Vol. 44: 17–27, 2001 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published January 26

Recombinant vaccines against infectious hematopoietic necrosis virus: production by the *Caulobacter crescentus* S-layer protein secretion system and evaluation in laboratory trials

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ABSTRACT: We report the development of an IHNV vaccine produced by a new protein production system based on the bacterium *Caulobacter crescentus*. The subunit vaccines that were tested contain a 184 amino acid segment of the IHNV glycoprotein in different fusion arrangements with the *C. crescentus* S-layer protein. Relative percent survival of 26 to 34 % was demonstrated in rainbow trout fry for a vaccine that contained the 184 amino acid segment of the IHNV glycoprotein fused to the C-terminal one-quarter of the S-layer protein. Inclusion of the universal mammalian T-cell epitopes developed from the measles fusion protein or the tetanus toxin protein did not increase the effectiveness of the IHNV-G/S-layer recombinant protein.

KEY WORDS: Infectious hematopoietic necrosis virus · IHNV · *Caulobacter crescentus* · Fish virus · Rhabdovirus · Vaccines · S-layer · Type I secretion · T cell epitopes

INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus that produces a severe hemorrhagic disease in young salmonid fish including Atlantic salmon *Salmo salar*, rainbow trout *Oncorhynchus mykiss*, and Chinook salmon *Oncorhynchus tshawytscha*. IHNV infection of young fish, especially fry and alevins, results in massive destruction of the hematopoietic tissue, exopthalmia, and petechial hemorrhages that appear on the surface of the affected fish (Bootland & Leong 1999). In severe outbreaks, more than 90 % of the fish in a pond will die within a 2 to 3 wk period. The only control strategy available to farmers now is avoidance by destroying diseased animals and disinfecting eggs from potential carrier fish.

Vaccines for fish viruses have long been sought by the aquaculture industry to control disease outbreaks at fish rearing facilities. For trout and salmon farms, epizootics of IHNV can be devastating when they result in the loss of an entire season's production. The U.S. Trout Farmers Association has released production figures that show that commercial trout production declined in sales from $78 million in 1997 to $73.5 million in 1998 and losses increased from 6.79 to 7.84 million lbs ($3.08 to 3.39 \times 10^6$ kg) in that period. In 1998, 84 % of the trout losses were due to disease (Aquaculture Magazine Buyer's Guide '99, p. 34). The major disease-causing pathogen was IHNV. Thus, there is a critical need to develop a commercially

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viable vaccine against IHNV. Although several vaccines have been developed (Leong & Fryer 1993, Leong et al. 1997), none have been successful in field trials. The critical need for IHNV vaccines that are safe, easy to produce and administer, and inexpensive led us to examine the production of a recombinant IHNV vaccine in *Caulobacter crescentus*.

Caulobacter crescentus is a non-pathogenic gram-negative bacterium that elaborates a paracrystalline protein surface (S)-layer covering the cell surface (Smit et al. 1992). The S-layer protein monomer (RsaA) of 1026 amino acids is secreted by a Type I secretion mechanism which relies upon a C-terminal secretion signal that remains attached to the protein during the secretion process (Bingle et al. 1997a, Awram & Smit 1998). Once RsaA is secreted to the cell surface, the S-layer forms by a process of self-assembly. About 10 to 12% of the cell's protein synthetic capacity is devoted to S-layer production, and it is estimated that there are 40 000 interlinked RsaA monomers on the cell surface. Bingle et al. (1997a,b) have shown that this protein could be used to present different epitopes in large quantities on the bacterial surface or serve as a fusion vector for the secretion of the foreign protein into the culture medium. Preliminary work was reported on the secretion of a 109 amino acid segment of the IHNV surface glycoprotein (IHNV-G) fused to the last 242 amino acids of the S-layer protein (Bingle et al. 1997a). The present report examines the immunogenicity of different IHNV-G/S-layer fusion proteins in fish. It also presents some preliminary data on the effects of inserting universal mammalian T-cell epitopes into these fusion proteins as immunogens in fish.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α was used as a host for all recombinant DNA manipulations. This strain was routinely grown at 37°C for 18 to 24 h in Luria-Bertani (LB) medium. *Caulobacter crescentus* was grown at 30°C in peptone-yeast extract medium (PYE) (0.2% peptone, 0.1% yeast extract, 0.01% $CaCl_2$, 0.02% $MgSO_4$). When necessary, antibiotics were included in the media at the following concentrations: Streptomycin (Sm), 50 µg ml^{-1}, Ampicillin (Ap), 50 µg ml^{-1}, and Chloramphenicol (Cm) 20 µg ml^{-1}. Plasmid DNA was isolated from *E. coli* using the alkaline lysis method for pUC-based plasmids (Ish-Horowitz & Burke 1981) or the boiling method for RSF1010-based plasmids (Holmes & Quigley 1981). Transformation of both *E. coli* and *C. crescentus* was carried out by electroporation as previously described (Gilchrist & Smit 1991). Electrotransformants were selected on solidified medium (agar concentration 1.2% w/v) containing the appropriate antibiotic(s).

Synthesis of IHNV-G DNA and T-cell epitope DNA. DNA encoding 109 and 184 amino acid segments of the IHNV-G gene was PCR amplified with Taq DNA polymerase (Life Technologies, Burlington, ON, Canada) and a Techne PH3 thermal cycler (Mandel Scientific Co., Guelph, ON, Canada). Plasmid pG8 (Koener et al. 1987) was used as the template (Table 1). The primers were designed so that the PCR product would contain a 5′ *Xho*I restriction site and a 3′ *Stu*I site, with respect to the coding sequence and synthe-

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source
Bacterial strains		
Caulobacter crescentus		
JS4000	Spontaneous RsaA$^-$ mutant of strain CB2 (ATCC 15252). Formerly CB2A	Smit & Agabian (1984)
Escherichia coli		
DH5a	recA$^-$, endA	Gibco BRL Life Technologies
Plasmids		
pUC8	pBR322-derived cloning/*lacZ*-based expression vector; Ap	Vieira & Messing (1982)
pKT215	RSF1010-derived cloning vector; Cm, Sm	Bagdasarian et al. (1981)
pWB9KSAC	pKT215-derived expression vector incorporating the *rsaA* promoter; Cm, Sm	Bingle et al. (1997a)
pUC9CXS	pUC9 with a modified multiple cloning site. Ap, Cm	Bingle et al. (1997a)
pUC8: *rsaA*242C	pUC8 carrying a 1.3 kb *Bam*HI/*Hind*III fragment encoding the final 242 C-terminal amino acids of RsaA, Ap	Bingle et al. (1997a)
pUC8: *rsaA*336C	pUC8 carrying a 1.6 kb *Bam*HI/*Hind*III fragment encoding the final 336 C-terminal amino acids of RsaA, Ap	Bingle et al. (2000)
pWB9KSAC:*rsaA*ΔP (*Msp*I69*Bam*HI)	*rsaA*ΔP carrying a *Bam*HI linker insertion at a site corresponding to amino acid 69 of RsaA; Cm, Sm	Bingle et al. (1997b)
pG8	Source of IHNV G-protein gene	Xu et al. (1991)

sized at the Nucleic Acid/Protein Service (NAPS) Unit at the University of British Columbia. The PCR products were digested with *Xho*I and *Stu*I (Life Technologies) and purified by agarose gel electrophoresis (Sambrook et al. 1989) and a QIAEX II agarose gel extraction kit (Qiagen Inc., Chatsworth, CA, USA). The gel-purified fragments were then ligated into digested and gel purified pUC9CXS (Fig. 1a) with T4 DNA ligase (Life Technologies) by standard methods (Sambrook et al. 1989). Because the 184-amino acid segment of IHNV-G contains an internal *Stu*I site, it was cloned as a partial digest.

Oligonucleotides encoding 2 different T-cell epitopes were designed and synthesized (NAPS) such that upon annealing of the complemetary oligonucleotides, the coding dsDNA would have termini compatible with *Xho*I and *Stu*I sites. The oligonucleotides for the tetanus toxin T-cell epitope (amino acid, aa 830–844 encoding the P2 sequence QYIKANSKFIGITEL) (Valmori et al. 1992) were 5′-TCGACAGTACATCAAGGCCAACTCGAAGTTCATCGGCATCACCGAGCTGG-3′ and 5′-CCAGCTCGGTGATGCCGATGAAGTTCGAGTTGGCCTTGATCTACTG-3′. The oligonucleotides for the measles F protein T-cell epitope (aa 288–302

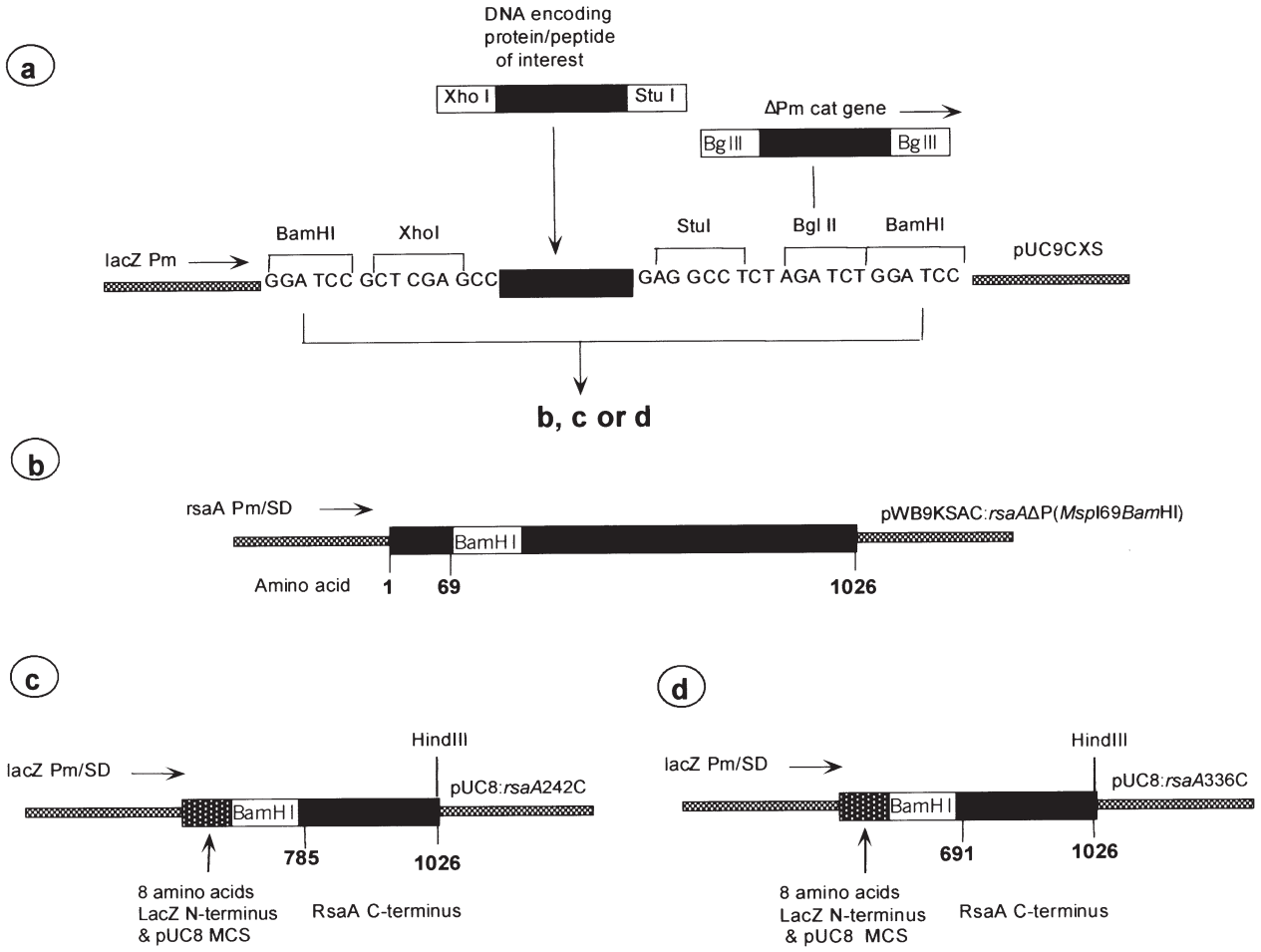

Fig. 1. Creation of RsaA fusion proteins. (a) To provide DNA encoding a heterologous protein with *Bam*HI termini for fusion to *rsaA*, the DNA of interest is first passed through pUC9CXS, a pUC9 derivative carrying a modified multiple cloning site and a promoterless chloramphenicol resistance ($Cm^r\Delta P$) gene (Bingle et al. 1997a). The polylinker encodes 5 restriction sites: *Bam*HI, *Xho*I, *Sal*I, *Stu*I, and *Bgl*II. The $Cm^r\Delta P$ gene was inserted into the *Bgl*II site so that its expression is directed by the *lacZ*a promoter (Pm). The DNA of interest is provided with *Xho*I/*Stu*I termini and inserted into the *Xho*I/*Stu*I sites of pUC9CXS in the same orientation as the $Cm^r\Delta P$ gene. It is then excised as a *Bam*HI fragment and ligated into the *Bam*HI linker site of the appropriate *rsaA* fusion plasmid (b, c or d). The $Cm^r\Delta P$ gene is used to select for insertion events with the correct orientation. Afterwards, the $Cm^r\Delta P$ gene is removed using *Bgl*II, and the plasmid sealed. (b) pWB9KSAC:*rsaA*ΔP(*Msp*I69*Bam*HI), a pKT215 derived vector with the *rsaA*ΔP carrying a *Bam*HI linker insertion at a site corresponding to amino acid 69 of RsaA. (c) pUC8:*rsaA*242C, pUC8 carrying a 1.3 kb *Bam*HI/*Hind*III fragment encoding the final 242 C-terminal amino acids of RsaA. (d) pUC8:*rsaA*336C, pUC8 carrying a 1.6 kb *Bam*HI/*Hind*III fragment encoding the final 336 C-terminal amino acids of RsaA. For introduction into *Caulobacter crescentus*, the pUC based plasmids (c or d) carrying the DNA of interest is fused to broad-host-range vector pKT215 at their common *Hind*III sites. Bold numbers indicate the amino acid position relative to full-length RsaA

encoding the MVF sequence LSEIKGVIVIHRLEGV) (Panina-Bordignon et al. 1989) were 5′-TCGAGCCC-TGTCGGAGATCAAGGGCGTCATCGTCCACCGCC TGGAGGGCGTCG-3′ and 5′-CGACGCCCTCCAGG-CGGTGGACGATGACGCCCTTGATCTCCGACAGG-GC-3′. The annealed oligonucleotides were inserted into *Xho*I/*Stu*I digested pUC9CXS as described above.

Fusion protein construction. The insertion of IHNV-G DNA into pUC9CXS (above) provided the fragments with *Bam*HI termini and a *cat* gene tag. The gene *rsaA*, which encodes the RsaA protein, has been cloned and earlier reports have described the in-frame insertion of *Bam*HI linkers at various sites in a promoterless *rsaA* gene (*rsaA*ΔP) (Bingle et al. 1997a,b). Several of these *Bam*HI sites were used to receive the IHNV-G/T-cell DNA. The *cat* gene tag was included to select for insertion events as well as monitor the orientation of the inserted IHNV-G DNA (Bingle et al. 1997a,b); following insertion of the IHNV-G/T-cell DNA into an *rsaA Bam*HI site, the *cat* gene tag was removed.

To create 'full-length fusion proteins', *Bam*HI fragments carrying IHNV-G DNA and a *cat* gene tag were excised from plasmid pUC9CXS (Fig. 1a), gel purified and inserted into the *Bam*HI site of pWB9KSAC: *rsaA*ΔP(*Msp*I69*Bam*HI) (Table 1, Fig. 1b), corresponding to an insertion at aa 69 of the full-length RsaA protein. In this construct, expression was driven by the rsaA promoter.

In addition to full-length RsaA, truncated versions of the *rsaA*ΔP gene have been created, encoding 242 and 336 C-terminal portions of RsaA (Bingle et al. 1997b, 2000); these truncated genes were inserted into pUC8 forming pUC8:*rsaA*242C and pUC8:*rsaA*336C (Table 1, Fig. 1c,d). In these constructs, synthesis of the C-terminal fragments of RsaA was driven by *Escherichia coli lac* transcription and translation initiation. As with the 'full-length' *rsaA*ΔP, DNA encoding the IHNV-G segments or the T-cell epitopes, each tagged with the *cat* gene was released from pUC9CXS by *Bam*HI digestion and inserted into the *Bam*HI site of pUC8: *rsaA*242C or pUC8:*rsaA*336C. After receiving IHNV-G and/or T-cell epitope inserts, each of the pUC8-based plasmids was ligated to pKT215 via their common *Hind*III sites for introduction into *Caulobacter crescentus* (Bingle et al. 1997a, 2000). Fusion proteins composed of an N-terminal IHNV-G/T-cell epitope portion and a C-terminal RsaA portion are referred to as 'C-terminal fusion proteins'.

The use of pUC9CXS allowed the creation of '*Bam*HI modules' that could be placed in any order at any single *Bam*HI site. After insertion of one *Bam*HI module into an *rsaA Bam*HI site, the *cat* gene was removed leaving behind a single *Bgl*II site (Fig. 1). Because of the compatibility between the cohesive ends of *Bgl*II and *Bam*HI, this *Bgl*II site was used to repeatedly receive additional *Bam*HI fragments carrying an internal *Bgl*II site. This approach was used to construct RsaA fusion proteins carrying several IHNV-G sequences and/or T-cell epitopes (Fig. 2). The construction of all plasmids was confirmed by DNA sequence analysis (NAPS).

Recovery of aggregated fusion proteins from the growth medium. Plasmids carrying recombinant genes encoding fusion proteins were introduced into *Caulobacter crescentus* JS4000 via electroporation (Gilchrist & Smit 1991). Electrotransformants were selected on solidified PYE containing Sm (50 µg ml^{-1}). Colonies were transferred to 5 ml liquid PYE medium containing 2 µg ml^{-1} of Cm (Bingle & Smit 1990) and grown overnight at 30°C in a rotary shaker in 5 ml of PYE medium. The entire 5 ml culture was then transferred to a 2800 ml Fernbach flask with 1275 ml of M_{11} HIGG medium, a modification of M_6 HIGG medium (Smit et al. 1981) containing 5 mM imidazole; 2 mM potassium phosphate, 0.15% glucose, 0.15% L-glutamate (monosodium salt), 1% modified Hunter's mineral base and 0.58 mM $CaCl_2$, and grown for a further 72 to 96 h on a G3 gyratory shaker (New Brunswick Scientific, New Brunswick, NJ) at 55 rpm. The aggregated fusion proteins were recovered from culture medium by sieving through nylon mesh (diagonal pore dimension 350 µm) draped over a 12.5 cm diameter Buchner funnel (Bingle et al. 1997a). The aggregates were rinsed with water and collected for either SDS-PAGE or vaccine formulation.

Yields of aggregated fusion proteins for 6 of the vaccine candidates were estimated in a similar manner. Aggregates were recovered from the culture fluids by filtration of each 1275 ml culture through a 4.5 cm diameter nylon mesh disk housed in a filtration apparatus with an O-ring seal. The aggregated protein which collected on the nylon mesh was washed 3 times with water by centrifugation (about 1 min, 12 000 × *g*), lyophilized and the dry weight of the fusion proteins was determined (Table 2).

SDS-PAGE and Western immunoblot analysis. Protein samples were solubilized by mixing with equal amounts of 8 M urea in 100 mM Tris-HCl, pH 8.0, followed by the addition of sample loading buffer. The samples were then analyzed on a 12% polyacrylamide gel by the discontinuous gel method of Laemmli (1970). Two identical gels were prepared. One gel was stained for total protein in Gel-Code Blue reagent (Pierce Chemical Inc., Rockford, IL, USA) and dried on cellulose film. The dried gel was scanned with a Molecular Dynamics Personal Densitometer (Molecular Dynamics, Sunnyvale, CA, USA). Molecular weights of the protein bands were calculated with FragmeNT Analysis version 1.1 (Molecular Dynamics) (Fig. 3a). The proteins from the second gel

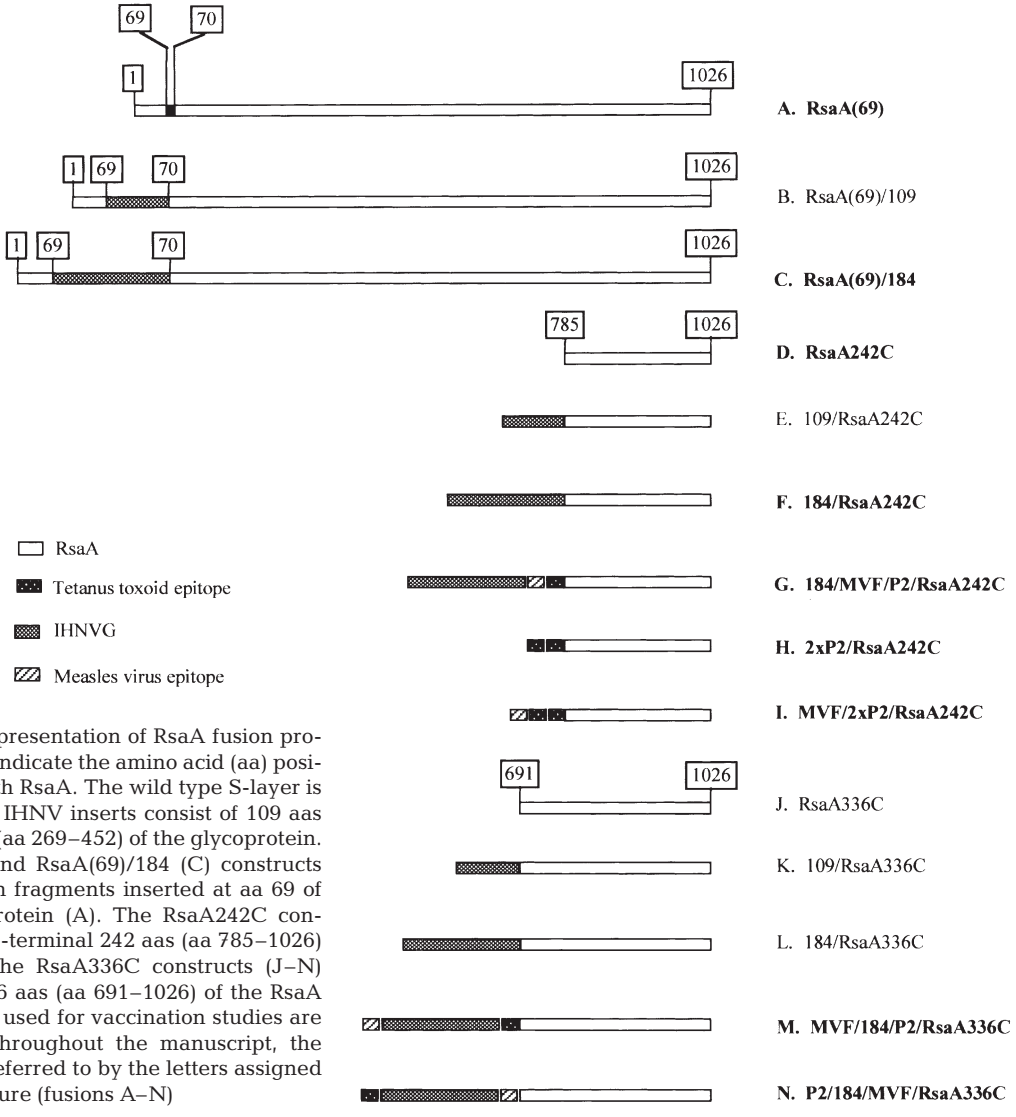

Fig. 2. Diagrammatic representation of RsaA fusion proteins. Numbered boxes indicate the amino acid (aa) position relative to full-length RsaA. The wild type S-layer is 1026 aas in length. The IHNV inserts consist of 109 aas (aa 335–443) or 184 aas (aa 269–452) of the glycoprotein. The RsaA(69)/109 (B) and RsaA(69)/184 (C) constructs have IHNV glycoprotein fragments inserted at aa 69 of the full length RsaA protein (A). The RsaA242C constructs (D–I) carry the C-terminal 242 aas (aa 785–1026) of the RsaA protein. The RsaA336C constructs (J–N) carry the C-terminal 336 aas (aa 691–1026) of the RsaA protein. Fusion proteins used for vaccination studies are shown in bold type. Throughout the manuscript, the fusion proteins will be referred to by the letters assigned in this figure (fusions A–N)

were electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane for immunoblotting. Following transfer, the membrane was blocked with Tris-buffered saline plus 2% BSA and 0.1% Tween-20 (TBBT). The blot was probed with polyclonal rabbit anti-IHNV at a 1:500 dilution in TBBT for 2 h and washed with TBBT. FITC-conjugated polyclonal goat anti-rabbit antibody (Sigma-Aldrich, St. Louis, MO, USA) was applied to the membrane at a 1:2500 dilution in TBBT for 2 h, washed in TBBT and scanned on the FMBioII fluorescence imaging system (Hitachi Software Engineering America Ltd., San Bruno, CA, USA) (Fig. 3b).

Formulation of vaccines. For the killed IHNV vaccine, type 1 IHN virus was harvested from infected epithelioma papulosum cyprini (EPC) cells and inactivated with 0.01 M binary ethyleneimine for 18 h at

Table 2. Yield (mg dry wt l^{-1} of culture) of various RsaA:IHNVG fusion protein aggregates from *Caulobacter crescentus* culture supernatants. Fusion protein letters A–L are defined in Fig. 2. Standard error (SE) is calculated as SE = standard deviation/$n^{0.5}$. NA: not applicable

Fusion	Average	SE	Passenger	
			mg l^{-1}	mol l^{-1}
Full length fusions (rsaA promoter)				
A	245	16	NA	NA
B	117	4	11	1.0
C	57	3	9	0.5
C-terminal fusions (lacZ promoter)				
D	48	21	NA	NA
E	86	22	25	2.3
F	54	7	22	1.2
J	261	32	NA	NA
K	95	17	22	2.0
L	35	14	12	0.7

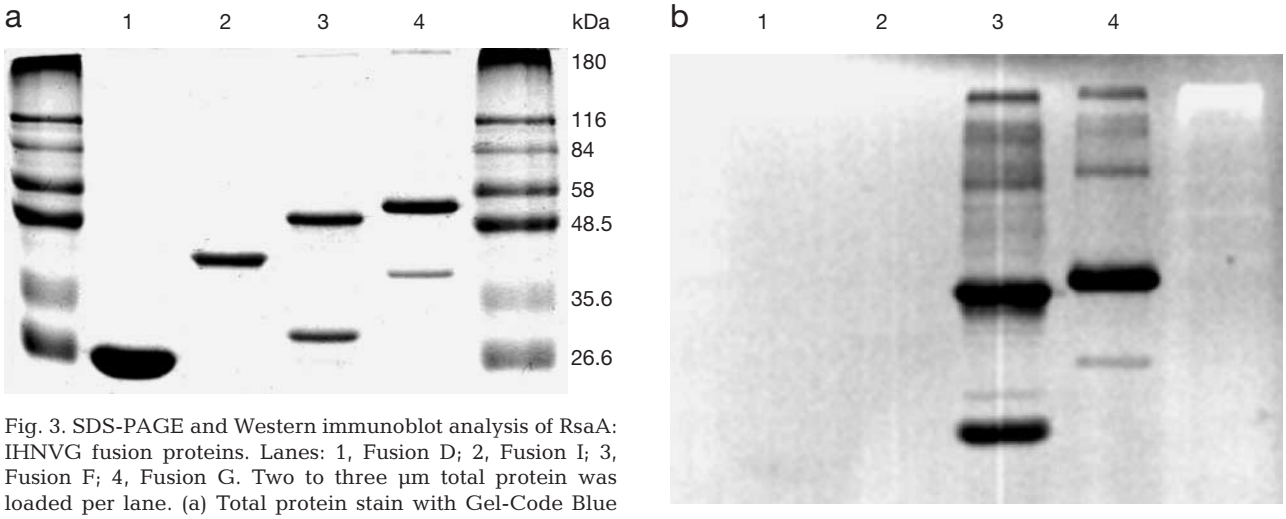

Fig. 3. SDS-PAGE and Western immunoblot analysis of RsaA: IHNVG fusion proteins. Lanes: 1, Fusion D; 2, Fusion I; 3, Fusion F; 4, Fusion G. Two to three µm total protein was loaded per lane. (a) Total protein stain with Gel-Code Blue (Pierce). (b) Immunoblot. Primary antibody was polyclonal rabbit anti-IHNV that had been preabsorbed with lysed *Caulobacter crescentus* cells. Secondary antibody was FITC conjugated goat anti-rabbit

25°C. Inactivated virus was emulsified with 4 parts water and 1 part proprietary mineral oil adjuvant (Microtek, Victoria, BC, Canada) to a final concentration of approximately 3×10^6 tissue culture infective dose ($TCID_{50}$) per ml or 1.5×10^5 $TCID_{50}$ per 50 µl vaccine dose.

Recombinant protein aggregates were washed twice with sterile distilled water and then solubilized with an equal volume of sterile 8 M urea. After overnight dialysis (8000 molecular weight, MW, cutoff) against sterile distilled water, the solubilized proteins were filter sterilized through a 0.45 µm filter. The proteins were then emulsified with water and adjuvant (Microtek, Victoria, BC, Canada) to a concentration of 10 pmol per dose (calculated from apparent MW of the recombinant protein on SDS-PAGE gels) corresponding to 5.6 to 27 µg ml^{-1}.

The fusion H-Alhydrogel® for the IHNV + H vaccine was prepared in a similar manner, except the protein was mixed with Alhydrogel (Cedar Lane Laboratories, Toronto, Canada) at a ratio of 20% (v/v) before emulsion with adjuvant and water.

Production of IHNV challenge virus. Chinook salmon embryo (CHSE-214) cells were maintained as previously described (Engelking & Leong 1981) and used to grow IHNV. The type 2 IHNV used for challenge was isolated from rainbow trout at the Rangen Research Laboratories, Idaho, USA (Hsu et al. 1986, Drolet et al. 1993). Virus was propagated at a multiplicity of infection of 0.01 at 17°C for 7 d. Then the culture supernatant was collected and centrifuged at $2500 \times g$ for 10 min at 4°C to remove cell debris. The clarified supernatant was distributed into 13-ml tubes and frozen at –80°C. The viral titer was estimated by determining the plaque titer for a tube of frozen virus (Burke & Mulcahy 1980).

Fish immunization and challenge. Rainbow trout fry were held in aquaria supplied with 12°C specific pathogen-free well water (2.2 l min^{-1}) at the Oregon State University Center for Salmon Disease Research. The fish were held in a room where photoperiod was controlled by sunlight and all of the experiments were carried out from January through August. All fish were fed ad libitum for the entire experiment. The fish with an average mass of 0.3 to 0.5 g were anesthesized by immersion in 50 to 100 ppm tricaine-methanesulphonate (MS-222) and vaccinated by intraperitoneal injection as previously described (Engelking et. al. 1991). Each fish received a dose of approximately 10 pmol of recombinant protein in a volume of 50 µl in the first trial, and in subsequent trials the injection volume was reduced to 25 µl but the dose was held constant at approximately 10 pmol of protein. After vaccination the fish were held for 30 d in 12°C specific pathogen-free well water. Holding densities during this immune development period was approximately 0.01 to 0.02 kg m^{-3}. At the end of the 30 d period, the fish were distributed into replicate tanks so that replicates within a treatment contained the same number of fish per tank. The number of fish per replicate group ranged from 30 to 50. Fish were then exposed to 10^4 or 10^5 plaque-forming units, PFU, IHNV ml^{-1} in static water for a period of 5 h, at a density of 50 fish per 2 l of water. Water flow was restored after 5 h and mortalities were recorded daily for 30 d after virus exposure.

Relative percent survival (RPS) was calculated using the following formula (Ellis 1988):

$$RPS = 1 - \frac{\%\ \text{vaccinated mortality}}{\%\ \text{control mortality}} \times 100$$

Statistical methods. Cumulative mortality was recorded for each replicate tank at 30 d post challenge. Data for each trial were first tested for extra-binomial variation between replicates using Fisher's exact test (Ramsey & Schafer 1997) to determine if use of the standard binomial model was adequate. Where appropriate, the replicates within each treatment were pooled for calculation of a p-value using Pearson's chi-squared analysis (Ramsey & Schafer 1997) and pair-wise comparisons were made between all groups. Otherwise, quasi-likelihood analysis was employed to compensate for the extra-binomial variation between replicate tanks (Ramsey & Schafer 1997).

Fisher's exact and quasi-likelihood analyses were performed using Statistical Analysis Systems for Windows, release 8.0 (SAS Inc., Cary, NC, USA). Pearson's chi-squared analysis was performed using Microsoft Excel 97 (Microsoft Corp., Bellvue, WA, USA).

RESULTS AND DISCUSSION

A subunit vaccine for IHNV consisting of the viral glycoprotein produced from purified virus (Engelking & Leong 1989) or as a recombinant protein in *Escherichia coli* (Gilmore et al. 1988, Xu et al. 1991) had been shown previously to be highly effective as a vaccine in rainbow trout. The recombinant protein contained a sequence of 184 aas of IHNV G protein from aa 270 to 453 fused to tryptophan synthetase. Although highly effective in laboratory trials where only small quantities of the vaccine were required, the vaccine produced for large field trials was inconsistent. Efforts to produce the *E. coli* vaccine in large fermentation batches resulted in the unsatisfactory formation of IHNV-G-TrpE fusion protein inclusion bodies in the bacteria. The work did identify an immunodominant domain that was reactive with neutralizing monoclonal antibody (Xu et al. 1991) and induced protective immunity in fish vaccinated with a 109 amino acid segment (aa 335 to 443) of the viral G protein.

The laboratory success of the *Escherichia coli* vaccine indicated that a recombinant protein would be an effective vaccine for IHNV, if produced in an appropriate expression system. The secretion of proteins fused to the S-layer protein of *Caulobacter crescentus* offered an attractive alternative for the production of the IHNV vaccine. Several plasmids were constructed containing the S-layer protein gene, *rsaA*, or a fragment of that gene, fused to the 109 or 184 aa region of the IHNV G protein gene (Fig. 2). These fusion proteins were examined as possible vaccine candidates.

Yield of aggregated fusion proteins

Earlier reports on the production of the IHNV-G 109 aa segment fused to the 242 aa C-terminal end of RsaA (Fusion E) indicated that the fusion protein was secreted from *Caulobacter crescentus* at concentrations as high as 10% of the total protein in stationary phase culture (Bingle et al. 1997a). The current study, aimed to determine if an increase in passenger protein yield could be brought about by using more of the RsaA C-terminus (336 aas) or even full length RsaA itself.

When the IHNV-G sequences, either encoding the 109 or 184 aa insert, were fused to the full-length RsaA gene near its C-terminus (Fusions B and C), a reduction in the amount of aggregated protein recovered from the culture fluids as compared to Fusion A was observed (Table 2). The size of the IHNV-G insert had an impact on the reduction; when the size of the insert was increased by approximately 2-fold from 109 to 184 aas, the yield of aggregated fusion protein dropped by 50%. This relationship meant that the yield of the IHNV-G portion of the fusion protein remained constant when expressed in terms of dry weight. However, on a molar basis twice as much of Fusion B was recovered from the culture fluids as compared to Fusion C.

In comparison to the fusion proteins with full-length RsaA, the yield produced with only the C-terminus of RsaA (Fusions D, E, F, J, K, and L) exhibited considerable variation between production batches. This was probably due to the fact that the reduced amount of RsaA in the fusion protein made it less capable of aggregating. Despite this, the yields of aggregated RsaA336C fusion proteins (Fusions J, K, and L) exhibited the same general trend, i.e. insertions decreased production and the larger 184 aa insert had a greater effect on the reduction of fusion protein production. Fusions D, E, and F showed a different yield pattern. The addition of the IHNV-G insert either had little effect on the yield of aggregated protein on a weight basis (Fusion F) or led to an increase in yield (Fusion E). The molar yield of Fusion E, however, was approximately 2 times that of Fusion F (Table 2).

We also tested the use of smaller portions of the RsaA C-terminus (166, 134, 119 and 82 aas) for the secretion of the 109 and 184 amino acid INHV-G segments using a suite of plasmids similar in design to those used here (see Bingle et al. 2000). These experiments showed that while both of the IHNV-G segments could be recovered in aggregated form from the growth medium when fused to the C-terminal 166, 134, and 119 amino acids of RsaA, yields were much lower than those obtained using the 242 and 336 amino acid portions of RsaA. Thus, the use of these smaller constructs for vaccine production was abandoned.

SDS-PAGE and immunoblot analysis of RsaA:IHNV-G fusion proteins

An analysis of the different fusion proteins produced in this study indicated that the fusion proteins were secreted into the culture medium as nearly pure protein aggregates (Fig. 3). Fusion D migrated as a 26 kDa protein (Fig. 3a, lane 1). Fusion I containing the C-terminal 242 aas of RsaA plus 2 copies of the tetanus toxin and 1 copy of the measles virus F protein universal T-cell epitopes migrated at 43 kDa (Fig. 3a, lane 2). The Fusion F preparation showed 2 proteins: one migrating at 50 kDa and another migrating at 30 kDa (Fig. 3a, lane 3). The 30 kDa protein consisted of the RsaA C-terminus bearing a short N-terminal extension consisting of 31 IHNV-G aas (31/RsaA242C). A similar protein bearing 21 IHNV-G aas (21/RsaA242C) was found in a previous study in which the secretion of Fusion E was evaluated (Bingle et al. 1997a). It was hypothesized that 21/RsaA242C was either a proteolytic cleavage product of the RsaA:IHNV-G fusion protein or possibly an internal translation initiation product originating in the IHNV-G sequence. The possibility of internal translation initiation was an attractive hypothesis because the N-terminal aa of both 21/RsaA242C and 31/RsaA242C was encoded immediately after the same methionine residue and an IHNV-G-derived N terminal cleavage product was not found in the aggregated protein recovered from the culture fluids. Resolution of this issue was important in the present context because proteolytic cleavage of RsaA fusion proteins would complicate the use of the *Caulobacter crescentus* S-layer protein secretion system.

To test the idea that the 31/RsaA242C was derived from internal translation initiation, pUC8:*rsaA*242C (carrying the 184 aa sequence of the IHNV-G DNA inserted at its *Bam*HI site) was digested with *EcoRI* (Fig. 1). The resulting termini were polished with Klenow fragment of DNA polymerase I (New England Biolabs, Mississauga, ON, Canada) and the plasmid was circularized by ligating the blunt ends together. The result of these manipulations was to introduce a +1 reading frameshift so the RsaA:IHNV-G fusion sequence could not be translated from the *lacZ* ribosome-binding site. When this modified plasmid was fused to pKT215 and introduced into *Caulobacter crescentus*, an aggregated protein was produced and found to be composed entirely of 31/RsaA242C (unpubl.). This result cannot be explained by proteolytic cleavage but is consistent with internal translation initiation. Similar results were obtained when plasmid, pUC8:*rsaA*336, carrying the 184 IHNV-G *Bam*HI DNA insertion was modified as described above. In this case, the internal translation initiation product possessed a higher molecular mass because a larger portion of the RsaA C-terminus was used in the constructed plasmid.

SDS-PAGE analysis of Fusion G also yielded 2 protein bands of 53 and 43 kDa (Fig. 3a, lane 4). The 43 kDa band can also be explained by internal translation initiation in the IHNV-G portion of the fusion protein. Its larger molecular mass can be accounted for with the additional MVF and P2 sequences.

When an identical gel, containing the same samples as shown in Fig. 3a, was blotted and probed with antibody to IHNV, only those proteins carrying IHNV-G sequences demonstrated immunoreactivity (Fig. 3b). The immunoblot revealed some higher molecular mass species not readily seen on the stained gels. The apparent molecular masses of these proteins suggested that they could be dimers. Although RsaA possesses no cysteine residues, the 184 aa IHNV-G segment does possess one such residue, which could potentially react with another cysteine residue to produce a disulfide-linked dimer. This was confirmed by omitting β-mercaptoethanol from the SDS-PAGE sample-loading buffer. In this case, the higher molecular mass bands were much more prominent when the reducing agent was omitted (data not shown). Thus the protein preparations consisted of, at least, monomeric and dimeric F fusion protein, as well as RsaA242C carrying 31 aas of IHNV-G resulting from internal translation initiation. Although only Fusions D, F, G, and I are shown in Fig. 3, the functional construction of each fusion protein used in this report was verified by SDS-PAGE and immunoblot analysis.

Evaluation of the fusion proteins as potential vaccines

A number of different RsaA:IHNV-G fusion proteins were evaluated in laboratory trials as vaccines against IHNV. Rainbow trout fry with an average mass of 0.3 to 0.5 g were used for this trial because larger fish do not die from virus infection at the levels required to assess protection. As the fish age, they become more resistant to the lethal effects of IHNV infection. Also, numerous experiments have shown that fish can be protected by vaccination against IHNV at 0.4 to 1 g (Anderson et al. 1996). Early experiments indicated that we should concentrate our efforts on the RsaA:IHNV-G fusion proteins containing the 184 aa segment of the viral glycoprotein. The fusion proteins described in Table 3 were evaluated over the course of 3 laboratory trials. The trials were extensive and for every trial, experimental samples were run in replicate tanks of 30 to 50 fish per tank at virus challenge doses of 10^4 to 10^5 infectious doses ml^{-1} of water (diluted from a virus stock of 2×10^8 PFU ml^{-1}, as determined by plaque assay) (Burke & Mulcahy 1980). This is a high chal-

Table 3. Relative percent survival (RPS) and cumulative percent mortality (CPM) after IHNV challenge. RPS is calculated as RPS = [1 – (percent vaccinated mortality/percent control mortality)] × 100 (Ellis 1988). In Trials 1 and 2, RPS was calculated relative to the adjuvant alone controls. For Trial 3, RPS was calculated relative to the PBS injected controls. Replicate tanks for each treatment group were run in duplicate for Trial 1 and triplicate for Trials 2 and 3 with 30 to 50 fish per replicate tank. Letters A–N refer to the fusion protein codes in Fig. 2. Standard error is calculated as SE = standard deviation/$n^{0.5}$

Treatment	Trial 1		Trial 2		Trial 3	
	CPM ± SE	RPS	CPM ± SE	RPS	CPM ± SE	RPS
Killed IHNV	53.4 ± 1.1	24.2	–	–		–
Killed IHNV + H	44.0 ± 1.2	37.5	–	–	–	–
A	69.6 ± 14.1	1.2	–	–	–	–
C	66.3 ± 1.1	5.9	–	–	–	–
C + H	69.7 ± 5.8	1.2	–	–	–	–
D	–	–	–	–	34.0 ± 2.0	12.1
F	46.4 ± 5.2	34.2	38.6 ± 11.3	31.5	28.7 ± 1.8	25.9
F + H	46.2 ± 3.8	34.4	41.2 ± 12.7	26.9	–	–
G	–	–	–	–	29.3 ± 2.9	24.1
I	–	–	–	–	38.7 ± 6.7	0.0
M	–	–	43.1 ± 11.3	23.6	–	–
N	–	–	40.6 ± 8.6	28.1	–	–
PBS	–	–	53.7 ± 3.1	4.8	38.7 ± 5.3	0.0
Adjuvant alone	70.5 ± 7.3	0	56.4 ± 3.8	0.0		–

lenge dose since virus titers reached at the height of an epizootic in holding ponds are rarely above 0.02 to 0.2 PFU ml^{-1} (Watanabe et al. 1988). The trials were designed to test the recombinant vaccines under the most stringent conditions. Adjuvant alone or PBS was used as the negative control in each trial. A control vaccine consisting of adjuvant plus killed IHNV was also included in the study. The data is presented as cumulative percent mortality (CPM) and RPS in Table 3 and this permits some comparison between the different trials. The data from Trial 1 is also shown in Fig. 4 as cumulative percent mortality for each replicated treatment.

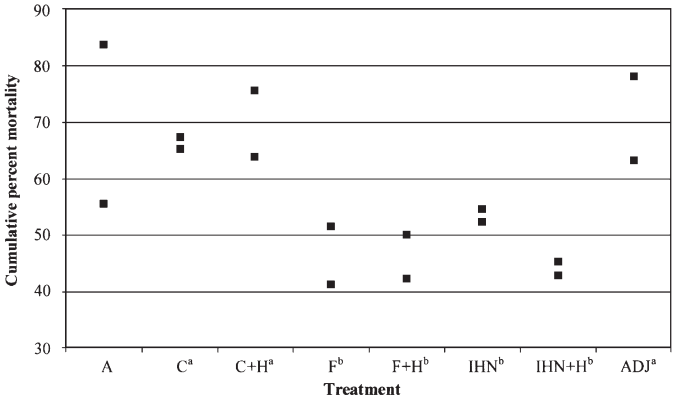

Fig. 4. Cumulative percent mortality at 30 d post challenge. IHNV challenge titer = 10^4 PFU ml^{-1}. Groups labeled with the same superscript were not significantly different from each other. The Fusion A treatment group showed evidence of extra-binomial variation and was therefore excluded from this analysis. ADJ: adjuvant

The level of protection afforded by vaccination with *Caulobacter crescentus* synthesized IHNV-G/S fusion protein was limited. The differences we observed between vaccinated and unvaccinated treatment groups were statistically significant in Trial 1 only (Fig. 4). In the first trial there was little evidence of extra-binomial variation, except in the Fusion A treatment group; therefore, this treatment group was not examined further. Of the remaining groups, F, F+H, killed IHNV (IHN), and killed IHNV+H (IHN+H) were significantly different from the adjuvant alone (ADJ) control ($p \leq 0.05$) (Fig. 4). Extra-binomial variation was evident in the second trial and quasi-likelihood analysis did not show significant treatment effects. The third trial showed no evidence of extra-binomial variation; however, Pearson's chi-squared analysis did not reveal significant treatment effects after pooling of replicates.

In all 3 trials, the adjuvant alone or PBS negative control fish reached cumulative mortalities ranging from 38.7 to 70.5% (Table 3). The killed IHNV in ADJ (positive control) provided some protection (RPS 24.2%) in Trial 1 (Table 3). In all 3 trials, the most efficacious recombinant vaccine was Fusion F: Trial 1, RPS 34.2% ($p = 0.004$); Trial 2, RPS 31.5%; and Trial 3, RPS 25.9% (Table 3). The inclusion of the universal mammalian T-cell epitopes did not seem to increase the protective immunity induced by the IHNV-G/RsaA fusion proteins G, M or N. Rearrangement of the fusion sequence did not have any effect and may have even decreased the immunoprotective capacity of Fusions M and N. These studies also show that construction of the fusion protein is important since insertion into a complete *rsaA* gene did not produce an IHNV-G

fusion protein capable of inducing protective immunity in trout.

Universal or 'promiscuous' T-cell epitopes that enhance the immunogenicity and overcome genetic restriction of the immune response have received considerable attention recently as vaccine immunopotentiators. In particular, the human T-cell epitopes for measles virus fusion protein and for tetanus toxin have been used successfully to augment peptide vaccines for mouse mammary tumor virus (Astori & Kraehenbuhl 1996) and Human T-lymphotropic virus (Kaumaya et al. 1993, Lairmore et al 1995). When the universal T-cell epitopes for tetanus toxin and measles virus fusion protein were tested as enhancers in fish for the recombinant protein IHNV vaccines, we found no augmentation of protection. The only effect we observed was with Fusion H suspended in Alhydrogel and mixed with the killed IHNV vaccine in oil-in-water adjuvant (Table 3, Fig. 4), the observed protection increased from 24.2% RPS (killed IHNV) to 37.5% RPS (killed IHNV + H) ($p = 0.22$). The significance of this finding is unclear and must be repeated with other universal T-cell epitopes and with different killed/peptide vaccines.

These studies suggest *Caulobacter crescentus* mediated production of viral proteins for vaccines may be an alternative to *Escherichia coli* and yeast protein production. Studies are continuing on different fusion protein constructs with the entire IHNV-G gene sequence at the N-terminus of the S-layer protein. Also, the identification of fish specific T-cell epitopes has been initiated.

Acknowledgements. We thank Clifford Pereira for his assistance with the statistical analysis of the data. The research was supported in part by the US Department of Agriculture (the Western Regional Aquaculture Center) (grant 92-38500-7195, project no. 92080441), by an Oregon Sea Grant with funds from the Office of Sea Grant, National Oceanic and Atmospheric Administration, Department of Commerce (grant NA89 AA-D-SG108, project R/FSD-16; grant NA36RG451, project R/FSD-23, and amendment no. 2), and by the Canadian National Science and Engineering Research Council Technology Partnership Program. Oregon Agriculture Experiment Station Technical Paper 11718.

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Editorial responsibility: Carey Cunningham, Aberdeen, Scotland, UK

Submitted: March 15, 2000; Accepted: October 16, 2000
Proofs received from author(s): December 29, 2000