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Topographical distribution of antimicrobial genes in the zebrafish intestine

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ABSTRACT

The zebrafish is increasingly being utilized to study aspects of the conserved innate intestinal immunity of vertebrates. In mammals, some antimicrobial proteins are synthesised by specialised immune cells that appear to have no equivalent in zebrafish. To delineate foci of antimicrobial protein production along the zebrafish intestine, we examined the antero-posterior expression gradients of antimicrobial genes. Quantitative PCR revealed distinct expression gradient profiles, with the mid-intestine exhibiting elevated expression of several genes such as *dual oxidase* and the *defensin beta-like* and *peptidoglycan recognition protein* families. This region also presented with the most numbers of leukocytes and endocytic cells, supporting a specialised immunological role. Conversely, expression of the *Dr-RNase* family was prominent in the anterior intestine. Expression of the zebrafish β-defensin family was examined in adult zebrafish tissues. Strong expression of *defensin beta-like 1* was detected in the swim bladder of zebrafish from the larval stage of development through to adults.

1. Introduction

The metazoan intestine is in constant contact with a potentially overwhelming number of microbes. The host contributes to the shaping of the intestinal microbiota, dictating nutritional capacity and colonisation resistance to enteric infection, through the production of antimicrobial factors secreted by cells of the adaptive and innate immune systems. The β-defensin, dual oxidase (Duox), peptidoglycan recognition protein (Pglyrp) and RNase-A families are important mediators of innate antibacterial immunity that have been identified in *Danio rerio* (zebrafish) (Chang et al., 2007; Cho and Zhang, 2007; Flores et al., 2010; Li et al., 2007; Niethammer et al., 2009; Zou et al., 2007).

Positioned as an intermediate between the simple invertebrate and the more complex mammalian experimental systems, the zebrafish has become an important animal model for human disease studies. Furthermore, the zebrafish has emerged as a tractable model organism for the study of the interrelated fields of host-microbe interaction and digestive function (Dahm and Geisler, 2006; Flynn et al., 2009; Hama et al., 2009; Kanther and Rawls, 2010). While the leukocytic components of the zebrafish immune system have been well characterised (Lieschke and Trede, 2009), much less is known about the immune capacity of the zebrafish intestinal tract. Early studies have revealed anatomical and functional conservation between the zebrafish and mammalian intestine (Bates et al., 2007; Flores et al., 2008; Ng et al., 2005). Additionally, studies of gnotobiotic zebrafish have revealed an evolutionarily conserved and early capability of the zebrafish intestine to shape the composition of its intestinal microbiota (Rawls et al., 2006, 2004). While these studies have demonstrated evolutionary conservation of the outcomes of host-microbe interactions, there remains a paucity of information regarding the spatial expression of immunity genes within the zebrafish intestine to shed light on the potential mediators of these interactions.

The presence of specialised immune lineages in the vertebrate intestine distinguishes it from the invertebrate enteric immune system. Two important cellular features of the vertebrate intestine are intestinal follicle-associated epithelial cells involved in luminal antigen sampling and Paneth cells, an important source of antimicrobial gene expression in the small intestine (Sansonetti, 2004). While the equivalent of M-cell structures have recently been identified in teleosts (Fuglem et al., 2010), identification of Paneth cells remains elusive (Ng et al., 2005; Wallace et al., 2005). Evidence for the presence of specialised intestinal cell types in teleosts, coupled with experimental tractability, make them excellent candidates for the investigation of the evolution of intestinal immunity.

As previous studies of zebrafish antimicrobial gene expression have focused on broad expression in the intestine (Chang et al., 2007; Cho and Zhang, 2007; Li et al., 2007; Quarto et al., 2008; Zou et al., 2007), we have undertaken a topographical survey of antimicrobial gene expression along the length of the zebrafish intestine with the aim of identifying regions with specialised immune activity. Herein we identify distinct gradients of antimicrobial gene expression and leukocyte distribution along the anterior-posterior axis of the zebrafish intestine. Our data implicate the mid-intestine

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of the zebrafish to be an immunologically specialised region in the larval through to adult stages of development.

2. Materials and methods

2.1. Animals

Adult zebrafish were kept in a 14 h light, 10 h dark cycle fish facility and fed twice daily with artemia and once daily with dry feed. Zebrafish embryos were obtained from natural spawnings and raised at 28.5 °C in embryo medium (E3) (Westerfield, 2000). Research was conducted with approval from The University of Auckland Animal Ethics Committee.

2.2. Zebrafish dissections

Zebrafish were euthanized in ice cold water (Wilson et al., 2009). The abdominal cavity was opened by incision; organs were dissected and immediately immersed in Trizol for RNA extraction. Segmentation of the intestine was carried out manually by dividing the intestine as indicated in each experiment.

2.3. Neutral red staining

Neutral red granules are readily endocytosed by endocytic cells in the zebrafish larval intestine (McLeish et al., 2010). Neutral red was added to fish water to a final concentration of 2.5 μg/ml for 24 h before dissection (Herbomel et al., 2001).

2.4. Expression analysis, cloning and probe synthesis

Total RNA was isolated from zebrafish embryos using Trizol (Invitrogen) according to manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA with SuperScript® III (Invitrogen) for RT-PCR or with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) for quantitative RT-PCR. The primer pairs used for RT-PCR are shown in Table 1. Quantitative (q)PCR was carried out with iTaq SYBR Green Supermix With ROX (Bio-Rad) in an ABI PRISM® 7900HT Fast sequence detection system (Applied Biosystems). PCR cycling conditions were 50 °C for 2 min, 95 °C for 2 min and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Analysis used the comparative CT method with ef1α as the endogenous control.

PCR products were cloned using TOPO® cloning into pCR®II-TOPO® (Invitrogen) for sequence confirmation and use as a transcription template for riboprobe synthesis.

2.5. Whole-mount in situ hybridisation

The expression vectors containing cloned genes were linearized by restriction enzyme digest, purified using a Purelink™ PCR purification kit (Invitrogen) and *in vitro* transcribed using SP6 and T7 RNA polymerase enzymes to synthesize antisense and sense digoxygenin (DIG)-11-UDP (Roche)-labeled riboprobes. Transcripts were detected using alkaline phosphatase cleavage of BM purple substrate (Roche) as described (Thisse and Thisse, 2008). Stained specimens were mounted in paraffin, sectioned and counter stained with Nuclear Fast Red (Vector Labs) prior to imaging with a Leica DMR compound microscope equipped with a DFC420C camera.

2.6. Imaging

Specimens were imaged and photographed with a Leica MZ16 FA stereomicroscope and a Leica DFC490 camera.

Table 1
Primers used for PCR in this study.

Gene	Sequence (5′–3′)
ccl20 qPCR	CATTTTCCACACCGTGACAGG GCTCATCGTCTTCGTCTTCATTG
defbl1 cloning	GCCATCATCTGAAGAATCCAAC CTTGTGCTTGTATGCTTTACCG
defbl1 qPCR	AGGATGCAGCCTCATTCTCTTT TGAAGCCCCAGAGCATATTTATC
defbl2 qPCR	CTTTTTGCAGGGAATGTGCAT CCGTACCCACACGTCCAATT
defbl3 qPCR	TGGTTATCGTGGCTGCCC CGCAAATGTTAAAATCGCAAAG
duox qPCR	ATGGGTTCATTTGAGCTACTTTTG CTGCAGCCCCTCGATCGT
ef1a qPCR	TGCCTTCGTCCCAATTTCAG TACCCTCCTTGCGCTCAATC
fabp2 qPCR	TCAACGGGACCTGGAAAGTC CCCATTTGTTCCATGAACTTCTC
fabp6 qPCR	CTCCGCTCAATCAACACCAA TGAGATTCGGTTTCCCACTTG
gata5 qPCR	ACCAAAGATGCCCAAAACCA GGAGACGTAGCGCCAGACA
il17a qPCR	GGTCAAATGAATGAACCATGT TGCAAGCCACCATAAGAAAGC
il17c qPCR	CAATCACTCCGAAGTCACTTTTGAT CGACAACCAGAAGAATAATAATCCAA
il17d qPCR	GCGAACACCGGTCAATCTG GCTGGATCATGGGAGATTCTGTAC
il1b qPCR	ATCAAACCCCAATCCACAGAGT GGCACTGAAGACACCACGTT
il8 qPCR	TGTTTTCCTGGCATTTCTGACC TTTACAGTGTGGGCTTGGAGGG
lyzC qPCR	GTGAAAATGGACGGGCTGAA CTTTGTTTGCGCTGCTCACA
mmp9 qPCR	CATTAAAGATGCCCTGATGTATCCC AGTGGTGGTCCGTGGTTGAG
mpx qPCR	TCAATATGAGGACGCCGTTTCT GAATGCGATTGGAAACCAGTCT
nod1 qPCR	CAGACCAGGTACGCAAAATCTTG TGAAATACGCCGAGCATTCC
nod2 qPCR	GTTATGGTGGGCAAGGACAGA CATTGGCCTGTCAGCCAGTA
pglyrp2 qPCR	TGCAGGAGGATTTCACCATTC CTGGGCAGCTGGTGGTTACT
pglyrp5 qPCR	GACACACAAACACCGTGGACAT CCCCATCCTCTGCCTTCATA
pglyrp6 qPCR	TGGCATTGCTGAAGCCACTA GCTCTCCACAGCGCTAATGAA
rnasel2 qPCR	CGCATTGTCAATACCGTGGTA GCCTTTATCACAACGCAAAACA
rnasel3 qPCR	TTGTGCTGCTGCTCCTTTGT CGCCTTATTTCTGCTGGTTGA
rnasel4 qPCR	TCACTCGGAGATCGTACATCTTTC CGGGTCTTCATGATCAATTGC
sdh qPCR	GAGTCTCCAATCAFTATCCAGTAGTAGA CACTGTGTGCGAGCGTGTTG