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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Viral hemorrhagic septicemia virus (VHSV) and infectious hematopoietic necrosis virus (IHNv) are important fish rhabdoviruses which cause severe epizootics among most salmonid species. 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,
TABLE 1. Oligodeoxynucleotide primers used for PCR cloning of viral G proteins

<table>
<thead>
<tr>
<th>Virus and primer</th>
<th>Sequence</th>
<th>Position</th>
<th>Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHSV</td>
<td>5'-ATGGAATGGAACACTTTTTTT-3'</td>
<td>1-21</td>
<td>Sense</td>
</tr>
<tr>
<td>VGR</td>
<td>5'-TCAGACGCTGACATCCTTGA-3'</td>
<td>1505-1524</td>
<td>Antisense</td>
</tr>
<tr>
<td>IHNV</td>
<td>5'-ATGATCCACCCGCTCATT-3'</td>
<td>1-21</td>
<td>Sense</td>
</tr>
<tr>
<td>IGR</td>
<td>5'-CGGTGGTGGAGAATCAC-3'</td>
<td>1492-1512</td>
<td>Antisense</td>
</tr>
</tbody>
</table>

- *Data for VHSV and IHNV primers are from the work of Thiry et al. (35) and Koener et al. (18), respectively.
- *Numbering of nucleotides according to the open reading frame.

20, 27) as well as a complex of lipopolysaccharide and glycerophospholidcholesterol 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 IHNV or VHSV glycoprotein epitopes. When used as vaccines in rainbow trout, these constructs are shown to provide protective immunity against A. salmonicida, IHNV, 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 gonadal cell line RTG-2 was used for propagation of virus strains. The history of VHSV strain F113, respectively.

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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 provided greater protection than the commercially available bacterin administered by immersion, although intraperitoneally injected bacterin was more protective (Fig. 1).
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 IHNV 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* and *A. salmonicida* (Fig. 3). The vector chosen was pAT18/19, which contains the broad-host-range pAMβ1 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 IHNV G gene, a BglII-PstI fragment containing 747 bp of the G-gene region encoding the C terminus was cloned into the *BamHI*-PstI 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 *BamHI*, blunted with Klenow fragment, and then digested with *EcoRI*. The 1.5-kb band containing the VHSV G gene was then ligated into pBluescript SK digested with *EcoRI* and *SmaI*. 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 *EcoRV*, which left 961 bp of the VHSV G gene remaining. This new construct was then digested with *BamHI* and *NruI* 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 *PvuII* frag.
FIG. 4. Expression of VHSV and IHNV G-protein fragments from pATVHS and pATHIN in E. coli JM109. Lane 1, pATVHS with no induction; lane 2, pATVHS with 1 mM IPTG; lane 3, pATHIN with no induction; lane 4, pATHIN 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 (pPATHIN). Numbers on the left are molecular size markers, in kilodaltons.

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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, 105R, 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 A449LMB has a kanamycin resistance cassette in the asa4 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 defense 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

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Challenge</th>
<th>Mortality (%)</th>
<th>RPS</th>
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<tbody>
<tr>
<td>A440(pATVHS), live</td>
<td>IHNV</td>
<td>39</td>
<td>NA</td>
</tr>
<tr>
<td>A440(pATHIN), live</td>
<td>IHNV</td>
<td>23</td>
<td>41</td>
</tr>
<tr>
<td>A440(pATHIN), inactivated</td>
<td>IHNV</td>
<td>31</td>
<td>20</td>
</tr>
<tr>
<td>A440(pPATHIN), live</td>
<td>VHSV</td>
<td>50</td>
<td>NA</td>
</tr>
<tr>
<td>A440(pATVHS), live</td>
<td>VHSV</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>A440(pATVHS), inactivated</td>
<td>VHSV</td>
<td>34</td>
<td>32</td>
</tr>
</tbody>
</table>

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

RPS = (1 - [%mortality(vaccinated)/%mortality(control)]) × 100.

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 pX3L (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(pATIHN)] 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(pATIHN) 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 construct. 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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