & MacKenzie 1993, Malmberg 1993, Dalgaard et al. 2003, 2004, Heinecke & Buchmann 2006, Lindenstrøm et al. 2006). The response patterns of fish against gyrodactylids can be divided into 3 groups: (1) innately resistant, (2) susceptible but finally responding and (3) susceptible with no activation of responses (Bakke et al. 2002). Theoretically a series of immunological effector mechanisms (both humoral and cellular) could be involved in the response of a fish host against monogeneans (Buchmann 1999, Buchmann & Lindenstrøm 2002), but experimental evidence on this topic is limited. Lindenstrøm et al. (2006) found that susceptible Scottish salmon overexpressed interleukin-1β (IL-1β), in the skin during the initial phase of *G. salaris* infections compared to less susceptible Baltic salmon.

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This was interpreted as an inappropriate response because the cytokine-induced mucus secretion merely favoured the propagation of the parasites, which suggested that one or more other factors participate in the final elimination of parasites in the responding Baltic salmon. Further, Collins et al. (2007) recognised the gene for FIP2 to be upregulated in susceptible salmon upon infection but the exact function of the gene is still to be determined. In the present work we present evidence that genes associated with cellular responses such as *INF*γ, *Mx* and *MHC I* are expressed during the response phase of Baltic salmon, but not in East Atlantic salmon, whereas no evidence for involvement of immunoglobulin could be detected in any of the strains.

MATERIALS AND METHODS

Fish. East Atlantic salmon eggs from the Danish River Skjernå were hatched and fry reared to the age of 4 mo at the Danish Center for Wild Salmon, Jutland, and subsequently brought to the experimental university facility, University of Copenhagen, Frederiksberg. Baltic salmon eggs from the River Ume Älv in Sweden were hatched at this university facility and fry were kept for 4 mo until experimentation. The 2 fish groups had body lengths of 4 to 6 cm. All fish were acclimatised in four 120 l tanks for 1 wk before the experiment.

Parasites. A laboratory stock of the Norwegian form of *Gyrodactylus salaris* from the River Lærdalselva was propagated in our laboratory on Scottish salmon for 4 yr before experimentation.

Water and fish tanks. The water used was a 50:50 mixture of deionised water and municipal water (Frederiksberg County), which was dechlorinated and kept aerated for 1 wk before use. The 120 l tanks were kept aerated and with internal biofilters (Eheim) in thermostat controlled rooms at 12 to 13°C with a 12 h light:12 h dark cycle.

Experimental design. A total of 240 salmon divided into 4 groups (2 experimental and 2 control tanks) were used for the experiment. Thus, 120 salmon were exposed to parasites. This was done by subjecting a group of 60 River Ume Älv fish to 3000 parasites for 24 h and a group of 60 River Skjernå fish to 3000 live parasites. The infection was performed by placing the fish with infected salmon fins carrying parasites in 120 l aquaria with lowered water levels. Another 120 fish (2 × 60 control fish) were kept and handled similarly, but without inducing infection (sham infection with uninfected fins).

Parasite counting. Subsamples (5 specimens) of fish in all 4 groups were examined weekly. Following anaesthesia of fish in MS-222 (50 mg l^{-1}) the parasites were counted on all body regions (Buchmann & Uldal 1997) under a dissection microscope (7 to 40 × magnification). The infection level was expressed as mean intensity (mean number of parasites per infected fish).

Sampling for molecular analysis. At Days 0, 1, 14, 21, 28, 35 and 42 a total of 5 individuals from each tank were sampled for molecular analysis. This was done before sampling for parasite counting was performed to prevent any effect on gene expression due to handling. Fish were anaesthetised and killed in MS-222 (200 mg l^{-1}). Fin tissue samples cut immediately from the individual fish were conserved in RNA*later*™ (Sigma).

Isolation of RNA and cDNA synthesis. RNA was isolated from sonicated fin tissue with GenElute™ Total RNA kit (Sigma). Removal of genomic DNA was conducted with Deoxyribonuclease I (Sigma) and finally the quality was checked with 1% agarose electrophoresis of 2 µl of the RNA. An amount of 400 ng RNA was used in each 20 µl reaction for cDNA synthesis with TaqMan® Reverse Transcription Reagents (Applied Biosystems). The cDNA was diluted by addition of 180 µl RNase-free water.

Real-time quantitative PCR (RQ-PCR). A total of 2.5 µl of the diluted cDNA was used as template in a 12.5 µl RQ-PCR reaction using Jumpstart™*taq*ReadyMix™ (Sigma cat. no. D7440) in the Mx3000P™ real-time PCR system (Stratagene). Cycle conditions for all reactions were 1 cycle of pre-denaturation at 94°C for 2 min, and 40 cycles with denaturation at 94°C for 30 s. Annealing and elongation were in 1 step at 60°C for 1 min with endpoint measurement. Expression analyses were performed for the immune relevant genes encoding INFγ (GenBank accession number AY795563), CD8 α (GenBank accession number AY693393), Mx isoforms 1, 2 and 3 (GenBank accession numbers for Mx1: U66475, Mx2: U66476, Mx3: U66477), MHC I (conserved region of 10 sequences presented by Grimholt et al. 2002) and IgM (GenBank accession number S48658). Primers, probes (designed from the above mentioned sources) and $MgCl_2$ concentrations used are listed in Table 1. Elongation Factor α1 (GenBank accession number AF321836) was used as the housekeeping gene as it has been shown to be the least regulated gene amongst several others (Ingerslev et. al. 2005).

Analysis of RQ-PCR data. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method by Livak & Schmittgen (2001). Change in threshold cycle number (ΔC_t) was calculated as the difference in the C_t between the target gene and the housekeeping gene for each individual. The means of the control groups (uninfected groups for each sampling point for each salmon strain) were calculated. For each salmon strain the $\Delta\Delta C_t$

Table 1. Primers, probes and applied $MgCl_2$ concentrations used in the reactions for detection of gene expression in fins of salmon using real-time PCR

Gene	Probe (5′-3′)	Forward (5′-3′)	Reverse (5′-3′)	$MgCl_2$ (mM)
CD8 α	CAACTCGACTTGCTGGGCCA	AATCAATGGTAACGCGCTTG	TGGCTGTGGTCATTGGTGTA	3.5
IFNγ	TTGATGGGCTGGATGACTTTAGGA	AAGGGCTGTGATGTGTTTCTG	TGTACTGAGCGGCATTACTCC	5.5
IgM	ACCGACAGGGACAGCATGGG	ACTGTCCATGCAGCAACACC	CTCCAACGCCATACAGCAGA	3.5
MHC I	TGGTGTCCTGGCAGAAAGACGG	GCGACAGGTTTCTACCCCAGT	TGTCAGGTGGGAGCTTTTCTG	3.5
Mx 1,2,3	CAACTGGAGGAACCAGCAGTCAAGA	TTGAGGTGATGGTGAAAGACC	GCTCTGAGCCAGCAGTAAGAA	5.5

values were then calculated as the difference between the C_t of the infected individual and the average of the uninfected group at the sampling point. $\Delta\Delta C_t$ values for each group at each sampling point were averaged and standard deviation calculated before calculating folds as $2^{-\Delta\Delta Ct}$. The 95% confidence intervals of the fold value was then calculated according to Livak & Schmittgen (2001). If the fold value was >1, the fold was denoted as a fold increase (positive values); if it was <1, the negative reciprocal was calculated and the result denoted as a fold decrease (negative values), and if the fold value was equal to 1, the target gene was considered unregulated. The Mann-Whitney *U*-test was used to detect differences between groups using a 5% probability level.

RESULTS

The infection level of *Gyrodactylus salaris* on the 2 salmon strains followed the same course for the first 4 wk post-infection (p.i.). However, the parasite load on Baltic salmon from River Ume Älv fell drastically during the following 3 wk to a few parasites per host at Day 42. In contrast, the parasite population on the East Atlantic River Skjernå salmon increased markedly to several hundred parasites per fish during this period (Table 2). When a number of genes in fin tissue from the 2 salmon strains were tested for regulation during the course of infection, the responding Baltic salmon showed upregulation of the genes encoding INFγ, Mx proteins, CD8α and MHC I during large parts of the period (Table 3). *INFγ* was generally (but not significantly) downregulated in River Skjernå salmon. In contrast this gene was upregulated in Ume Älv salmon from Days 14 to 35. *Mx* genes were also upregulated in River Ume Älv salmon at Day 28. The gene encoding CD8α was generally weakly (and non-significantly) downregulated in Danish salmon in contrast to a weak but not significant upregulation in Swedish salmon. Both fish strains showed a nonsignificant upregulation of CD8α at Day 42. The *MHC I* gene was generally upregulated in River Ume Älv salmon with significant values at Days 14 and 42. No regulation of immunoglobulin genes were detected in any of the strains (Table 3).

DISCUSSION

The notion that at least most of the Baltic strains of Atlantic salmon, with one exception (Bakke et al. 2004), are less susceptible to *Gyrodactylus salaris* infection compared to the East Atlantic strains (Bakke et al. 1990, Bakke & MacKenzie 1993, Dalgaard et al. 2003, 2004, Lindenstrøm et al. 2006, Heinecke & Buchmann 2006) is supported by the present investigation. This study showed a clear antiparasitic response in River Ume Älv salmon after 4 wk of infection, whereas the Danish River Skjernå salmon from the western part of Denmark exhibited a continous increase in the parasite load during the entire period. The mechanisms involved in the response have not been finally resolved. An inappropriate (and possibly parasite-promoting) hyperactivation of *IL-1β* genes in susceptible Scottish salmon was found by Lindenstrøm et al. (2006) and

Table 2. *Gyrodactylus salaris* infecting *Salmo salar*. Course of infection in River Skjernå salmon (East Atlantic strain) and River Ume Älv salmon (Baltic strain) during 42 d post-infection. Data are expressed as number of parasites per fish (mean ± SD) (5 fish per sample). Values in **bold** indicate significant differences ($p < 0.05$) between the 2 strains (Mann-Whitney *U*-test)

	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
River Skjernå	20.2 ± 14.3	15.6 ± 12.1	26.8 ± 13.3	44.0 ± 19.3	75.0 ± 18.4	248.3 ± 35.8	326.7 ± 163.8
River Ume Älv	12.8 ± 10.4	20.2 ± 11.2	34.6 ± 15.8	54.6 ± 11.9	72.4 ± 29.3	**24.4** ± 13.5	**5.2** ± 1.8

Table 3. *Gyrodactylus salaris* infecting *Salmo salar*. Gene expression in fins of Swedish (Baltic strain) and Danish (East Atlantic strain) salmon during infection with *G. salaris*. Values are given as fold increase or decrease, with 95% confidence intervals in brackets (5 fish per sample). Values in **bold** designate significant difference ($p < 0.05$) from control groups within the same hoststrain (Mann-Whitney *U*-test)

Gene	Strain	Day 0	Day 1	Day 14	Day 21	Day 28	Day 35	Day 42
CD 8 α	River Skjernå	–1.4 [–4.4; 2.3]	+1.7 [–1.4; 4.2]	+1.0 [–3.1; 3.3]	–1.7 [–3.7; 1.3]	–1.0 [–1.7; 1.7]	–1.5 [–2.6; 1.1]	+3.7 [1.7; 7.9]
	River Ume Älv	+1.8 [–1.1; 3.7]	+2.0 [–1.1; 4.3]	+3.2 [1.7; 5.9]	+1.3 [–1.1; 2.0]	–1.1 [–3.1; 2.4]	+1.6 [–1.0; 2.4]	+5.2 [1.2; 22.4]
INFγ	River Skjernå	–1.8 [–4.7; 1.5]	–2.5 [–5.5; –1.1]	+1.2 [–3.3; 5.0]	+1.2 [–1.8; 2.4]	+2.0 [1.1; 3.4]	–1.5 [–1.8; –1.2]	+1.6 [–1.1; 2.9]
	River Ume Älv	–1.0 [–2.1; 1.9]	+1.4 [–2.6; 5.4]	**+11.5** [4.5; 29.4]	**+5.3** [3.4; 8.1]	+4.0 [–1.3; 20.2]	**+3.2** [1.6; 6.5]	–1.2 [–3.4; 2.5]
IgM	River Skjernå	+1.6 [–1.0; 2.4]	–1.1 [–2.2; 1.8]	+1.2 [–2.0; 2.9]	–1.2 [–2.1; 1.4]	+1.3 [–1.2; 2.0]	–1.9 [–2.6; –1.3]	–1.2 [–1.3; –1.1]
	River Ume Älv	+1.4 [–1.1; 2.0]	–1.1 [–1.5; 1.3]	–1.3 [–3.0; 1.8]	+1.1 [–1.5; 1.8]	–1.8 [–4.2; 1.4]	**+1.2** [–1.2; 1.6]	–1.6 [–1.9; –1.4]
MHC I	River Skjernå	–1.5 [–2.5; 1.2]	–1.7 [–4.2; 1.5]	–1.7 [–2.8; 1.0]	–1.2 [–1.9; 1.3]	–1.0 [–1.3; 1.3]	+1.2 [–1.4; 1.9]	+1.3 [1.2; 1.5]
	River Ume Älv	+1.0 [–1.6; 1.6]	+2.5 [–1.1; 6.8]	**+5.7** [2.7; 12.0]	+1.9 [–1.1; 3.7]	+2.2 [1.1; 4.2]	+2.2 [–1.2; 6.1]	**+4.7** [1.9; 11.4]
Mx 1,2,3	River Skjernå	+1.3 [–1.6; 2.5]	–2.5 [–5.5; –1.1]	+1.2 [–1.7; 2.5]	–1.8 [–2.5; –1.3]	–1.3 [–1.8; 1.0]	+1.6 [1.2; 2.1]	+1.6 [1.4; 1.8]
	River Ume Älv	–1.3 [–2.7; 1.6]	+1.5 [–1.2; 2.6]	+1.3 [–1.3; 2.3]	+2.5 [–1.5; 9.3]	**+5.0** [2.6; 9.6]	+2.5 [1.2; 5.3]	+2.2 [1.1 ; 4.3]

a similar role of the *FIP2* gene has been suggested by Collins et al. (2007). However, the decisive factors which reduce the parasite population on the fish fins (the main microhabitat of *G. salaris*, Heinecke & Buchmann 2006) remain unknown. The gene expression results shown here indicate that the genes encoding INFγ, Mx, and MHC I could play a role. These genes were found upregulated in the responding Baltic salmon at least during part of the period when the parasite population decreased on the hosts. Expression of genes encoding INFγ (T-cell produced cytokine) especially suggests that T-cell-like reactivity takes part in the reaction against *G. salaris*. Thus, INFγ is known to be produced by activated T-lymphocytes and natural killer (NK)-cells and enhances the expression of MHC I (markers for nucleated cells), and promotes CD8 positive T-cell responses and induces production of Mx proteins (Goodburn et al. 2000). Therefore, the results of this study could either be explained by immigration of T-cells, T-cell equivalents or NK-cells from the vascular system to the infected fin epidermis or could be interpreted as increased expression of resident T-cells or NK-cells in fin tissue. This would result in a relatively higher presence of RNA encoding INFγ, Mx and MHC I. No regulation of immunoglobulin production in any of the fish strains was found following infection, and this supports the immunochemical work by Buchmann et al. (2004) that showed (using western blot techniques) that no increased specific immunoglobulin production in salmon skin could be found after *G. salaris* infection. A number of other immune relevant genes including those encoding the T-cell receptor (alpha chain), IL-10, MHCII and CRP also were tested in this study. However, no differences (or trends towards differences) between the 2 salmon strains were found with respect to these genes (data not shown). The *Il-1β* gene was not studied in this work but should be included in future more detailed investigations. Thus, the findings presented here call for further validation and extended studies of gene expression in skin and fin tissue obtained from responding and non-responding salmon strains following infection with *G. salaris*. Further, the exact location of these above-mentioned factors in the fin tissue (and specific cells) at various time points after infection should be determined and tools for the study of additional immune relevant genes should be developed.

Acknowledgements. The investigation was supported by the Danish Research Councils and the Danish Ministry of Environment. The authors are indebted to Dr. Tor Atle Mo, The National Veterinary Institute, Oslo, Norway, who originally provided the Lærdalselva strain of *G. salaris* to the senior author's laboratory.

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Editorial responsibility: Dieter Steinhagen, Hannover, Germany

Submitted: November 11, 2006; Accepted: January 18, 2007
Proofs received from author(s): May 22, 2007