arTs Università degli Studi di Trieste
Archivio della ricerca – postprint

F-type lectin from serum of the Antarctic teleost fish *Trematomus bernacchii* (Boulenger, 1902): Purification, structural characterization, and bacterial agglutinating activity

Mariano Dara [a], Piero Giulio Giulianini [b], Chiara Manfrin [b], Maria Giovanna Parisi [a,*], Daniela Parrinello [a], Claudia La Corte [a], Gerardo R Vasta [c], Matteo Cammarata [a,*]

[a] *Marine Immunobiology Laboratory, Department of Earth and Marine Sciences, University of Palermo, Palermo, Italy*
[b] *Department of Life Sciences, University of Trieste, Building Q - room 306, Trieste, Italy*
[c] *Department of Microbiology and Immunology, University of Maryland School of Medicine, UMB, IMET, Suite 236, Columbus Center, 701 East Pratt Street, Baltimore, MD 21202, USA*

ARTICLE INFO

Editor: Chris Moyes

Keywords:
Lectins
F-type lectin
Antarctic fish
Trematomus bernacchii
Bacterial agglutination

ABSTRACT

The increasing availability of sequenced genomes has enabled a deeper understanding of the complexity of fish lectin repertoires involved in early development and immune recognition. The teleost fucose-type lectin (FTL) family includes proteins that preferentially bind fucose and display tandemly arrayed carbohydrate-recognition domains (CRDs) or are found in mosaic combinations with other domains. They function as opsonins, promoting phagocytosis and the clearance of microbial pathogens.

The Antarctic fish *Trematomus bernacchii* is a Perciforme living at extremely low temperatures (−1.68 °C) which is considered a model for studying adaptability to the variability of environmental waters. Here, we isolated a Ca^{++}-independent fucose-binding protein from the serum of *T. bernacchii* by affinity chromatography with apparent molecular weights of 32 and 30 kDa under reducing and non-reducing conditions, respectively. We have characterized its carbohydrate binding properties, thermal stability and potential ability to recognize bacterial pathogens. In western blot analysis, the protein showed intense cross-reactivity with antibodies specific for a sea bass (*Dicentrarchus labrax*) fucose-binding lectin. In addition, its molecular and structural aspects, showing that it contains two CRD-FTLs confirmed that *T. bernacchii* FTL (*Tb*FTL) is a *bona fide* member of the FTL family, with binding activity at low temperatures and the ability to agglutinate bacteria, thereby suggesting it participates in host-pathogen interactions in low temperature environments.

1. Introduction

Although teleost fish express most components of typical mammalian adaptive immune responses, it is well-established that a significant portion of their immediate response against infectious challenges is carried out by innate immune defence mechanisms. Accordingly, during the past few years, interest in the structural-functional aspects of fish innate immune responses, particularly lectins, toll-like receptors and complements, has grown at an exponential rate (Magnadóttir, 2006; Vasta et al., 2011; Cammarata et al., 2016; Elumalai et al., 2019; Sahoo, 2020). Lectins are carbohydrate-binding proteins present in essentially all living organisms and are involved in immune responses as well as in other biological processes. Most lectins are multivalent proteins capable of recognizing and binding carbohydrate moieties by specific domains (carbohydrate-recognition domains; CRDs) (Drickamer, 1999); they also participate in various biological functions (Kuhlman et al., 1989; Cooper et al., 1994), including innate and adaptive immune responses (Arason, 1996; Drickamer and Dodd, 1999; Vasta and Ahmed, 2008). Because lectins may display CRDs in combination with other domains, they not only recognize carbohydrates on the surfaces of potential pathogens, but also mediate several effector functions, including the opsonization of microbial pathogens, immobilization, and agglutination, as well as

* Corresponding authors.
E-mail addresses: mariano.dara@unipa.it (M. Dara), giuliani@units.it (P.G. Giulianini), cmanfrin@units.it (C. Manfrin), mariagiovanna.parisi@unipa.it (M.G. Parisi), daniela.parrinello@unipa.it (D. Parrinello), claudia.lacorte@unipa.it (C. La Corte), gvasta@som.umaryland.edu (G.R. Vasta), matteo.cammarata@unipa.it (M. Cammarata).

Accepted 7 June 2021

complement pathway and phagocyte activation (Russell and Lumsden, 2005). Based on the presence of conserved amino acid sequence motifs in their CRDs, their calcium requirements and structural folding, animal lectins have been classified into several families, such as C-, P-, I-, and L-type lectins, galectins, pentraxins and others (Arason, 1996; Vasta et al., 2004). Among these, the F-type lectin family is characterized by the binding of fucose, a unique sequence motif in its CRD (F-type CRD), and a novel structural fold (Odom and Vasta, 2006; Bianchet et al., 2010; Bianchet et al., 2002). The F-type CRD can be associated with other domains (pentraxin, C-type lectin, or "sushi" domains), yielding complex multidomain proteins (Vasta et al., 2004).

Lectins from most families, including C- and R-type lectins and galectins, have been isolated from tissues and organs of teleost fish species, and various defence functions have been identified, including opsonization, enhancement of intracellular respiratory burst, and activation of bactericidal activity (Arason, 1996; Jensen et al., 1997; Listinsky et al., 1998; Suzuki et al., 2003; Russell and Lumsden, 2005; Cammarata et al., 2016). F-type lectins (FTLs) have also been identified and characterized from the serum of several teleost fishes, such as eels (*Anguilla* sp.), striped bass (*Morone saxatilis*), gilt-head bream (*Sparus aurata*), the European bass (*Dicentrarchus labrax*) (Cammarata et al., 2016). Although F-type lectins have been proposed to mediate roles as molecular recognition factors in innate immunity, the experimental evidence is fragmentary and the detailed mechanisms of their activity have not been fully elucidated (Vasta et al., 2004).

Among teleost fish, the Arctic and Antarctic species present unique research opportunities because of the extreme environments they live in. Notothenioidei is a sub-order within the Perciformes that has radiated to become the most numerous and widespread fish taxon in the Antarctic region, where it encompasses at least 90% of the biomass of the fishes populating this environment (Wells and Eastman, 1994). During the past few years, studies have placed emphasis on their environmental adaptations, particularly on their ability to avoid freezing and survive at extremely low temperatures (−1.68 °C) (Devries, 1982). Furthermore, because the Perciformes are a closely related groups, the physiological consequences of ecological adaptations between species can be evaluated without the difficulties posed by comparing fishes with different phylogenetic backgrounds. In this regard, they constitute an excellent study model for investigating structural and functional adaptations of proteins to low temperatures. For example, biochemical features, such as protein amino acid differences among species, have been useful tools for investigating evolutionary processes and relationships, and for identifying events associated with speciation (Macdonald et al., 1988).

Trematomus bernacchii, an Antarctic notothenioid fish, has been the species of choice as a model system for investigations focused on adaptability to climate change and environmental contamination, particularly the potential warming and acidification of environmental waters, as well as its detoxification capabilities for environmental pollutants (Santovito et al., 2012; Laura et al., 2017). Studies have been carried out on immunoglobulin genes, individuating structural and functional modifications and adaptations to the low temperature of the Southern Ocean (Oreste and Coscia, 2004; Coscia et al., 2010; Coscia et al., 2011).

Unlike immunoglobulins, lectins do not generate diversity in recognition by genetic recombination and, therefore, additional interest has arisen in the germline-encoded diversity of the lectin repertoires, including their allelic variation or polymorphisms, the presence of tandem gene duplications and multigene families, the formation of chimeric structures by exon shuffling, additional mechanisms for expanding the ligand recognition spectrum by alternative splicing, and the structural basis for the potential "plasticity" of their carbohydrate binding sites (Vasta et al., 2011).

Our interest in this species has centred on its innate immune recognition capacity mediated by lectins in these extreme low-temperature conditions. In this study, we initiated the characterization of a two-CRD, 32 kDa FTL (*Tb*FTL) that we isolated from *T. bernacchii* serum, including its carbohydrate binding properties, thermal stability, structural aspects as compared to available FTL structures and potential ability to recognize bacterial pathogens. Our results confirmed that is a *bona fide* member of the FTL family, has binding activity at low temperatures and can agglutinate bacteria, thereby suggesting its potential participation in host-pathogen interactions in the Antarctic low temperature aquatic environment.

2. Materials and methods

2.1. Chemicals, molecular biology reagents and bacteria

Chemicals and reagents were purchased from Sigma-Aldrich (USA). *Escherichia coli* (ATCC 25922) was obtained from Chrisope Technologies (Louisiana, USA).

Bacillus subtilis and *Kocuria rhizophila* (ATCC 9341) were supplied from the Molecular Microbiology and Biotechnology Laboratory of the University of Palermo (Italy).

2.2. Animals, collection of blood and preparation of serum from T. bernacchii

The sample collection and animal research conducted in this study comply with the regulations of the Italian Ministry of Education, University and Research concerning activities and environmental protection in Antarctica, as well as the Protocol on Environmental Protection to the Antarctic Treaty, Annex II, Art. 3.

Blood was collected, from 6 specimens, by caudal venepuncture from *T. bernacchii* caught in the Ross Sea near the Zucchelli Station (Terra Nova Bay, 74°41′42″S 164°06′50.4″E). The serum was collected by centrifugation at 2000 × *g* for 10 min and stored in liquid nitrogen until further processing upon arrival in Italy. Pools from specimens were used for different purifications.

2.3. Purification of T. bernachii serum F-type lectin

*Tb*FTL was isolated by affinity chromatography. Twenty ml of diluted serum (1:10 in Tris Buffered Saline, TBS) were loaded on a column containing L-fucose-agarose and equilibrated with TBS, following the method reported by Cammarata et al. (2007) at a constant temperature (10 °C). The column was washed with 1.0 M NaCl, followed by TBS (10 volumes) at a 0.5 ml/min flow rate. Elution of *Tb*FTL was carried out with 20 ml of 50 mM L-fucose in TBS at the same flow rate. The protein concentration was monitored for each fraction. The fractions containing the isolated protein, collected after fucose elution, were dialyzed against TBS using a cellulose tube (Sigma-Aldrich, MWCO 12400 Da) overnight for sugar removal from the samples. The dialyzed fractions were tested for hemagglutinating activity (HA) toward rabbit erythrocytes, and those that exhibited the highest activity were pooled and analysed by electrophoresis.

2.4. Protein content estimation

Protein content was estimated using the Bradford solution (Bradford, 1976), reading the sample's absorbance at 595 nm. Bovine serum albumin was used as a standard.

2.5. Hemagglutination activity

Rabbit and sheep erythrocytes (rabbit and sheep red blood cells: RBC) were supplied by the Istituto Zooprofilattico della Sicilia (Italy). They were washed three times by centrifugation at 500 ×*g* for 10 min at 4 °C in phosphate buffered saline (PBS) and resuspended at 1% in TBS containing 0.1% (*w/v*) pig gelatine. A volume (25 μl) of serum or a volume of the dialyzed purified *Tb*FTL were 2-fold serially diluted in TBS-gelatine in 96-well round-bottom microtiter plates (Nunc,

Denmark), and an equal volume of erythrocyte suspension was added. The hemagglutinating activity titer (HAT) was measured after incubation for 1 h at 10 °C. HAT is expressed as the reciprocal of the last dilution showing clear agglutination (Ballarin et al., 2008).

The HA of serum and isolated fractions was assayed against RRBC in the presence of potential inhibitor carbohydrates: L(−) fucose, D(+) fructose, D(+) glucose, D(+) galactose, L(−) rhamnose, carried out using decreasing concentrations (from 200 mM to 0.09 mM in TBS pH 7.4, 1% gelatine) (Ballarin et al., 2008).

Divalent cation requirement for serum HA was examined by adding $CaCl_2$ and $MgCl_2$ to the assay medium to obtain 3 mM for each final concentration. Ethylenediaminetetraacetic acid (EDTA,10 mM) or Ethylene glycol bis (2-aminoethyl) tetraacetic acid (EGTA, 10 mM) were used to examine the effect of Ca^{2+} *versus* Mg^{2+} depletion.

To examine the thermolability, serum samples or purified fractions were incubated at 1, 4, 7, 10, 15, 20, 36 and 50 °C for 20 min before testing the HA. To investigate the activity at temperatures close to 0 °C, the serum and pure molecule agglutination assays were carried out at 0 °C and 4 °C, and *E. coli* and Rabbit RBC were employed as targets.

2.6. Bacterial suspensions

Escherichia coli, *Bacillus subtilis* and *Kocuria rhizophila* bacteria were grown to log phase in tryptic soy broth containing 3% NaCl at 25 °C, with continuous shaking (120 rpm) in a Gallenkamp incubator. We determined the relationship between cell number by plate count and cultures and absorbance evaluation (600 nm) to standardize the number of cells during all the experiments. Bacteria were killed by heat (120 °C, 1 atm for 20 min). After centrifugation at 6000 ×*g* for 15 min at 4 °C, the heat-killed bacteria were washed three times with sterile PBS, suspended in PBS containing 0.1% (*w/v*) gelatine to obtain 1×10^9 cells/ml, and stored at 4 °C until use.

2.7. Fluorescent bacteria preparation

Centrifuged bacteria (10,000 ×*g*, 5 min, 4 °C) were resuspended in sodium bicarbonate buffer and diluted. 0.2 µl of FITC (fluorescein isothiocyanate) stock solution and 10 mg/ml FITC in DMSO (Dimethyl sulfoxide) were added to the bacteria suspension and incubated under agitation for 30 min in the dark at room temperature. Bacteria were washed 4 times with HBSS (Hank's balanced salt solution) to remove excess FITC and resuspend it to the final concentration.

2.8. Bacterial agglutination assay and fluorescent bacteria observation

For the agglutination assay, bacteria were washed three times in sterile PBS, suspended in PBS containing 0.1% (w/v) gelatine to obtain 1×10^7 bacteria/ml and dispensed in 96-well plates. Plates were incubated at 18 °C overnight. A volume (25 µl) of serum or dialyzed isolated fractions were 2-fold serially diluted in TBS-gelatine in 96-well flat-bottom microtiter plates (Nunc, Denmark), and an equal volume of bacterial suspension was added. The bacteria agglutinating titer (BAT) was measured after overnight incubation at 10 °C and expressed as the reciprocal of the highest dilution showing clear agglutination (Ballarin et al., 2008).

Fluorescent *E. coli* labelled by FITC was utilized to observe and better visualize the agglutinated bacteria. Fluorescent bacteria and the serum or dialyzed isolated fractions were mixed, dispensed on microscope slides, covered with cover slides, and incubated in a humid chamber overnight at room temperature.

After the incubation, the slides were observed under Nomarsky differential interference contrast microscopy (Diaplan, Leika, Wetzlar). FITC-labelled bacteria appear bright green when observed under UV light.

2.9. Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE 10%) was carried out following the method of Laemmli (1970), under different experimental conditions. Samples were treated with 5% beta-mercaptoethanol at 95 °C for 15 min, 5% beta-mercaptoethanol at room temperature, in absence of beta-mercaptoethanol at room temperature and, finally, in absence of beta-mercaptoethanol at 95 °C for 15 min.

To evaluate molecular size, gels were calibrated with low-range standard proteins (Bio-Rad, Richmond, CA). Proteins were stained with Coomassie Brillant Blue R250.

2.10. Antibody preparation

Anti-*Dicentrarchus labrax* fucose binding lectin (*Dl*FBL) antibodies were prepared and assayed according to Cammarata et al. (2001). Briefly, antibodies were produced in rabbits by injections of *Dl*FBL isolated from SDS-PAGE slices. Slices were suspended in distilled water and fragmented by passing them repeatedly through a syringe. The coarse suspension of lectin was used for rabbit immunization by Medprobe (Norway).

For greater certainty and to check for specificity, the antiserum was absorbed with *Dl*FBL overnight at 4 °C and centrifuged at 27,000 ×*g* for 30 min at 4 °C.

2.11. Western blot analysis

SDS-PAGE gels were soaked in transfer buffer (20 mM Tris, 192 mM glycine, 10% methanol, pH 8.8) for 10 min and proteins were transferred for 75 min at 0.8 mA/cm^2 to a nitrocellulose sheet in a semi-dry blotting bath (Biorad, USA). The filter was soaked in blocking buffer (PBS containing 3% BSA and 1% Tween 20) for 1.5 h. After three washes with PBS-T, the nitrocellulose sheet was incubated with anti-*Dl*FBL (1:800 in PBS) for 1 h, then washed 4 times with blocking buffer and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit sheep IgG (Sigma; 1:1000 in blocking buffer). To reveal the bands, four washes with PBS for 15 min were performed, and the filter was treated with 3 ml of 3,3′,5,5'-Tetramethylbenzidine (TMB) liquid substrate system.

Negative control and antibody specificity were checked by incubating the *Tb*FTL electroblotted on the membrane and incubated with pre-immune serum and pre-absorbed serum.

2.12. Phylogenetic analysis

The deduced amino acid sequence (NCBI accession number: XP_034001797) was obtained from the publicly available head kidney transcriptome (ID: PRJNA263718) (Gerdol et al., 2015) (Qiagen, Denmark) by performing a BLAST search using the *Dl*FBL sequence as the query. The obtained sequence was submitted to multiple alignments using the CLUSTAL X program v.2.1(28). A phylogenetic tree was constructed by the Neighbor-Joining method (NJ), considering 1000 bootstrap hits using CLC Sequence Viewer Version 8.0. The computation of theoretical protein features from the peptide sequence was performed with the ProtParamTool (Gasteiger et al., 2005). The tertiary structure of *Tb*FTL was modelled using the crystallographic structure of the *M. saxatilis* F-type lectin as a template (Bianchet et al., 2010).

2.13. N-terminal sequencing

The purified lectin was run by SDS-PAGE in reducing conditions, then the band was transferred (10 V for 30 min) by electrophoresis onto a polyvinylidene difluoride (PVDF) Immobilon membrane (Millipore, Bedford, MA, USA). The membrane was treated with Coomassie blue R-250 (0.1% Coomassie blue R-250, 40% methanol, 1% glacial acid acetic)

and rinsed extensively with 50% methanol and distilled water. The membrane portions containing proteins were excised and applied to a protein sequencer (Shimadzu PPSQ30). The sequence was determined by automated Edman degradation using the TFA conversion provided by the Biochemistry Institute of the Italian National Research Council (CNR, Naples, Italy).

3. Results

3.1. Hemagglutinating activity of the T. bernacchii serum

Fish sera (7.20 ± 0.9 mg/ml) were assayed for HA ($n = 6$), showing a Ca^{2+}- and Mg^{2+}-independent agglutinating activity toward rabbit erythrocytes (RRBC) (HAT 64-128) and, to a lesser extent, sheep erythrocytes (HAT 16-32) at 10 °C. The presence of $MgCl_2$ or $CaCl_2$ (5 mM), EGTA or EDTA (10 mM, in presence or absence of $CaCl_2$ or $MgCl_2$) in sera (HA 64–128) did not significantly affect HA. The data are summarized in Table 1.

3.2. Lectin purification, features

Hemagglutination-inhibition tests allowed us to characterize the sugar specificity of lectin(s) potentially present in serum (Table 2). Among the sugars tested, L-fucose inhibited hemagglutination at the lowest concentration (1.75 mM). Based on this information, we proceeded with the purification by using a fucose-agarose column. Fig. 1 shows a typical affinity chromatography purification profile of 20 ml of diluted serum carried out at 8 °C. HA was found in the dialyzed fractions.

3.3. SDS-PAGE analysis

The purified fractions eluted from the column (0.38 ± 0.05 mg/ml) were analysed by SDS-PAGE under different conditions. Electrophoresis on SDS-PAGE under non-reducing conditions revealed a single 30.0 ± 0.7 kDa component (Fig. 2A, lane 1), whereas under reducing conditions (5% beta-mercaptoethanol, 95 °C for 15 min) the apparent mass was 32.0 ± 0.5 kDa (Fig. 2A, lane 2).

3.4. Western blot

Western blot analysis (Fig. 2B) of the isolated fractions showed that *Dl*FBL antibody (anti-*Dl*FBL) cross-reacted with the purified *Tb*FTL, both under reducing and non-reducing conditions (lane 2-no reducing condition, and lane 3-reducing condition). The purified *Dl*FBL used as the positive control showed an intense band of the expected mobility (lane 1-no reducing condition, and lane 4-reducing condition). No cross reaction appeared on the membrane treated with pre-immune serum (Fig. 2C, lane 5 and 6). The same result, no reaction, was obtained by incubating the membrane with the adsorbed serum.

3.5. Thermal stability

The purified protein maintained stable HA toward RRBC (HAT: 128–256) at 1, 4, 7, 10, 15 °C, with a small increase at 20 °C, but was rapidly inactivated by incubation at temperatures over 36 °C for 30 min (Fig. 3). In the experiments carried out at low temperatures (0 and 4 °C), both the serum and the purified fractions induced the agglutination targets, both erythrocytes and bacterial cells (Table 3). The HA tests were carried out in triplicate. 2-way ANOVA was conducted to determine the effects of temperature, comparing the HA activity of the three different lectins (Fig. 3). Asterisks indicate significance among the HA activity (** $P < 0.0001$; * $P < 0.05$). Blue asterisks indicate statistical significance between *Tb*FTL HA and *Dl*FBL HA, green asterisks between *Tb*FTL HA and SaFBL. Error bars indicate the standard deviation.

Table 2
Inhibition of hemagglutinating activity of the *T. bernacchii* fucose binding lectin (*Tb*FTL) against RRBC by various carbohydrates.

Inhibitor sugars	Minimum inhibiting concentration (mM)
L-fucose	3.15–1.75
D-galactose	NI
L-rhamnose	NI
D-fructose	NI
D-glucose	NI

(NI = No inhibition).

3.6. Bacterial agglutination

The bacterial agglutinating activity of the purified lectin was demonstrated by a bacterial agglutination assay. In the presence of lectin, the bacteria utilized in the tests (*Escherichia coli*, *Bacillus subtilis* and *Kocuria rhizophila*) showed a measurable clear agglutination, confirming the bacterial recognition and cross-linking capabilities of the molecule. BATs from the bacterial agglutination assay were 8–16, 8–32 and 32 toward *Escherichia coli*, *Bacillus subtilis* and *Kocuria rhizophila*, respectively. Results are summarized in Table 4. The assay results are shown in Fig. 4, as observed under a light microscope (A, B), as well as the fluorescent bacteria (labelled by fluorescein isothiocyanate, FITC) under Nomarsky differential interference contrast microscopy (Diaplan, Leika, Wetzlar) (Fig. 4 C, D).

3.7. Amino acid sequence of the isolated lectin

The protein identified by SDS-PAGE under non-reducing conditions was transferred to PVDF membrane and sequenced. The determined N-terminal sequence was YQNVAMRGKA. A GenBank search with the N-terminal and internal peptides revealed identities with fucolectin of *T. bernachii* and a similarity to other F-type lectin family members.

3.8. Primary structure

The transcriptome of *T. bernacchii* (accession number: PRJNA263718) contains the complete nucleotide sequence of 1351 nucleotides, with a 930 open reading frame corresponding to a putative fucose-binding lectin. The deduced protein sequence reveals a cleavage site of the 19-residue signal peptide at the N-terminal between Thr19 and Tyr20; the signal peptide was predicted by the SignalP 4.1 online tool (Petersen et al., 2011). The putative translation start site is 191 bp downstream of the 5'-UTR (untranslated region), and a 196-nucleotide 3'-UTR spans from the stop codon to the polyadenylation site. The deduced mature protein sequence (Fig. 5) is 310 amino acids long, with a calculated molecular mass of 32.16 kDa and an isoelectric point of 5.21. The deduced size for the mature protein agrees with the size of the lectin previously isolated from the serum through column affinity chromatography and visualized through SDS-PAGE. Like other FTLs,

Table 1
Hemagglutination titer of the specimens toward rabbit or sheep erythrocytes in presence of ions or chelating agents.

	Buffer (Tbs)	$MgCl_2$ (5 mM)	$CaCl_2$ (5 mM)	EDTA (10 mM)	EDTA (10 mM) $CaCl_2$ (5 mM)	EGTA (10 mM)	EGTA (10 mM) $MgCl_2$ (5 mM)
Rabbit erythrocyte HAT	64–128	64–128	64–128	64–128	64–128	64–128	64–128
Sheep erythrocyte HAT	16–32	16–32	16–32	16–32	16–32	16–32	16–32

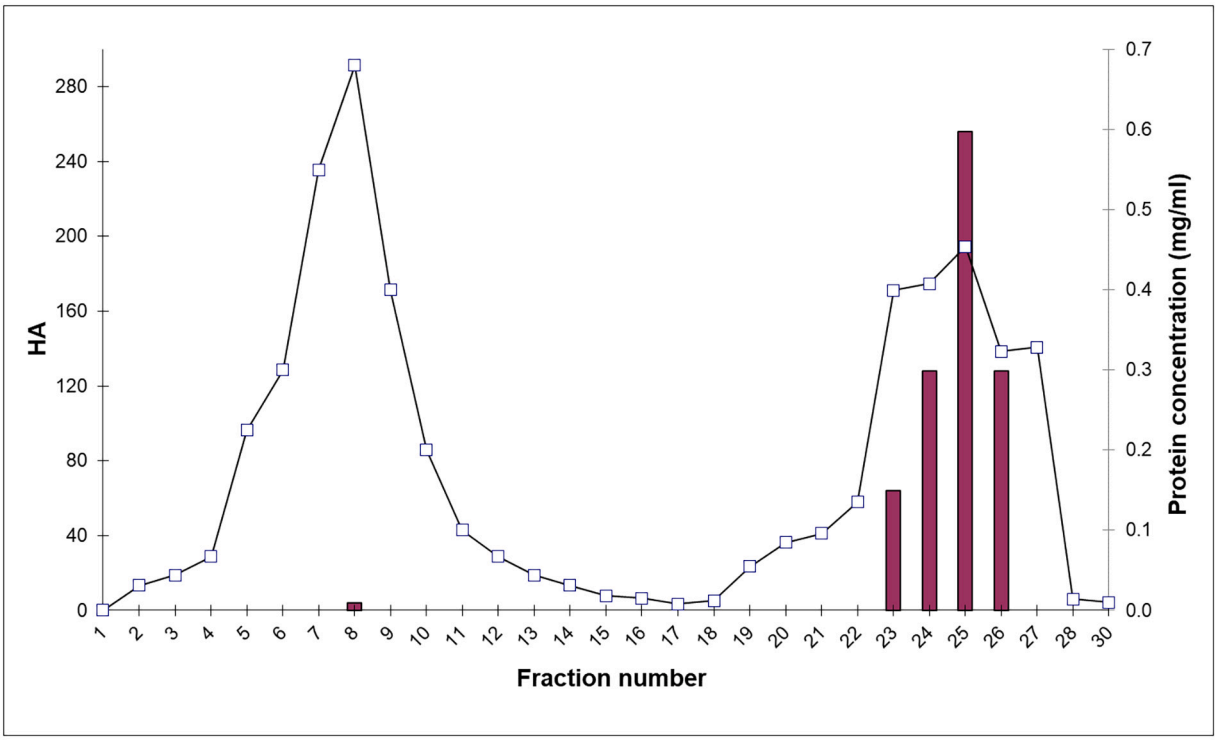

Fig. 1. Purification profile.
Affinity chromatography purification profile of *T. bernacchii* serum flow through a fucose-agarose column. Protein concentration (□); Hemagglutinating activity (HA; red bars). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

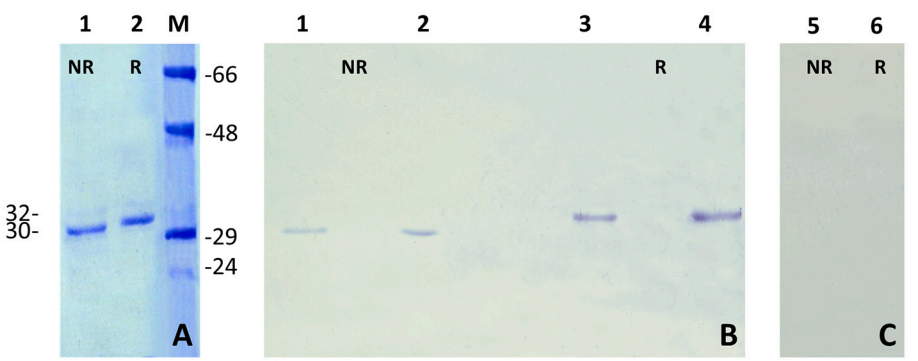

Fig. 2. SDS-PAGE and western blot of *T. bernacchii* purified lectins (*Tb*FTL).
Purified protein (2 μg per lane) was subjected to SDS-PAGE (10%) under reducing conditions (95 °C for 15 min, 5% beta-mercaptoethanol) (Fig. 2A, lane 2), and non-reducing conditions (room temperature, absence of beta-mercaptoethanol) (Fig. 2A, lane 1). Molecular weights (kDa) of markers are on the right (lane M); on the left are the molecular weights of the reduced and non-reduced protein, 32 and 30 KDa respectively. The gel was stained with Coomassie blue (Fig. 2A). *Tb*FTL subjected to SDS-PAGE under reducing conditions was electrotransferred to a nitrocellulose membrane for western blotting (Fig. 2B). The nitrocellulose sheet was treated with anti-*D. labrax* F-lectin primary antibody and horseradish peroxidase-conjugated anti rabbit IgG. *Dl*FBL was utilized as control. Lane 1 *Dl*FBL under non-reducing conditions (NR); lane 2 *Tb*FTL under non-reducing conditions (NR); lane 3 *Tb*FTL under reducing conditions (R); lane 4 *Dl*FBL under reducing conditions (R). Fig. 2C shows *Tb*FTL under non-reducing conditions (NR, lane 5) and under reducing conditions (R, lane 6) blotted on nitrocellulose membrane; cross reaction after incubation with pre-immune serum is not shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

*Tb*FTL is most likely a secreted protein. Correspondence with the purified lectin was confirmed by the first ten N–terminal amino acids sequenced (highlighted in yellow).

3.9. Primary structure analysis, phylogenetic analysis, homology modelling and comparison with CRD structures from other FTLs

Basic Local Alignment Search Tool (BLAST) analysis showed that the *Tb*FTL deduced amino acid sequence presents close homologies with vertebrate binary F-lectins and revealed that *Tb*FTL is a binary tandem domain FTL with the N- and C-CRDs connected by a 15 amino acid peptide linker. The *Tb*FTL protein sequence revealed that this lectin, like the *M. saxatilis* FTLs, possesses two tandemly arrayed CRDs. Odom and Vasta isolated two fucose-binding lectins of 30 and 32 kDa (MsaFBP32 and MsaFBP32II) from the serum and liver of the striped bass (*M. saxatilis*), each carrying two tandem CRDs that exhibit the F-type carbohydrate recognition motif and the typical F-type structural fold established for the *A. anguilla* agglutinin (AAA) (Odom and Vasta, 2006;

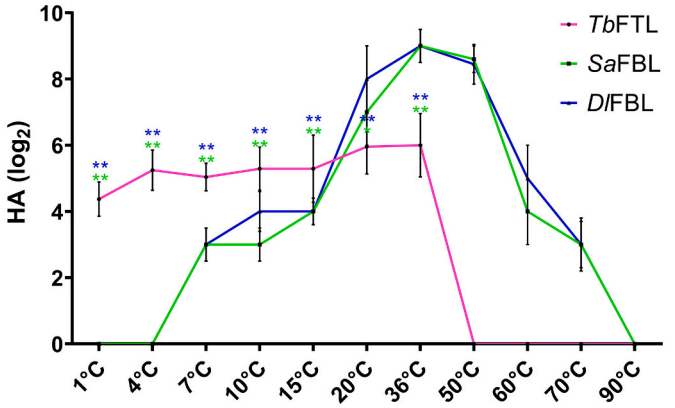

Fig. 3. Temperature effect.
The HA of the purified molecule was tested using rabbit erythrocytes and maintained stable activity after treatment at 1, 4, 7, 10, 15 °C, with a small increase at 20 °C, but was blocked by treatment at temperatures above 36 °C for 30 min. Blue and green lines indicate the HA of F-lectins from the serum of warmer water fishes (respectively, the Perciformes *Dicentrarchus labrax* and *Sparus aurata*). 2-way ANOVA was conducted to determine the effects of temperature, comparing the HA activity of the three different lectins. Asterisks indicate significance among the HA activity (** $P < 0.0001$; * $P < 0.05$). Blue asterisks indicate statistical significance between *Tb*FTL HA and *Dl*FBL HA, green asterisks between *Tb*FTL HA and SaFBL. Error bars indicate the standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Agglutinating activity toward bacteria and erythrocytes (BAT, HAT) of serum and *Tb*FTL at 0 and 4 °C. Data are expressed as Log_2 of the agglutination titer ± the standard deviation.

		Agglutination/Cp (log_2) ± SD	
		E. coli	*Rabbit RBC*
0 °C	Serum	3.5 ± 0.5	9.5 ± 0.5
	*Tb*FTL	3 ± 0.5	6 ± 0.5
4 °C	Serum	3.5 ± 0.5	9.5 ± 0.5
	*Tb*FTL	2.5 ± 0.5	6 ± 0.5

Table 4
Bacterial agglutinating activity of *Tb*FTL toward different bacteria. Agglutinating activity is expressed as agglutination titer and as Log_2 of the agglutination titer ± the standard deviation.

Bacterial	BAT	Agglutination/Cp (Log_2) ± SD
Escherichia coli	8–16	3 ± 0.5
Bacillus subtilis	8–32	4 ± 1
Kocuria rhizophila	32	5 ± 0

Bianchet et al., 2010; Bianchet et al., 2002). There are relevant biochemical and structural similarities between *Tb*FTL and the two-binary tandem CRD F-type lectins isolated from the striped bass and European bass (Odom and Vasta, 2006; Bianchet et al., 2010).

The *Tb*FTL deduced amino acid sequence presents high sequence identity with the fucose-binding lectins isolated and characterized in various fish species within the Perciformes (Supplemental Fig. 1), such as *Sander lucioperca*, *Perca flavescens*, *Oplegnathus fasciatus*, *Larimichthys crocea*, *Parambassis ranga*, *Anabas testudineus*, *Oreochromis niloticus*, *Astatotilapia calliptera*, *Amphiprion ocellaris*, *Sphaeramia orbicularis*, *Cottoperca gobio*, *Sparus aurata*, *Dicentrarchus labrax* and *Morone saxatilis*.

The substitutions highlighted (in cyan) in the figure (Figure Supplemental Fig. 1) are: D^{17}; $SS^{21–22}$; $SH^{24–25}$; $NN^{33–34}$; Q^{36}; P^{58}; I^{65}; R^{78}; D^{89}; A $T^{93–94}$ R^{103}; $DSNYLE^{105–110}$; D^{130}; H^{145}; M^{165}; T^{168}; $PG^{177–178}$; K^{190}; $EE^{217–218}$; E^{235}; F^{237}; S^{242}; GVG^{-256}; D^{259}; K^{261}; D^{282}.

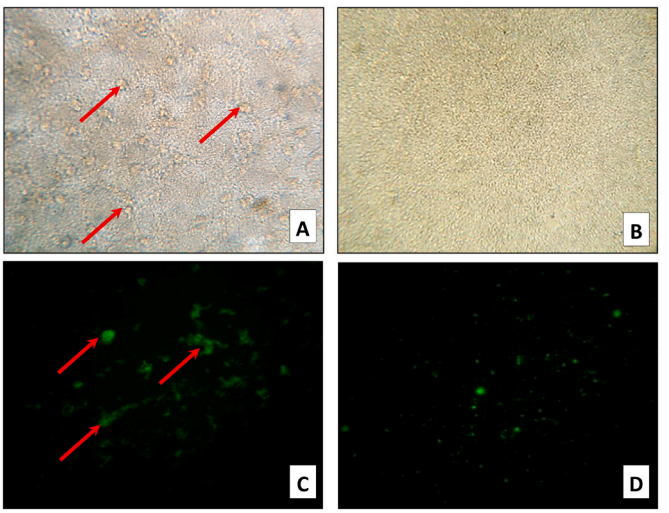

Fig. 4. Bacterial agglutination.
*Tb*FTL displays the ability to agglutinate bacterial targets. *E. coli* agglutinated in the presence of *Tb*FTL (A). *E. coli* suspended in TBS gel (B). Fluorescent *E. coli* agglutinated in the presence of *Tb*FBL (C), and fluorescent *E. coli* suspended in TBS gel (D). The red arrows indicate the agglutinated bacteria. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In the amino acid sequence of *Tb*FTL, there is a noticeably increased presence of positively and negatively charged amino acids, usually involved in the formation of salt bridges, as compared to the FTL homologues from other mesophile Perciformes (Asp^{17}, Hys^{25}, Arg^{78}, Asp^{89}, Arg^{103}, Asp^{105}, Glu^{110}, Asp^{130}, Hys^{145}, Lys^{190}, Glu^{217}, Glu^{218}, Glu^{234}, Asp^{259}, Lys^{261}, Asp^{282}).

A detailed sequence analysis of binary fish FTL CRDs (Fig. 6) showed highly conserved amino acid sequences in their N-CRDs or C-CRDs, with the N-CRDs being closer to the *Anguilla sp* CRD cluster. Conserved Cys residues can form two disulphide bonds (Cys^{40}–$Cys^{137}$and Cys^{244}-Cys^{260}); an additional bond between two consecutive cysteine residues (Cys72 and Cys73) in N-CRD can be formed, as in AAA, which participates in ligand binding by hydrophobic interaction with the sugar. The canonical triad (His^{42}, Arg^{69}, Arg^{76}) of basic amino acids involved in sugar binding are present in both *Tb*FTL N-CRD and C-CRD, participating in the interaction with Phe^{35} and Cys^{72} residues. The amino acids responsible for cation (Ca^{2+}) ligation are Asn^{171}, Asp^{174}, Asn^{176}, Ser^{185}, Cys^{288}, Glu^{289}. Multiple alignment by BLAST with 14 F-type lectins isolated and characterized from Percomorpha fishes with percentage identity of at least 60%, clearly demonstrated that the unique amino acid substitutions characterize *Tb*FTL in non-phylogenetic manner and may be related to rapid cold adaptation. The fishes and the NCBI Reference Sequence of each protein are summarized in Table 5.

The tertiary structure modelled on the *M. saxatilis* template (3cqo.1.A) revealed that the marked substitutions are mainly located at the ends of the two CRDs, close to the sugar binding sites (Fig. 7a, in the red ellipses).

The 3D structure is shown in Supplemental Fig. 2 and the elaboration of electrostatic surface charges for *Ms*FBP32 and *Tb*FTL, realized by Swiss-Pdb Viewer (Guex and Peitsch, 1997), are shown in Fig. 7(b,c). The different coloured spots on the surface are utilized to represent the molecular surface potentials (blue, red and white are for positive, negative, and neutral potentials, respectively).

4. Discussion

The Notothenioidei are a group of teleost fish used as a model species to investigate evolutionary adaptations to low environmental temperatures, including protein modifications, detoxification mechanisms and

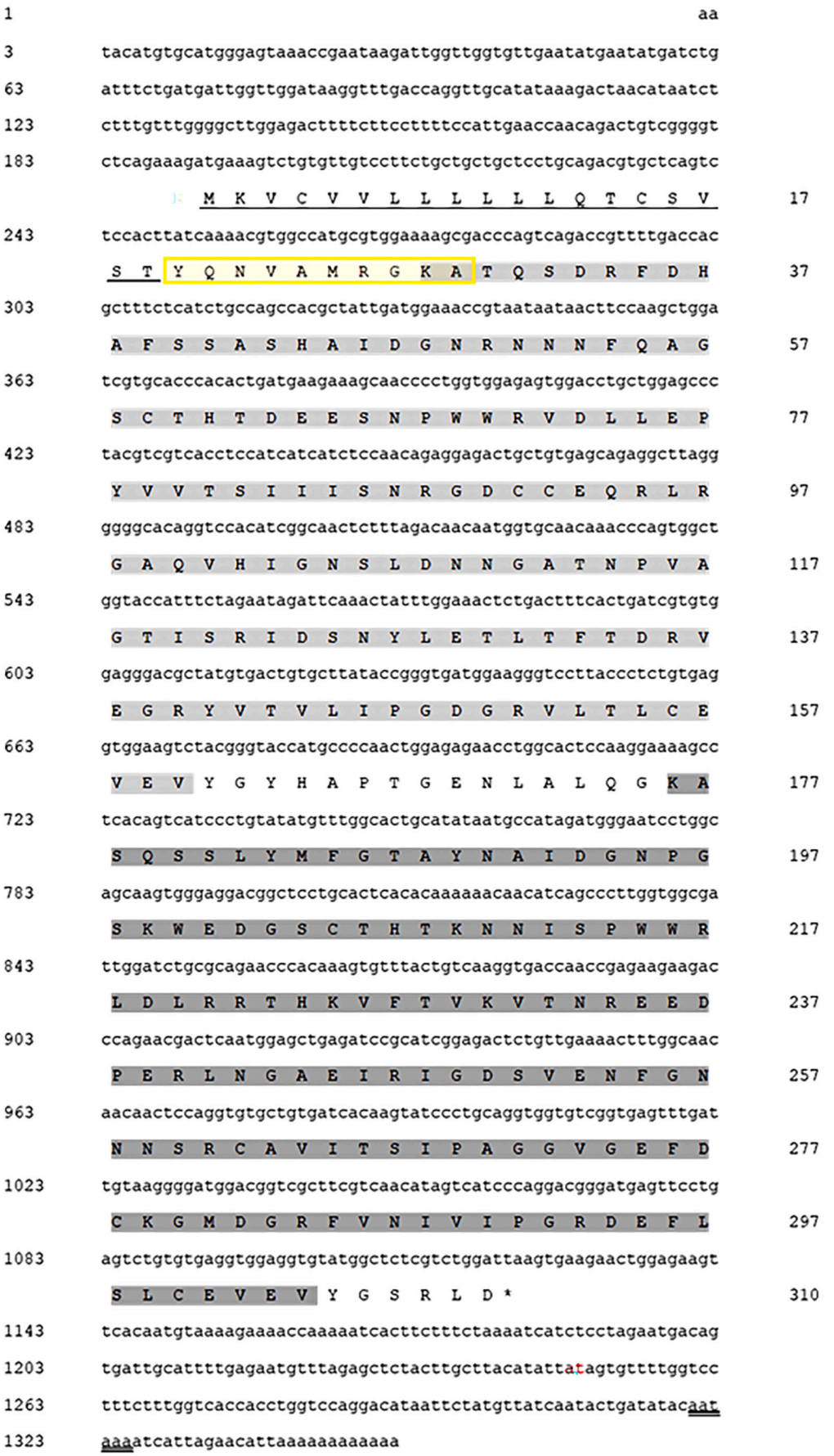

Fig. 5. cDNA and the amino acid sequence of *Tb*FTL.
The cleaved signal peptide is indicated by underlined amino acids. The chemically sequenced peptide is highlighted in yellow. The in-frame stop codon is marked with an asterisk. The polyadenylation motif is double underlined. The two CRDs are highlighted in grey (N-CRD is light grey and the C-CRD dark grey). On the left the number of the nucleotides are indicated, and on the right are the number of amino acids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

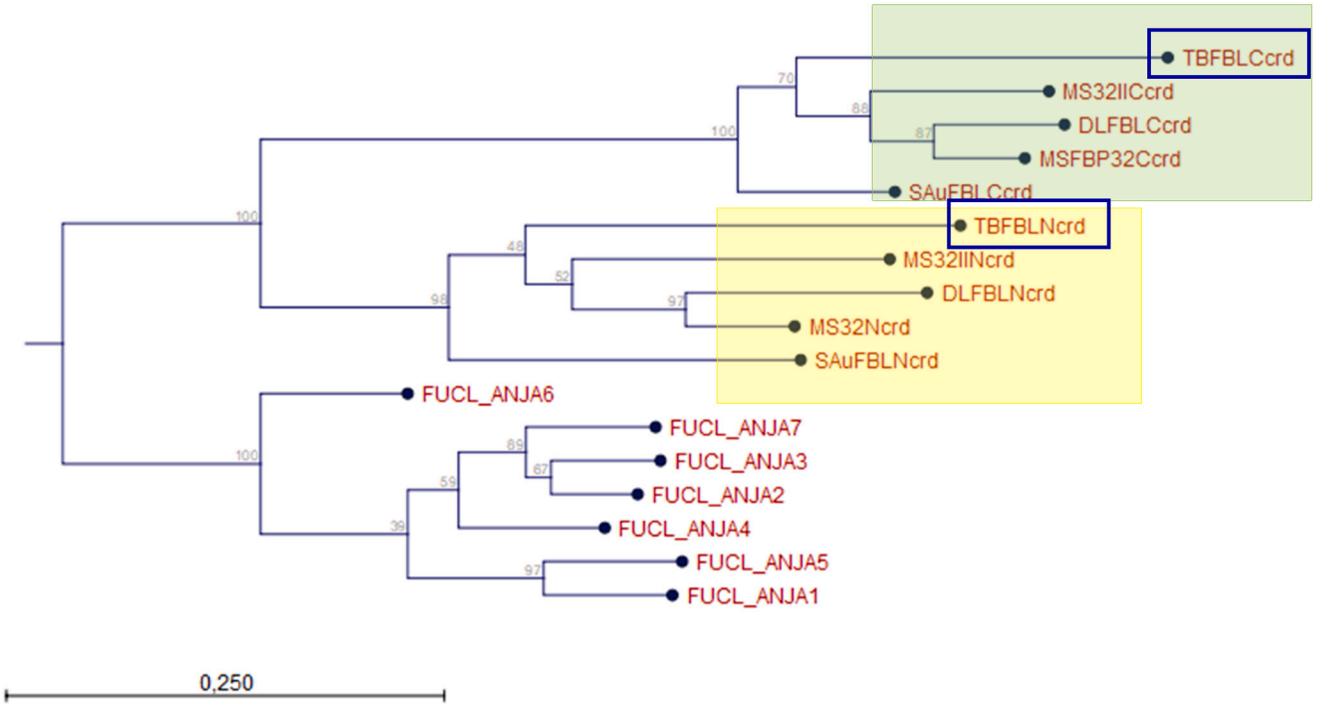

Fig. 6. Analysis of genetic distance between FBP CRD domains.
The phylogram was created by NJ analysis using the CLC Sequence Viewer Version 8.0. Distances were corrected for multiple substitutions, and gap positions were excluded. Bootstrap values are percentages from 1000 iterations. The yellow box contains the FBP N-CRDs, whereas the green box contains the C-CRDs. The *Tb*FTL CRDs are in the blue rectangles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Lectins alongside their GenBank accession numbers aligned with the *Tb*FTL amino acid sequence. The theoretical isoelectric point (pI) and molecular weight (Mw; kDa) were calculated using the Compute pI/Mw tool.

Fish name	NCBI reference sequence	Protein name	% identity	Ip/Mw
Sander lucioperca (Sander)	XP_031135299.1	fucolectin-1-like	79.51%	5.27 / 31.81
Perca flavescens (Perca)	XP_028435873.1	fucolectin-1	77.74%	5.29 / 31.96
Oplegnathus fasciatus (Oplegnathus)	BAK38714.1	F-type lectin	71.33%	5.39 / 32.34
Larimichthys crocea (Larimichthys)	AEL33635.1	fucolection 1	71.02%	5.81 / 32.09
Morone saxatilis (Morone)	ABB29997.1	FBP32	68.90%	6.16 / 32.11
Dicentrarchus labrax (Dicentrarchus)	ACF94293.1	fucose binding lectin	68.55%	5.83 / 32.21
Parambassis ranga (Parambassis)	XP_028281935.1	fucolectin-3-like	68.20%	5.34 / 32.16
Anabas testudineus (Anabas)	XP_026226610.1	fucolectin-4-like	68.90%	5.99 / 31.69
Oreochromis niloticus (Oreochromis)	XP_003450308.1	fucolectin-6 isoformX1	67.14%	6.10 / 32.27
Astatotilapia calliptera (Astatotilapia)	XP_026041500.1	fucolectin-1-like	67,01%	6.42 / 31.80
Amphiprion ocellaris (Amphiprion)	XP_023132500.1	fucolectin-3-like	68.55%	5.54 / 31.53
Sphaeramia orbicularis (Sphaeramia)	XP_030013491.1	fucolectin-5-like	66.43%	5.29 / 32.20
Cottoperca gobio (Cottoperca)	XP_029307766.1	fucolectin-1-like	66.90%	7.71 / 31.98
Sparus aurata (Sparus)	AIT83009.1	fucolectin	64.66%	7.74 / 31.50

immunoglobulin-mediated adaptive immune recognition (Oreste and Coscia, 2004; Coscia et al., 2010). Here, we have focused on innate immune recognition mediated by lectins.

The monosaccharide L-fucose is present as a non-reducing terminal sugar in a wide variety of pro- and eukaryotic glycans (Staudacher et al., 1999). The CRD number in the F-lectin family has undergone diversification. In contrast with the single-CRD F-lectins from eels, in teleosts the binary homologues may have diversified through gene duplication and speciation events, such as the binary CRD in Perciformes, in which *T. bernacchii* is included, or the four-tandem CRD F-lectins that are unique to the Salmoniformes (Odom and Vasta, 2006).

Our initial hemagglutination analysis of *T. bernacchii* serum, followed by binding-inhibition tests performed with various sugars, strongly suggested the presence of a fucose-binding lectin. The inhibition assays with test monosaccharides demonstrated that the binding sites recognize L-fucose. Affinity chromatography on a fucose-agarose resin enabled the isolation of a 32 kDa lectin that we designated *Tb*FTL; we characterized its binding properties and compared it to other similar lectins from related Perciformes, in order to identify potential *T. bernachii* adaptations to low temperatures (Cammarata et al., 2016). The purified lectin was estimated at approximately 2.6% of the total serum protein content. The electrophoretic mobility of the purified *Tb*FTL under non-reducing conditions revealed a major 30 kDa band. Under reducing conditions, the mobility of the major band shifted to about 32 kDa, and this increased size is probably due to polypeptide linearization as an effect of intrachain bridge reduction, in agreement to other FTLs (Bianchet et al., 2002; Bianchet et al., 2010; Odom and Vasta, 2006).

When incubated with either Gram-negative or Gram-positive bacteria, both the crude *T. bernacchii* serum and the purified *Tb*FTL showed intense agglutinating activity, which suggests a potential role of *Tb*FTL in immune defence responses (Bianchet et al., 2010; Cammarata et al., 2016).

Sequence analysis of the available transcriptome revealed the

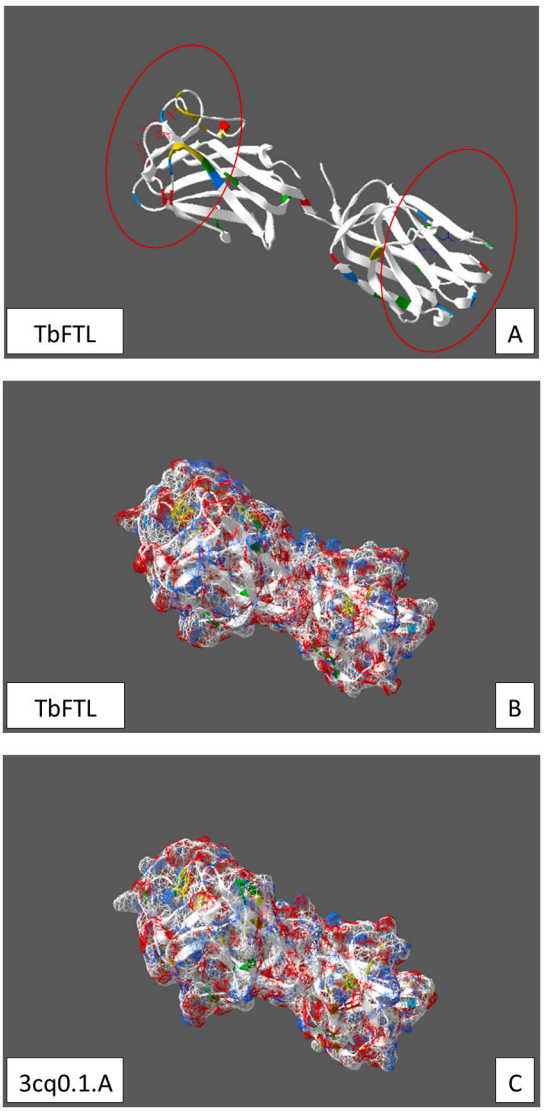

Fig. 7. 3D model of *Tb*FTL; 3D representation of electrostatic charges on protein surfaces.
*Tb*FTL modelled on the on the basis of the cDNA sequence (A); structure of *Tb*FTL evidencing the amino acid substitutions: the coloured segments mark the substitute amino acids. Colours: green = nonpolar amino acid; yellow = uncharged polar amino acid; cyan = negative charged polar amino acid; red=positive charged polar amino acid. The red ellipses indicates the sugar binding sites in the CRDs.
Graphic representation of the electrostatic charges on the protein surfaces of *Tb*FTL and MsFBP32 (*Morone saxatilis* fucose-binding protein32; 3cqo.1.A) (B, C). The images were realized using the Swiss-PdbViewer. The colours blue, white and red on the molecular surface indicate positive, negative, and neutral potentials, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

presence of a single complete nucleotide sequence with an open reading frame corresponding to a putative FTL. The identity of the FTL transcript with the purified *Tb*FTL was confirmed by N-terminal sequencing of the latter. BLAST analysis showed that the deduced amino acid sequence of the transcript has close homologies with vertebrate binary FTLs and indicated that *Tb*FTL is a typical binary tandem domain FTL with the N- and C-CRDs connected by a 15 amino acid peptide linker. Thus, in addition to similar structural features, *Tb*FTL is also expected to display common biochemical properties of FTLs, like those of the European bass (Cammarata et al., 2001) and striped bass (Odom and Vasta, 2006). The binding-inhibition analysis of the purified *Tb*FTL revealed preference for fucose, providing further support for the inclusion of this protein in the FTL family. This was further confirmed by the cross-reactivity of *Tb*FTL to anti-*Dl*FBL specific antibodies. As was the case for other members of the FTL family (Odom and Vasta, 2006), the binding of *Tb*FTL to a carbohydrate ligand did not require divalent cations, as the activity was modified by the addition of $CaCl_2$, $MgCl_2$ or EDTA. Although a divalent cation is present in the FTL subunit, it does not participate in interactions with the carbohydrate ligand but appears to have a structural role (Odom and Vasta, 2006; Bianchet et al., 2010; Bianchet et al., 2002). It is possible that the EDTA concentration or the length of the treatment was not sufficient to remove the divalent cation present in *Tb*FTL. In summary, our observations suggest that *Tb*FTL may be included in the fucolectin family: (1) *Tb*FTL can bind fucose in a cation-independent manner; (2) *Tb*FTL shares *Tb*FTL and *Dl*FBL epitopes, as observed by its cross-reactivity with anti-*Dl*FBL antibodies; (3) the *Tb*FTL amino acid sequence has high sequence identity with the FTL family (Odom and Vasta, 2006; Bianchet et al., 2010; Honda et al., 2000).

It is noteworthy that, unlike other FTLs characterized to date, the HA of *Tb*FTL is unstable at temperatures above 36 °C, an observation highly unusual for fish lectins. Although in this study we have not investigated this observation further, it could be speculated that this unusual *in vitro* behaviour might reflect a property related to the biological function of *Tb*FTL *in vivo* at low environmental temperatures.

It is important to individuate the emergent features of the interaction and characteristics of this network, such as cooperativity and anti-cooperativity, in order to understand the adaptation mechanisms. Observing the multiple alignments and highlighting the amino acid substitutions along the sequence, we can discuss the electrostatic interactions and salt bridges in terms of cold adaptation modifications of *Tb*FBL, particularly with regard to residues that could establish electrostatic interactions and salt bridges between a pair of oppositely charged residues (Asp or Glu with Arg, Lys or His). In this context, there is a noticeably increased presence of amino acids usually involved in salt bridge formation along the primary amino acid structure of *Tb*FBL. Experimental evidence supports the notion that salt bridges may facilitate water penetration across the hydrophobic core and increase protein flexibility (Parvizpour et al., 2017). By comparing the locations of salt bridges in the crystal structures of citrate synthase from thermophilic, mesophilic and psychrophilic organisms, Kumar and Nussinov (2002) concluded that thermophilic and psychrophilic citrate synthases are more similar to each other and contain a larger number of salt bridges than their mesophilic homologue. The main difference is the distribution in the tertiary structure of the folded protein of the salt bridges and the relative networks disposed closer to the active site. In the folded protein, the distribution of the substitutions is mainly positioned in the extremities of the CRDs, around the sugar recognition sites. Considering this evidence, we might hypothesize that these modifications in the protein sequence could favour sugar binding at lower temperatures, as we observed regarding *Tb*FTL HA close to 0 °C. However, despite the nature of the amino acid substitutions and their position in the folded protein, the analysis of surface electrostatic potential did not reveal any major changes. Thus, unlike the reported adaptation of immunoglobulins to the Antarctic low temperatures that include specific modifications, such as high protein glycosylation and flexibility (Oreste and Coscia, 2004), *Tb*FTL maintains a structure similar to the FTLs of mesophilic Perciformes. However, the HA of this lectin at temperatures close to 0 °C and its rapid inactivation at temperatures above 36 °C are unique to *Tb*FTL and suggest that the FTLs in both mesophilic and Antarctic fish do not require significant structural changes to function at lower temperatures. Additional studies will need to be oriented toward defining the structural features in *Tb*FTL that are responsible for its thermal adaptation, and indeed our current efforts are aimed at investigating this aspect.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpb.2021.110633.

Funding

This research did not receive any specific funding from public, commercial, or not-for-profit sector agencies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Dr. Umberto Oreste for his critical reading and helpful suggestions. This study was funded by the FFR-Cammarata (PJ_RIC_FFABR_2017_004312 MC) and FFR-Parisi (PJ_RIC_F-FABR_2017_161753 MGP) for Scientific Research from the University of Palermo, Italy.

References

Arason, G.J., 1996. Lectins as defence molecules in vertebrates and invertebrates. Fish Shellfish Immunol. 6, 277–289. https://doi.org/10.1006/fsim.1996.0029.

Ballarin, L., Cammarata, M., Cima, F., Grimaldi, A., Lorenzon, S., Malagoli, D., Ottaviani, E., 2008. Immune-neuroendocrine biology of invertebrates : a collection of methods. Invertebr. Surviv. J. 5, 192–215.

Bianchet, M.A., Odom, E.W., Vasta, G.R., Amzel, L.M., 2002. A Novel Fucose Recognition Fold Involved in Innate Immunity, p. 9. https://doi.org/10.1038/nsb817.

Bianchet, M.A., Odom, E.W., Vasta, G.R., Amzel, L.M., 2010. Structure and specificity of a binary tandem domain F-Lectin from striped bass (*Morone saxatilis*). J. Mol. Biol. 401, 239–252. https://doi.org/10.1016/j.jmb.2010.06.018.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. https://doi.org/10.1016/0003-2697(76)90527-3.

Cammarata, M., Vazzana, M., Chinnici, C., Parrinello, N., 2001. A serum fucolectin isolated and characterized from sea bass *Dicentrarchus labrax*. Biochim. Biophys. Acta Gen. Subj. 1528, 196–202. https://doi.org/10.1016/S0304-4165(01)00193-3.

Cammarata, M., Benenati, G., Odom, E.W., Salerno, G., Vizzini, A., Vasta, G.R., Parrinello, N., 2007. Isolation and characterization of a fish F-type lectin from gilt head bream (*Sparus aurata*) serum. Biochim. Biophys. Acta Gen. Subj. 1770, 150–155. https://doi.org/10.1016/j.bbagen.2006.09.015.

Cammarata, M., Parisi, M.G., Vasta, G.R., 2016. Evolution and Immune Function of Fish Lectins, in: Lessons in Immunity. Elsevier, pp. 239–256. https://doi.org/10.1016/B978-0-12-803252-7.00018-7.

Cooper, D., Butcher, C.M., Berndt, M.C., Vadas, M.A., 1994. P-selectin interacts with a β2-integrin to enhance phagocytosis. J. Immunol. 153 (7), 3199–3209.

Coscia, M.R., Varriale, S., De Santi, C., Giacomelli, S., Oreste, U., 2010. Evolution of the Antarctic teleost immunoglobulin heavy chain gene. Mol. Phylogenet. Evol. 55, 226–233. https://doi.org/10.1016/j.ympev.2009.09.033.

Coscia, M.R., Varriale, S., Giacomelli, S., Oreste, U., 2011. Antarctic teleost immunoglobulins: more extreme, more interesting. Fish Shellfish Immunol. 31, 688–696. https://doi.org/10.1016/j.fsi.2010.10.018.

Devries, A.L., 1982. Biological antifreeze agents in coldwater fishes. Comp. Biochem. Physiol. A 73, 627–640. https://doi.org/10.1016/0300-9629(82)90270-5.

Drickamer, K., 1999. C-type lectin-like domains. Curr. Opin. Struct. Biol. 9, 585–590. https://doi.org/10.1016/S0959-440X(99)00009-3.

Drickamer, K., Dodd, R.B., 1999. C-type lectin-like domains in *Caenorhabditis elegans*: predictions from the complete genome sequence. Glycobiology 9, 1357–1369. https://doi.org/10.1093/glycob/9.12.1357.

Elumalai, P., Rubeena, A.S., Arockiaraj, J., Wongpanya, R., Cammarata, M., Ringø, E., Vaseeharan, B., 2019. The role of lectins in finfish: a review. Rev. Fish. Sci. Aquacult. 1–18. https://doi.org/10.1080/23308249.2018.1520191.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., Bairoch, A., 2005. Protein identification and analysis tools on the ExPASy server [WWW document]. In: The Proteomics Protocols Handbook. https://doi.org/10.1385/1592598900.

Gerdol, M., Buonocore, F., Scapigliati, G., Pallavicini, A., 2015. Analysis and characterization of the head kidney transcriptome from the Antarctic fish Trematomus bernacchii (Teleostea, Notothenioidea): a source for immune relevant genes. Mar. Genomics 20, 13–15. https://doi.org/10.1016/j.margen.2014.12.005.

Guex, N., Peitsch, M.C., 1997. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18, 2714–2723. https://doi.org/10.1002/elps.1150181505.

Honda, S., Kashiwagi, M., Miyamoto, K., Takei, Y., Hirose, S., 2000. Multiplicity, structures, and endocrine and exocrine natures of eel fucose-binding lectins. J. Biol. Chem. 275, 33151–33157. https://doi.org/10.1074/jbc.M002337200.

Jensen, L.E., Hiney, M.P., Shields, D.C., Uhlar, C.M., Lindsay, A.J., Whitehead, A.S., 1997. Acute phase proteins in salmonids: evolutionary analyses and acute phase response. J. Immunol. 158, 384–392.

Kuhlman, B.Y.M., Joiner, K., Ezekowitz, R.A.B., 1989. The human mannose-binding protein functions as opsonine. J. Exp. Med. 169, 1733–1745.

Kumar, S., Nussinov, R., 2002. Close-range electrostatic interactions in proteins. Chembiochem 3, 604–617.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685. https://doi.org/10.1038/227680a0.

Laura, A.E., Evan, M.H., Sean, P.P., 2017. The effects of elevated temperature and ocean acidification on the metabolic pathways of notothenioid fish. Conserv. Physiol. 5 (1), cox019 https://doi.org/10.1093/conphys/cox019.

Listinsky, J.J., Siegal, G.P., Listinsky, C.M., 1998. α-L-Fucose: a potentially critical molecule in pathologic processes including neoplasia. Am. J. Clin. Pathol. 110, 425–440. https://doi.org/10.1093/ajcp/110.4.425.

Macdonald, J.A., Montgomery, J.C., Wells, R.M.G., 1988. Comparative physiology of Antarctic fishes. Adv. Mar. Biol. 24, 321–388. https://doi.org/10.1016/S0065-2881(08)60076-0.

Magnadóttir, B., 2006. Innate immunity of fish (overview). Fish Shellfish Immunol. 20, 137–151. https://doi.org/10.1016/j.fsi.2004.09.006.

Odom, E.W., Vasta, G.R., 2006. Characterization of a binary tandem domain F-type lectin from striped bass (*Morone saxatilis*). J. Biol. Chem. 281, 1698–1713. https://doi.org/10.1074/jbc.M507652200.

Oreste, U., Coscia, M.R., 2004. Investigations on *Trematomus bernacchii* immunoglobulins at gene and protein level. Antarct. Sci. 16, 45–50. https://doi.org/10.1017/S0954102004001804.

Parvizpour, S., Razmara, J., Shamsir, M.S., Illias, R.M., Abdul Murad, A.M., 2017. The role of alternative salt bridges in cold adaptation of a novel psychrophilic laminarinase. J. Biomol. Struct. Dyn. 35, 1685–1692. https://doi.org/10.1080/07391102.2016.1191043.

Petersen, T.N., Brunak, S., Von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786. https://doi.org/10.1038/nmeth.1701.

Russell, S., Lumsden, J.S., 2005. Function and heterogeneity of fish lectins. Vet. Immunol. Immunopathol. 108, 111–120. https://doi.org/10.1016/j.vetimm.2005.08.004.

Sahoo, B.R., 2020. Structure of fish toll-like receptors (TLR) and NOD-like receptors (NLR). Int. J. Biol. Macromol. 161, 1602–1617. https://doi.org/10.1016/j.ijbiomac.2020.07.293.

Santovito, G., Piccinni, E., Boldrin, F., Irato, P., 2012. Comparative study on metal homeostasis and detoxification in two Antarctic teleosts. Comp. Biochem. Physiol. 155, 580–586. https://doi.org/10.1016/j.cbpc.2012.01.008.

Staudacher, E., Altmann, F., Wilson, I.B.H., März, L., 1999. Fucose in N-glycans: from plant to man. Biochim. Biophys. Acta Gen. Subj. 1473, 216–236. https://doi.org/10.1016/S0304-4165(99)00181-6.

Suzuki, Y., Tasumi, S., Tsutsui, S., Okamoto, M., Suetake, H., 2003. Molecular diversity of skin mucus lectins in fish. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 136, 723–730. https://doi.org/10.1016/S1096-4959(03)00178-7.

Vasta, G.R., Ahmed, H., 2008. Animal Lectins: a functional view. In: Animal Lectins. CRC Press, Boca Raton, Fl. https://doi.org/10.1201/9781420006971.

Vasta, G.R., Ahmed, H., Odom, E.W., 2004. Structural and functional diversity of lectin repertoires in invertebrates, protochordates and ectothermic vertebrates. Curr. Opin. Struct. Biol. 14, 617–630. https://doi.org/10.1016/j.sbi.2004.09.008.

Vasta, G.R., Nita-Lazar, M., Giomarelli, B., Ahmed, Hfc, Du, S., Cammarata, M., Parrinello, N., Bianchet, M.A., Amzel, L.M., 2011. Structural and functional diversity of the lectin repertoire in teleost fish: relevance to innate and adaptive immunity. Dev. Comp. Immunol. 35, 1388–1399. https://doi.org/10.1016/j.dci.2011.08.011.

Wells, R.M.G., Eastman, J.T., 1994. Antarctic Fish Biology: Evolution in a Unique Environment. Copeia. https://doi.org/10.2307/1446701.