Recombinant Infectious Hematopoietic Necrosis Virus and Viral Hemorrhagic Septicemia Virus Glycoprotein Epitopes Expressed in *Aeromonas salmonicida* Induce Protective Immunity in Rainbow Trout (*Oncorhynchus mykiss*)

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Fragments of the glycoprotein genes of viral hemorrhagic septicemia virus (VHSV) and infectious hematopoietic necrosis virus (IHNV) were cloned into a bacterial broad-host-range expression vector under the control of the *plac* promoter. Western blot (immunoblot) analysis with monoclonal antibodies specific to the glycoproteins demonstrated the inducible expression of the fusion proteins in *Escherichia coli*. *Aeromonas salmonicida* is the causative agent of furunculosis in salmonid fish. It was confirmed that an avirulent strain of *A. salmonicida*, A440, which contains a deletion in the structural gene for the paracrystalline surface protein array, will provide protective immunity against furunculosis when used as a live attenuated vaccine. The plasmid-encoded viral epitopes were then mobilized into A440 for use as a shuttle system for the expression of fragments of the glycoprotein genes of IHNV and VHSV. Vaccination of rainbow trout with A440 containing the viral epitopes resulted in the development of protective immunity against both VHSV and IHNV. This indicates that the use of cloned fragments of the glycoproteins and the use of *A. salmonicida* as a shuttle system constitute a feasible approach to fish vaccine development.

Viral hemorrhagic septicemia virus (VHSV) and infectious hematopoietic necrosis virus (IHNV) are important fish rhabdoviruses which cause severe epizootics among most salmonid fish. VHSV replicates in rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta fario*), brook trout (*Salvelinus fontinalis*), grayling (*Thymallus thymallus*), chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*), and whitefish (*Coregonus* sp.) (33). Epidemiological studies (11) indicate that VHS is primarily a disease of pike (*Esox lucius*). VHS has been reported in a number of European countries, and in 1988, VHSV was first isolated from chinook salmon (*O. tshawytscha*) and from coho salmon (*O. kisutch*) on the west coast of North America (7, 17). Isolation of VHSV from Pacific cod (*Gadus macrocephalus*) in Alaska (24) gave rise to the belief that the virus had been enzootic in the North Pacific Ocean for some time (2). IHNV is enzootic in sockeye salmon (*Oncorhynchus nerka*) populations on the west coast of North America but also causes severe epizootics among stocks of chinook salmon and rainbow and steelhead trout. In 1977, IHNV was introduced into Japan (32), and since 1987 the virus has been detected in France, Italy, and Belgium (3, 5, 21a). In the summer of 1992, IHNV was isolated from rainbow trout in Germany (10). A further distribution of the disease within European salmonids is to be expected. Neither virus has been classified fully, because there are no serological relationships to other members of the hitherto-classified genera of the family. There is, however, at least one common epitope on VHSV and IHNV viruses which is detectable with polyclonal and monoclonal antibodies (9).

Despite intensive investigations into the development of vaccines against VHSV and IHNV, a licensed vaccine has yet to

be developed. Because of safety considerations, attenuated viral vaccines for fish are not allowed in many countries since, in most cases, outflow from hatcheries and farms remains untreated. A vaccine must be administered to young fish in the hatchery prior to the possibility of their being exposed to virus in infected areas. Recent studies have focused on the use of recombinant viral proteins as candidates for vaccines, with particular attention being given to the glycoprotein (G protein). Rhabdoviral glycoproteins are membrane-associated proteins that form spike-like projections on the surface of mature virions (23) and have apparent molecular masses of 57 kDa. Cloning and nucleotide sequencing of cDNAs of IHNV and VHSV glycoprotein genes have been described (18, 35). Studies have shown that portions of the IHNV G protein expressed in *Escherichia coli* are protective for rainbow trout and that the VHSV G protein is immunogenic in rainbow trout (22, 46).

Aeromonas salmonicida is a gram-negative, facultatively anaerobic, rod-shaped bacterium that is an economically significant pathogen of fish (40, 41). Typical strains of *A. salmonicida* are the causative agent of furunculosis in salmonid fish, whereas atypical strains cause a variety of symptoms in other fish species. The paracrystalline surface protein array (A-layer) of *A. salmonicida* is composed of tetragonally arranged protein subunits which assemble to form a complete layer around the cell. Previous studies have shown that the secretion and assembly of the A-layer is essential for virulence, as S-layer mutants are no longer resistant to phagocytosis by macrophages (13, 14, 43) and are more sensitive to serum killing (25). The S-layer also protects against the action of proteases and binds certain porphyrins, immunoglobulins, and a range of extracellular matrix proteins (42, 44). Other factors which are believed to contribute to the ability of *A. salmonicida* to cause disease are a variety of extracellular proteases (20, 29) and hemolysins (12,

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TABLE 1. Oligodeoxynucleotide primers used for PCR cloning of viral G proteins

Virus and primer ^a	Sequence	Position ^b	Orientation
VHSV			
VG1	5'-ATGGAATGGAACACTTTTTC-3'	1-21	Sense
VGR	5'-TCAGACCGTCTGACTTCTGGA-3'	1505-1524	Antisense
IHN			
IG1	5'-ATGATCACCACTCCGCTCATT-3'	1-21	Sense
IGR	5'-CCGGTTTGCCAGGTGATACAT-3'	1492-1512	Antisense

^a Data for VHSV and IHN primers are from the work of Thiry et al. (35) and Koener et al. (18), respectively.

^b Numbering of nucleotides according to the open reading frame.

20, 27) as well as a complex of lipopolysaccharide and glycerophospholipid:cholesterol acyltransferase (GCAT), which is thought to serve as a major toxin (21).

This study dealt with the construction of an avirulent *A. salmonicida* strain which can express plasmid-encoded IHN or VHSV glycoprotein epitopes. When used as vaccines in rainbow trout, these constructs are shown to provide protective immunity against *A. salmonicida*, IHN, and VHSV. As such, they represent the first vaccines which protect against both bacterial and viral pathogens of fish.

MATERIALS AND METHODS

Virus strains and culture conditions. The rainbow trout gonad cell line RTG-2 was used for propagation of virus strains. The history of VHSV strain F13, which belongs to serogroup F1, and the growth conditions used have been described previously (9). Additional VHSV strains used were reference strains F1, He, Klappmille, Rindsholm (obtained from the European Union reference laboratory, Aarhus, Denmark), and 23.75 (obtained from P. de Kinkelin, Jouy-en-Josas, France). IHN was obtained from the Clear Springs Trout Company, Buhl, Idaho. Additional IHN strains used were WRAC, SRCV, and RB (obtained from the Hatfield Marine Science Center, Newport, Oreg.).

Bacterial strains and plasmids used. *E. coli* strains used were S17.1 (34), HB101 (6), and JM109 (47). The *A. salmonicida* strains used were MT16 (wild type) and A440 (ATCC 14174). *E. coli* strains were grown in tryptic soy broth (TSB) or Luria broth at 37°C. *A. salmonicida* was grown in TSB at 20°C. When required, agar was added to the media at a final concentration of 1.5% (wt/vol). For *E. coli*, erythromycin was used at a final concentration of 150 µg/ml. For *A. salmonicida*, erythromycin was used at a final concentration of 30 µg/ml. The vectors used in this study to express portions of the viral G-proteins were pAT18 and pAT19 (39). Cloning vectors used were pSP73 (Promega, Heidelberg, Germany) and pBluescript II (Stratagene).

Bacterial transformation and conjugation. Transformation of competent *E. coli* cells was as described by Sambrook et al. (30). Plasmid constructs containing viral DNA were mobilized from *E. coli* S17.1 to *A. salmonicida* A440 by conjugation. A 5-ml suspension of *E. coli* containing plasmids was cultured to mid-log phase (approximately 5 h) and then washed and resuspended in 20 µl of fresh TSB medium. An overnight culture of A440 cells was washed in TSB and concentrated 100-fold, and 20 µl of this was added to the *E. coli* suspension. The conjugation mixture was placed on a tryptic soy agar plate and incubated at 20°C for 3 h. The conjugation mixture was then resuspended in 1 ml of TSB, serially diluted, and plated on tryptic soy agar plates containing the appropriate antibiotics.

RT-PCR of VHSV G and IHN G genes. Prior to cDNA synthesis, virus strains were cloned by 8 endpoint dilutions in RTG-2 cells. G genes from IHN and VHSV were cloned and sequenced by using primers derived from published sequences (18, 35). Primers were synthesized on an Applied Biosystems synthesizer. For cDNA synthesis, RTG-2 cells were infected at a multiplicity of infection of 0.1. Three days after infection, total RNA was extracted following the method of Chomczynski and Sacchi (8). Single-stranded cDNA was synthesized from 5 mg of total RNA with a pair of primers (10 pmol each) shown in Table 1 and 8 U of avian myeloblastosis virus reverse transcriptase (RT) according to the protocol recommended by the supplier (Promega; 1 h at 42°C). Following dissociation of RNA-DNA hybrids at 95°C for 3 min further, 8 U of avian myeloblastosis virus RT was added to the reaction mixture for another incubation period of 1 h. Five microliters from these preparations was used for PCR. Amplification of DNA was performed in 100-µl reaction volumes, containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 1 µl of a 40 mM de-

oxynucleoside triphosphate mixture, 1 U of *Taq* polymerase (Promega), and 10 pmol each of sense and antisense primer. The temperature was raised to 95°C for 5 min. PCR amplification involved 25 cycles of 45 s at 94°C, 1 min at 55°C, and 1.5 min at 72°C. PCR products were purified from agarose gels with GeneClean (Bio 101, La Jolla, Calif.).

DNA cloning and sequencing techniques. The viral cDNA was cloned into the vector pSP73 (Promega) with T4 DNA ligase. The complete nucleotide sequences of the inserts were determined by the dideoxynucleotide sequencing method (31) with Sequenase, version 2.0 (Amersham, Braunschweig, Germany), or by automated sequence analysis with the *Taq* DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Inc.) on an Applied Biosystems model 373A automated sequencer, as recommended by the manufacturer. Small-scale plasmid preparations were performed with the Wizard Minipreps DNA purification system (Promega). Routine DNA manipulations were performed as described by Sambrook et al. (30), and restriction enzymes were utilized as recommended by the manufacturers.

Vaccination and challenge studies. Rainbow trout (*O. mykiss*), 14 cm long (approximately 30 to 40 g each), were used for vaccination trials with IHN and VHSV. For IHN, 20 to 25 fish were used for each protection study, and in the case of VHSV, 32 to 38 fish were used. *A. salmonicida* A440 containing the plasmid constructs was used for vaccination either as live or as formalin-killed (0.5% formalin for 24 h) cells. Cultures were grown overnight in TSB and then induced with 1 mM isopropyl-thio-β-D-galactoside (IPTG) for 4 h prior to vaccination. The methods of delivery used were immersion bath and spray. All vaccines were based on whole-bacterium cultures. For immersion, the vaccines were diluted 1/10 in water and fish were immersed for 30 min. Optimum dilution of vaccine and time of immersion have not been determined. For spray vaccination, the vaccines were diluted 1/10 in water and fish were sprayed for 50 s. Vaccinated fish were maintained at a water temperature of 12°C for 12 weeks (VHSV) or 8 weeks (IHN). Infection with either VHSV or IHN was carried out by cohabitation with infected rainbow trout. Relative percent survival (RPS) values were calculated according to the method of Amend (1).

In the case of vaccine trials against furunculosis, experimental fish (*O. mykiss*, approximately 10 to 14 g each) were maintained at 13°C (±1°C) in a continuous flow of dechlorinated city water. Protection assays were performed by the method of Newman and Majnarić (26), and 70 fish were used for each immunization. Fish were vaccinated by immersion in 5 × 10⁷ A440 cells in 0.85% (wt/vol) saline for 15 min with aeration. Commercial bacterin (MICROSAL; Micrologix) was administered both by immersion for 1 min in a 10% (wt/vol) solution in 0.85% (wt/vol) saline and by intraperitoneal injection. For injection, the bacterin was diluted 1/10 with 20% (vol/vol) adjuvant in 0.85% (wt/vol) saline. Vaccinated fish were challenged with *A. salmonicida* MT16 after 6 weeks by immersion with 1 × 10⁸ cells. Fish were monitored for 18 days after challenge.

SDS-PAGE and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (19). Protein samples were stacked in 4.5% acrylamide at 10 mA and then separated in 12.5% acrylamide at 20 mA. Proteins were transferred to nitrocellulose or nylon membranes. Western blotting (immunoblotting) was carried out as described previously (38) with monoclonal antibodies to the IHN G and VHS G proteins (antibodies IHN and VIIIIF6, respectively), diluted 1/1,000.

RESULTS

Vaccination against furunculosis with *A. salmonicida* A440. The *A. salmonicida* strain chosen for use as a delivery system for viral epitopes was A440. A440 has a 1,410-bp deletion in the *vapA* gene which renders it avirulent (16). To confirm that *A. salmonicida* A440 could serve as a basis for a potential anti-*A. salmonicida* vaccine strain (36) and viral antigen delivery system under the conditions used in this study, rainbow trout were vaccinated with live *A. salmonicida* A440 by immersion. After 6 weeks, the immunized fish were challenged with the heterologous virulent *A. salmonicida* strain MT16. A440 was shown to elicit protection against MT16 with a survival rate greater than 70% (Fig. 1). A440 provided greater protection than the commercially available bacterin administered by immersion, although intraperitoneally injected bacterin was more protective (Fig. 1).

Amplification of VHSV and IHN G gene RNA by RT-PCR. RNAs extracted from RTG cells 3 days after infection were subjected to RT-PCR with the primers pairs VG1-VGR and IG1-IGR (Table 1). VG1 and VGR amplify the complete G gene of VHSV beginning from the ATG start codon, whereas IG1 and IGR amplify the complete G gene of IHN. Figure 2 shows the products obtained in these reactions after separation by agarose gel electrophoresis. The IHN and VHSV G-gene

FIG. 1. Percent survival of rainbow trout (*O. mykiss*) challenged with *A. salmonicida* MT16. Column 1, control, unvaccinated fish; column 2, fish vaccinated with commercial bacterin by bath immersion; column 3, fish vaccinated with commercial bacterin by intraperitoneal injection; column 4, fish vaccinated with *A. salmonicida* A440 by bath immersion. Seventy fish were used for each treatment. Results are means for three experiments (plus standard errors). Means under horizontal lines of the same level are not significantly different. ($P > 0.05$, analysis of variance by the T method).

bands were isolated from the agarose gel and cloned into pSP73. The complete nucleotide sequence of the two clones in pSP73 was determined and compared with published sequences (GenBank accession numbers X59148 [VHS] and M16023 [IHN]) (18, 35) to confirm the nature of the PCR products (data not shown).

Generation of plasmid constructs expressing viral G-gene epitopes. The DNA sequences of the IHN and VHSV G genes were used to determine complete restriction enzyme maps of the clones. This allowed the identification of restriction enzyme sites suitable for cloning fragments of the two G genes into vectors which would allow their expression in *E. coli*

FIG. 2. Ethidium bromide-stained agarose gel showing results of RT-PCR of IHN and VHSV glycoprotein genes. Lane 1, lambda DNA cut with *Hind*III; lane 2, IHN G gene amplified with primers IG1 and IGR; lane 3, VHSV G gene amplified with primers VG1 and VGR. Numbers on the left are DNA molecular size markers, in kilobases.

FIG. 3. Construction of pATVHS and pATIHN. Vectors used are indicated on the right. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; EV, *Eco*RV; N, *Nru*I; P, *Pvu*II; Ps, *Pst*I; S, *Sma*I. *plac* is the *lacZα* promoter.

and *A. salmonicida* (Fig. 3). The vector chosen was pAT18/19, which contains the broad-host-range pAMB1 origin of replication, which allows its use in *A. salmonicida*, as well as the pUC origin of replication, which is functional in *E. coli*, and also the transfer origin of the IncP plasmid RK2. The pAT18/19 vectors also encode erythromycin resistance, which is useful for selection in both *E. coli* and *A. salmonicida*, as well as the *lacZ* promoter in front of the pUC18/19 multiple cloning site. In the case of the 1,524-bp IHN G gene, a *Bgl*II-*Pst*I fragment containing 747 bp of the G-gene region encoding the C terminus was cloned into the *Bam*HI-*Pst*I sites of pAT18. This resulted in an in-frame fusion to the *lacZα* gene on the vector (Fig. 3). In the case of the 1,521-bp VHSV G gene, the gene cloned in pSP73 was linearized with *Bam*HI, blunt ended with Klenow fragment, and then digested with *Eco*RI. The 1.5-kb band containing the VHSV G gene was then ligated into pBluescript II SK digested with *Eco*RI and *Sma*I. This resulted in an in-frame fusion with the *lacZα* gene from the vector. The 3' region of the G gene which encodes the hydrophobic C terminus was removed by digestion and religation with *Eco*RV, which left 961 bp of the VHSV G gene remaining. This new construct was then digested with *Bam*HI and *Nru*I and treated with Klenow fragment prior to religation to delete a highly hydrophobic 285-bp fragment from the region encoding the N terminus, which encodes the leader sequence. This resulting construct in pBluescript contained an open reading frame of 676 bp of the VHSV G gene cloned in frame with *lacZα*. The entire open reading frame was cloned as a 1.2-kb *Pvu*II frag-

FIG. 4. Expression of VHSV and IHNV G-protein fragments from pATVHS and pATIHN in *E. coli* JM109. Lane 1, pATVHS with no induction; lane 2, pATVHS with 1 mM IPTG; lane 3, pATIHN with no induction; lane 4, pATIHN with 1 mM IPTG. Monoclonal antibodies were diluted 1/1,000. The open arrowhead indicates a band with an apparent molecular mass of 28 kDa (pATVHS), and the solid arrowhead indicates a band with an apparent molecular mass of 25 kDa (pATIHN). Numbers on the left are molecular size markers, in kilodaltons.

ment into the *Sma*I site of pAT19. The orientation of the insert, with respect to the vector copy of *plac*, was examined by restriction analysis, and constructs in which the expression of the open reading frame was under the transcriptional control of both the *plac* promoter in the insert and the *plac* promoter in pAT19 were chosen. The constructs generated are shown in Fig. 3.

Expression of viral epitopes in *E. coli*. *E. coli* JM109 containing the plasmids was inoculated from overnight cultures into fresh Luria broth containing erythromycin (150 µg/ml) and grown at 37°C for 3 h. At this point the *plac* promoters on the plasmids were induced by the addition of 1 mM IPTG, and this was followed by incubation at 37°C for a further 4 h. The cells were then harvested by centrifugation, resuspended in loading buffer, and run on an SDS-PAGE gel. Following electrophoresis, the separated proteins were transferred to nitrocellulose and analyzed by Western blotting with monoclonal antibodies to the IHNV G and VHSV G proteins. This resulted in the identification of a band with an apparent molecular mass of 25 kDa from JM109(pATIHN) and a band with an apparent molecular mass of 28 kDa from JM109(pATVHS) (Fig. 4). These apparent molecular masses correspond to those deduced from the nucleotide sequence (24.5 and 27 kDa, respectively).

Vaccination of rainbow trout and viral challenge infections. The constructs pATIHN and pATVHS were mobilized into *A. salmonicida* A440 by conjugation. Induced exconjugant cultures were administered to fish by spraying. Since it is frequently very difficult to obtain sufficiently high levels of mortality with fingerlings in experimental waterborne challenges with IHNV and VHSV, infection of vaccinated fish was performed by cohabitation with infected rainbow trout, which also mimics the natural route of viral transmission. This challenge infection was performed 8 weeks (IHNV) and 12 weeks (VHSV) after vaccination. A440(pATIHN) gave rise to protection against IHNV; the live vaccine had an RPS of 41 and was twice as effective as the formalin-treated bacterin (Table 2). A440(pATVHS) was even more effective against VHSV infection, with live vaccine giving rise to an RPS of 50 (Table 2). Vaccination by bath immersion of rainbow trout was less protective than vaccination by spraying in the case of both IHNV and VHSV (data not shown).

DISCUSSION

This study describes the first use of an avirulent *A. salmonicida* strain both as a delivery system for the immunization of fish against two economically significant viral diseases and as a means of providing protection against an economically significant bacterial disease. In recent times, the aquaculture industry has faced a number of serious problems with both bacterial and viral pathogens. Among the pathogens most frequently

isolated in salmon hatcheries and farms, as well as from wild stocks, are *A. salmonicida* and the rhabdoviruses IHNV and VHSV (3, 5, 7, 10, 17, 23, 33, 40). In particular, this study shows that such a method of vaccination is effective against viral infection due to cohabitation with infected fish, which mimics the natural route of IHNV and VHSV transmission.

Bacterin vaccines are available for *A. salmonicida* and have been used commercially for a number of years, but these strains are ill defined. The recent characterization of a live attenuated *A. salmonicida* strain, which can be administered either by immersion or by intraperitoneal injection and provides excellent protection in trials, suggests that this approach is a viable alternative to injected bacterins (37). In this recent study, a spontaneous *A. salmonicida* mutant, 10SR, which exhibited a number of phenotypic characteristics, including a disorganized surface layer and a range of carbon metabolic defects, was isolated. This avirulent mutant was found to elicit protective immunity when used as a vaccine against furunculosis in rainbow trout (37). The choice of an *A. salmonicida* strain for use as an attenuated vaccine must be made with caution. While attenuated strains are the best means of protecting against *A. salmonicida*, a recent study has demonstrated that the disruption of the *A. salmonicida* surface layer can lead to an enhancement of virulence (28). *A. salmonicida* A449L-MB has a kanamycin resistance cassette in the *asoA* gene which disrupts the integrity of the outer surface of the cell, resulting in an increase in systemic virulence. The *A. salmonicida* strain used in this study, A440, is avirulent and affords protection against furunculosis (36). A440 is unable to synthesize the A-protein subunits of the A-layer, and PCR studies have indicated that a deletion of approximately 1,410 bp has occurred in the structural gene for the A protein, *vapA* (16). The lack of a surface layer prevents A440 from surviving the effects of the fish defence systems, and so it is unable to colonize the host tissues. Avirulent strains of *A. salmonicida* survive in the host fish for approximately 48 h after infection, after which time viable bacterial cells can no longer be isolated (37). However, this is clearly sufficient time for a protective immune response to be triggered. With the ultimate objective of a rationally designed attenuated furunculosis vaccine in mind, A440 appears to be an ideal candidate as a strain with which to begin. *A. salmonicida* possesses a number of endogenous plasmids (4), and so to avoid the possibility of complementation due to transfer of DNA between strains, it will be necessary to generate A440 strains which contain other defined mutations in genes which play a role in virulence and are preferably located in different regions of the chromosome. Likely candidates will include genes encoding previously identified proteases (20, 29), hemolysins (12, 20, 27), or other factors believed to be important for pathogenicity.

TABLE 2. Vaccination of rainbow trout against IHNV and VHSV with *A. salmonicida* A440 containing recombinant G-gene plasmids^a

Vaccine	Challenge	Mortality (%)	RPS ^b
A440(pATVHS), live	IHNV	39	NA
A440(pATIHN), live	IHNV	23	41
A440(pATIHN), inactivated	IHNV	31	20
A440(pATIHN), live	VHSV	50	NA
A440(pATVHS), live	VHSV	25	50
A440(pATVHS), inactivated	VHSV	34	32

^a Results are representative of four experiments for IHNV and three experiments for VHSV.

^b RPS = $\{1 - [\% \text{ mortality}(\text{vaccinated})/\% \text{ mortality}(\text{control})]\} \times 100$.

^c NA, not applicable.

Vaccines which offer good protection against viral pathogens combined with cost-efficient administration have not been successfully developed as yet. Because of the difficulties in treating fish infected with IHNV or VHSV, methods of disease control to date have had to rely on various strategies of disease prevention and fishery management (45). These include control of water quality, disinfection of eggs and equipment, and destruction of infected fish. The use of traditional vaccines such as killed or attenuated viruses has also raised a number of economic and epidemiological safety considerations (22). Recent studies have focused on recombinant DNA methods to express viral antigens which are capable of stimulating host immunity to the disease and are relatively inexpensive to produce by conventional fermentation technologies. The key viral protein appears to be the glycoprotein in both IHNV and VHSV. Studies have shown that recombinant IHNV G protein fragments are capable of providing protection against the virus (15, 46). When fragments of the gene were cloned and expressed in *E. coli*, effective protection was obtained, particularly with C-terminal portions of the G protein. Immersion in lysates of *E. coli* containing pXL3 (amino acids 270 to 453) was found to protect rainbow trout at RPS values of 81 to 95, depending on the viral challenge dose (46). The expression of other fragments of IHNV G in *E. coli* in this study gave rise to RPS values of 25 to 66. The level of protection in this study [RPS = 41 for live A440(pATIHNV)] cannot be directly compared with the values obtained in the study by Xu et al., as the methods of viral challenge were very different.

This study demonstrates that a recombinant VHSV G protein can provide protective immunity against VHSV infection. Lorenzen et al. expressed the VHSV G protein without its hydrophobic N-terminal leader sequence in the expression vector pCMHVG-1 and found that the purified protein was immunogenic in rainbow trout (22). The purified and renatured portion of the VHSV G protein was able to elicit VHSV-specific antibodies and neutralizing antibody activity when injected into rainbow trout. However, attempts by that group to induce antibody formation or protective immunity by immunization with bacterial cell lysates or inclusion bodies containing a truncated VHSV G protein (amino acids 225 to 507) were unsuccessful (22).

The G proteins of IHNV and VHSV are normally glycosylated, but expression in *E. coli* or *A. salmonicida* results in the production of nonglycosylated proteins. The role of the carbohydrate associated with the G proteins of IHNV and VHSV in pathogenesis or in the stimulation of an immune response is not understood at this stage. However, Lorenzen et al. have demonstrated that monoclonal antibodies to the VHSV G protein, which are able to neutralize VHSV in cell culture and in aquarium challenge conditions, are directed against carbohydrate-free determinants (22).

The levels of protection against the viral pathogens obtained with A440(pATIHNV) and A440(pATVHS) are promising at this stage. Fine-tuning of the vaccines as well as the vaccination process, including the use of adjuvants or optimization of the route of vaccination, should lead to increased levels of protection. In these studies, the induction of the *plac* promoters controlling the expression of the recombinant IHNV and VHSV G proteins in A440 was performed immediately before vaccination. However, this induction is lost immediately after vaccination, and therefore, little or no new viral antigen is synthesized by live A440 after vaccination. One obvious future improvement on this system of viral antigen delivery will be the cloning of a constitutive *A. salmonicida* promoter to control the expression of the recombinant viral G genes. Similarly, the insertion of the cloned viral epitopes into the *A. salmonicida*

chromosome under constitutive expression would increase the stability of the constructs. This would allow the continued synthesis of the G-gene proteins even after vaccination, at least when a live attenuated *A. salmonicida* strain is used as the delivery system. The levels of protection against furunculosis obtained with attenuated vaccines can be significantly improved if booster vaccinations are performed (35a), and this may also prove to be the case with regard to protection against viral diseases with cloned fragments of the IHNV and VHSV G proteins.

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