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The rainbow trout TLR9 gene and its role in the immune responses elicited by a plasmid encoding the glycoprotein G of the viral haemorrhagic septicaemia rhabdovirus (VHSV)

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ABSTRACT

The aim of this work was to improve the knowledge about the factors contributing to the immunogenicity of the DNA vaccines based on the viral haemorrhagic septicaemia virus glycoprotein G gene, through identifying the rainbow trout Toll-like receptor 9 (*Omtlr9*) gene that curiously contains an insertion of an incomplete transposon at the 5′-end of the third intron. Concerning the role played by this receptor in the fish innate defence, in response to the injection of a plasmid (pAE6) encoding or not the viral haemorrhagic septicaemia rhabdovirus (VHSV) glycoprotein G gene (pAE6-G), the presence of *Omtlr9* transcripts remained unchanged in the fish secondary lymphoid organs while was highly increased at the injection site (muscle). The level of *Omtlr9* transcripts correlated with those of cluster of differentiation 83 (*cd83*) and CXC chemokine receptor 4 (*cxcr4*), suggesting the recruitment of dendritic-like cells into the muscle as the source of *Omtlr9* expressing cells. Transcription of tumour necrosis factor-alpha (*tnfα*) and interleukin-6 (*il6*) genes, two cytokines directly related to TLR9 induction with unmethylated CpG oligodeoxynucleotides (CpG ODNs), was solely observed in head kidney and spleen of the fish immunised with pAE6-G. Thus, the glycoprotein G of VHSV could be more implicated in triggering the pathways for TNF-α and IL6 production than the recognition of the unmethylated CpG motifs of the plasmid backbone by OmTLR9. Therefore, our results seem to indicate that OmTLR9-mediated recognition of plasmid DNA is not the key of the innate immune recognition of the adjuvant elements of fish DNA vaccines.

1. Introduction

Due to the economic and social impact of viral infections on aquaculture, and to the lack of efficient therapeutic agents against fish pathogens, vaccination seems the most adequate method to control viral diseases of aquacultured fish. In order to develop optimal vaccines for aquaculture, it is important to understand the mechanisms of their actions on the immune system in terms of efficacy as well as safety. In particular, the innate immune recognition of the adjuvant elements of vaccine formulations had been shown to be critical for its immunogenicity (Ishii et al., 2008; Pulendran and Ahmed, 2006). In the case of DNA vaccines, unmethylated cytosine–phosphodiester–guanosine (CpG) motifs within plasmid backbone have been considered to be 'built-in' adjuvants, owing to their apparent ability to activate the innate immune system by means of the Toll-like receptor (TLR) 9 (Krieg and Davis, 2001; Kurath, 2005; McCluskie and Krieg, 2006; Tonheim et al., 2008; van Duin et al., 2006).

TLR9 belongs to a family of pattern-recognition receptors (PPRs), the TLRs, that recognize specific pathogen associated molecular patterns (PAMPs) shared by many bacteria, viruses and fungi (Aderem and Ulevitch, 2000; Medzhitov and Janeway, 2000) and initiate signalling events leading to the activation and orchestration of innate host defences. To date, the *tlr9* gene has been identified from the genome of invertebrates (Ferrandon et al., 2007) and vertebrates, including several fish species such as pufferfish (Oshiumi et al., 2003), zebrafish (Jault et al., 2004), gilthead seabream (Franch et al., 2006), Japanese flounder (Takano et al., 2007), Atlantic salmon (Skjaeveland et al., 2008), and four seabreams (Chen et al., 2008).

Although TLR9 was first cloned and identified as a receptor for unmethylated CpG dinucleotides which are abundant in bacterial DNA (Hemmi et al., 2000), recent evidences suggest that TLR9 can also recognize diverse patterns of DNA that do not have typical CpG motifs (Baldari, 2008), and even non-DNA cyclic compounds such as heme polymers, raising fundamental questions: what does TLR9 recognize, and how does it do so? (Ishii and Akira, 2006). In fact,

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the TLR9 ligand activity of plasmid DNA as well as the molecular and/or cellular mechanisms underlying the adjuvant effects and element(s) of DNA vaccines has not been fully clarified (Babiuk et al., 2004; Ishii et al., 2008; Tonheim et al., 2008; Ulmer et al., 2006).

In this context, the aim of this work was to improve the knowledge about the factors contributing to the immunogenicity of the DNA vaccines based on the fish rhabdovirus glycoprotein G gene, the most efficient vaccines against viral diseases in fish to date (Chico et al., 2009; Lorenzen and LaPatra, 2005). For that, we first identified the rainbow trout *tlr9* (*Omtlr9*) gene and next we analysed the role played by *Omtlr9* in the fish innate immune response induced by the intramuscular injection of a plasmid (pAE6) encoding or not the viral haemorrhagic septicaemia virus glycoprotein G (G) gene (pAE6-G).

Omtlr9 gene characterization showed that it strikingly contains an insertion of an incomplete transposon at the 5′-end of the third intron. After fish immunisation with pAE6 and pAE6-G, the level of *Omtlr9* transcripts was highly increased in muscle and correlated with those of cluster of differentiation 83 (*cd83*), a dendritic cell activation marker (Kutzler and Weiner, 2004), and CXC chemokine receptor 4 (*cxcr4*), a chemokine receptor highly expressed in dendritic cells (Penna et al., 2002), suggesting that the recruitment of dendritic-like cells, and not the myocites, is the real source of *Omtlr9* gene transcripts at the injection site. Besides, transcription of tumour necrosis factor-alpha (*tnfα*) and interleukin-6 (*il6*) genes was solely observed in head kidney and spleen of the fish immunised with pAE6-G, suggesting that probably the glycoprotein G of VHSV, and not the recognition of unmethylated CpG motifs of the plasmid backbone by OmTLR9, might be triggering the pathways for TNF-α and IL6 production. All together, these results seem to indicate that OmTLR9 could not be a key receptor for DNA-vaccine-induced immunogenicity.

2. Materials and methods

2.1. Fish collection and DNA immunisation

Rainbow trout fingerlings of ∼5 cm obtained from a VHSV-free commercial farm (Lillogen, Leon, Spain), were maintained at the University Miguel Hernandez (UMH) facilities at 12–14 °C, with a re-circulating dechlorinated-water system, and fed daily with a commercial diet (Trouw, Leon, Spain). Prior to experiments, fish were acclimatized to laboratory conditions during 2 weeks.

Fish DNA immunisation was carried out following procedures previously described (Falco et al., 2008; Tafalla et al., 2007; Chico et al., 2009). A batch of fishes was then separated as control group, and two other batches were separated for DNA intramuscular injection (i.m.) with an empty plasmid (pAE6 (Cheng et al., 2002; Brocal et al., 2006)) and a plasmid encoding the VHSV-G gene (pAE6-G (Chico et al., 2009)). At 3 days post-injection (p.i.), three fish from each group were sacrificed by overexposure to MS-222 and muscle (site of injection), head kidney and spleen biopsies were sampled.

2.2. RNA extraction and cloning

Total RNA was extracted from spleen as previously described (Falco et al., 2008) and stored at −80 °C until used. PCR primers designed for other salmonid species (Table 1; Skjaeveland et al., 2008) were used to firstly amplify a fragment of the rainbow trout *tlr9* (*Omtlr9*) gene. The PCR product was directly sequenced and verified against the NCBI database. *Omtlr9* gene-specific primers were then designed from this sequence (Table 1). Rapid amplification of 5′- and 3′-cDNA ends (RACE) method was utilized to obtain the full sequence of the *Omtlr9* RNA by SMART™ RACE cDNA amplification kit (Clontech), following manufacture's instruc-

Table 1
Primer sequences used for RACE-PCR and gene full sequencing.

Primer name	Sequence	T_a (°C)	Reference/accesion no.
Homology cloning			
*SsTLR9*fw	5′-CATTGGCATTGCGTTTTCTCTACC-3′	60	Skjaeveland et al. (2008)
*SsTLR9*rv	5′-ACCTCCTCCGCCCCACGTTCTCCAG-3′		
RACE			
OmTLR9 5′ RACE GSP	5′-CCCCTTTGACTGCTCCTGTGACACTT-3′	68–72	FJ594277
OmTLR9 3′ RACE GSP	5′-CATCCCAGCCGTAGAGGTGCCTGAGTAG-3′		
Gene full sequencing			
OmTLR9 fw	5′-GGCTTTTCTCTCTCCATGCTT-3′	53	FJ594277
OmTLR9 rv	5′-TGCATGTTATCCAACTCTTTCA-3′		

GSP: gene-specific primers; T_a: annealing temperature; *SsTLR9*: *Salmo salar* Toll-like receptor 9; *OmTLR9*: *Onchorhynchus mykiss* Toll-like receptor 9.

Table 2
Primer sequences used for gene expression analysis.

Primer name	Sequence	T_a (°C)	Reference/accesion no.
tlr9 fw	5′-CCCCTTTGACTGCTCCTGTGACACTT-3′	60	FJ594277
tlr9 rv	5′-CATCCCAGCCGTAGAGGTGCCTGAGTAG-3′		
cd83 fw	5′-TAGCTGCCTCTGTGCAAGGT-3′	58	Ohta et al. (2004)
cd83 rv	5′-ACCCTGTCTCGACCAGTTTG-3′		
cxcr4 fw	5′-GTGCATGTGATCTACACCATC-3′	50	Daniels et al. (1999)
cxcr4 rv	5′-GAGCTGTGGCAAACACTATGT-3′		
Tnf-alpha fw	5′-TTCGGGCAAATATTCAGTCG-3′	60	Lindenstrom et al. (2004)
Tnf-alpha rv	5′-GCCGTCATCCTTTCTCCACT3-3′		
il-6 fw	5′-TTTCAGAAGCCCGTGGAAGAGA-3′	58	Iliev et al. (2007)
il-6 rv	5′-TCTTTGACCAGCCCTATCAGCA-3′		
gapdh fw	5′-ATGTCAGACCTCTGTGTTGG3-3′	52	Wang et al. (2004)
gapdh rv	5′-TCCTCGATGCCGAAGTTGTCG-3′		

tlr9: Toll-like receptor 9; *cd83*: cluster of differentiation 83; *cxcr4*: CXC chemokine receptor 4; *Tnf-alpha*: tumour necrosis factor-alpha; *il-6*: interleukin-6; *gapdh*: glyceraldehyde 3-phosphate dehydrogenase.

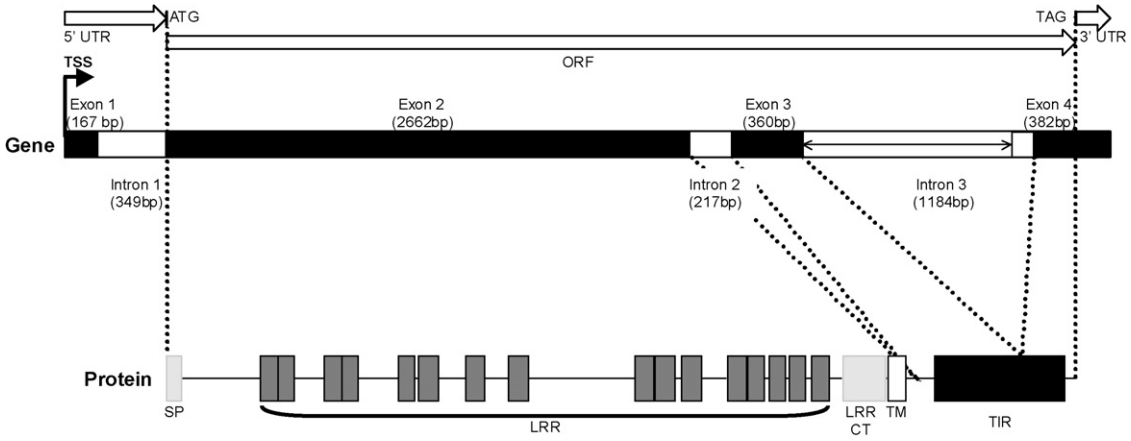

Fig. 1. Diagram of the rainbow trout TLR9 gene and its deduced protein sequence (GenBank accession number: FJ594277, ATG position: 521, stop codon position: 5144). Upper diagram, gene. Lower diagram, protein. Both diagrams are connected with dot lines to indicate the position of introns. TSS (transcription start site), 5′ and 3′ UTRs (Untranslated regions), putative ORF (Open reading frame), exons (black boxes) and introns (white boxes) are indicated. Double horizontal arrowhead indicates transposon localization in the intron 3. Exon and intron base pairs (bp) are indicated in parenthesis. Protein domain abbreviations: SP: signal peptide, LRR: leucine-rich repeat, LRR-CT: C-terminal leucine-rich repeat, TM: transmembrane, TIR: Toll-interleukin 1 receptor.

```
Om      ------mqgygvllclcllppavrtTN--PKFFPCDSNADSS---VVNCSRRALTRIPVI 49
Ss      ------mqgygvllclclltpavrtTN--PKFFPCDSNADSS---VVNCSRRALTRIPVI 49
Sa      mhaistmamlntivilcqllqltrtIN--TSFFPCDTDMNTT---YVDCSDRPLKRVPII 55
Po      --mllamavlrnilifgqllslvrtQN--VKFFPCDSDENAT---TVDCCGRSLKKVPTI 53
Tr      ----------mrflilcqllpivssIN--PIFLPCDTDANTS---RVDCSDRPLRRVPLI 45
Dr      -----mfgpmvslilllnqfqlfaASH--PQFYPCESHFTKDGHINVDCQHRRLSKVPRF 53
Hs      mgfcrsalhplslllvqaimlamtlaLGTLPAFLPCELQPHGL----VNCNWLFLKSVPHF 56
                  ::          :      * **: .        *:*    *  :* :

Om      CSAS----VLSLDLSYTGIQQVGKDAFSGVPNLQKLSMTRNCLPSHMRALGSPSCHVEIH 105
Ss      CSAS----VLSLDLSYTGIQQVGKDAFSGVPNLQKLSMTRNCLPSHMRALGSPSCHVEIH 105
Sa      KSES----VLSLSLSWTKIQQVGSDDLSGLKNLRTLEIVGNCLPGRLRDYSDRSCKMEIH 111
Po      KSNT----VVSINLSQNKIHHVEKDAFAGVLNLQTLKIIYNCQPSRLRALDNDSCNMEIH 109
Tr      KSDT----VTSLRLSRTKIRHLWQYDLSGVPNLRAFTMADNCQPSSMKASLHD-CEVKID 100
Dr      TSPS----VISLNLNNNHIHRIKGDAFSGLPNLKYLSLMWNCISDRLKEARWPLCSVNID 109
Hs      SMAAPRGNVTSLSLSSNRIHHLHDSDFAHLPSLRHLNLKWNCPPVGLSPMHFP-CHMTIE 115
           :    * *: *. . *:::    :: : .*: : :  ** .  :       * : *.

Om      KDAFMCLKNLSTLLLEGNSLTSPLPRLPDSLEVLNLESNHLYNITQP--LGTPNLKQLLL 163
Ss      KDAFMCLKNLSTLLLEGNSLTSPLPRLPDSLEVLNLESNHLYNITQP--LGTPNLKQLLL 163
Sa      DDAFRSLWNLTFANLSGNSLTR-IPWLPESLKILDLQENCISHIIQP--LKTPNLEALYL 168
Po      PQVFKSLHNLTFLYLSGNSLKS-IPWLPETLKVLDLQNNCIFQITQP--LKTPNLEGLFL 166
Tr      HGVFRNLSLLQVLNLSGNSLTS-LPKLPANLRVLNLQRNHLFNIVEP--LKTPHLQELYL 157
Dr      PDAFVGLKNLTSLQLAGNSLKM-IPPLPKQLEVLGLEFNNIFNIVKP--LGTPQLKQLLL 166
Hs      PSTFLAVPTLEELNLSYNNIMT-VPALPKSLISLSLSHTNILMLDSASLAGLHALRFLFM 174
          .*  :  *    *  *.:   :* **  *  *.*. . :  : ..       *. * :

Om      TMNCYYGNPCNQSFHIDEAVFRELTQLQTLTLGFNNVTSIPPGMPPSLKSLD-LNENKIS 222
Ss      TMNCYYGNPCNQSFHIDEAVFRELTQLQYLTLGFNNVTSIPPGMPPSLKRLD-LNENKIS 222
Sa      SKNCFYANPCGQPFNISEEVFKGLPKLKNLTLGYNNLTAIPIGLPRSLELLD-LRENTIT 227
Po      TKNCFYANPCYQSFYISEMVFRELHKLKNLTLGYNNLTSIPKGLPPSLDESGYLRENTIT 226
Tr      TKNCFYANPCQQSLEISKTVFRELSRLKTLTLAYNNLTSVPEGLPSSLEILD-LSENTIT 216
Dr      SKNCFYANPCHQPYFINSSVFQDLPELLNLTLSYNNLTAIPSYLPGSLESLD-LRENTID 225
Hs      DGNCYYKNPCRQALEVAPGALLGLGNLTHLSLKYNNLTVVPRNLPSSLEYLL-LSYNRIV 233
          **:* *** *.  :   .:  * .*  *:* :**:* :*  :* **.    *  * *

Om      VLDEWAFANLTQLCKLNLGWNCQRCDHAAQPCFPCFNNTPLDINPHAFYAQNNSLKFLSL 282
Ss      VLNEWAFANLTQLCQLNLGWNCQRCDHAAQPCFPCFNNTPLDINPRAFYAQNNSLKFLSL 282
Sa      EVLEGAFANLTFLHHLILEWNCQRCDHAARPCFPCPDNRPLKLHSKSFYAENSSVTLLSL 287
Po      KILNGTFANLTMLKYLSLEWNCQRCDHAARPCFPCPGLKPLDLYSNSFYAESSSIIFLSL 286
Tr      EVLEGAFADLTHLRELNLEWNCQRCDRAARPCFPCPRHLPLQLHSKSFYANKSTLTYLNL 276
Dr      HINKESFANLRNLRHLNLGWNCQRCDHASDPCFPCPNNQSLDLHQDAFLDQRDSLVSLHL 285
Hs      KLAPEDLANLTALRVLDVGGNCRRCDHAPNPCMECPRHFP-QLHPDTFS-HLSRLEGLVL 291
         :    :*:*  *  * :  **:***:*. **: *     . .:   :*  . . :  * *
```

Fig. 2. Amino acid sequence alignment of rainbow trout (Om, *Oncorhynchus mykiss*) TLR9 deduced protein to zebrafish (Dr, *Danio rerio*) TLR9, human (Hs, *Homo sapiens*) TLR9, japanese flounder (Po, *Paralichthys olivaceus*) TLR9, gilthead seabream (Sa, *Sparus aurata*) TLR9, atlantic salmon (Ss, *Salmo salar*) TLR9, fugu rubripes (Tr, *Takifugu rubripes*) TLR9. Sequence gaps are indicated with hyphens, identical residues with an asterisk, conserved substitutions with a double dot, and semi-conserved substitutions with a dot. Signal peptides are pointed out with small letters. LRR domains are indicated with bold letters and LRRCT domains with an underline. Transmembrane domains are highlighted with a dotted underline, and the TIR domains are boxed. The two CXXC motifs, that are important for CpG binding and gene regulation, are indicated by a gray box. Black arrows indicate the conserved amino acid residues in the CpG-DNA-binding domain. Open brackets indicate salmonid specific insertions. Rounded rectangle indicates TIR domain boxes important for ligand adaptor.

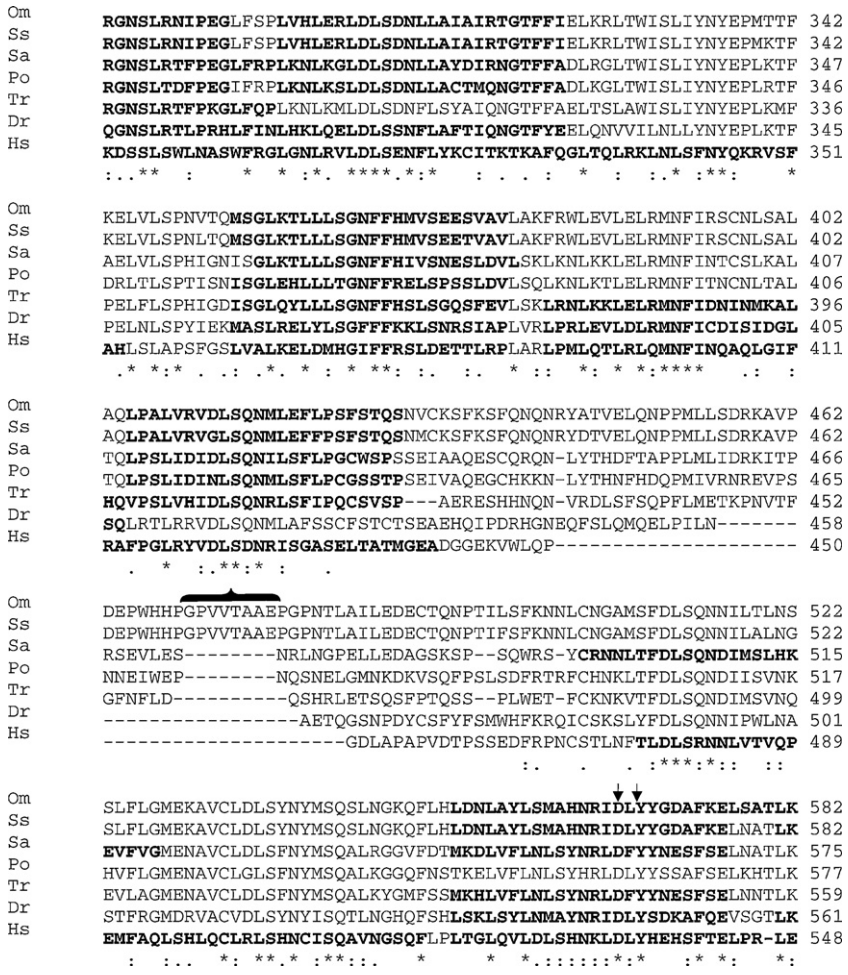

Fig. 2. (*Continued*)

tions. PCR amplified fragments were gel purified and cloned into the TOPO® TA Cloning kit (Invitrogen), following manufacture's instructions. Next, the vector plus the *Omtlr9* cDNA fragment was sequenced. Genomic DNA was extracted from muscle using a standard phenol–chloroform protocol (Sambrook et al., 1989). The primers employed in *Omtlr9* gene full sequencing are listed in Table 1. The nucleotide sequences of the *Omtlr9* RNA and DNA are deposited in the GenBank (accession no. FJ594277).

2.3. Sequence analyses

Full length OmTLR9 protein sequence was aligned against human, zebrafish, fugu, Japanese flounder, gilthead seabream and Atlantic salmon full length TLR9 protein sequences (GenBank accession numbers: EAW65192, AAI63628, AAW69377, BAE80691, AAW81698 and ABV59002, respectively). Multiple sequence alignment was performed using ClustalW2 (Larkin et al., 2007) (http://www.ebi.ac.uk/clustalw2/). The structure of the deduced amino acid sequence of rainbow trout TLR9 was characterized using the SMART program (Letunic et al., 2006) (http://smart.embl-heidelberg.de/). Sequence similarities were identified using BLASTN (Altschul and Koonin, 1998) (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4. RT-PCR

RNA was extracted from different rainbow trout tissues as indicated before and cDNA synthesis carried out following procedures previously described (Falco et al., 2008; Tafalla et al., 2007; Chico et al., 2009). PCR amplification reactions (25 μl) were performed using 0.5 μl dNTP mix (10 mM each), 0.2 μl Taq DNA polymerase (Roche, Barcelona, Spain), 2.5 μl Taq 10× buffer, 0.5 μl of each primer (20 μM) (Table 2) and 2.5 μl of cDNA. A parallel PCR with primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Wang et al., 2004) (Table 2) was performed with all samples as a housekeeping gene to standardise the RT-PCR results, using conditions previously described (Falco et al., 2008; Tafalla et al., 2007). PCRs were carried out in a GeneAmp® PCR System 2700 cycler (Applied Biosystems). PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide. A 100-bp DNA ladder (BIORON) was used as a size marker. Band intensities were quantified using Scion Image software (Scion Corp.). Relative gene transcription was calculated as follows: intensity of target gene band/intensity of its corresponding GAPDH band.

3. Results

3.1. Omtlr9 gene

Full length *Omtlr9* gene (GenBank accession no. FJ594277) was 5321 bp (Figs. 1 and 2). Untranslated (UTR) regions were 520 bp (5′ UTR) and 175 bp (3′ UTR), respectively. The *Omtlr9* gene is composed of four exons and three introns. Introns are located in positions 168 bp (before the start codon), 3179 bp (between the transmembrane (TM) and the Toll-interleukin 1 receptor (TIR) domain, and 3756 bp (within the TIR domain). Intron/exon distribution was dif-

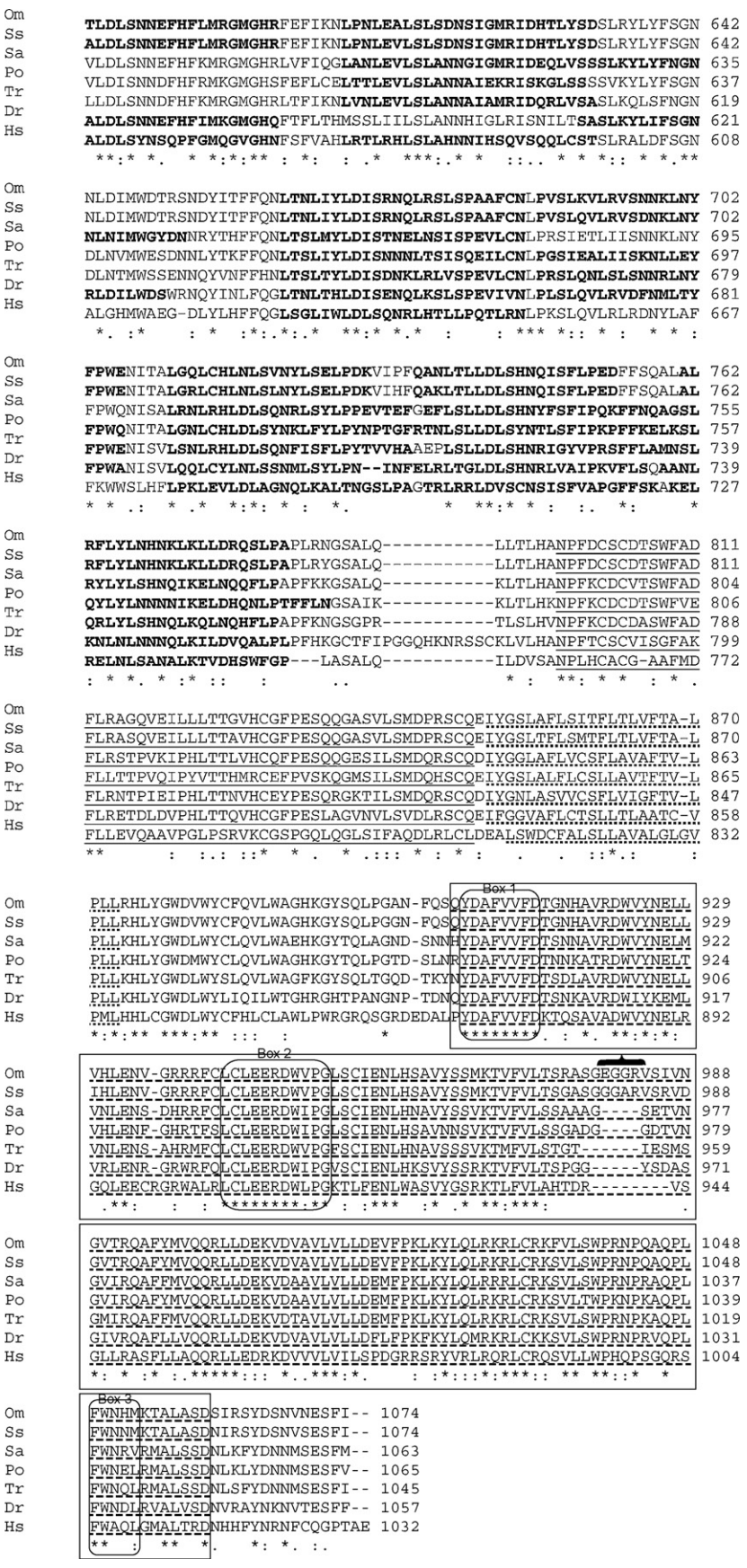

Fig. 2. (*Continued*)

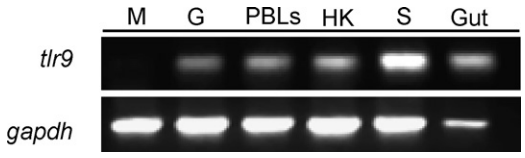

Fig. 3. Constitutive expression of *Omtlr9* gene in different tissues of healthy rainbow trout measured by RT-PCR. M, muscle; G, gills; PBLs, peripheral blood leucocytes; HK, head kidney; S, spleen; Gut, gut.

ferent from salmon *tlr9* (Skjaeveland et al., 2008) and fugu *tlr9* (Oshiumi et al., 2003), which had only one intron located in the TIR domain. Japanese flounder (Takano et al., 2007) and gilthead seabream (Franch et al., 2006) *tlr9* genes presented two introns, one located close to the signal peptide and another within the TIR domain, showing in these cases an intron distribution more similar to *Omtlr9*.

The third intron presented at its 5′-end an insertion of an incomplete transposon of 1080 bp (GenBank accession no. FJ594277), which showed high homology (sequence identity >85%, query coverage >65%) with Sleeping Beauty 10 (SB10) transposon (Ivics et al., 1997), but lacked the transposase amino terminal and the 5′ ITR (inverted terminal repeat) (data not shown). As well, a BLASTN search revealed transposon high sequence similarity (i.e. sequence identity >85%, query coverage >74%) with transposons within the rainbow trout IgH.A (Ig heavy-chain) locus, major histocompatibility complex (MHC) Class I region, ABCB2 region, and IgD gene. Moreover, the transposon sequence was found to be present in two of the 3′ RACE clones sequenced.

3.2. Sequence analysis of OmTLR9 predicted protein

The OmTLR9 deduced amino acid sequence (GenBank accession no. ACC93939) has a coding region of 1074 amino acid residues. Protein domain prediction analysis revealed a potential signal peptide of 19 residues, 16 leucine-rich repeat domains (LRD) conforming the functional ectodomain (ECD), a C-terminal cap (LRR-CT), a TM region located between residues 851 and 873, and an intracellular TIR domain (Fig. 2).

The TLR9 functional sites identified in mammals are shown to be conserved in rainbow trout, as it has been previously shown for other teleost TLR9s (Chen et al., 2008; Franch et al., 2006; Skjaeveland et al., 2008; Takano et al., 2007). The OmTLR9 CpG-DNA-binding domain contains the two essential amino acid residues for interacting with CpG-DNA (Rutz et al., 2004), an aspartic acid (Asp) and a tyrosine (Tyr), positioned in the LRR 9 (Asp^{567} and Tyr^{569}) (Fig. 2, black arrows). OmTLR9 has also two CXXC motifs (gray box, Fig. 2), region important for CpG binding and gene regulation, conserved in mammals and teleosts (Bell et al., 2003). Three TIR domain boxes, functionally fundamental for ligand adaptor and downstream signalling (Yamamoto et al., 2004), appeared highly conserved in OmTLR9 and other teleosts (rounded rectangle, Fig. 2). Two insertions were identified as specific for salmonids, since they were only present in rainbow trout and salmon TLR9, one of eight residues in the ECD, between LRR 9 and 10, and another of four residues in the TIR domain (open brackets, Fig. 2).

The deduced OmTLR9 protein sequence showed 96% identity to Atlantic salmon ortholog, between 50 and 60% identity to gilthead seabream, Japanese flounder, fugu, and zebrafish orthologs, and 36% identity to human ortholog.

3.3. Tissue distribution of Omtlr9 RNA transcripts revealed by RT-PCR

RT-PCR was performed using total RNA extracted from different trout tissues in order to investigate the tissue distribution of *Omtlr9* gene in healthy rainbow trout. Constitutive gene transcription of *Omtlr9* was mostly detectable in gills, PBLs, head kidney, spleen and gut but undetectable or detectable at very low levels in muscle (Fig. 3).

3.4. Omtlr9 and immuno-related gene transcript levels in fish immunised with pAE6 and pAE6-G

Immunisation with plasmids pAE6 and pAE6-G did not increase the presence of *Omtlr9* transcripts in head kidney and spleen by day 3 p.i., compared to non-immunised fish, but it increased ~5-fold at the site of injection (muscle) (Fig. 4). Since the presence of the *Omtlr9* transcripts in muscle could be due to the recruitment of TLR9-expressing cells into the site of injection, we tried to identify the source of TLR9-expressing cells at the injection site, by evaluating the transcription level of the mature dendritic cell markers *cd83*, and *cxcr4*, both of them identified in several fish species (Alabyev et al., 2000; Daniels et al.,

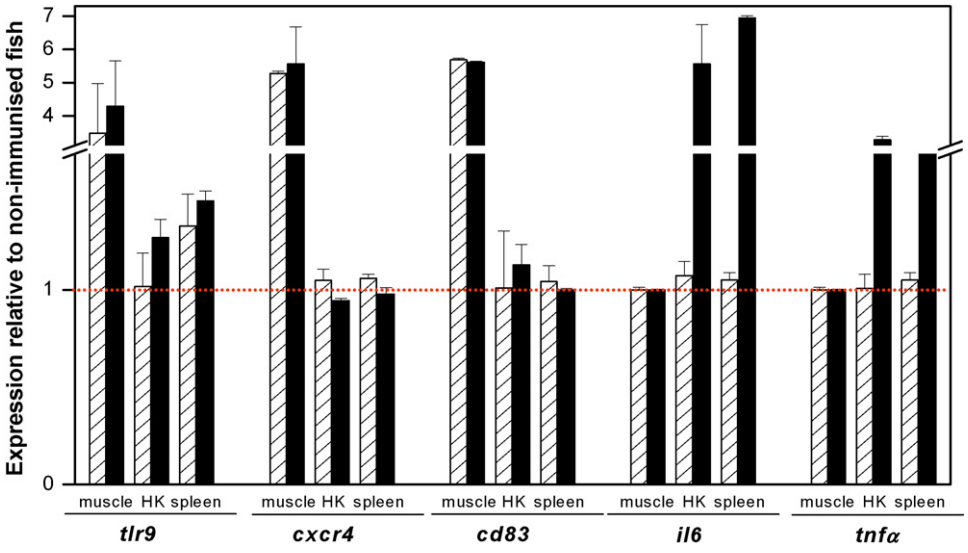

Fig. 4. TLR9 and TLR9-related genes transcript levels after DNA vaccine injection with pAE6 (hatched bar) and pAE6-G (black bar). Data represent mean ± S.E. of gene transcripts in muscle, spleen and head kidney (HK) of immunised individuals, relative to non-immunised individuals, and normalized to GAPDH gene transcripts. Horizontal line points out similar expression levels between control and immunised fish.

1999; Ohta et al., 2004). A direct correlation was found between *Omtlr9* transcripts and this of *cd83* and *cxcr4*, thus suggesting that fish dendritic-like cells seem to be the TLR9-expressing cells preferably recruited at the injection site, at early time post-immunisation.

Both TNF-α and IL6 production are included among the TLR9-dependent cytokine responses (Ahmad, 2007). For that, the presence of *tnfα* and *il6* transcripts was analysed in muscle, head kidney and spleen of the fish immunised with pAE6 and pAE6-G. Transcription of *tnfα* and *il6* was solely observed in spleen (increment of ~6.5-fold *tnfα* and ~7-fold *il6*) and head kidney (increment of ~7-fold *tnfα* and ~5.5-fold *il6*) of the fish injected with pAE6-G plasmid (Fig. 4). No transcription of *tnfα* or *il6* was observed in any of the tissues of the fish immunised with pAE6, or in the muscle of the fish immunised with any of the plasmids.

4. Discussion

In the present study, a rainbow trout cDNA corresponding to the TLR9 protein was cloned and sequenced. The *Omtlr9* gene, composed of four exons and three introns, presented an intron/exon distribution similar to Japanese flounder (Takano et al., 2007) and gilthead seabream (Franch et al., 2006) *tlr9* genes, but different from salmon *tlr9* (Skjaeveland et al., 2008) and fugu *tlr9* (Oshiumi et al., 2003). The *Omtlr9* gene presented at the 5′-end of the third intron an insertion of an incomplete copy of a transposon which showed high homology with SB10 transposon. Similarly, Tc1-like transposons are known to be present in other genes implicated in salmonid fish defence response, signal transduction and/or regulation of transcription (Krasnov et al., 2005). The *Omtlr9* transposon-like sequence was found to be transcribed in two of the 3′ RACE clones sequenced which could be explained by the finding of an alternative 5′ GT splicing site at transposon position 1078–1079. Whether or not the presence of this incomplete copy of a transposon might reduce/impair the *Omtlr9* gene transcripts functionality remain to be determined since the possibility of involvement of transposon transcripts in the regulation of gene expression in salmonid fish has not been yet studied (Krasnov et al., 2005). In fact, the role of transposon transcripts in the regulation of gene expression has been only reported in yeast (Schramke and Allshire, 2003).

The OmTLR9 predicted protein was similar to mammalian and other teleost TLR9s since it presented all the TLR9 specific domains and motifs critical for their activity (Bell et al., 2003; Rutz et al., 2004; Takano et al., 2007). Therefore, OmTLR9 functionality in response to its specific PAMPs was expected to be similar to that observed in mammalian and teleost TLR9s.

Omtlr9 was predominantly transcribed in spleen, head kidney, PBLs, gut and gills, showing low or undetectable levels of transcription in muscle. Therefore, *Omtlr9* constitutive transcription pattern was similar to that previously described for other teleost fish species (Chen et al., 2008; Franch et al., 2006; Jault et al., 2004; Oshiumi et al., 2003; Skjaeveland et al., 2008; Takano et al., 2007).

After fish immunisation with the pAE6 and pAE6-G plasmids, *Omtlr9* transcripts did not increase in head kidney and spleen, by day 3 p.i. In contrast, the levels of *Omtlr9* gene transcripts in muscle, a non-lymphoid tissue, were highly increased compared to the muscle of control fish (non-immunised fish), and this increment was similar in response to both plasmids. Hence, the immune cells recruited into the site of injection as a consequence of the DNA immunisation may be the source of *Omtlr9* transcripts in muscle at early times post-immunisation. The recruitment of TLR9-expressing cells has been also observed in tissues of *Edwardsiella tarda* infected fish (Takano et al., 2007). Transcripts corresponding to the *cd83* and *cxcr4* genes showed identical gene up-regulation levels to *Omtlr9*. *cd83* is the best-known cell surface marker for fully mature human DCs and it is strongly up-regulated during DCs maturation (Prechtel and Steinkasserer, 2007; Prechtel et al., 2007). The induction of *cd83* on DCs/antigen presenting cells (APCs) surface is an important step in the initiation of adaptive immunity and will favour a cellular response (Strandskog et al., 2008). After intramuscular DNA vaccine injection, our findings suggested an up-regulation of the OmTLR9 receptor in the DCs recruited to the site of injection, triggering DCs maturation and posterior cellular response in secondary lymphoid organs. In mammals, APCs are key inducers of immunity, as they are the pivotal mediators of immune responses between resident somatic cells and T-cells in the lymph nodes.

TNF-α and IL6 are two cytokines directly related to TLR9 induction after DNA immunisation with unmethylated CpG ODNs (Cognasse et al., 2008). IL6 in mammals acts as both a pro-inflammatory and anti-inflammatory cytokine. It is produced by a diverse group of cells, including T-cells, B-cells, macrophages, fibroblasts, neurons, endothelial and glial cells (Iliev et al., 2007). B-cells TLR9 engagement to CpG ODNs is followed by sustained IL6 production (Cognasse et al., 2008). Our findings nevertheless showed that *il6* and *tnfα* transcripts production was solely up-regulated in secondary lymphoid organs (head kidney and spleen) of trouts immunised with the pAE6-G plasmid, however, the pAE6 plasmid was unable to induce *il6* gene transcription, indicating that OmTLR9 could not be acting as plasmid DNA receptor and consequently triggering its signalling till IL6 production. Therefore, the glycoprotein G of VHSV seems to be more related to TNF-α and IL6 production than the plasmid backbone recognition by OmTLR9. Obviously, further studies are needed to corroborate this hypothesis. To conclude, the present work gives further insight on the involvement of fish TLR9 in the recognition plasmid DNA, demonstrating that although the immunogenicity of DNA vaccines may vary due to many factors such as the quality of plasmid DNA, injected sites, injection methods or modification of CpG motifs within plasmid DNA (Chico et al., 2009; Gurunathan et al., 2000), our results support previous findings (Babiuk et al., 2004; Ishii and Akira, 2006) indicating that that TLR9-mediated recognition of plasmid DNA might be not essential for optimal DNA vaccination.

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