Sustainable development of aquaculture relies on preventing and controlling diseases, for which the use of vaccines has been of major importance. Despite vaccination efficiently controlling many bacterial diseases in salmonid aquaculture, viral infections still remain a major problem. Although commercial vaccines are available against infectious pancreatic necrosis (IPN) and pancreas disease (PD), all are non-replicating vaccines based on inactivated whole virus (IPNV and PD-virus) and recombinant subunit vaccines (IPNV-VP2), and the general understanding has been that these vaccines confer suboptimal protection (Salgado-Miranda et al., 2013). A key question regarding development of protective vaccines against viral infections in fish and higher vertebrates is to understand the mechanisms underlying the difference between inferior protection attained by inactivated vaccines, and the strong immunity often induced by replicating variants (Robert-Guroff, 2007). Replicating vaccines possess the advantage of mimicking an actual infection and therefore inducing T-cell-mediated immunity, whereas inactivated (non-replicating) protein antigens elicit mostly B-cell-mediated humoral responses (Lundstrom, 2012). Concerns regarding reversion to virulent virus and ecosafety have, however, hindered the use of live vaccines in aquaculture.

The use of alphaviruses as vector vaccines is an attractive technology for the generation of novel and improved vaccines, as these vectors express high levels of recombinant proteins in a broad spectrum of hosts, including many mammalian and insect cells (Lundstrom, 2001; Rayner et al., 2002; Riezebos-Brilman et al., 2006). The alphavirus genome is a positive-stranded RNA capped at the 5’ end and polyadenylated at the 3’ end, with a size of approximately 11.7 kb (Strauss & Strauss, 1994). The genome structure of alphavirus is composed of two ORFs. The first ORF encodes a polypeptide containing four non-structural proteins (nsp1–4), which forms the replicase complex involved in RNA replication and transcription. The second ORF is driven by a 26S subgenomic promoter, and expresses the structural proteins (CP-E3-E2-6k-E1) responsible for encapsidation of viral RNA and assembly into enveloped particles (Strauss & Strauss, 1994).

Compared to the classical live-attenuated form, where the virus infects the animal without causing disease, recombinant replicon vaccines have several advantages in terms of safety. By removing the genes that encode the viral structural genes, and replacing them with vaccine antigens, these viral vectors can replicate their genomes but are not productively infectious, as they are unable to make new virus particles (Lundstrom, 2005). The use of plasmid-encoded viral replication systems is believed to induce a strong immune response, through intracellular antigen expression and the ability of the replicating RNA to stimulate innate antiviral signals (Leitner et al., 2002), including generation of double-stranded RNA intermediates, which
Fig. 1. (a) Supernatant from cells transfected with pSAV3-HHFL and pSAV3-FL was collected at the indicated time points, and viral titration was performed. (b) Design of modified SAV3 replicon constructs for improved protein expression. Ag, antigen (in this study: EGFP/luciferase/IPNV segA); E, translational enhancer; 2A, FMDV 2A peptide; RZ, hepatitis D ribozyme. (c) Expression of EGFP in CHH-1 cells transfected with either pRep-EGFP or pRep-E2A-EGFP was quantified by flow cytometry. Each data point represents the mean intensity of EGFP per cell when 35,000 transfected cells from each individual transfection were analysed. (d) Quantification of total EGFP expression in the cells transfected with either pRep-E2A-EGFP or
cines, reducing the cost of vaccine production (Berglund et al., 1998). Several alphaviruses such as Sindbis virus (SINV), Semliki Forest virus (SFV) and Venezuelan equine encephalitis virus (VEEV) have all been developed as potential replicon based vaccine vectors carrying heterologous antigens, and shown to elicit protective immune response in animals (Bennett et al., 2000; Frolov et al., 1996; Lundstrom, 1997, 2009; Zhou et al., 1995). The SFV replicon has been developed to express antigens in fish cells; however, this is with limitations because of very low protein expression at low temperatures (15°C and below) (Phenix et al., 2000). In contrast, salmonid alphavirus (SAV) causes natural infections in farmed salmonids at low temperatures, and therefore represents an alternative replicon based vector vaccine. Recent published studies have indeed shown that a SAV replicon based vaccine was functional in cells originating from fish, mammals and insects, and at temperatures ranging from 4 to 37°C (Olsen et al., 2013). Furthermore, the SAV based replicon vaccine carrying the haemagglutinin-esterase (HE) gene of infectious salmon anaemia virus (ISAV) has been proven to protect Atlantic salmon against ISAV challenge when delivered by intramuscular injection (Wolf et al., 2013a), while intraperitoneal immunization with the same dose induced no protection (Wolf et al., 2014). However, SAV replicons provide low to modest protein production compared to what is reported from terrestrial alphavirus replicons (Olsen et al., 2013). Modifications to the construct for improved levels of protein expression would therefore be valuable, as it is well known that antigen dose in fish vaccines is well correlated with protection (Munang’andu et al., 2013). The objective of the present study was to develop a SAV3 replicon vaccine vector, based on a previously constructed infectious full-length cDNA clone (Guo et al., 2014), and to evaluate different modifications to the constructs for enhanced expression of heterologous protein.

Initially, the effect of inserting a hammerhead (HH) ribozyme sequence, as described previously (Guo et al., 2014), to ensure precise cleavage at the 5’ end was evaluated. Chinook salmon embryonic (CHSE-214) cells were transfected (Fugene HD transfection reagent, Roche) with an infectious full-length SAV3 clone, with or without the HH ribozyme sequence fused at the 5’ end (pSAV3-HHFL and pSAV3-FL, respectively), and incubated at 15°C. Cell supernatant was collected at 2, 4, 8, 9, 11 and 13 days post-transfection (dpt), followed by titration of rescued virus on CHSE-214 cells by the method of Spearman and Kärber (Miller & Ulrich, 2001). Recovery of recombinant virus in pSAV3-HHFL transfected cells started at 4 dpt (1.0 × 10^3 TCID_{50} ml^{-1}), and generated a peak titre of 2.1 × 10^7 TCID_{50} ml^{-1} at 13 dpt (Fig. 1a). In contrast, infectious virus recovered from pSAV3-FL was delayed until 11 dpt (4.7 × 10^6 TCID_{50} ml^{-1}), and end point titres were reduced 1000-fold compared with the construct containing the HH ribozyme. It has previously been reported that inclusion of a HH ribozyme sequence is indispensable for rescue of SAV2 by reverse genetics (Moriette et al., 2006). Our result confirmed the important role of the HH ribozyme, yet we also observed that recovery of virus from pSAV3-FL (non-HH) cDNA was feasible, albeit giving lower titres.

Based on the findings above, the full-length cDNA clone with an inserted HH sequence at the 5’ end was used as a template for construction of a SAV3 replicon vector. The structural genes were deleted by PCR splicing, using primers F1/R1 and F2/R2 (Table 1). At the same time, two restriction sites, AgeI and Ascl were introduced downstream of the viral subgenomic promoter and upstream of the 3’UTR to facilitate cloning of insert DNA. To evaluate the functionality of the SAV3 replicon, the EGFP marker gene was inserted into the SAV3 replicon at the AgeI and Ascl sites, generating pRep-EGFP (Fig. 1b). It has previously been shown for SFV replicons that the first 102 nt of the capsid gene, including the initiating AUG, function as a translational enhancer. When the capsid gene segment was fused to the heterologous gene, expression of the corresponding fusion protein was found to be at the same level as viral structural proteins during WT SFV infection (Sjöberg et al., 1994). Despite only 56% sequence similarity between SAV3 and SFV within the N-terminal end of the capsid protein (data not shown), we decided to examine whether this enhancing property is evolutionarily conserved in SAV3. The N-terminal 102 nt of the SAV3 capsid gene were therefore inserted into pRep-EGFP. To obtain an accurate 5’ end of the expressed antigen, the 2A peptide sequence derived from foot-and-mouth disease virus (FMDV) was placed immediately downstream of the translational enhancer sequence, generating the construct pRep-E2A-EGFP (Fig. 1b). The FMDV 2A peptide executes translational skipping, and thus prevents the formation of a peptide bond between enhancer and antigen (Ryan & Drew, 1994), so producing authentic EGFP. Two replicon constructs, pRep-EGFP and pRep-E2A-EGFP, were then transfected into Chinook salmon heart (CHH-1) cells (Herath et al., 2009) by electroporation (Neon transfection system, Invitrogen) and incubated at 15°C. Expression of EGFP, in the cells transfected with or without E2A in the construct, was detectable from 2 dpt using a fluorescence microscope.
At 4 dpt, a number of EGFP positive cells were observed, yet the intensity of EGFP per cell could not be quantified by fluorescence microscopy. Therefore, in order to clarify the role of E2A on EGFP expression levels, cells electroporated with either pRep-E2A-EGFP or pRep-EGFP were trypsinized, spun down and suspended in PBS before analysis with a flow cytometer (Guava easyCyte HT system, Merck Millipore). The results showed that the transfection rate of two replicon constructs by electroporation was similar (data not shown), while the mean intensity of EGFP per cell was significantly higher in the E2A+ group (Fig. 1c), showing the benefit of introducing the N-terminal 102 bases of the SAV3 viral capsid gene as a translation enhancer.

Furthermore, it has been shown that a hepatitis D virus ribozyme (HDV-RZ) sequence placed immediately downstream of the poly(A) sequence enhances expression levels of the reporter gene in a SINV replicon vector (Yamanaka & Xanthopoulos, 2004). HDV-RZ self-cleaves at its 5’ end, and therefore generates authentic 3’ termini of viral RNA molecules, which again facilitate the successful initiation of RNA replication in transfected cells. To evaluate whether HDV-RZ can improve expression levels of inserted genes in SAV3 replicon vectors, HDV-RZ sequence (Wen et al., 1999) was introduced into the pRep-E2A-EGFP vector by stepwise fusion PCR using the primers listed in Table 1. The constructed plasmid (Fig. 1b), pRep-E2A-EGFP-RZ, together with pRep-E2A-EGFP, were separately transfected into CHH-1 cells using Fugene HD transfection reagent (Roche), and total fluorescence intensities of EGFP were measured by a microplate reader (Tecan GENios). The total intensities of EGFP were not different (P > 0.05) for cells transfected with pRep-E2A-EGFP or pRep-E2A-EGFP-RZ (Fig. 1d), suggesting the exact 3’ end sequence of SAV3 RNA molecules is not a crucial factor for viral replication.

We have previously documented that CHH-1 cells are the optimal cell line for the generation of recombinant SAV3 (Guo et al., 2014) between three susceptible cell lines, Chinook salmon embryo (CHSE-214), Bluegill fry (BF-2) and CHH-1. To determine whether expression of foreign antigen using the SAV3 replicon system is cell-dependent, the pRep-E2A-EGFP vector was transfected into the same cell lines. Compared to CHH-1 cells, the EGFP expression in CHSE-214 and BF-2 cells was delayed, and in this regard we prolonged the incubation time up to 10 days before evaluating the intensity of fluorescence. The result showed that EGFP expression in CHH-1 cells was significantly higher compared to CHSE-214 and BF-2 cells (Fig. 1e). For validation of the results above, the EGFP gene was replaced with another reporter gene, luciferase. The activity of expressed luciferase was assayed with the luciferase reporter assay system (Promega) before luminescence was detected by a microplate reader (Tecan GENios). When comparing the ability of the three transfected cell lines to produce the gene of interest, luciferase activity gave similar results to EGFP expression. These results further support our previous study (Guo et al., 2014), where we found that viral infection of CHH-1 cells results in limited induction

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Table 1. Primers used for plasmid construction

<table>
<thead>
<tr>
<th>Primers</th>
<th>Designation</th>
<th>Primer sequence (5’ – 3’)*</th>
<th>Restriction site</th>
</tr>
</thead>
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<tr>
<td>F1-F</td>
<td>ACCTGTTATATGGTGAGCAAGGGCGAGGAG</td>
<td>AgeI/AscI</td>
<td></td>
</tr>
<tr>
<td>F1-R</td>
<td>AGGTTCAGGGGGAGGTGTGGGAGGF2-F</td>
<td>SpeI</td>
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<tr>
<td>F2-F</td>
<td>GGCGCGCCATATTATATTACCGGTGGTTGGTTGAGAGTATGATGCAGAAAATATTAAG</td>
<td>AscI/AgeI</td>
<td></td>
</tr>
<tr>
<td>F2-R</td>
<td>GGCGCGCCATATTATATTACCGGTGGGCCCAGGGTTGGACTCGACGTCTCCCGCAAGCTTAAGAAGGTCAAAATTCTTAGTGCGCGGCCGGTACGG</td>
<td>AscI/AgeI</td>
<td></td>
</tr>
<tr>
<td>EGFP-F</td>
<td>ATATTACCGGTATGGTGAGCAAGGGCGAGGAG</td>
<td>AgeI</td>
<td></td>
</tr>
<tr>
<td>EGFP-R</td>
<td>ATATTGGCGCGCCTTACTTGTACAGCTCGTCCATG</td>
<td>AscI</td>
<td></td>
</tr>
<tr>
<td>LUC-F</td>
<td>ATATTACCGGTATGGAAGACGCCAAAAACAT</td>
<td>AgeI</td>
<td></td>
</tr>
<tr>
<td>LUC-R</td>
<td>ATATTGGCGCGCCTTACTTTCCGCCCTTCTTGGCC</td>
<td>AscI</td>
<td></td>
</tr>
<tr>
<td>SegA-F</td>
<td>ATATTACCGGTATGAACACAAACAAGGCAACCG</td>
<td>AgeI</td>
<td></td>
</tr>
<tr>
<td>SegA-R</td>
<td>ATATTGGCGCGCCTTACACCTCAGCGTTGTCTCC</td>
<td>AscI</td>
<td></td>
</tr>
<tr>
<td>RZ-F</td>
<td>GATCACATGGTCCTGCTGGAGTTCGTGACCRZ-R1</td>
<td>BmtI</td>
<td></td>
</tr>
<tr>
<td>RZ-R1</td>
<td>TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTTATATTGAAAATTTTAAAACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RZ-R2</td>
<td>CCGCGAGGAGGTGGAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDV-RZ</td>
<td>GGCGCGCCATATTATATTACCGGTGGGTTTCATCGCACGTCCACTCGGATGGCTAAGGGAG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Sequences for restriction sites are underlined.
of interferon, Mx and ISG15, and consequently higher amounts of viral products are generated post-transfection, which interestingly also applies to luciferase. To what extent the same mechanisms apply remains elusive.

We then moved on to test the capability of the replicon system to express the complete structural genes from a heterologous virus of importance in salmonid aquaculture, IPNV. The pRep-E2A-EGFP vector was engineered to carry the complete structural proteins of IPNV, replacing the EGFP gene, and this was designated pRep-E2A-IPNsegA. As IPNV infection in CHH-1 cells has not previously been described, IPNV was first inoculated onto CHH-1 cells. A significant cytopathic effect was evident from day 5 post-infection by phase-contrast microscopy (Fig. 2a). Immunostaining of cells infected by IPNV using a rabbit anti-IPNV primary antibody (Munang’andu et al., 2013b) gave a strong signal in fluorescence microscopy, confirming the successful expression of IPNV protein in CHH-1 cells (Fig. 2b). We finally tested the expression of IPNV structural proteins produced by the pRep-E2A-IPNsegA construct, and the result showed evident expression of IPNV structural proteins at 5 dpt (Fig. 2d). As expected, the percentage of positive cells was lower compared to infected cells (Fig. 2b), in line with the replicon not being able to spread from initially transfected cells.

A recently published study has shown the potential of a similar SAV3 based replicon vaccine for protection against ISAV infection (Wolf et al., 2013). However, levels of produced antigen are of pivotal importance for vaccine efficacy, and expression levels from this construct were low to moderate (Olsen et al., 2013). This study reports the importance of inserting a translational enhancer in the construct, as antigen expression levels were suboptimal when the heterologous gene was located immediately after the viral subgenomic promoter. This important finding underscores the potential use of the improved SAV3 replicon vectors for fish vaccine development, though future immunization studies should be conducted to evaluate the impact on protection against challenge.

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