*Article*

# Sea Bass Immunization to Downsized Betanodavirus Protein Displayed in The Surface of Dna-Damaged Repair-Less Bacteria

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**Abstract:** This work describes practical immunization of European sea bass (*Dicentrarchus labrax*) juveniles against viral nervous necrosis virus (VNNV), a *betanodavirus* causing worldwide mortalities in many fish species. Protection was obtained with the so called spinycterin vehicles consisting in irreversibly DNA-damaged DNA-repair-less *E.coli* displaying at their surface a downsized antigen. In this work we, **i)** maximized bacterial expression levels by downsizing the C protein to a fragment ($frgC_{91-220}$) containing most of its antigenicity, **ii)** developed an scalable autoinduction bacterial media based in soy-bean increasing membrane display and reproducibility, **iii**) enriched surface expression by screening different anchors from several prokaryotic origins (anchor+$frgC_{91-220}$), **iv**) preserved $frgC_{91-220}$ antigenicity by inactivating bacteria by irreversible DNA-damage by means of Ciprofloxacin, and **v)** increased safety using a repair-less *E.coli* strain as spinycterin chassis. These second generation of spinycterins protected fish against VNNV challenge with partial (Nmistic+$frgC_{91-220}$) or 100 % (YBEL+$frgC_{91-220}$) protection, in contrast to those fish immunized with $frgC_{91-220}$ spinycterins. The proposed spinycterin platform has high levels of environmental safety and cost effectiveness, thus providing potential for small fish vaccines for sustainable aquaculture.

**Keywords:** VNNV; mass-immunization; sea bass; recombinant bacterins; spinycterins; DNA-damaged; repair-less

## 1. Introduction

Viral encephalopathy and retinopathy caused up to 100 % mortalities in juveniles of more than 40 finfish species including those most important to the European marine aquaculture industry such as sea bass and sea bream (*Sparus aurata*) [1,2]. All these diseases are caused by viral nervous necrosis viruses (VNNV) which belong to the *Nodaviridae* family within the *betanodavirus* genus [3,4]. VNNVs are non-enveloped particles of icosahedral symmetry enclosing two single-stranded, positive sense RNAs. One of the RNAs encodes an RNA-dependent RNA polymerase, while the other encodes their capsid protein (C protein). According to C gene-derived protein sequences, *betanodavirus* isolates from Europe, Asia and Japan could be classified into 4 genotypes, but displaying only 19-23 %

sequence differences among them [5,6]. Most C proteins of geographically-related betanodaviruses share up to 98-99 % of their sequence.

Different types of VNNV killed vaccines have been described [7], including those made with inactivated virus [8,9], VLP virus-like particles [10-12], recombinant C proteins [13,14], or synthetic peptides derived from the C protein [15]. However, the efficacies of most of those vaccines were evaluated after injection of relatively medium-size young fish, while the highest mortality occurred at the juvenile/larval stage, a size too small to be immunized by injection. Vaccination by oral delivery have been also reported using inactivated bacteria encapsulating dsRNA, chitosan conjugated DNA [7] or most recently by using large amounts of alive recombinant bacteria expressing the C protein sequence mixed in the feed [16]. Although the use of recombinant bacteria will be most appreciated for large scale oral vaccination by avoiding stressful, labour intensive and costly delivery, the release of alive genetically modified organisms (GMOs) will have practical problems. Thus, the presence of recombinant DNA and antibiotic resistance genes in alive GMOs will raise numerous safety concerns for sustainable aquaculture.

To explore dead alternatives to alive recombinant bacteria, we have improved here a previously reported platform consisting in inactivated recombinant bacteria using surface display of downsized viral antigens (called spinycterins) [17]. Such spinycterins were obtained by genetic fusion of selected prokaryotic anchor-motifs to the N-terminal part of small linear immunodominant viral fragments. Despite the high reduction of antigenicity caused by traditional crosslinking inactivation by formaldehyde, successful production of anti-viral antibodies were demonstrated by immersion of ultrasound-treated zebrafish and/or carps in spinycterins expressing downsized CyHV-3 herpesvirus in their surfaces [17]. Among the safety advantages, the spinycterin inactivated condition may allow also for lyophilization and/or addition into feeds, contributing also to bypass the low temperature-dependence of fish vaccines. However, several fine-tunning details are still required for the practical use of spinycterins for small fish vaccines. First, there are not yet any evidences that they will work against VNNV. Second, formaldehyde inactivation caused a ~ 80 % of antigenicity loss and the yields of some the anchor fusions were low or inhibited bacterial growth. Third, safety concerns may still remain when handling and releasing to the environment large amounts of recombinant bacteria and those need to be minimized even when using dead GMOs. Therefore, improvements in the above mentioned concepts were explored for large-scale manufacturing of spinycterins for practical small fish vaccination.

Downsizing of the C protein of VNNV was performed to increase its expression levels on recombinant *Escherichia coli* while maintaining their immunogenicity potential [18,19]. The prokaryotic membrane anchor-motifs fused to the downsized C protein in this work, included those used before [17] and the P9 anchor-motif identified in the envelope of phage ϕ6 [20]. Because of the importance of nodaviruses in the aquaculture of commercially important fish species, like sea bass and sea bream, we choose one of them (sea bass) to test protection against VNNV challenge. To preserve immunogenicity of spinycterins, several alternative methods to formaldehyde inactivation were explored. Among the many alternatives described before, bactericidal drugs appeared to be an attractive possibility since they allow for 100 % of preservation of antigenicity, while maintaining intact the recombinant bacterial morphology and their inherent adjuvanticity. Described bactericidal drugs have been targeted to double-stranded DNA (i.e., bacterial DNA gyrase inhibitors), bacterial DNA-dependent RNA synthesis (i.e., rifampycins), cell-wall envelopes (wall synthesis inhibitors), and/or bacterial protein translation (synthesis inhibitors) [21]. Therefore, we explored the possibilities of some of these drugs to irreversibly inactivate surface displaying bacteria in a cost-efficient manner. In addition, to increase safety during recombinant *E.coli* manipulation, large-scale production, induction of expression, and delivery to the environment, we explored the BLR(DE3) strain defective in DNA-repair. In contrast to the BL21(DE3) *E.coli*, their derived BLR(DE3) cannot repair double DNA strand breaks, nor revert antibiotic-dependent ROS oxidation damage, making their recombinants more susceptible to DNA inactivation methods [22]. Additionally, the BLR(DE3) strain is resistant to tetracycline (TetR) which is more convinient for large-scale manufacturing because it makes possible to reduce any possible contaminant bacterial growth. Furthermore, BLR(DE3) requires Isoleucine

(Ileu$^-$) in the culture media to grow [23], opening the possibility to develop antibiotic-independent recombinant selective methods to reduce possibilities to spread resistant genes. All these characteristics make BLR(DE3) highly advantageous for large scale production, but it is not yet known whether BLR(DE3) can be used to produce spinycterins.

The results obtained in this work, showed that a new bacterial culture media containing soy rather than milk hydrolysates made anchor-antigen expression more reproducible by delayed autoinduction eliminating the IPTG requirement. In addition, Ciprofloxacin inactivation preserved all antigen immunogenicity and irreversibly damaged the DNA of the recombinant bacteria, thus increasing safety during both manipulations and delivery of the resulting spinycterins. Finally, BLR(DE3) could be a good substitute for BL21(DE3), adding another safety level for spinycterin production for small fish vaccines. Therefore, irreversibly DNA-damaged recombinant BLR(DE3) displaying downsized viral antigens may be used as new spinycterin vehicles for VNNV antigens in an environmental safer way. This second version of spinycterins may not only contribute to move ahead the state-of-the art of small fish viral vaccinology but also other veterinary vaccination procedures.

## 2. Materials and Methods

### *2.1. Construction of downsized VNNV coat sequence and genetic fusion to prokaryotic anchor-motifs.*

The frgC$_{91\text{-}220}$ sequence (amino acid residues 91-220) was derived from the C coat protein of viral nervous necrosis virus (VNNV) isolate AY284959 from *D.labrax* [18,19]. The C derived sequence was fused downstream of 6 different bacterial membrane anchor-motif sequences (Table 1). The different modular constructs were genetically fused by arbitrary flexible linkers (GlicSerGliSer, GSGS). All the corresponding DNA sequences were chemically synthesized (GeneArt, Regensburg, Germany) and subcloned into the pRSET prokaryotic expression plasmid which adds poly-histidine tails (polyH) at their C-terminal ends. Therefore, the general formula of the resulting recombinant constructs was: $H_2N$-anchor + GSGS + frgC$_{91\text{-}220}$ + GSGS + polyH-COOH. The purified plasmids were then transfected by the $CaCl_2$ method into *E.coli* BL21(DE3) or BLR(DE3) strains and grown in either TB or SB media (Table 2) at 37 ºC.

### *2.2. Induction of protein expression and inactivation of antigen surface-displaying recombinant bacteria.*

The BL21(DE3) *E.coli* strain has been widely used for recombinant protein expression including previously reported spinycterins for fish immunization to CyHV-3 herpesvirus [17]. The BLR (DE3) is a transposon derivative of the BL21(DE3) strain lacking recombinase A (RecA$^-$)[24], having tetracycline resistance and requiring isoleucine for growth [23] [F_ ompT hsdSB(rB_ mB_) gal dcm (DE3) D(srl _ recA)306::Tn10 (TetR)] (Novagen). To produce spinycterins, BL21(DE3) or BLR(DE3) recombinant bacteria were grown overnight by strong agitation at 37 ºC in 40 ml of TB or SB medium (see composition in Table 2) with 100 µg/ml of ampicillin for the BL21(DE3) strain or 100 µg/ml ampicillin, and 12.5 µg/mL tetracyciline for the BLR(DE3) strain. To induce recombinant protein expression, either 0.5 mM IPTG were added every 2 h twice in TB media or autoinduction was allow to proceed during 2-3 days in SB media. The resulting bacteria were washed in PBS, and adjusted to a final concentration of $10^{10}$ cfu / ml. To irreversibly damage their DNA, Ciprofloxacin was added at 50 µg / ml, and incubated at room temperature for 2 h with agitation. The resulting spinycterins were finally washed with PBS, 20 % glycerol added to preserve their morphology and kept at -20ºC until used.

### *2.3. Characterization of the expression of pRSET-anchor+frgC$_{91\text{-}220}$ coded proteins*

Confirmation of the frgC$_{91\text{-}220}$ expression was performed in spinycterin pellets boiled in buffer containing SDS and ß-mercaptoethanol. 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 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).

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 with the diaminobenzidine DAB stain.

### *2.4. Assay of pRSET-anchor+frgC$_{91-220}$ enrichment on the bacterial surface.*

To assay for frgC$_{91-220}$ enrichment on the bacterial surface, the spinycterins were first "shaved" by partial digestion with 1 % of trypsin during 2 h at 37 ºC. Control undigested spinycterins were incubated in parallel with PBS. Trypsin-dependent partial digestion levels were then estimated by polyacrylamide gel electrophoresis (PAGE) and by ELISA.

To assay by PAGE, the amounts of recombinant bands after Coomassie blue staining of PAGE were compared between trypsin-digested and control undigested spinycterins. The resulting Coomassie-stained PAGE bands were densitometrized in Image J 1.41o (http://rsb.info.nih.gov/ij). The percentage of the recombinant protein bands relative to the total protein bands were first normalized by the formula, optical density of the recombinant band / total optical density of the corresponding spinycterin. The optical densities were then expressed relative to the optical density of frgC$_{91-220}$ spinycterins by the formula, 100 x % of the anchored recombinant protein / % of frgC$_{91-220}$.

To assay by ELISA, several dilutions of the trypsin-digested and control undigested spinycterins were used to coat wells of polystyrene plates of Maxisorb 96-wells by overnight incubation to dryness at 37 ºC. The coated solid-phases were then blocked by overnight 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, Che. Co.). After washing, the wells were incubated with peroxidase-labeled anti-polyH monoclonal antibody (Sigma Che. Co.) in 50 µl per well during 60 min. 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. Percentage of poliH binding was calculated by the formula, 100 x Absorbance after trypsin digestion / Absorbance of undigested samples. The percentage of absorbance was then expressed relative to frgC$_{91-220}$ spinycterins by the formula, 100 x absorbance of the anchored recombinant protein / absorbance of frgC$_{91-220}$.

### *2.5. Immunization with surface-displaying bacterins by intraperitoneal injection and harvest of blood plasma from the immunized fish*

Groups of 15-20 fingerling sea bass (*D. labrax*) of ~10 g of body weight were acclimatized for 30 days to sea water aquaria closed-circuits of 60 l each. Two independent aquaria for each antigen were injected with 100 µl of PBS (two independent intraperitoneal injections of 50 µl each per fish) containing $10^8$ cfu of frgC$_{91-220}$, Nmistic+frgC$_{91-220}$ or YBEL+frgC$_{91-220}$ spinycterins. Non-infected, infected with empty pRSET plasmid and non-immunized controls were also included. The fish were fed with commercial pellets and maintained at 26 ºC during 30 days. Viral challenge was performed by intramuscular injection of 100 µl of the VNNV strain 475-9/99 (2 x $10^4$ $TCID_{50}$ per ml) of the RGNNV genotype provided by the Istituto Zooprofilacttico delle Venize (Italy) isolated from *D.labrax* {Bovo, 1999 #26088}. Mortalities were recorded daily. Relative percentage of survival was calculated by the formula, 100 – percentage of mortality in the spinycterin-immunized VNNV-challenged fish / percentage of mortality in the non-immunized VNNV-challenged fish.

### *2.6. Ethic statement on fish handling*

Fish care and challenge experiments were reviewed and approved by the CSIC National Committee on Bioethics under approval number ES360570202001/16/FUN01/PAT.05/tipoE/BNG, 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 200mg/ml to euthanize moribund fish by an overdose of MS222 (200 mg/ml) to minimize suffering.

## 3. Results

### *3.1. Selection of the frgC$_{91-220}$ sequence from the betanodavirus C coat protein*

To select an appropriatted VNNV antigen for sea bass vaccination, the C-coat protein was chosen because is the only target for fish neutralizing antibodies. Since a minimal antigen size favors expression on *E.coli*, and by taking into account the additional increment of molecular size because of the fusion with a prokaryotic membrane anchor as required for surface display, we studied the best way to downsize the C protein. Since targeted epitopes of both neutralizing monoclonal antibodies and serum samples from VNNV-infected survivor sea bass were first mapped at positions 1–32 (signal peptide), 91–162 and 181–212 [19][25], the fragment extending from the 91 to the 220 amino acid positions was chosen for optimal antigenic expression on *E.coli*. FrgC$_{91-220}$ contained most of the C shell domain (S-domain) located between residues 52-213 [26] (Figure 1A and B), the highest hydrophilic peaks of exposed amino acid residues on the viral surface, three cysteines, all the Ca++ binding sites implicated in subunit-subunit C-interactions in the viral shell structure [26] and most of the neutralizing B-cell epitopes mentioned above [19][25]. By choosing the frgC$_{91-220}$, we avoided the highly hydrophobic 1-32 signal peptide which is deleterious to *E.coli*. We also avoided the 223-331 region that contains most of the sequence variability among VNNV isolates [5], and provided a frgC$_{91-220}$ that would serve as immunogen for a wider number of VNNV isolates. Finally, to reduce *E.coli* inclusion bodies with reduced-immunogenicity, the three cysteins were mutated to serines during the gene synthesis.

To increase bacterial membrane expression, several prokaryotic anchor-motifs were genetically fused to frgC$_{91-220}$. Those included, Nmistic, Mistic, NTD, P9, YAIN and YBEL [20,27-34]. Most of this anchor-motifs were already described when fused to a CyHV-3 herpesvirus fragment [17], but the genetic fusion to P9 [20] is described here for the first time. The P9 protein of 90 amino acids constitutes the major envelope of phage ϕ6. It was proposed as an N-terminal anchor alternative because it facilitated the integration of 11 of 14 target proteins into the *E.coli* cell membrane [20]. Therefore, the general formula of the resulting recombinant constructs were $H_2N$-prokaryotic membrane anchor-motif + GliSerGliSer (GSGS) linker + frgC$_{91-220}$ + GSGS linker + polyHis(H)-COOH (Figure 2). The expected molecular weights of the recombinant fusion products with the selected prokaryotic anchors varied from 20 to 34.8 KDa while the frgC$_{21-220}$ without anchors was 16.2 KDa (Table 1).

### *3.2. Autoinduction media to improve anchor-fused frgC$_{91-220}$ expression*

Previous results revealed partial or total inhibition of expression in some of the anchors in fused constructs with herpesviral proteins coded in pRSET plasmids under the control of T7/lactose promoters in *E.coli* [17]. Most probably the presence of lactose in the casein hydrolizates of LB / TB media contributed to too early autoinduction and toxicity during the growth phase, like in other T7/lac promoter expression systems [35,36]. To reduce early autoinduction, the casein hydrolysate broth (LB / TB) was substituted by soy-bean hydrolisate broth (SB). Because of the small amounts of galactose in SB, a T7/lac promoter inducer weaker than lactose, soy-bean hydrolisates enriched in glucose may be used for delaying autoinduction to the stationary phase thus increasing yields and reproducibility [35,37]. In addition to containing no lactose, because of its plant origin SB contains no mammalian infectious contamination, which is important for large scale production and release into the environment. After numerous tests, the composition of the SB media optimized for maximal and reproducible yields of autoinducible expression of anchored frgC$_{91-220}$ resulted in the formula shown in Table 2.

Transformed *E.coli* BL21(DE3) clones selected from SB agar plates in the presence of ampicilin were grown in small scale 2 ml SB cultures. All the 6 anchors were efficiently expressed in *E.coli* when using SB media (Figure 3A), in contrast to the varibility previously observed when grown

in TB [17]. PAGE densitometry estimations of each of the anchor+frgC$_{91-220}$ at their expected molecular weights (Table 1) varied between 4.1 to 8.4 % (n=3) of the total stained protein in each of the *E.coli* extracts, while the estimate of the frgC$_{91-220}$ in the absence of any anchors was 4.5 ±1.3 % (Figure 3B). The YBEL+frgC$_{91-220}$ spinycterins showed the highest expression level (8.4 ± 3.2 %). The presence of polyH detected in the corresponding Western blots identified the corresponding bands, which correlated with the expected molecular weights of the anchor+frgC$_{91-220}$ protein products (Figure 3C).

We then selected one of the lower and the higher molecular weight constructs (Nmistic and YBEL, respectively) to escale up production for additional testing. The small scale up confirmed that slighly lower but similar yields could be produced in 40 ml cultures. Thus, 2.4 ± 0.7 %, 2.7 ± 0.3 and 4.6 ± 2.1 % of stained protein were estimated for the frgC$_{91-220}$, Nmistic+frgC$_{91-220}$ and the YBEL+frgC$_{91-220}$ constructs, respectively (not shown).

### *3.3. Inactivation of recombinant E.coli by DNA-damage*

To be safely handled and released to the environment, the bacteria expressing the anchor+frgC$_{91-220}$, need to be irreversibly inactivated. However, inactivation methods should be found to improve the loss of antigen reactivity shown before by traditional methods employing formaldehyde [17]. After exploring the results obtained during preliminary experiments, methods damaging the bacterial DNA appeared among the most convenient (not shown). To select for a suitable compound to irreversibly damage the DNA of *E.coli*, several selected antibiotics and/or base analogs (Oxolinic acid, Levofloxacin, Ciprofloxacin, BrdU, Fluoracin, Thioguanine, Rifampicin, and Mitomicin C) were screened for inhibition of replication of YBEL+frgC$_{91-220}$ spinycterins made in BL21(DE3) *E.coli*. According to the corresponding survival concentration-dependent curves, the most active compounds were Ciprofloxacin (CPFX) and 5-Fluoracin (Figure 4). CPFX was selected for further studies because its bacteriostatic / bactericidal effects have been thoroughly studied and it was easily available at a low prize. Furthermore, in contrast to some of the other compounds, up to 1600 μg per ml did not affect the levels of expression, as estimated by Coomassie-blue staining of the resulting spinycterin extracts after overnight incubation (not shown).

### *3.4. Recombinant expression levels were lower when using the DNA-repair deficient BLR(DE3) E.coli strain*

To comparatively study the expression levels of the recombinant display constructs in the BL21(DE3) and the repair-less BLR(DE3) *E.coli*-derived spinycterins. both were grown in parallel 40 ml cultures. Results showed a higher expression level of 40 ml culture in comparison with the 2 ml cultures (Figure 5). However, while the frgC$_{91-220}$ was similarly expressed in the two strains (14.3 ± 4.5 % and 12.3 ± 3.7 %, respectively, n=2), the expression level of the corresponding Nmistic+frgC$_{91-220}$ and YBEL+frgC$_{91-220}$ was lower when produced in BLR(DE3) than in the BL21(DE3) strain (5.4 ± 0.4 and 12.4 ± 0.5, respectively in BLR compared to 17.7 ± 0.2 and 36.3 ±5.4, respectively in BL21, n=2).

To test for the irreversibility of CPFX inactivation, the BLR(DE3) bacteria were treated with several concentrations of CPFX and the viability of the resulting spinycterins tested by both overnight growth in TB-ampicillin-tetracycline plates and several days overgrowth in TB-ampicillin-tetracycline liquid medium. No colonies nor growth, respectively could be detected after treatment with 1 μg / ml of CPFX, in contrast to untreated spinycterins with initial inoculum of $10^9$ cfu of BLR(DE3) Nmistic+frgC$_{91-220}$ or YBEL+frgC$_{91-220}$ spinycterins (not shown). This CPFX dosage was ~100-fold lower than the inactivation obtained for the BL21(DE3) strain (Figure 4), most probably due to the DNA repair deficiency of the BLR(DE3) strain.

### *3.5. Surface expression of Nmistic+frgC$_{91-220}$ and YBEL+frgC$_{91-220}$*

To estimate the relative surface enrichment of frgC$_{91-220}$ in the selected spinycterins, PAGE and ELISA experiments were carried out after partially "shaving" the corresponding spinycterin surfaces by limited trypsin digestion. By any of these methods, the higher surface exposure of frgC$_{91-220}$ should be more susceptible to trypsin digestion and therefore would show a lower stained-band by PAGE

and poliH- absorbance by ELISA. Surface exposure of frgC$_{91-220}$ estimated by comparing the Coomassie-blue stained bands of anchor+frgC$_{91-220}$ with those from frgC$_{91-220}$ spinycterins, showed that the most exposed levels were obtained for the YBEL+frgC$_{91-220}$ (55.5 ± 5.3 % band intensitiy after trypsin digestion) and Nmistic+frgC$_{91-220}$ (79.05 ± 5.8 %) spinycterins (Figure 6A), suggesting that the YBEL+frgC$_{91-220}$ have the highest surface exposure. The carboxy-terminal poliH binding of frgC$_{91-220}$ to peroxidase labelled anti-polyH antibodies after trypsin digestion estimated by ELISA, showed that again the best levels of surface expression were obtained for the YBEL+frgC$_{91-220}$ spinycterins (60.4 ± 13.5 % poliH binding after trypsin digestion) compared to the Nmistic+frgC$_{91-220}$ (83.6 ± 40.1 %) (Figure 6B). Both results confirmed that the surface exposure of frgC$_{91-220}$ was higher in YBEL+frgC$_{91-220}$ than in Nmistic+frgC$_{91-220}$ spinycterins.

### *3.6. In vivo immunization against VNNV using spinycterins obtained in DNA-damaged DNA-repair deficient E.coli expressing anchor+frgC$_{91-220}$*

After the evidences for inactivation by DNA-damage and for relative enrichment in surface expression were shown for the selected spinycterins, those were intraperitoneally injected to duplicated groups of fingerling sea bass. After the VNNV challenge, total mortalities adding the results for the two aquaria per spinycterin resulted in 25.7 % of the non-immunized VNNV-challenged controls. Similar results were obtained for the fish injected with the empty pRSET plasmid. The relative percent survival was 100 % in sea bass injected with YBEL+frgC$_{91-220}$ spinycterins, while it was of 62.3 % in Nmistic+frgC$_{91-220}$. In contrast, a survival of 2.7 % was recorded for those fish injected with frgC$_{91-220}$ spinycterins (Figure 7). A correlation between surface exposure and protection was suggested by all these results.

## 4. Discussion

This work describes 100 % protection of sea bass juveniles against VNNV challenge. This high level of protection was obtained with newly developed irreversibly DNA-damaged DNA-repair-less spinycterins. The selected spinycterins displayed in their surface a synthetically-made cystein-free downsized C$_{VNNV}$ N-terminal antigen (frgC$_{91-220}$) because that fragment contained most of the epitopes targeted by fish neutralizing antibodies (B-cell epitopes). The frgC$_{91-220}$ was fused to several bacterial membrane anchors to screen for the one with higher membrane expression. A correlation between the levels of surface expression and protection was demonstrated by comparing YBEL+frgC$_{91-220}$ (higher frgC$_{91-220}$ surface display and full protection) and Nmistic+frgC$_{91-220}$ (lower frgC$_{91-220}$ surface display and partial protection). Those protections were obtained despite the selected N-terminal frgC$_{91-220}$ being located outside the most important shell protrusion C-terminal domain (P domain) in both VLP [38] and whole virus [26], which could be expected to be more antigenic as based only on structural criteria (Figure 1B).

On the other hand, to improve spinycterin yields, reproducibility and safety, the following strategies were combined: a novel scalable autoinduction media for *E.coli* expressing recombinant proteins under the control of the T7/lac promoter, inactivation through an irreversible DNA-damage alternative to traditional formaldehyde inactivation, and a DNA repair-less *E.coli* strain as chassis.

A nobel auto-induction media was developed based on previous reports to reduce possible toxicity [35,36,39]. Thus, we have previously found that the expression of recombinant anchor-proteins in *E.coli* under the T7/lac promoter control were partially or totally inhibited, due to early toxicity during the growth phase [17]. Residual lactose present in the casein hydrolyzate of the LB / TB media used may habe been responsible for that early autoinduction of *E.coli* BL21(DE3) causing reduction of the yields and irreproducibility [35]. Therefore, a series of experiments were undertaken to reduce early autoinduction that lead us to the development of the so called SB media, a media based on soy-bean rather than casein hydrolyzates and containing glucose to further inhibit early autoinduction. Using SB, the highest expression levels by PAGE / Western blotting were obtained for the YBEL+frgC$_{91-220}$ construct among 6 other anchors. Similar results were previously reported for YBEL+frgII$_{CyHV3}$ [17]. In contrast to the yields obtained with the previous TB media, the levels obtained with SB were now similar for 6 anchor-motifs despite of being originated from diverse

sources (*E.coli*, *B.subtilis*, phage). Nevertheless, the YBEL+frgC$_{91-220}$ construct still remained with the highest level of expression.

After optimization of the bacterial culture media, experiments were focused in finding inhibitors of DNA replication by searching for an alternative to the bacterial inactivation method by formaldehyde which destroyed ~80 % of the frgII$_{CyHV3}$ immunogenicity [17]. Among the inactivators screened, the DNA replication targeting their supercoiling steps appeared to be the best because it was well known. The best drugs for the present purposes were found among the quinolone family of antibiotics. Quinolones target DNA gyrase or topoisomerase II (Gram-negative bacteria) and topoisomerase IV (Gram-positive bacteria), by interacting with double stranded DNA, and stopping DNA cleavage and strand rejoining. Additionally, quinolone bactericidal effects are induced by harmful hydroxyl radicals or ROS [40]. The above mentioned topoisomerases are essential in bacteria but absent in higher eukaryotes, making them an attractive possibility for the present purposes. The best studied gyrase is that from *E.coli*, which has A and B subunits. The A subunit cleaves DNA, while the B subunit hydrolizes ATP for rejoining the DNA strands. Inhibition of cleavage by stabilisation of the covalent gyrase-DNA complex (gyrase poisoning), shows concentration-dependent bacteriostatic or bactericidal effects [41]. For instance, the Ciprofloxacin (CPFX) quinolone exhibits a bacteriostatic reversible activity at low concentrations and an irreversible bactericidal activity at high concentrations [42,43]. First generation quinolones derived from nalidixic/oxolinic acids are rarely used today because of their toxicity to eukaryotic cells. Second (i.e., Ciprofloxacin), third (i.e., Levofloxacin) and fourth (i.e., Gemifloxacin) generation quinolones are clinically used today. After numerous experiments, Ciprofloxacin was selected for this work because of its high activity at low concentrations, its irreversible DNA-damage (bactericidal) and its low cost. Because this is an area of intensive research, new quinolones may appear in the future to cause irreversible DNA-damage of recombinant bacteria.

Even though some fish vaccines based in eukaryotic expression plasmids (i.e., DNA vaccines) have been recently approved, their use is still highly controversial in Europe [44]. Therefore, using immune-relevant viral protein antigens rather than DNA may be a more practical alternative. Furthermore, protein antigens coded in prokaryotic rather than eukaryotic vectors like those employed for DNA vaccines, offer safer environmental alternatives. In addition, the maintainance of spinycterin morphology, adds some adjuvant-effects to the immunization [45-48], while allowing easier mass delivery and lower production costs. Spinycterins may be looked as a method to reduce the generation of low-immunogenicity inclusion bodies very often found when trying to express whole heterologous proteins in recombinant bacteria. Although, recent results suggested that isolated nanopellets derived from bacterial inclusion bodies may also be immunogenic [49], their practical use would need additional purification steps lossing bacterial morphology and their adjuvant properties and most probably, they will require too high concentrations. Most recently, however, intact recombinant bacteria carrying their inherent adjunvanticity and coding for the whole C$_{VNNV}$ protein has been reported to induce low levels of sea bass antibodies and protection when orally delivered [16]. Furthermore, injection of $10^{10}$ cfu of recombinant bacterial extracts per fish fully protected against VNNV challenge[16]. In this context, there are some practical advantages of the DNA-damaged recA- *E.coli* alternative when coding for surface-displaying downsized viral antigens (spinycterins) in comparison with recombinant wild type *E.coli* coding for the whole C$_{VNNV}$ protein. For instance, as shown in this work, only $10^{8}$ cfu per fish of injected morphologically-intact YBEL+frgC$_{91-220}$ spinycterins fully protected against VNNV challenge. Furthermore, spinycterins would be better accepted in aquaculture because they are safer due to their irreversible DNA-damaged and DNA-repair deficient *E.coli* vehicle. Other advantages are due to the antigen downsizing concept of spinycterins such as a higher epitope density for a given mass of bacteria, and the possibility to use with mixes of different pathogen antigenic sequences in a single delivery. Additionally, because of its isoleucine deficiency, the BLR *E.coli* strain opens up the possibility of developing antibiotic-free selection methods to eliminate any antibiotic resistance sequences and thus further increase environmental safety.

**Table 1.** Anchor-motifs fused to the N-terminus of frgC91-220 and resulting molecular weights

| Name | AccNum | KDa | references |
|---|---|---|---|
| **none+frgC$_{91-220}$** | AY284969 | 16.2 | [50] |
| **Mistic+frgC$_{91-220}$** | AY874162 | 28.9 | [30,51] |
| **Nmistic+frgC$_{91-220}$** | AY874162 | 20.0 | [30,51] |
| **NTD+frgC$_{91-220}$** | AJ516945 | 18.4 | [33] |
| **P9+frgC$_{91-220}$** | M12921 | 25.6 | [20] |
| **YAIN+frgC$_{91-220}$** | NP_414891 | 26.3 | [34] |
| **YBEL+frgC$_{91-220}$** | NP_415176 | 34.8 | [34] |

**AccNum**, Gene Bank accession numbers. **frgC$_{91-220}$**, amino acid residues 91-220 from the C coat protein of viral nervous necrosis virus VNNV (sequence accession number AY284959) [18,19]. The anchor-motif+GSGS+frgC$_{91-220}$+GSGS+polyH DNA sequences were designed, synthesized, cloned into pRSET, used to transform *E.coli* and autoinduced in SB media. **KDa**, expected molecular weight of the recombinant proteins. **N-mistic** and **Mistic**, 33 N-terminal and 110 amino acid anchor-motif from the Mistic gene from *Bacillus subtilis*. **NTD**, N-terminal domain of 21 amino acid anchor-motif of the exosporal BclA protein from *Bacillus anthracis*. **P9**, 90 amino acid anchor from the coat-protein of bacteriophage ϕ6. **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*.

**Table 2.** of *E.coli* culture media.

| Component | Concentration,% | TB | SB |
|---|---|---|---|
| **Yeast extract** | 2.4 | X | X |
| **Glycerol** | 0.8 | X | X |
| **$KHPO_4$** | 0.9 | X | X |
| **$KH_2PO_4$** | 0.2 | X | X |
| **Tryptone** | 1.2 | X | - |
| **Soybean hydrolysate** | 4.8 | - | X |
| **Glucose** | 0.3 | - | X |

Products were from Sigma Che. Co.

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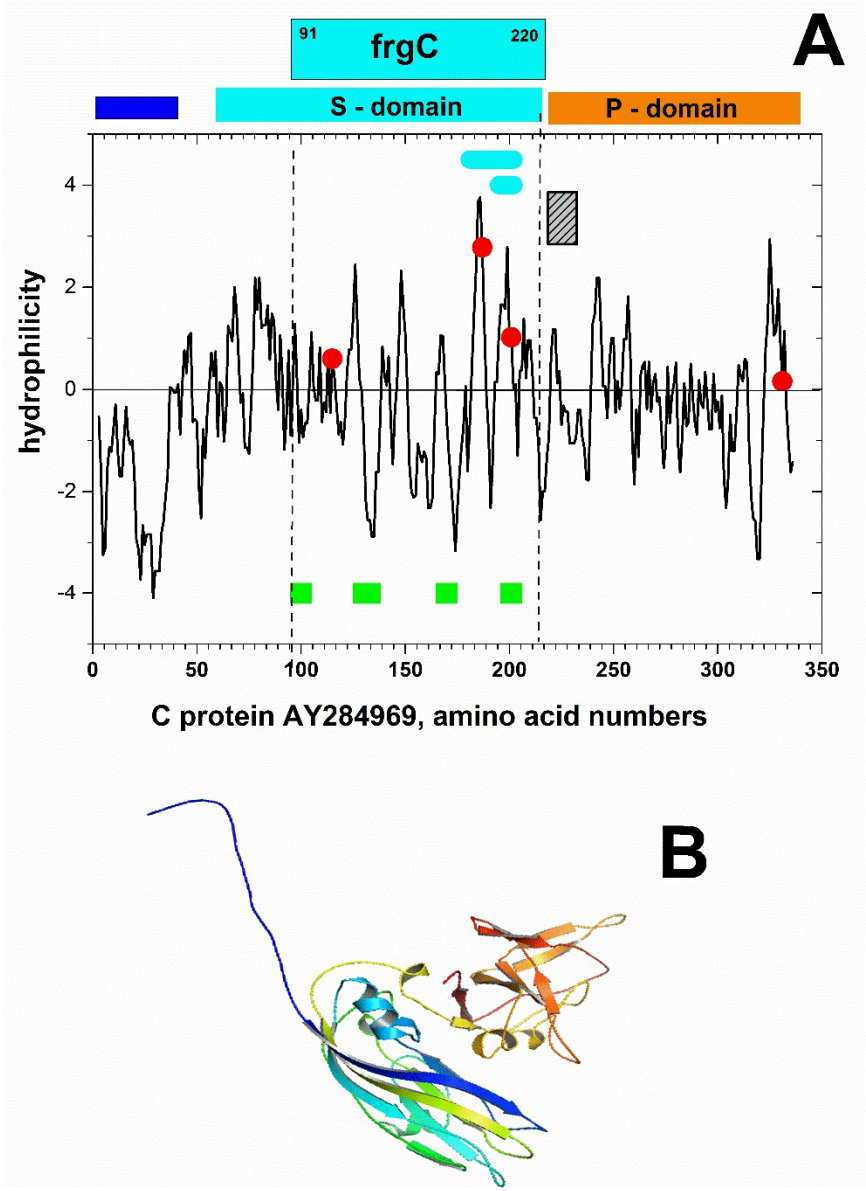


**Figure 1.** Scheme of the properties of frgC$_{91-220}$. **A)** Hydropathicity plot of the whole coat protein C from the *D.labrax* encephalitis virus isolate DL-040899-IL **(**AY284969) obtained using Clone Manager vs 9. Shell (S, blue rectangle) and protrusion (P, red rectangle) domains located in residues 52-213 and 221-338, respectively, according to X-ray data [26]. **Blue top rectangle**, frgC$_{91-220}$ (amino acid residues 91-220). **Blue horyzontal lines inside the plot**, neutralizing B-cell epitopes localized by pepscan mapping targeted by sera from sea bass surviving VNNV and by neutralizing monoclonal antibodies [19] or by alanine-scanning mutagenesis [18]. **Red circles**, cysteine positions which were mutated to serines in the recombinant frgC$_{91-220}$. **Green squares**, Ca++ binding sites for subunit-subunit interactions in the betanodavirus shell structure [26]. **Gray hatched rectangle**, highest protein sequence variability among betanodavirus isolates corresponding to the 223-331 positions [5]. **B)** Scheme of the tridimensional structure of the C protein of the AY284969 isolate. The automatically predicted modelled structure of the C protein of the AY284969 isolate (Swiss model server), selected the 4WIZ.3.A sequence as the best template; it was derived from a Grouper Nervous Necrosis Virus isolate [26] with a 99.11 % of sequence identity .

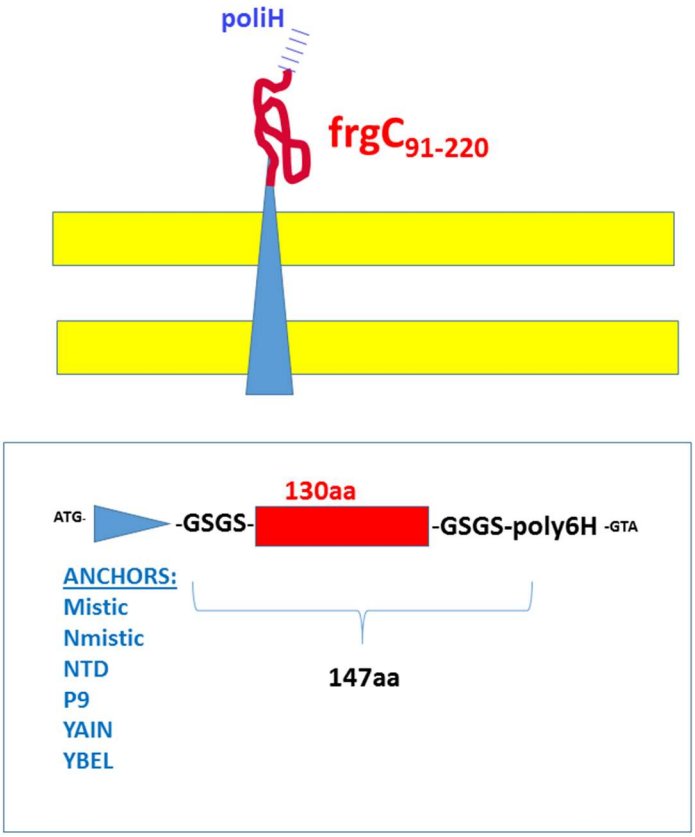


**Figure 2.** Scheme of the genetically fused constructs for bacterial surface expression of frgC$_{91-220}$. The nucleotide sequences corresponding to the frgC$_{91-220}$ were fused downstream to each of the 6 bacterial membrane anchor-motif sequences described in Table 2, bracketed by an arbitrarily chosen flexible linker (coding for GliSerGlicSer, GSGS). All the corresponding synthetically fused DNA sequences (GeneArt, Regensburg, Germany) were cloned into the pRSET prokaryotic expression plasmid which adds 6 Histidine tails (polyH) at their C-terminal ends. **Red**, FrgC$_{91-220}$. **Blue triangles**, Anchors. **Yellow**, schematic bacterial membrane. **Blue lines**, C-terminal polyH tail.

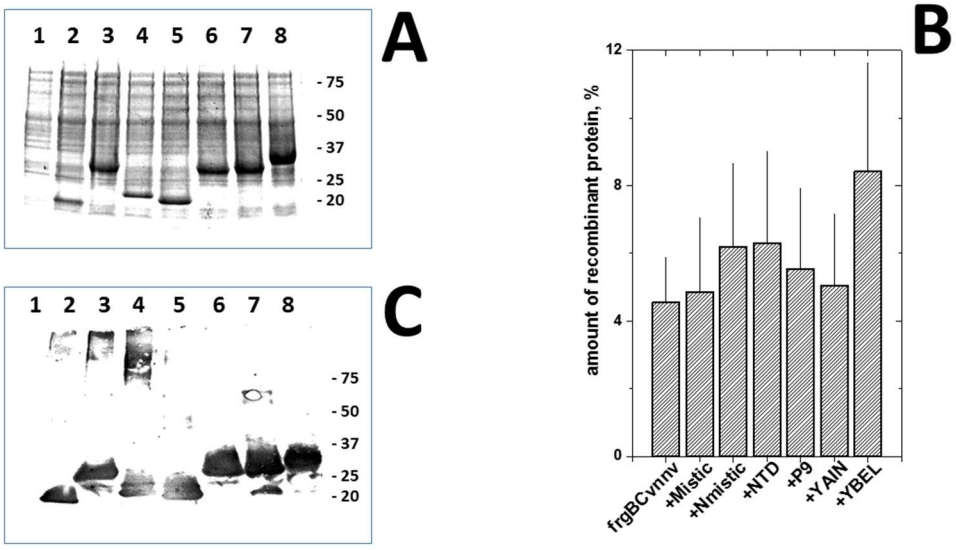


**Figure 3.** Coomassie-blue staining (A,B) and Western blotting (C) of anchor-motif+frgC$_{91-220}$ spinycterins grown and autoinduced in SB medium. (**A**) BL21 (DE3) *E.coli* coding for anchor-motif+frgC$_{91-220}$+polyH recombinant proteins were grown in autoinduction SB medium overnight. Bacteria were pelleted and their extracts analysed by Coomassie-blue staining. One representative

experiment is represented. (**B**). Densitometry of the anchor-motif+frgC$_{91-220}$ recombinant bands stained by Coomassie by Image J 1.41o software (http://rsb.info.nih.gov/ij). Means and standard deviations are shown (n=3). (**C**) Western blotting of gels transferred to nitrocellulose membranes, stained with peroxide-labeled anti-polyH monoclonal antibody and visualized with DAB as described [17]. One of 3 experiments was represented. **Numbers to the right of the gels**, KDa positions of molecular weight markers. The anchor-motifs of the recombinant *E.coli* in A and C corresponded to lanes: **1**, empty plasmid. **2**, frgC$_{91-220}$. **3**, Mistic+frgC$_{91-220}$. **4**, Nmistic+frgC$_{91-220}$. **5**, NTD+frgC$_{91-220}$. **6,** P9+frgC$_{91-220}$, **7**, YAIN+frgC$_{91-220}$. **8**, YBEL+frgC$_{91-220}$.

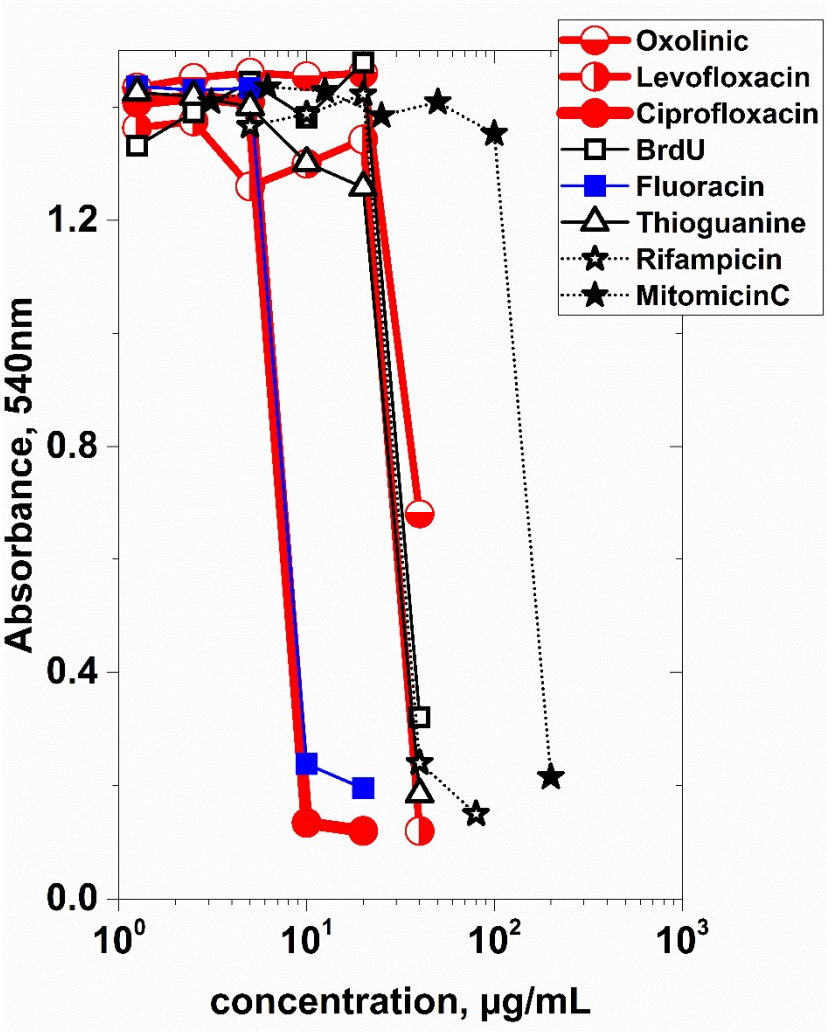


**Figure 4.** Selection of drugs for irreversible DNA-damage for *E.coli* BL21(DE3) inactivation. To select for a suitable drug to irreversibly inhibit *E.coli* BL21(DE3) replication without altering their immunogenicity, several antibiotics and/or base analogs were tested for YBEL+frgC$_{91-220}$ spinycterin replication. The recombinant bacteria (3 x $10^{10}$ cfu / ml) were exposed overnight to several concentrations of the selected antibiotics in 150 µl of TB with continuous agitation. After 2 washes with PBS, 10 µl of the suspensions were inoculated into 100 µl of fresh TB medium. Bacterial growth was estimated by absorbance at 540 nm after overnight incubation with agitation at 37 ºC. **Upper-half open red circles**, Oxolinic acid. **Left-half open red circles**, Levofloxacin. **Solid red circles**, Ciprofloxacin. **Open squares**, 5-Bromo deoxiuridine. **Solid blue squares**, 5-Fluoracin. **Open triangles,** 6-Thioguanine. **Open stars**, Rifampicin. **Solid starts**, Mitomicin C.

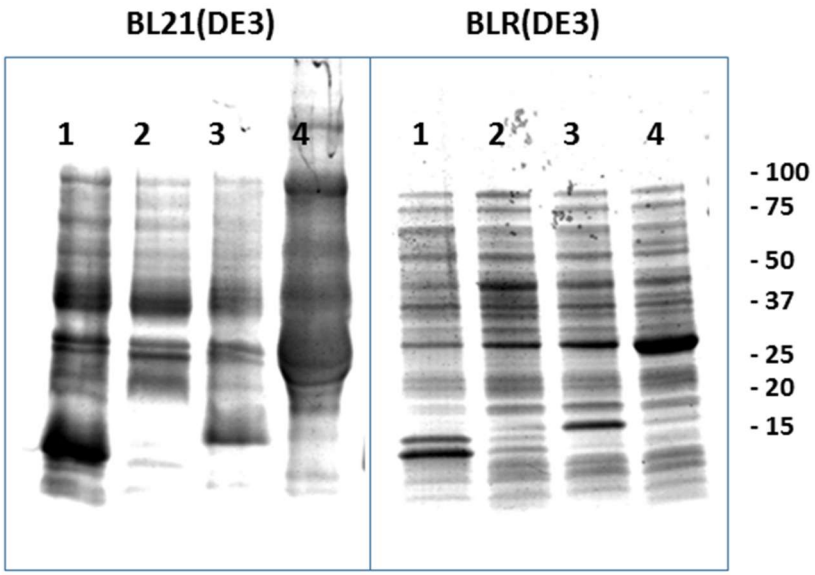


**Figure 5.** Coomassie-blue staining of anchor-motif+frgC$_{91-220}$ obtained in large amounts in BL21(DE3) and BLR(DE3) *E.coli* strains. *E.coli* coding for anchor-motif + GSGS +frgC$_{91-220}$+GSGS+polyH recombinant proteins were obtained in either BL21 (DE3) or in the repair-deficient *recA*- BLR(DE3) *E.coli* strains and grown in SB medium overnight. The *E.coli* BL21 (DE3) were grown at 37 ºC and induced with IPTG at 24 ºC for 2 h. The *E.coli* BLR(DE3) were grown and autoinduced for 4 days at 37 ºC. The resulting suspensions were incubated at 24 ºC for 2h with 125 µg / ml of CPFX for irreversible inactivation to generate spinycterins. Extracts were analysed as described in Figure 3. One of 2 experiments was represented. **Numbers to the right of the gels**, KDa positions of Coomassie blue stained molecular weight markers. The anchor-motifs of the spinycterins corresponded to lanes: **1**, frgC$_{91-220}$. **2**, empty pRSET plasmid. **3**, Nmistic+frgC$_{91-220}$. **4**, YBEL+frgC$_{91-220}$.

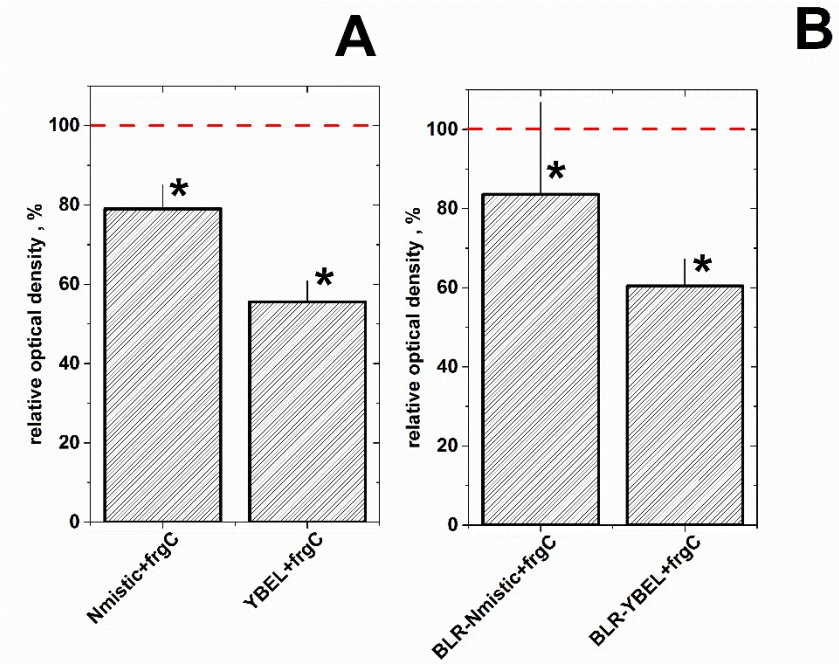


**Figure 6.** Estimation of frgC$_{91-220}$ surface enrichment by partial trypsin digestion of spinycterins followed by PAGE (A) and ELISA (B) analysis. **(A)** To assay by PAGE, the amounts of the corresponding stained bands were compared between trypsin-digested and control undigested spinycterins. The optical densities were first normalized by the formula, optical density of the recombinant bands / total optical density of each spinycterin extract. The normalized optical densities

were then calculated relative to the optical density obtained in the frgC$_{91-220}$ spinycterin bands by the formula, 100 x (% of anchor+frgC$_{91-220}$ / % of frgC$_{91-220}$). Means and standard deviations (n=3) were represented. **(B)** To assay by ELISA, 96-well plates were coated with trypsin-digested or control undigested spinycterins. The amount of exposed poliH tails was stimated by binding to peroxidase-conjugated anti-polyH monoclonal antibodies. Percentage of poliH binding was calculated by the formula, 100 x (Absorbance after trypsin digestion / Absorbance of undigested samples). The percentage of absorbance was then calculated relative to the frgC$_{91-220}$ spinycterins by the formula, 100 x (absorbance of anchor+frgC$_{91-220}$ / absorbance of frgC$_{91-220}$. Means and standard deviations (n=3) were represented. **Red horyzontal dashed lines,** mean optical density (A) and absorbance (B) of frgC$_{91-220}$. *, significatively different from frgC$_{91-220}$ spinycterins as determined by the Student t-test ($p<0.05$).

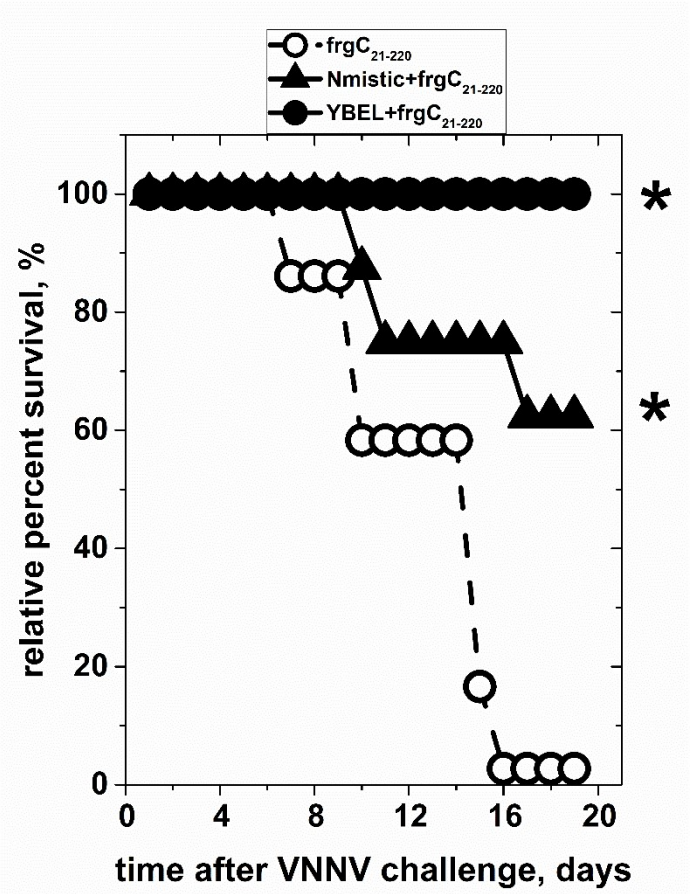


**Figure 7.** Protection to VNNV challenge of sea bass juveniles after intraperitoneal injection of frgC$_{91-220}$, Nmistic+frgC$_{91-220}$ or YBEL+frgC$_{91-220}$ spinycterins. Two independent aquaria per group each containing 15-20 fingerling sea bass (*D. labrax*) of ~10 g of body weight were injected with $10^8$ cfu of frgC$_{91-220}$, Nmistic+frgC$_{91-220}$ or YBEL+frgC$_{91-220}$ spinycterins. Non-infected, injected with empty pRSET plasmid and non-immunized controls were also included. Viral challenge was performed by intramuscular injection of VNNV (2 x $10^4$ TCID$_{50}$/ml). Mortality in the non-immunized VNNV-challenged group controls was 25.7 %. Relative percentages of survival were calculated by the formula 100 – (percentage of mortality in the spinycterin-immunized VNNV-challenged fish / percentage of mortality in the non-immunized VNNV-challenged fish). *, significantly different from the frgC$_{91-220}$ survival by the Log-Rank (Mantel-Cox) test at the $p<0.05$ level. Open circles, fish injected with frgC$_{91-220}$ spinycterins. Closed triangles, fish injected with Nmistic+frgC$_{91-220}$ spinycterins. Closed circles, fish injected with YBEL+frgC$_{91-220}$ spinycterins.

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