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Fish mass immunization against virus with recombinant "spiny" bacterins

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ABSTRACT

Bacterins obtained from recombinant bacteria displaying heterologous antigens in its surface coded by prokaryotic rather than eukaryotic expression plasmids (here called "spiny" bacterins or spinycterins), have been used to increase fish immunogenicity of recombinant viral protein fragments. To explore their immunogenicity, five bacterial-specific membrane anchor-motifs characterized in the literature (Nmistic, Mistic, NTD, YAIN and YBEL) were genetically fused to the immunorelevant cystein-free 117 amino acid fragment II from the ORF149 of cyprinid herpes virus 3 ($frgII_{CyHV3}$). The fusion of anchor-motifs to the N-terminus of $frgII_{CyHV3}$ enriched expression in *E.coli* outer membranes as demonstrated by ELISA, immunofluorescence and flow cytometry of formaldehyde-fixed recombinant bacteria (spinycterins). Unconventional low-intensity ultrasound inducing mucosal micropores in a reversible non-harmful manner was used before carp or zebrafish immersion on spinycterin suspensions as a practical delivery alternative to fish-to-fish injection. After ELISA screening for anti-$frgII_{CyHV3}$-specific antibodies of spinycterin-immunized fish plasma, the YBEL constructs were identified as the most immunogenic in both carp and zebrafish, correlating with one of the best expressed recombinant proteins as demonstrated by Western blot and surface enriched as demonstrated by ELISA and flow cytometry. The use of prokaryotic expression plasmids to express viral immunorelevant protein fragments in traditionally used fish vaccination bacterins should reduce the environmental concerns raised by DNA vaccination based on eukaryotic expression plasmids. Therefore, spinycterins may be a useful alternative to develop safer fish viral vaccines and mass vaccination methods.

1. Introduction

A practical method for mass delivery of vaccines to small fish will be worldwide appreciated, since only in Europe, annual vaccination requires ~4000 millions of fish-to-fish injections [1]. Injectable fish vaccines contain either killed bacteria (bacterins) or recombinant gene-coded viral antigens (DNA vaccines) [2–4]. While efficient in inducing durable protection, fish-to-fish injection induces low mucosal immunization, is labour intensive, causes fish stress and cannot be used in small fish. In addition, environmental and safety concerns have been raised to viral DNA vaccines [5] because they contain eukaryotic expression plasmid vectors under the control of human viral promoters [6]. On the other hand, alternatives to injection, such as immersion-vaccination have been successful only for some killed bacteria (i.e.: bacterins from *Aeromonas, Yersinia,* or *Vibrio*) [7], while vaccination by oral delivery has been reported in very few cases [8–10]. Therefore, one of the research frontiers on fish viral vaccinology is focused on finding environmentally safer, easy to manage and low-cost large-scale mass delivery procedures [1].

Enrichment expression in outer bacterial membranes of heterologous proteins rather than in inclusion bodies was selected in this work to screen for novel and practical fish viral vaccine vectors because bacterial surface display is a very expansive field [11] that has not yet been applied to fish immunization. Bacterial outer membrane expression or chemical attachment to membranes of suitable antigens can be obtained by diverse methods, but to show a first proof-of-concept, genetic fusion of prokaryotic anchor-motifs to the N-terminal part of small linear antigens have been selected for this work. Because most efficient membrane display in *E.coli* surfaces have been dependent on the bacterial anchor-motif fused to them, five alternatives were selected here among those successfully reported in the literature: Nmistic, Mistic, NTD, YAIN and YBEL. Mistic is a 13 KDa 110 amino acid protein from *Bacillus*

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subtilis. When the Mistic protein was fused to the N-terminus of heterologous enzymes ("misticated" proteins), those were overexpressed at the bacterial outer membranes rather than at their inclusion bodies [12–14] maintaining its functionalities [13–15]. Downsizing the Mistic protein to a 32 amino acid fragment was possible after studying several chimeric Mistic versions made with orthologous genes from several bacterial species [16,17]. Another anchor-motif alternative for best *E.coli* membrane display, consisted in a 21 amino acid fragment (NTD) of the *B. subtilis* exosporal BclA protein fused to the N-terminus of some enzymes [18]. Enzyme activity, outer membrane fractionation and microscopy, confirmed that the NTD-fused enzymes were active and enriched in the surface of the recombinant *E.coli* when compared to intracellular expression. Finally other selected anchor-motif examples came from YAIN (98 amino acids) and YBEL (120 amino acids), two short hydrophilic bacterial proteins with non-globular alpha-helical predicted structures favoring lipid membrane interactions [19]. A comparative study using YAIN, YBEL and/or Mistic fused to the N-terminus, C-terminus or to both termini of 14 different heterologous proteins, resulted in high levels of expression in outer membranes of 12 of the proteins only when fused to their N- rather than to their C-terminus [19]. Overexpression of YAIN/YBEL fusion proteins enriched their localization into the recombinant *E. coli* membranes while reduced their accumulation as inclusion bodies.

Although both a signal peptide and a transmembrane domain are generally required for expression in lipid bilayer biological membranes, the efficiency of the above mentioned prokaryotic outer membrane anchor-motifs did not required such domains, nor in the Mistic [16,17], nor in the YAIN/YBEL [19]. The enrichment of expression into the outer membrane was even reduced when a traditional signal peptide was added to the NTD anchor-motif [18], as shown also by other prokaryotic anchor-motifs [20]. The mechanisms for bacterial outer membrane anchoring remain mostly unknown.

Viral epitopes were selected as immunorelevant antigens, because viral infections cause most of the highest economic impacts on fish farming. However, even though immune-relevant antigenic proteins are much more easy to identify in viruses than in bacteria, their corresponding purified recombinant whole proteins are usually not immunogenic to fish. Thus, viral protein highly hydrophobic signal peptides and transmembrane regions, numerous glycosylation sites and 10–20 cystein-conformation dependent 3D structures, are difficult to reproduce in recombinant bacteria. Because the immunorelevance of some cystein-free viral protein fragments has been described in fish [21–23] and plasma from survivors of viral infections contain IgM antibodies against viral linear epitopes [22,24,25] in addition to conformational neutralizing epitopes [26–28], viral small protein fragments could be use as an alternative immunogen. Therefore, for this work, the N-terminal (amino acid 42–149) cystein-free domain of ORF149 of Cyprinid herpesvirus-3 ($frgII_{CyHV3}$) preferentially targeted by IgM from carp populations surviving infection [23], was selected to be fused to the above mentioned bacterial anchor-motifs. Once obtained and characterized, the recombinant bacteria were inactivated by conventional formaldehyde-fixing to develop the here called "spiny" bacterins or spinycterins.

For spinycterin delivery, unconventional low-intensity ultrasound was used to induce fish mucosal micropores in a reversible non-harmful manner prior to bath immersion on spinycterin suspensions. At critical frequencies (KHz) and low-intensities (W/cm^2) [29,30], ultrasound is used in commercial trout farming to mass-label fish with fluorescent calcein with no alteration of well being, nor enhanced susceptibility to pathogens, nor reduced growth rates (Dr.Scott LaPatra, personal communication). Fish ultrasound immersion (FUSI) increased the uptake of bacterins into fish epidermis [31]. Consequently, low-intensity pulsed-FUSI followed by immersion in *A.salmonicida* bacterins lowered side effects while protected carp against bacterial infection [32–34]. Most recently, similar FUSI mass protocols enhanced *A.salmonicida* bacterin uptake by trout gills with absence of side effects and enhanced adjuvant activities [35,36] while previous pioneering work had reported low-intensity pulsed-FUSI DNA vaccination of trout against a rhabdovirus [37,38]. However, mass delivery of recombinant bacterins expressing heterologous proteins as immunogens have not been yet tested by low-intensity pulsed-FUSI.

Results showed that some of the anchor-motif constructs tested in this work were efficiently expressed and enriched in the *E.coli* outer membranes. The immunorelevant epitopes fused to the anchor-motifs were partially maintained after fixing with formaldehyde to induce $frgII_{CyHV3}$-specific plasma IgM antibodies in FUSI-immunized carp and zebrafish. Therefore, recombinant bacterins may be used as surface-display vectors (spinycterins) of different pathogen antigens in an environmental safe way, provided they induce a reproducible and practical immunization. The spinycterin concept contains a unique combination of ideas which upon further fine tuning may move ahead the state-of-the art of fish viral vaccinology.

2. Methods

2.1. Fish virus and cells used for the experiments

The Cyprinid Herpesvirus 3 (CyHV3), affecting carp (*Cyprinus carpio*) was replicated in cells from common carp brain (CCB), following the procedures described before [23].

2.2. Construction of recombinant $frgII_{CyHV3}$ and fusion to bacterial anchor-motifs

The mRNA of the ORF149 protein (NC009127) of the CyHV3-U reference strain was used for the design, construction, expression and purification of its frgII (amino acid 42–159) as described before [23]. On the other hand, 5 bacterial membrane anchor-motif sequences were selected to be expressed as fusion proteins to the N-terminus of $frgII_{CyHV3}$ as indicated in detail in Table 1. All the corresponding synthetic DNA sequences (GeneArt, Regensburg, Germany) were cloned into the pRSETa prokaryotic expression plasmid adding poly-histidine tails (polyH) at their C-terminal ends to become anchor-motif + GSGS (linker) + $frgII_{CyHV3}$ + GSGS (linker) + polyH recombinants. Controls included pRSET (control1), pRSET-$frgII_{CyHV3}$ (control2) [23] and pRSET-irrelevant 9 KDa protein followed by an stop codon but fused to $frgII_{CyHV3}$ (control3). The purified plasmids were then transfected into *E.coli* BL21 and grown at 37 °C. Bacteria from the recombinant pRSET-$frgII_{CyHV3}$ were induced with IPTG at 25 °C and their extracts separated by Ni^{2+} affinity chromatography to obtain purified $frgII_{CyHV3}$ protein to coat ELISA solid-phases [23]. Gradient 4–20% SDS polyacrylamide gel electrophoresis showed >95% purity of purified frgII $_{CyHV3}$ (not shown).

2.3. Inactivation of recombinant bacterins (spinycterins)

E.coli BL21 recombinant bacteria were grown overnight by strong agitation at 37 °C in TB medium with 100 μg/ml of ampicillin in 80 ml batches. Temperature was reduced to 25 °C and 0.5 mM IPTG added every 2 h twice and then maintained in agitation overnight. The resulting bacteria were washed in PBS, titrated in TB-ampicillin by colony forming units (cfu), adjusted to a final concentration of 10^{10} cfu/ml and inactivated by agitation overnight in 1.1% formaldehyde. The resulting spinycterins were finally

Table 1
Bacterial anchor-motifs fused to the N-terminus of $frgII_{CyHV3}$.

Name	Bacterial anchor-motifs	$+frgII_{CyHV3}$	AccNum	KDa	ref
control1	no anchor-motif, no $frgII_{CyHV3}$	–	————————	4.0	———
control2	no anchor-motif	+	————————	13.8	[23]
control3	irrelevant protein + stop codon	+	————————	9.7	———
Nmistic	33 N-terminal aa Mistic gene	+	AY874162	17.5	[15,48]
Mistic	110 aa Mistic gene	+	AY874162	26.4	[15,48]
NTD	21 aa NTD from BclA gene	+	AJ516945	15.9	[18]
YAIN	91 aa from *E.coli* gene	+	NP_414891	23.9	[19]
YBEL	160 aa from *E.coli* gene	+	NP_415176	32.3	[19]

aa, amino acids. **AccNum**, Gene Bank accession numbers. The DNA sequences of the bacterial anchor-motifs were fused (+) to the N-terminus of $frgII_{CyHV3}$ through a double Glycine-Serine (GSGS) linker. A carboxy-terminal part of 6 Histidines (polyH tail) was added following another GSGS linker, resulting in the final anchor-motif + GSGS + $frgII_{CyHV3}$ + GSGS + polyH recombinant proteins. The DNA sequences were chemically synthesized and cloned into the pRSET plasmid. BL21 *E.coli* bacteria were transformed with the recombinant plasmids and IPTG induced to express the recombinant proteins. **KDa**, expected molecular weight of the recombinant proteins. **control1**, pRSET empty plasmid. **control2**. pRSET-$frgII_{CyHV3}$ [23]. **Control3**, irrelevant protein + stop codon + $frgII_{CyHV3}$. **N-mistic**, 33 N-terminal anchor-motif from the mistic gene from *Bacillus subtilis* [15,48]. **Mistic**, 110 amino acid Mistic anchor-motif gene from *Bacillus Subtilis* [15,48]. **NTD**, N-terminal domain of 21 amino acid anchor-motif of the exosporal BclA protein from *Bacillus anthracis* [18]. **YAIN**, 91 amino acid anchor-motif hydrophilic regulatory protein of the frmR operon of *E.coli*. **YBEL**, 160 amino acid anchor-motif hydrophilic HTH-type transcriptional regulator DUF1451 family protein from *E.coli* [19].

washed with PBS, and kept at 4 °C until used. Plaquing in TB-ampicillin plates and/or liquid medium did not detected any residual bacterial colonies in aliquots corresponding to 10^9 cfu of spinycterins.

2.4. Immunization by low-intensity pulsed ultrasound prior to bath immersion in spinycterins

Groups of 5–9 fingerling carp (*Cyprinus carpio*) of ~10 g of body weight or 10–12 zebrafish (*Danio rerio*) of ~2 g of body weight were placed into an ultrasound bath (Selecta, Spain) with 800 ml of aquarium water with strong aeration. Carp were exposed to 6 pulses, 25 s per pulse with 1 min intervals between pulses, while zebrafish were exposed to 4 pulses, 7 s per pulse with 1 min intervals between pulses of 80 W/cm^2 ultrasound at 40 KHz frequency, following previously published procedures [34–37]. Each fish group was then immersed into 10^8 cfu spinycterin per ml during 30 min, released into 30 l closed-system aquaria and maintained at 26 °C during two months for carps and 1 month for zebrafish. No mortalities were recorded as a consequence of the ultrasound exposure.

2.5. Harvest of blood plasma from immunized fish

Anesthetized carp or zebrafish were bled by cutting the final end of their tails. Blood was collected at 4 °C in 500 μl for carp or 200 μl for zebrafish of sterilized anticoagulant media containing 0.64 g sodium citrate, 0.15 g EDTA, 0.9 g sodium chloride per 100 ml of water and 50 μg per ml of gentamicin. The ~10-fold diluted blood was immediately centrifuged at 1000 g for 3 min to obtain diluted plasma. Plasma samples were kept frozen at −20 °C until used.

2.6. Ethic statement on fish handling

Fish were handled following specific protocols approved by the Ethics Committee (authorization CEEA 2011/022) following the National Guidelines for type III experimentation (Annex X, permission RD53/2013) and the EU directive 2010/63/EU for animal experiments (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm). To record for possible mortality, the immunized fish were daily monitored 2–4 times during 2 or 1 month for carp or zebrafish, respectively. Methanesulfonate 3-aminobenzoic acid ethyl ester (MS222) was used at 40–50 mg/l to anesthetize the fish while obtaining their blood. The fish were then euthanized by an overdose of MS222 (200 mg/ml) to minimize suffering.

2.7. Characterization of the expression of pRSET-anchor + $frgII_{CyHV3}$ proteins expressed in recombinant E.coli by Western blotting

To assay for $frgII_{CyHV3}$ total expression on recombinant *E.coli*, the bacteria were analysed before inactivation with formaldehyde. Confirmation of expression of $frgII_{CyHV3}$ was performed in bacterial pellets boiled in buffer containing SDS and β-mercaptoethanol and briefly sonicated to reduce the size of contaminant bacterial DNA. The proteins separated in gels (SDS-precast 4–20% polyacrylamide gels from BioRad, Richmond, Vi, USA) were transferred to nitrocellulose membranes (BioRad, Richmond, Vi) and then blocked with dilution buffer (0.5% bovine serum albumin, 5% of skim milk, 0.1% Tween-20, 0.01% merthiolate, 0.005% phenol red in phosphate buffered saline pH 6.7). Blocked nitrocellulose membranes were then incubated with anti-polyH monoclonal antibody (Sigma Che. Co, St.Louis, Ms, USA) and with peroxidase-conjugated rabbit anti-mouse (RAM-PO) antibody. Bands were visualized by ECL (Bio-Rad). Similar Western-blot methods were used after fractionation experiments to isolate bacterial membranes according to the protocols described before [18].

2.8. Assay of $frgII_{CyHV3}$ epitope expression enrichment on the surface of spinycterins by ELISA

To assay for $frgII_{CyHV3}$ expression enrichment on the surface of recombinant *E.coli*, the bacteria were tested after inactivation with formaldehyde (spinycterins) to avoid antibody penetration into the cytoplasm. Therefore, polystyrene plates of Maxisorb 96-wells were coated with several dilutions of spinycterins. Solid-phases were then blocked for 60 min incubation with 100 μl per well of dilution buffer (0.5% bovine serum albumin, 0.1% Tween-20, 0.01% merthiolate, 0.005% phenol red in phosphate buffered saline pH 6.7) containing 10 μg per well of skimmed milk (Sigma). After washing, to detect $frgII_{CyHV3}$, the ORF149-specific monoclonal antibody (MAb) 11A4 purified by affinity chromatography on protein-A [23] was incubated in 50 μl per well during 60 min and then with horseradish labelled rabbit anti-mouse immunoglobulins (RAM-PO, Sigma Chem.Co, St.Louis, Mo). After washing 3 times, the colour reaction was developed by adding 50 μl of 1 mg/ml o-phenylenediamine in citrate buffer containing 3 mM H_2O_2. Absorbances were measured by reading at dual-wave lengths at 492–620 nm to correct for individual differences between wells. Means and standard deviations were then calculated from 2 to 3 independent experiments. The differences were considered significant at $p < 0.05$ (T-Student).

2.9. Assay of frgII expression on the surface of spinycterins by flow cytometry

To assay for frgII$_{CyHV3}$ expression enrichment on the surface of recombinant *E.coli* by flow cytometry, the recombinant *E.coli* were tested before (recombinant bacteria) and after inactivation with formaldehyde (spinycterins) to avoid antibody penetration into the cytoplasm. A high-throughput micro methodology [39] adapted to bacteria was used. Briefly, ~10^{10} cfu per ml of spinycterins in 100 μl were incubated with 4 μg/ml of protein-A purified anti-CyHV3 MAbs mixture [23] during 60 min. After washing by centrifugation, fluorescently labeled rabbit anti-mouse immunoglobulins were added and bacterial fluorescence analyzed in a BD FACS Canto II apparatus (Beckton Dickinson, San Agustin de Guadalix, Madrid, Spain) provided with a high throughput sampler (HTS). The number of fluorescent bacteria (frgII$_{CyHV3}$-expressing cells) over a threshold containing 95% (mean + 2 standard deviations) of fluorescent bacteria control1 or 3 (Table 1) were then determined. The percentage of frgII$_{CyHV3}$-expressing bacteria was calculated using the formula: 100 × number of bacteria with fluorescence above the threshold/total number of bacteria gated per well. Means and standard deviations were then calculated from 2 to 3 independent experiments. The differences were considered significant at $p < 0.05$ (T-Student).

2.10. Assay of frgII expression on the surface of spinycterins by immunofluorescence

To assay for frgII$_{CyHV3}$ expression enrichment on the surface of recombinant *E.coli* by immunofluorescence, the recombinant *E.coli* were tested after inactivation with formaldehyde (spinycterins) to avoid antibody penetration into the cytoplasm. About 10^{10} cfu per ml of spinycterins in 100 μl were incubated with 500-fold diluted anti-polyH monoclonal antibody from Sigma (St.Louis, Ms, USA) during 60 min. After washing by centrifugation, Alexa 488 fluorescently labeled rabbit anti-mouse immunoglobulins were added and bacterial fluorescence photographed with an Eclipse TE2000-E fluorescence inverted microscope provided with a Retiga Exi camera (Nikon Instruments Europe, Amsterdam, Netherlands) and with a TCS-SPE DMI 4000 confocal inverted microscope provided with LAS-AF software (Leica Microsystems CMS GmbH, Friedensplatz 3, Mannheim, Germany). Microphotographs were taken for all recombinant bacteria and representative micrographs selected for the Figures.

2.11. Assay of specific anti-frgII$_{CyHV3}$ antibodies in carp plasma by ELISA

Polystyrene plates coated to dryness with 2 μg per well of purified recombinant frgII$_{CyHV3}$ protein were blocked as described above. Fish plasma were 50 (carp) or 150 (zebrafish) -fold diluted in dilution buffer containing 2 mg/ml of *E.coli* extracts (supernatant from sonicated *E.coli*-BL21 centrifuged 10000*g* for 20 min) and incubated for 60 min. Then 50 μl per well of the above mentioned plasma and *E.coli* extract mix were incubated during 60 min with the frgII$_{CyHV3}$-coated solid phases. After washing, solid-phases were incubated with the corresponding MAb anti-carp IgM (Aquatic Diagnostics Ltd, Stirling, UK) [23], or MAb anti-zebrafish IgM (patent pending) and then with RAM-PO. Colour development and analysis was as indicated above.

3. Results

To demonstrate that the purified recombinant frgII$_{CyHV3}$ protein made in *E.coli* could be used to characterize the different anchor-motif + frgII$_{CyHV3}$ constructs and to follow up the induction of fish anti-frgII$_{CyHV3}$ antibodies, its binding to several monoclonal antibodies (MAbs) was first studied. To do that, binding of carp plasma IgM antibodies from healthy (Fig. 1, open circles) and survivors of CyHV3 infection (Fig. 1, gray circles) were compared using several ELISA formats. The recombinant frgII$_{CyHV3}$ protein was recognized by solid-phases coated with specific monoclonal antibody (MAb) 11A4 developed against CyHV3 and mapped to the N-terminus of its ORF149 [23], as shown by its specific binding to IgM antibodies from survivors but not to those from healthy carp (Fig. 1). Similar results were obtained when the mix of anti-CyHV3 MAbs described before [23] was used to coat the wells (not shown). The results were confirmed with solid-phases coated with Ni^{++} to bind the polyH tails of the recombinant frgII$_{CyHV3}$ protein (Fig. 1), demonstrating that frgII$_{CyHV3}$ exposed not only specific ORF149 epitopes but also those corresponding to its polyH tails. Furthermore, the frgII$_{CyHV3}$ protein was recognized also by IgM antibodies from carp plasma surviving a CyHV3 infection when solid-phases were coated with anti-carp IgM MAbs (anti-IgM8 and/or antiIgM2+3) to capture the corresponding IgM immunoglobulins, in contrast to captured IgM antibodies from healthy carp, irrelevant MAb or uncoated solid-phases (Fig. 1). The anti-ORF149 MAb 11A4, the mix of anti-

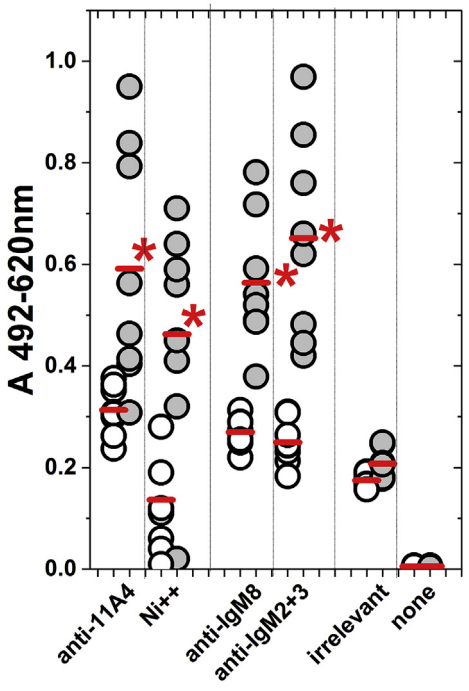

Fig. 1. Recombinant frgII$_{CyHV3}$ is specifically recognized by anti-CyHV3 antibodies from carp surviving CyHV3 infection when captured by monoclonal antibodies or Ni-coated plates.
Polystyrene plates were coated to dryness with 2 μg of protein-G purified anti-ORF149 MAb 11A4, anti-carp IgM MAb 8 or 2 + 3 (Aquatic Diagnostics Ltd, Stirling, UK), an irrelevant MAb or non-coated. Plates coated with Ni^{++} were used to capture polyH-expressing recombinant frgII$_{CyHV3}$ (Qiagen GmbH, Hilden, Germany). The MAb 11A4 coated solid-phases were incubated with 3 μg per well of purified recombinant frgII$_{CyHV3}$ during 60 min, washed and incubated with 50-fold diluted plasma from healthy carp or from carp surviving CyHV3 infection. The carp IgM was recognized by anti-carp MAbs and by RAM-PO. The Ni coated solid-phases were incubated with frgII$_{CyHV3}$ and then with MAb 11A4 and RAM-PO. The well coated with anti-carp IgM MAbs were incubated with plasma from carp before adding frgII$_{CyHV3}$ and recognized with peroxidase labelled anti-polyH antibody (Sigma Che Co, St.Louis, Mo, USA). **Horizontal red bars**, Means from 2 experiments, standard deviations omitted for clarity. **Open circles**, healthy carps (n = 8). **Gray circles**, CyHV3 survivor carp positive for neutralizing antibodies (n = 8). **Red** *, mean significantly different from control healthy carp as determined by the Student t-test ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CyHV3 MAbs and anti-polyH MAbs could detect different epitopes in the frgII$_{CyHV3}$ protein and therefore can be used to characterize anchor-motif + frgII$_{CyHV3}$ constructs and to follow up fish anti-frgII$_{CyHV3}$ antibodies.

To test for the *E.coli* expression of the recombinant anchor-motif constructs (pRSET-anchor-motif + GSGS + frgII$_{CyHV3}$ + GSGS + polyH), IPTG-induced *E.coli* extracts were analysed by Western blotting using anti-polyH antibodies before formaldehyde-fixing. After polyacrylamide gel electrophoresis (PAGE) under denaturing conditions, most recombinant *E.coli* extracts had similar profiles of Coomassie-blue stained protein bands, except those for the YBEL-fused construct which were dominated by a ~30 KDA stained band (Fig. 2A, lane 8). After the Western blotting, the highest polyH expression was obtained for the YBEL-fused construct (apparent molecular weight of ~30 KDa) (Fig. 2B, lane 8), followed by the Nmistic and Mistic-fused constructs (apparent molecular weights of ~25 KDa) (Fig. 2B, lanes 2 and 4, respectively). Only faint bands were recognized by anti-polyH for the NTD and YAIN-fused constructs (Fig. 2B, lanes 5 and 6, respectively), suggesting those constructs were expressed at lower levels than the rest. Although less likely, the inaccessibility of polyH tail epitopes could also explain their faint bands. Alternatively, the faint bands corresponding to the NTD (9.3 isoelectric point pI) and YAIN (8.2 pI) constructs and/or the apparent higher molecular weight of Nmistic (expected 17.5 KDA, 8.7 pI), may be caused by their lower transfer rates to the nitrocellulose membranes [40] or to abnormal running behavior on SDS PAGE [41], due to their higher pI when compared to Mistic (5.2 pI) or YBEL (5.9 pI) constructs. As expected, a faint band similarly migrating like the purified recombinant frgII$_{CyHV3}$ protein was detected on control2 (pRSET-frgII$_{CyHV3}$) (Fig. 2B, lane 3) and bands at < 10 KDa were detected for control1 and control3 which had no frgII$_{CyHV3}$ sequences (pRSET) or stopped expression (pRSET-stop + frgII$_{CyHV3}$), respectively (Fig. 2B, lanes 1 and 6, respectively). *E.coli* membrane fractionation experiments following previously published protocols [18], revealed similar relative levels of polyH expression in the outer membrane fractions than in total *E.coli* extracts (not shown).

After confirming that some of the anchor-motif constructs were expressed in the corresponding recombinant *E.coli*, the bacteria preparations were inactivated by formaldehyde to become spinycterins. ELISA, immunofluorescence and flow cytometry assays, were then used to evaluate their final enrichment in frgII$_{CyHV3}$ or polyH epitopes in bacterial surfaces.

Results from ELISA carried out in plates coated with a series of spinycterin dilutions and stained with anti-frgII$_{CyHV3}$ MAb 11A4 showed that while the best levels of bacterial surface expression could be detected in the YAIN- and YBEL constructs, those corresponding to Nmistic and Mistic were expressed at about the same level than unanchored frgII$_{CyHV3}$ (Fig. 3). In contrast, NTD constructs have similar low expression levels than control3, which included an stop codon to eliminate any frgII$_{CyHV3}$ expression (Fig. 3).

Staining with anti-polyH MAb was then used to evaluate bacterial surface expression on spinycterins by immunofluorescence. Spinycterin immunofluorescence at the optical microscope showed 3 pattern groups, absence of fluorescence for control3 (Fig. 4A and confocal insert), several degrees of intermediate fluorescence levels for most of the other constructs (Fig. 4B and insert) and high fluorescence for some of the constructs (Fig. 4C). Quantification of immunofluorescence levels by flow cytometry using a mix of anti-

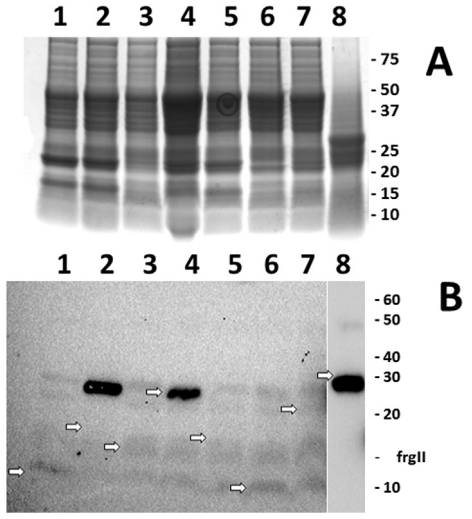

Fig. 2. Coomassie-blue staining (A) and Western blotting (B) of recombinant *E.coli* extracts revealed with anti-polyH monoclonal antibodies.
E.coli recombinants were IPTG-induced to express the recombinant proteins, and their extracts analysed by Coomassie-blue staining (A) and Western blotting (B) before formaldehyde inactivation. The extracts were boiled in SDS and β-mercaptoethanol, sonicated and electrophoresed in 2 parallel 4–20% polyacrylamide gradient gels (PAGE, BioRad). While one gel was stained with Coomassie (A), the other was transferred to nitrocellulose membranes, stained with anti-polyH monoclonal antibody (Sigma) and visualized by ECL. One of 3 experiments was represented. **Numbers to the right of the gel A**, KDa positions of Coomassie-blue stained molecular weight markers (BioRad). **Numbers to the right of the gel B**, bioluminescent molecular weight markers (Thermofisher). The anchor-motifs of the recombinant *E.coli* extracts (see Table 1 for a full description) were: **1**, control1. **2**, Nmistic. **3**, control2. **4,** Mistic. **5**, NTD. **6**, control3. **7**, YAIN. **8**, YBEL (10 times less ECL exposure than the rest of the gel). **FrgII**, frgII$_{CyHV3}$ protein purified from control2 recombinant *E.coli* by Ni^{++} affinity column (ProBond™, Invitrogen). **Horizontal white arrows**, molecular weights expected for anchor-motif + GSGS + frgII$_{CyHV3}$ + GSGS + polyH recombinant proteins.

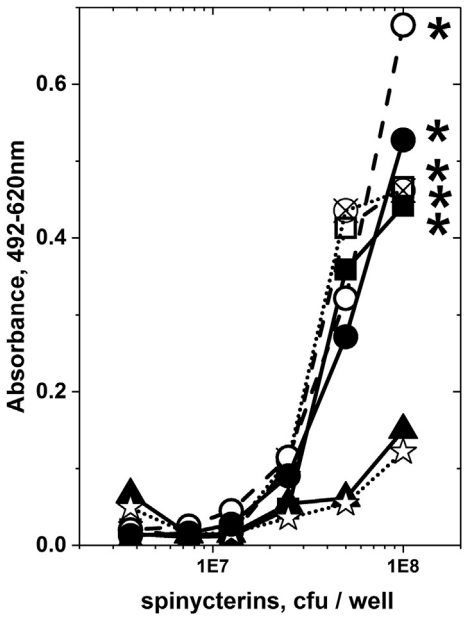

Fig. 3. Surface expression enrichment of frgII$_{CyHV3}$ epitopes by ELISA of recombinant *E.coli* cells after formaldehyde inactivation (spinycterins).
To assay for frgII$_{CyHV3}$ expression enrichment on the surface of recombinant *E.coli* cells by ELISA, the bacteria were tested after inactivation with formaldehyde (spinycterins) to avoid antibody penetration into the cytoplasm. The ELISA was carried out in plates coated with a series of spinycterin 2-fold dilutions and developed with anti-frgII$_{CyHV3}$ MAb 11A4 and RAM-PO. **Open circles**, YAIN (constructs containing anchor-motifs as described in Table 1). **Closed circles**, YBEL. **Open squares,** Nmistic. **Closed squares**, Mistic. **Closed triangles**, NTD. **Hatched open circles**, control2. **Open starts,** control3 (pRSET-stop + frgII$_{CyHV3}$). *, significantly different from control3 as determined by the Student t-test at the 10^8 cfu dilutions ($p < 0.05$).

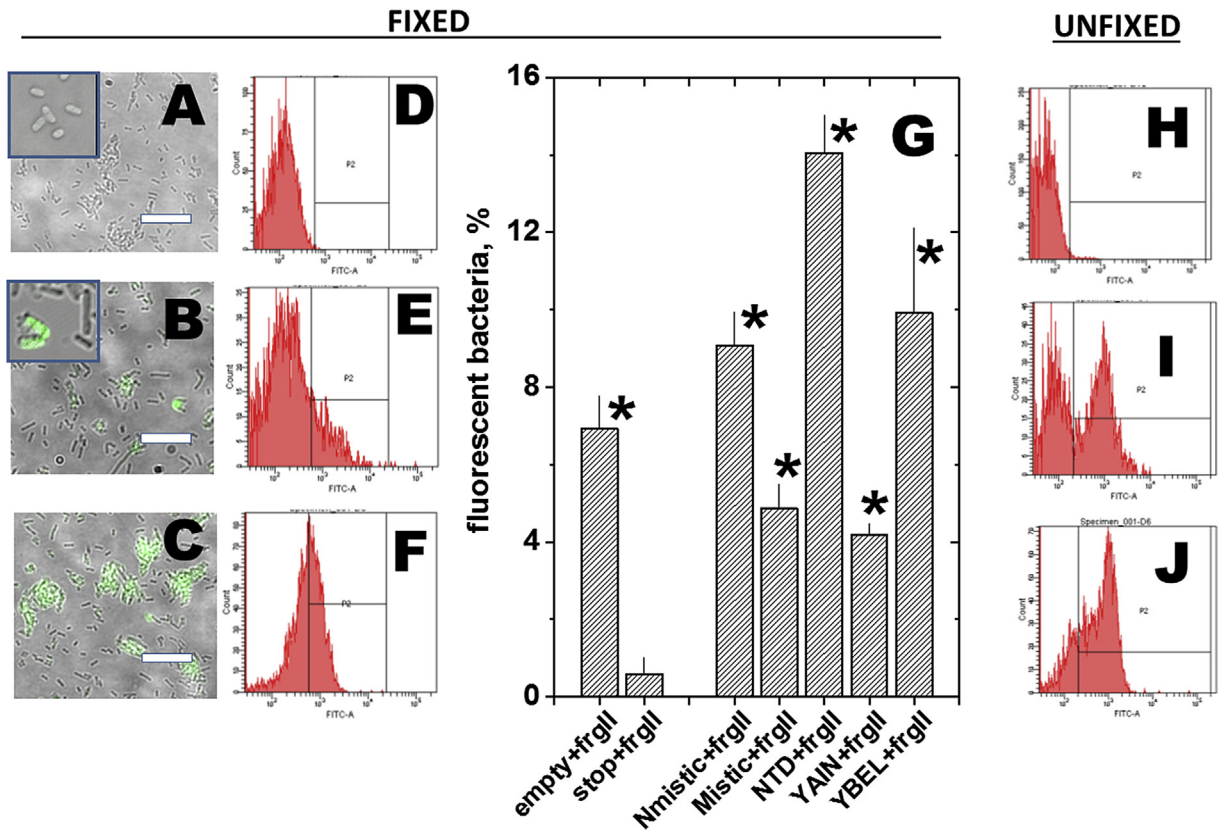

Fig. 4. Surface expression enrichment of frgII$_{CyHV3}$ recombinant proteins by fluorescence microscopy and flow cytometry of recombinant *E.coli* cells.
The panels show representative examples from 2 experiments of formaldehyde-fixed *E.coli* cells by optical (A, B, C) and confocal (A and B left upper inserts) fluorescent microscopy stained with anti-polyH monoclonal antibody and by flow cytometry (D, E, F, G) stained with a mix of anti-CyHV3 MAbs [23]. For comparative purposes, flow cytometry results from unfixed *E.coli* cells stained with a mix of anti-CyHV3 MAbs (H, I, J) are also represented. Optical (A, D) and confocal (insert A) representative fluorescent microscopies of formaldehyde-fixed recombinant *E.coli* cells from control3 (stop + frgII$_{CyHV3}$). Optical (B, E) and confocal (insert B) representative microscopies of formaldehyde-fixed recombinant *E.coli* cells with medium flow cytometry expression levels. Optical (C, F) fluorescent representative microscopy of formaldehyde-fixed recombinant *E.coli* cells with high flow cytometry expression levels. G, Flow cytometry quantitative results of formaldehyde-fixed *E.coli* cells. Means and standard deviations are represented (n = 2). *, significantly different from control3 (stop + frgII$_{CyHV3}$) by the Student t-test ($p < 0.05$). Optical representative fluorescence microscopy of unfixed *E.coli* cells from control3 and with medium and high flow cytometry expression levels (H, I, J, respectively).

frgII$_{CyHV3}$ MAbs, demonstrated that all 5 constructs including unanchored control2 (pRSET + frgII$_{CyHV3}$), contained frgII$_{CyHV3}$ epitope surface expression resulting in several degrees of enrichment levels compared to control3 (Fig. 4G, stop + frgII$_{CyHV3}$). The Nmistic, NTD and YBEL constructs have higher expression enrichment levels than unanchored frgII$_{CyHV3}$ (Fig. 4G). To note that the flow cytometry data were obtained with a mix of anti-CyHV3 MAbs rather than with MAb 11A4 in ELISA, suggesting that the NTD construct epitope to MAb 11A4 was removed by the formaldehyde fixing treatment (Fig. 3). However, the formaldehyde-fixing treatment also seems to partially remove epitopes in all constructs, because of the lower fluorescences of formaldehyde-fixed spinycterins (Fig. 4 D, E, F) when compared to those levels of unfixed recombinant bacteria (Fig. 4 H, I, J). Therefore, future work will require exploring alternative variables and/or methods for best preserving the immunorelevant epitopes expressed in the recombinant bacterial surface during inactivation while maximizing bacterial killing. The contrast between the high NTD construct expression by flow cytometry detected with mixed MAbs (Fig. 4G) and its lack of expression by Western blotting detected with anti-polyH (Fig. 2) or by ELISA with MAb 11A4 (Fig. 3) might be due to a low exposure of polyH tails or to formaldehyde inactivation of the 11A4 epitope, respectively. These data highlights the need for several MAbs or even polyclonal Abs to best characterize the relative levels of recombinant constructs due to possible differences in conformation which may influence exposure of different epitopes for each construct.

Once enrichment in surface expression was demonstrated for some spinycterins, those were delivered to groups of fingerling carp or adult zebrafish by bath-immersion after short-pulses of low-intensity ultrasound treatments. Two months later, frgII$_{CyHV3}$-specific IgM antibodies were detected in plasma from carp immunized with spinycterins coding for Nmistic, Mistic, YAIN and YBEL anchor-motifs in 3 of 8, 2 of 8, 1 of 8 and 4 of 5 fish, respectively. In contrast, plasma from the controls not expressing frgII$_{CyHV3}$ (pRSET and pRSET-stop + frgII$_{CyHV3}$) were negative for anti-frgII$_{CyHV3}$ IgM antibodies. In addition, only 1 of 6 plasma was positive when unanchored frgII$_{CyHV3}$ was used for carp immunization (Fig. 5A). On the other hand, one month after immunization, frgII$_{CyHV3}$-specific IgM antibodies were detected in plasma from zebrafish immunized with spinycterins coding for Nmistic, Mistic, NTD, YAIN and YBEL anchor-motifs in 2 of 10, 4 of 11, 2 of 10, 3 of 11 and 6 of 10 fish, respectively. In contrast, plasma from the controls not expressing frgII$_{CyHV3}$ (pRSET and pRSET-stop + frgII$_{CyHV3}$) were negative for anti-frgII$_{CyHV3}$ IgM antibodies and 5 of 10 plasma were slightly positive when unanchored frgII$_{CyHV3}$ was used for zebrafish immunization (Fig. 5B).

4. Discussion

This work describes the first evidence for successful antibody induction in small carp and adult zebrafish against an immune-

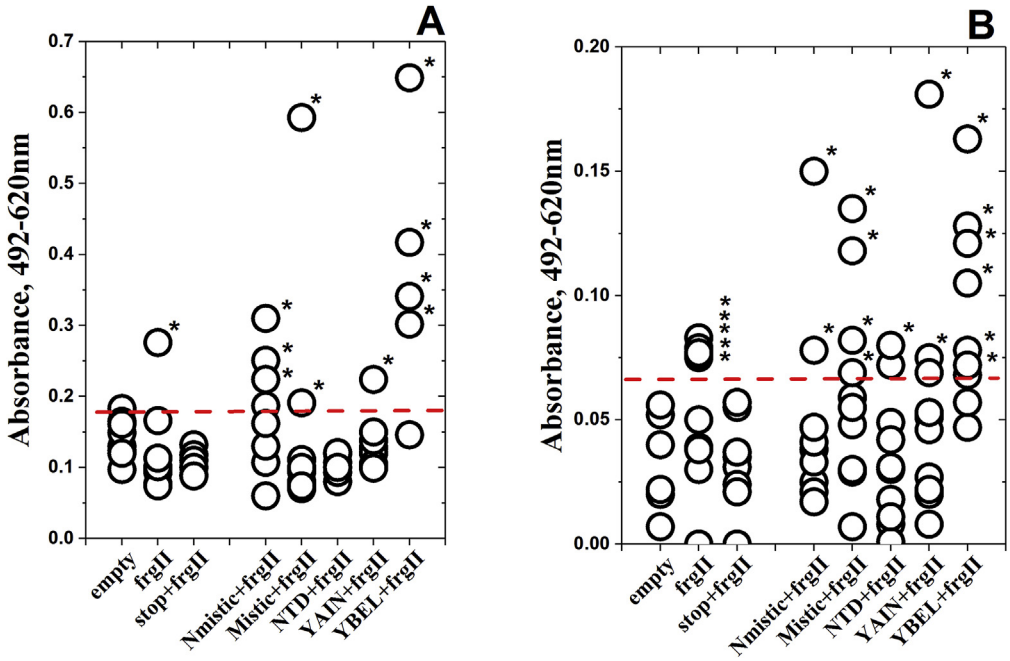

Fig. 5. Anti-frgII$_{CyHV3}$-specific plasma antibodies raised in carp (A) and zebrafish (B) after immunization with spinycterins.
Fingerling carp and adult zebrafish were immunized after low-intensity multiple-pulses of ultrasound exposure followed by 30 min bath-immersion in a suspension containing 10^8 cfu per ml of each of the spinycterins described in Table 1. Fish were then released to 30 l aquaria and maintained at 26 °C during 2 months for carp or 1 month for zebrafish. Plasma were then harvested from the immunized fish, 50 (carp) or 150 (zebrafish) -fold diluted and assayed by ELISA on plates coated with Ni^{++} affinity-purified frgII$_{CyHV3}$. Means were represented from 2 experiments, standard deviations omitted for clarity. *, Individual plasma higher than the mean + 2 standard deviations of the corresponding plasma immunized with control3 (stop + frgII$_{CyHV3}$).

relevant viral protein fragment using inactivated recombinant bacteria enriched in expression of that antigen in their surface (spinycterins). To enrich in surface expression and to increase safety, the surface expression was obtained by coding the recombinant sequences fused to anchor-motifs in prokaryotic rather than eukaryotic expression plasmids. Therefore, to obtain these results, the following strategies have been combined for the first time: **i)** Recombinant bacterins enriched in displaying an heterologous immune-relevant cystein-free protein fragment in its surface to increase the low immunogenicity of viral proteins made in bacteria, **ii)** Prokariotic rather than eukaryotic coded bacterial recombinants to increase safety and **iii)** Emerging mass delivery methods employing ultrasound immersion (FUSI) to increase practical application to small fish.

According to all the evidence analysed in this work, and taking into account the highest levels of anti-frgII$_{CyHV3}$ antibodies found in both carp and zebrafish, the best inducer of anti-frgII$_{CyHV3}$ IgM antibodies was the YBEL + frgII$_{CyHV3}$ construct, one of the constructs showing the highest level of protein expression by Western blotting and some of the best levels of enrichment in surface-expression of frgII$_{CyHV3}$ as shown by ELISA and flow cytometry.

Although after the legalization of an IHNV DNA vaccine in Canada [6] there have been no reports of IHN epizootics in vaccinated fish or negative effects for fish and/or consumers, the use of DNA vaccines based in eukaryotic expression vectors has been controversial due to safety concerns [42]. Recombinant bacterins coding for immune-relevant viral protein antigens in prokaryotic rather than eukaryotic vectors, are therefore, a safer alternative. In addition, the use of bacterins rather than DNA plasmids, adds some bacterial adjuvant effects to the immunization [32–34,36] while reducing production costs. However, because very often in such recombinant bacteria the heterologous viral whole proteins are expressed as inclusion bodies from which the immunogenicity of the protein is difficult to preserve, the use of whole bacteria expressing immunorelevant fragments in its surface recombinant protein purification was avoided, thus reducing additional costs. Although, some recent results suggest that bacterial inclusion bodies by themselves may also be used as carriers for immunogenic proteins in vaccines, they would also need purification steps. On the other hand, to obtain the necessary immunogenicity level for inclusion bodies may require too high concentrations [43].

In this context, the main practical advantages the proposed fish immunization with spinycterins are: **1)** Any type of bacterins, including spinycterins, should be accepted in aquaculture vaccination because being killed, bacterins have performed safety for the consumer, fish and environment during many years, **2)** By introducing surface expression enrichment of immunorelevant downsized protein fragments, the usual low-immunogenicity of most *E.coli*-made viral recombinant proteins may be enhanced, **3)** The use of whole bacteria will improve adjuvant activities to the recombinant protein responses, **4)** Because the viral antigens are fragmented and coded by prokaryotic rather than eukaryotic plasmids, they are safer and therefore would reduce environmental and consumer concerns, and **5)** The spinycterin concept is amenable to use with mixes of different pathogen antigenic sequences in a single delivery, to best tackle the wide spectrum of global circulating isolates and/or different pathogens.

Further refinement may be expected in the future to improve the described immunogenic efficiency of the anchor-motif fused constructs. For instance, because of possible bacterial toxicity, an increase in the levels of surface expression enrichment may be obtained using auto-induction media during the growth of the bacterial recombinants including selection of the best expressing clones [44–46], as already confirmed by preliminary results. In addition, other bacterial anchor-motifs or outer membrane proteins (OMP), most probably including some yet to be described, may

express their antigenic cargo in a higher number of recombinant bacteria and/or at higher levels of expression compared to inclusion bodies (in this work the maximal percentage of recombinant bacteria expressing $frgII_{CyHV3}$ epitopes after formaldehyde inactivation was estimated to be <20% despite higher levels of expression before inactivation). Therefore, many more methods and/or conditions for inactivating the recombinant bacteria should be screened and optimized to improve the preservation of immune-relevant viral epitopes enriched in the *E.coli* outer membranes. On the other hand, alternative immunorelevant viral antigens may be more efficiently expressed and/or conserved through the inactivation processes in the *E.coli* outer membranes. Finally, other bacteria specie may be best suited for membrane display (i.e., *lactobacillus*) [47], although *E.coli* has the advantages of the easy and wide variety of genetic engineering methodologies. Thus, although further investigation is still required to increase fish immunogenicity of spinycterins, this new strategy for small fish viral vaccination deserves that exploration.

Conflict of interest statement

None.

Acknowledgements

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