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Characterization of three salmon louse (*Lepeophtheirus salmonis)* genes with fibronectin II domains expressed by tegumental type 1 glands

Ewa Harasimczuk[a], Aina-Cathrine Øvergård[b], Sindre Grotmol[b], Frank Nilsen[b], Sussie Dalvin[a,*]

[a] SLCR Sea Lice Research Center, Institute of Marine Research, 5817 Bergen, Norway
[b] SLRC Sea Lice Research Center, Department of Biology, University of Bergen, Thormøhlensgt. 55, 5008 Bergen, Norway

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ABSTRACT

The salmon louse, *Lepeophtheirus salmonis* (Copepoda: Caligidae), is currently the most significant pathogen affecting the salmon farming industry in the Northern Hemisphere. Exocrine glands of blood-feeding parasites are believed to be important for the host-parasite interaction, but also in the production of substances for integument lubrication and antifouling. In *L. salmonis*; however, we have limited knowledge about the exocrine glands. The aim of this study was therefore to examine three genes containing fibronectin type II (FNII) domains expressed in *L. salmonis* tegumental type 1 (teg 1) glands, namely LsFNII1, 2 and 3. LsFNII1, 2 and 3 contains four, three, and two FNII domains respectively. Sequence alignment of LsFNII domains showed conservation of amino acids that may indicate a possible involvement of LsFNII domains in collagen binding. Ontogenetic analysis of *LsFNII1*, *2* and *3* revealed highest expression in pre-adult and adult lice. Localization of *LsFNII1*, *2* and *3* transcripts showed expression in teg 1 glands only, which are the most abundant exocrine gland type in *L. salmonis*. *LsFNII1*, *2* and *3* were successfully knocked-down by RNAi, however, alteration in gland morphology was not detected between the knock-down and control groups. Overall, this study gives first insight into FNII domain containing proteins in *L. salmonis*.

1. Introduction

The salmon louse, *Lepeophtheirus salmonis* is a marine obligate ectoparasite infesting salmonids belonging to the genera *Salmo, Oncorhynchus* and *Salvelinus* [1]. The lifecycle consist of eight developmental stages separated by ecdysis; two planktonic nauplius, one infective copepodid, two attached chalimus, two mobile pre-adult and one adult stage [2–4]. The salmon louse feeds on mucus, skin and blood [5], and can thereby cause light to severe skin lesions [6,7]. At present, salmon louse is the most severe disease problem in salmon aquaculture.

The fibronectin type II (FNII) domain is one of three types of internal repeats (type I, II and III), found within the multi domain glycoprotein fibronectin. FNII domains are approximately 60 amino acids long, and contain four conserved cysteines that forms disulfide bridges [8]. These bridges are essential for the function of the FNII domains that are in fibronectin located in the collagen-binding region. Here two FNII domains together with two flanking FNI domains binds to the α-chains of collagen and gelatin (denatured collagen) [9,10]. FNII domains have also been identified in other vertebrate proteins that binds collagen such as the matrix metalloproteinases (MMP) 2 and 9, bovine seminal plasma protein PDC-109, blood coagulation factor XII and mannose receptor of macrophages [11–15]. While the FNII domains of fibronectin are not capable of binding collagen alone [10], both MMP-2 and 9, which bind and degrade components of the extracellular matrix, each have three FNII domains that bind collagen/gelatin where one of those is capable of solely bind gelatin [16,17]. The bovine seminal fluid protein PDC-109 and its homologous also bind to collagen, despite the fact that the ligands of these proteins are phospholipids [18]. Moreover, the binding specificity of FNII domains present among the different proteins varies. For instance, the FNII domains present in the collagen binding region of fibronectin binds to native collagen type I and III, while FNII domains present in the mannose receptor binds to native collagen type I, III and IV collagen, while those of MMP-2 bind I, III and V [19,20,10]. Moreover, it has been suggested that the collagen binding property of the mannose receptor could play a role in clearance of collagen fragments or in mediating cell-matrix adhesions [19].

Since FNII domains have not been found in model invertebrate genomes as in *Caenorhabditis elegans* and *Drosophila melanogaster* they have been regarded as vertebrate specific [21,22]. Instead, invertebrates have kringle domains, suggested to be ancestral FNII

* Corresponding author.
E-mail addresses: Ewa.Harasimczuk@hi.no (E. Harasimczuk), Cathrine.Overgard@uib.no (A.-C. Øvergård), Sindre.Grotmol@uib.no (S. Grotmol), Frank.Nilsen@uib.no (F. Nilsen), Sussie.dalvin@hi.no (S. Dalvin).

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domains. However, FNII domains have recently been found in the genome of two freshwater planarian species *Dugesia ryukyuensis* and *Schmidtea mediterranea* (non-parasitic turbellarian) [21] and in *L. salmonis* [23]. In the *L. salmonis* genome, more than 200 copies of the FNII domain within more than 80 genes have been identified (www.licebase.org), making FNII the most expanded protein domain. In comparison, only five genes containing kringle domains have been found in the *L. salmonis* genome. Interestingly, a recent study showed that one gene, LsFNII1 that has four FNII domains, was expressed in tegumental type 1 glands (teg 1), which is the most abundant type of exocrine gland found in salmon louse [23]. Here, tegumental glands can be divided into type 1, 2 and 3 according to when they appear during development. Teg 1 glands can be detected already at the first planktonic larval stage with secretory ducts extending both dorsally and ventrally. As teg 1 glands also produce mucus and express astacin metallopeptidases [24,23], they have been suggested to lubricate the integument with anti-fouling agents [25,23].

The aim of the present study was primarily to explore the temporal and spatial expression of three genes with FNII domains, and investigate louse phenotype and histological morphology of knock-down animals. Since the genes were found to be expressed in teg 1 glands, cephalic teg 1 gland secretory pores were mapped, as the sites of secretion may give functional information of salmon louse proteins with FNII domains.

2. Material and methods

2.1. Animals

A laboratory strain of *L. salmonis* was raised on Atlantic salmon (*Salmon salar*) in tanks with salinity of 34‰ and temperature of approximately 10 °C. All experiments were conducted in accordance to Norwegian animal-welfare regulations. Prior to sampling, the fish was either sedated with a mixture of benzocaine (60 mg/L) and methomidate (5 mg/L) or killed by a blow to the head. Fish infected with dsRNA injected lice were kept in single tanks as described earlier by [26].

2.2. Collection of animals for analysis

Eggs were kept in flow-through incubators and cultivated to copepodids stages [27]. Copepodids 9 days post hatching were used to infest Atlantic salmon. All developmental stages of *L. salmonis* were collected in five biological replicates. Each replicate contained immature egg strings (light colored, n = 1), nauplius I −II and free-living copepodids (n ≈ 100), copepodids 2 and 4 days post infestation (DPI) respectively (n = 60), chalimus I (n = 30), chalimus II (n = 20), pre-adult or adult stages (n = 1).

2.3. RNA extraction and cDNA synthesis

All samples for RNA isolation were collected in RNA later (LifeTechnologies), kept at 4 °C overnight and stored at −20 °C. RNA was isolated using 1 ml TRI Reagent (Sigma Aldrich). Homogenization was carried out using 1.4 mm zirconium oxide beads (Precellys 24) for nauplius, copepodids and chalimus and 5 mm stainless steel beads for preadult and adult lice. The sample was homogenized for 2 × 2 min at 50 Hz with a tissueLyser LT (Qiagen). Phase separation was accomplished by adding 0.2 ml chloroform to the samples, and centrifuged at 12,000 x g for 15 min at 4 °C. Samples for ontogenesis were thereafter purified using RNeasy kits with DNase treatment preformed on the column. The water phase was withdrawn and mixed with 1 × volume of 70% ethanol and transferred to an RNeasy spin column. Further, RNA from immature eggs to preadult II stages was isolated using the RNeasy micro kit (Qiagen) while RNA from adult lice were isolated using RNeasy mini kit (Qiagen), according to supplier's instructions. RNA from adult RNAi treated animals was isolated using TRI Reagent (Sigma Aldrich) according to manufacturer's instructions, and DNase treated with DNaseI (Amplification Grade, Invitrogen). The amount and purity of the isolated RNA were measured with a Nanodrop Spectrophotometer (Nanodrop ND-1000). Extracted RNA was either kept at −80 °C until use or cDNA synthesis was performed directly.

cDNA synthesis for standard PCR was performed with the qScript cDNA synthesis kit (Quanta Bioscience) according to supplier's instructions, using 1 µg DNase treated total RNA. cDNA synthesis for quantitative RT-qPCR was performed using AffinityScript cDNA synthesis kit (Agilent Tecnologies). Each reaction consisted of 1 x cDNA synthesis mastermix, 100 ng Oligo dT, 50 ng Random primers, 0.5 U AffinityScript RT enzyme and 200 ng total RNA in a final volume of 10 µl. cDNA was diluted 1:10 in H_2O before storage at −20 °C.

2.4. PCR, RACE, cloning and sequencing

Candidate FNII genes were obtained from the salmon louse genome (www.licebase.org) based on InterProScan prediction on protein domains. Rapid amplification of 5′ and 3′ cDNA ends were performed using the SMARTer™ RACE cDNA Amplification Kit (Clonetech). RACE products were cloned using TOPO TA Cloning® Kit for sequencing (Invitrogen). Clones were further used as template in PCR reactions, using 10 µM M13 forward and reverse primer, 2 mM Mg^{2+}, 100 µM dNTP's, 1 × Green GoTaq® Flexi Buffer and 1.25 u of Go Taq Flexi DNA Polymerase (Promega), and run according to the suppliers recommendation. PCR products were purified with ExoSAP-it (Affymetrix) prior to sequencing at the sequence lab facility at the University of Bergen using BigDye Terminator 3.1 reagents (Applied Biosystems). To ensure amplification of the entire coding sequence, the three genes of interest were further sequences using LsFNII1_F and LsFNII1_R for the first gene, LsFNII2_F #1 and LsFNII2_R #3 for the second gene, and LsFNII3_F and LsFNII3_R for the third gene.

Sequences were analyzed and assembled using Vector NTI 10 (Invitrogen).

The three genes of interest were further BLASTed against the salmon lice genome in Licebase (www.licebase.org), in order to identify possible paralogs. ORFs were identified using Prediction of Translation Initiation ATG [28]. Protein domains were identified using InterPro database [29]. Sequence editing and alignment of selected FNII domains were performed using BioEDIT v. 7.2.3 [30]. For further prediction of protein structures the Phyre^2 protein fold recognition server was used [31].

2.5. Quantitative RT-qPCR

The RT- qPCR reaction was performed using 1 x PowerUp™ SYBR™ Green Master Mix (Applied Biosystems), 2 µl cDNA, 0.5 µM forward and reverse primer (Table 1) in a final volume of 10 µl per reactions. The efficiency for SYBR Green primers were checked by a five-point standard curve of 4-fold dilutions, and calculated by the equation $E\ \% = (10^{1/slope} - 1) \times 100$ [32]. The reaction set up was: initiation 50 °C for 2 min, holding 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, in addition of a melt curve analysis at 60–90 °C to check for primer/dimer formation on an Applied Biosystems 7500 Fast Real-Time PCR system. The RT- qPCR was always performed with two technical replicates. Moreover, a no template control (NTC) were used to monitor possible contamination and primer/dimer formation, and a minus reverse transcription control (−RT) were used to control for possible DNA contamination. The relative expression level was calculated using $2^{-\Delta Ct}$*100. Target genes were normalized using the salmon louse elongation factor 1 alpha (eEF1α) standard gene assay [33]. Primers used for real time RT-qPCR are listed in Table 1.

2.6. In situ hybridization

Adult female and male lice were fixated in phosphate buffered 4%