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ORIGINAL ARTICLE

Th2-biased immune response and agglutinating antibodies generation by a chimeric protein comprising OmpC epitope (323–336) of *Aeromonas hydrophila* and LTB

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Abstract *Aeromonas hydrophila* is responsible for causing fatal infections in freshwater fishes. Besides chemical/antibiotic treatment and whole-cell vaccine, no subunit vaccine is currently available for *A. hydrophila*. Outer membrane proteins of gram-negative bacteria have been reported as effective vaccine candidates. Peptide antigens elicit focused immune responses against immunodominant stretches of the antigen. We have attempted to characterize the immunogenicity of linear B-cell epitopes of outer membrane protein (OmpC) of *A. hydrophila* identified using in silico tools, in conjugation with heat-labile enterotoxin B (LTB) subunit of *Escherichia coli* as a carrier protein. Antisera against the fusion protein harboring 323–336 residues of the *Ah*OmpC (raised in mice) showed maximum cross-reactivity with the parent protein OmpC and LTB. The fusion protein displayed efficient GM_1 ganglioside receptor binding, retaining the adjuvanicity of LTB. Antibody isotype profile and in vitro T-cell response analysis, cytokine ELISA, and array analysis collectively revealed a Th2-biased mixed T-helper cell response. Agglutination assay and flow cytometry analysis validated the ability of anti-fusion protein antisera to recognize the surface exposed epitopes on *Aeromonas* cells, demonstrating its neutralization potential. Oral immunization studies in *Labeo rohita* resulted in the generation of long-lasting humoral immune response, and the antisera could cross-react with the fusion protein as well as both the fusion partners. Considering significant similarity among OmpC of different enteric bacteria, the use of *A. hydrophila* OmpC epitope$_{323–336}$ in fusion with LTB could have a broader scope in vaccine design.

Keywords *Aeromonas hydrophila* · OmpC · Epitope · LTB · B cell · Agglutination

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Introduction

Aeromonas hydrophila is a mesophilic, gram-negative motile rod-shaped bacterium, residing in different aquatic bodies including lakes, rivers, and marine, residual, and drinking water [1]. *Aeromonas* infects a wide range of hosts including fishes, domesticated pets, amphibians, reptiles as well as humans. Of these, fishes are the primary targets of this fatal infection, leading to colossal economic losses across the world. *A. hydrophila* and *Aeromonas veronii* have been characterized both as fish as well as human pathogens [2]. In fishes, *A. hydrophila* is responsible for both systemic and chronic infections, characterized by hemorrhagic septicemia, ulcers, scaly protusion, and swollen abdomen among other physiological symptoms [1, 3]. In humans, besides causing gastrointestinal, respiratory, and urinary tract infections, it has also been recognized as an opportunistic pathogen in patients

suffering from impaired immune conditions such as thalassemia, septic arthritis, diabetes, and many other chronic disorders [1, 4, 5].

Treatment strategies for *A. hydrophila* infections currently include antibiotic treatment [6] and live attenuated vaccine (Patent No.: US 7,988,977B2, August 2, 2011). These strategies have several drawbacks including risk of antibiotic resistance and increased antigenic load in the host along with probable reversion to original virulence, respectively. Thus, the search for a safe vaccine which elicits focused immune response against the pathogen still continues. *A. hydrophila* virulence is multifactorial involving different virulence factors, of which outer membrane proteins have been found to play an important role in host cell adherence and colonization [7]. Their high abundance in the bacterial cell, surface exposure, and interaction with the host cells make outer membrane proteins (OMPs) an attractive target for vaccine development [8]. Of the many OMPs, the role of proteins of the two-component regulatory system, i.e., OmpF, OmpC, and OmpR (involved in bacterial survival subsisting diverse osmolaric environments), in bacterial virulence has been studied extensively [9–14].

OmpC has been found to be able to initiate the classical complement pathway and display significant opsonophagocytic activity against infectious *Escherichia coli* strain [11]. Loss of virulence of *Shigella flexneri* harboring *ompC* and *ompB* mutants and restoration of virulence upon introduction of a plasmid harboring functional genes have also been reported [12]. Immunization with the *E. coli* rOmpC conferred protection upon challenge with different *E. coli* as well as *Shigella* strains [13]. The OmpC of *A. hydrophila* has also been shown to elicit Th2-biased immune response and generate OmpC-specific bacterial agglutinating antibodies post-immunization [14]. These data collectively demonstrate the role of OmpC in the virulence of different gram-negative bacteria.

Being present on the outer membrane, the exposed regions of these porins have a high probability of being recognized as antigenic epitopes. Once this antigenicity translates into immunogenicity, the surface-exposed epitopes can act as antigenic determinants [15–17] and can be used as peptide antigens in vaccine design. After pathogen invasion, B-cell-mediated immune response plays a key role in establishing a robust protective immune response [18], with the help of T-helper cells. The role of IgM and humoral immunity in providing protection and facilitating bacterial clearance has been reported for protein-based conjugate vaccines [19]. Immune response generation and immunological memory activation in fishes are dependent on the development and expansion of B lymphocytes and memory B cells [20]. Analysis of fish MHC-I and $CD3^+$ T-cell homologs suggests similar presentation of $CD8^+$MHC-I molecules in fishes and higher vertebrates [21]. In recent years, epitope-based vaccines have gained favor over a subunit vaccine approach; major advantages include introduction of functional moieties/isoforms of naturally occurring amino acids, multiple epitopes of similar/different antigenic source, elimination of sequences responsible for immunosuppression and antigenic shifts, thus offering focused immune response [22, 23]. However, these peptide vaccines cannot work as efficient immunogens due to their small size in protease-rich environments and need an appropriate mode of delivery, accomplished with the use of adjuvants or carriers [23]. Heat-labile enterotoxin B (LTB) subunit of *E. coli* has been demonstrated to stimulate both mucosal and systemic immune response effectively [24]. Adjuvanicity of LTB has been associated with increased expression of co-stimulatory and accessory molecules and GM_1 ganglioside receptor binding ability, leading to increased Th2-biased $CD4^+$ T-cell activation [25, 26]. Sharma and Dixit have earlier reported immunogenic and protective potential of a continuous immunodominant epitope of the OmpF of *A. hydrophila* in C-terminal fusion with LTB [17]. Since OmpC of *A. hydrophila* also generated an effective immune response [14], the present study was undertaken to evaluate the potential of an epitope-based vaccine comprising in silico predicted B-cell epitopes of *A. hydrophila* OmpC in translational fusion with LTB as the carrier molecule. The recombinant fusion proteins were assessed for their vaccine candidature on the basis of the immunogenic and protective response generated.

Materials and methods

Materials

Analytical grade chemicals were obtained from Sigma-Aldrich Chemical Co., USA, and SD Fine Chemicals Ltd., India, unless stated otherwise. Alkaline phosphatase (AP)-conjugated anti-his/anti-Fc anti-mouse antibodies and oligonucleotides were procured from Sigma-Aldrich Chemical Co., USA. Plasmid pET22b+ was from Novagen, USA. Plasmid pQE*LTB* harboring LTB gene was kindly gifted by Dr. L. C. Garg, National Institute of Immunology (NII), New Delhi, India [27]. DNA modification enzymes and molecular weight markers (DNA and protein) were from New England Biolabs, USA, and Thermo Scientific, USA, respectively. Bicinchoninic acid (BCA) protein assay kit and Ni^{2+}-NTA resin were purchased from G-Biosciences, USA.

Bacterial strains and animals

Different *Aeromonas* isolates used in this study [*A. hydrophila* (MTCC # 12301), *A. sobria* (MTCC # 1608), *A. liquefaciens* (MTCC # 2654), and *A. culicicola* (MTCC # 3249)] were acquired from the Microbial Tissue Culture Collection (MTCC), Chandigarh, India. *E. coli* DH5α and BL21(λDE3)pLysS cells were procured from GIBCO BRL, USA, and Novagen, USA, respectively. Female Balb/c mice

(4–6 weeks old) were from the Animal House Facility, Jawaharlal Nehru University (JNU), India. Animals were maintained on feed and water ad libitum. Animal usage for the study was approved by the Institutional Animal Ethics Committee, JNU, New Delhi (IAEC code # 12/2014). IAEC guidelines were followed for all animal procedures.

Cloning of *A. hydrophila* OmpC epitopes in fusion with LTB

Linear epitope prediction softwares (online IEDB analysis resource) were used for predicting potential B-cell epitopes of OmpC (NCBI accession no. CCO02590.1) [14]. To increase the reliability of prediction, OmpC sequence was screened using different softwares (Emini surface accessibility, Parker hydrophilicity prediction, Chou and Fasman beta turn prediction, Karplus and Sculz flexibility prediction, Bepipred Linear epitope prediction). Complementary oligonucleotides encoding the amino acid (aa) stretches corresponding to the predicted epitopes were synthesized from Sigma-Aldrich Chemical Co. (USA) with *Kpn*I and *Hin*dIII overhangs at 5′- and 3′-ends, respectively. These were annealed and cloned into pQE.*LTB* digested with the same enzymes. However, due to poor expression of the '*ltb-epitope*' gene products by *E. coli* M15 cells, these fragments were excised out from pQE.*LTB.ompC*$_{aa\text{-}aa}$ using *Sac*I and *Hin*dIII and subcloned into pET22b+ digested with the same enzymes [28]. The strategy for cloning of the oligonucleotides encoding the putative immunodominant epitopes as a C-terminal translational fusion with LTB is shown in Supplementary Fig. 1. The clones were confirmed by restriction enzyme digestion and automated DNA sequencing (DNA Sequencing Facility, University of Delhi, South Campus, New Delhi, India). Resultant plasmids were designated as pET.*LTB.ompC*$_{aa\text{-}aa}$, with 'aa-aa' defining the aa stretch of the epitope with respect to OmpC.

Expression and purification of the recombinant OmpC LTB-epitope fusion proteins

Expression and localization analysis of the fusion proteins was performed as described previously [17]. Briefly, recombinant plasmids harboring the '*ltb-epitope*' fragments were transformed into *E. coli* BL21(λDE3)pLysS cells. An overnight culture of the *E. coli* BL21(λDE3)pLysS cells harboring the recombinant plasmid was used to inoculate a secondary culture (1%) and grown at 37 °C at 200 rpm. Recombinant protein expression was induced with 1 mM isopropylβ-D-1-thiogalactopyranoside (IPTG) when the culture attained an A_{600} of ~0.6–0.8, and the culture was further incubated for 6 h. Cell pellets from the uninduced and induced cultures (1 ml each) collected by centrifugation (4 °C, 10 min, 6000 rpm, Eppendorf microcentrifuge) were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 2% SDS) and analyzed for expression by SDS-PAGE (14%). For recombinant fusion protein localization, soluble and insoluble fractions were prepared by sonication of the cell pellets followed by centrifugation at 4 °C for 30 min (13,000 rpm, Eppendorf microcentrifuge) and analyzed by SDS-PAGE (14%).

Recombinant fusion proteins were purified from solubilized inclusion bodies as described [17]. Briefly, induced cells from the log phase secondary cultures (1 l) of *E. coli* BL21(λDE3)pLysS cells harboring recombinant plasmids were harvested by centrifugation (4 °C, 10 min, 6000 rpm). After sonication and centrifugation of the sonicated cell lysate (4 °C, 30 min, 13,000 rpm), the pellet was resuspended in PENGU buffer (200 mM sodium phosphate buffer pH 7.3, 50 mM NaCl, 1 M urea, 1 mM EDTA) and washed thoroughly with the same by repeated resuspension and incubation (15 min each) followed by centrifugation (4 °C, 10 min, 13,000 rpm) three times. The pellet was then similarly washed three times with homogenization buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5% Triton-X-100). Final wash was given with 50 mM Tris-HCl, pH 8.0 containing 100 mM NaCl. The resultant pellet representing inclusion bodies was resuspended in solubilization buffer (10 mM Tris-HCl pH 8.0, 500 mM NaCl, 8 M urea), incubated at 4 °C for 1 h, and centrifuged at 4 °C for 20 min at 12,000 rpm. The supernatant (solubilized inclusion bodies) was used for recombinant protein purification.

Protein purification was carried out using Ni^{2+}-NTA affinity chromatography. Solubilized inclusion bodies were incubated with Ni^{2+}-NTA resin pre-equilibrated with wash buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 8 M urea) at 4 °C for 1 h. Nonspecifically bound proteins were removed by washing with 10 column volumes of wash buffer containing 20 mM imidazole and eluted with 150 mM imidazole in wash buffer. Different chromatographic fractions (analyzed by 14% SDS-PAGE) containing the purified proteins were pooled, dialyzed, and refolded using urea gradient dialysis method into 1× PBS (pH 7.4) [10]. Protein estimation was carried out using BCA protein assay as per the manufacturer's direction. The purified proteins were stored at −80 °C in small aliquots until further use. Parent protein rOmpC was purified from *E. coli* BL21(λDE3) cells as described earlier [14]. Purified rLTB used in the study was a kind gift from Dr. L. C. Garg, NII, New Delhi, India [27].

Western blot analysis

Western blotting was carried out essentially as described by Sharma and Dixit [17]. Different proteins/cell lysates resolved by SDS-PAGE (14%) were transferred onto a nitrocellulose membrane (Advanced Microdevices, Ambala, India) at 10 V constant voltage overnight using electrode transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% methanol).

The membrane was incubated in 2% BSA (in 1× PBST) at room temperature (RT) for 1 h to minimize non-specific binding. After each incubation step hereafter, the membrane was washed thrice with 1× PBST at RT for 10 min each. Post blocking, the membrane was incubated with primary antibody (anti-his/anti-fusion protein antisera raised in mice 1:10,000 in 1× PBS), followed by incubation with secondary antibody (AP-conjugated anti-Fc anti-mouse antibody raised in goat 1:10,000 in 1× PBS) at RT for 1 h. Immunoreactive bands were visualized using Western Blue substrate (Promega, USA). Double distilled water was used for terminating the color development reaction.

Immunization studies

Female Balb/c mice were immunized intraperitoneally ($n = 6$ mice/group, 15 μg protein/1× PBS per mice) in Complete Freund's adjuvant (1:1) for primary immunization and in Incomplete Freund's adjuvant (1:1) for the subsequent boosters at 14-day intervals. Mice were bled prior to immunization (pre-immune sera) and 7 days post each booster. Antisera were collected and stored essentially as described earlier [17].

For fish immunization studies, Indian major carp *Labeo rohita* was selected as a study model. Fish immunization methodology is described in detail in S1 appendix.

Enzyme-linked immunosorbent assay (ELISA) for antibody titer and antibody isotype profiling

Fusion protein- and parent protein-specific antibody titers in the anti-fusion protein antisera were determined by ELISA [17]. Different dilutions of the anti-fusion protein antisera were prepared for end point titer determination. To analyze the type of immune response, antigen-specific antibody isotypes in the primary anti-fusion protein antisera were determined using different horse radish peroxidase (HRP)-conjugated secondary antibodies (IgG1, IgG2a, IgG2b, IgA). Antisera drawn prior to immunization and from PBS-immunized mice were taken as negative controls.

Interaction of the fusion protein harboring the LTB and OmpC epitope with the GM_1 receptor was assessed using monosialoganglioside receptor (Sigma-Aldrich Chemical Co., USA, ≥ 95%). GM_1 receptor-coated immunoplates were incubated with recombinant fusion protein, rLTB (positive control) and BSA (negative control), followed by detection using anti-rLTB polyclonal antisera (1:5000 in 1× PBS) as primary antibody and goat AP-conjugated anti-rabbit secondary antibody by sandwich ELISA. Color was developed using *p*-nitrophenyl phosphate (PNPP) substrate in AP buffer, and absorbance at 450 nm was read using ELISA reader (Tecan, USA). The rLTB interaction with the GM_1 receptor was used as a positive control and evaluated using anti-rLTB antisera as the primary antiserum.

Lymphocyte proliferation assay

T-cell proliferation was assessed by XTT assay (Biological Industries, Israel) as per the manufacturer's protocol [17]. Immunized mice were sacrificed under anesthesia (using avertin tribromoethanol; as per Animal Care and Use Committee guidelines) 7 days post-secondary booster, and splenocytes were isolated as described earlier [17]. Splenocytes from the fusion protein immunized (15 μg/100 μl/mouse) and control mice (immunized with PBS) were stimulated (with PBS and fusion protein; 15 μg/ml) and cultured at 37 °C for 72 h under 5% CO_2 humidified atmosphere in flat bottom 96-well plates (Greiner, UK). Cell proliferation was measured every 24 h and absorbance was recorded at 450 nm using ELISA reader.

Cytokine ELISA

In vitro immune response was assessed by cytokine ELISA in supernatant of the cultured splenocytes isolated from the PBS- and fusion protein-immunized mice, stimulated with the fusion protein. Culture supernatant (50 μl) was collected every 24 h from the cultured splenocytes (set for the T-cell proliferation assay) and analyzed for the presence of IFN-γ and IL-4 using cytokine ELISA kit (Becton Dickinson Pharmingen, USA) as per the manufacturer's protocol [17].

Cytokine array

The culture supernatant collected 72 h post-stimulation with the fusion protein (15 μg/ml) was analyzed for a comprehensive cytokine/chemokine response using Proteome Profiler Antibody Array (Mouse Cytokine; cat. No. ARY006, R&D Systems, USA) as per the manufacturer's instructions [17]. Immunoreactive spots were developed using Chemi Reagent Mix (provided along with the kit). Images were captured using ChemiDoc, Biospectrum-500 (UVP Laboratory Products, USA), and densitometric analysis was carried out using VisionWorks®LS analysis software version 6.8 (UVP Laboratory Products, USA).

Agglutination assay

Antisera raised against the fusion proteins were investigated for their ability to agglutinate live *Aeromonas* cells. Secondary log phase cultures of different *Aeromonas* spp. were grown and harvested at 4 °C for 5 min at 6000 rpm as described earlier [17]. *Aeromonas* cells (1×10^8 cfu) were incubated with pre-immune sera and anti-fusion protein antisera (1:320) at 37 °C for 1 h. The cells were collected by

centrifugation as described above, washed with 1× PBS to remove any unbound antibodies, and resuspended in 1× PBS. Glass slide smears of the resuspended cells were visualized under microscope (Model Eclipse TE2000S, Nikon, USA) at ×40 magnification. Bacterial agglutination titer was determined as the final dilution exhibiting agglutination using microplate titer assay [29], with minor modifications. Different dilutions (1:160, 1:320, 1:480, 1:640, 1:800, 1:1000) of heat-inactivated serum in 1× PBS (containing Mg^{2+} and Ca^{2+}) were prepared in 96-well plates. Equal volume of formalin-killed *A. hydrophila* (adjusted to 1×10^8 cfu) was added to each well and incubated overnight at 25 °C. Plates were observed for bacterial lysis, containing both anti-fusion protein and anti-PBS antisera in separate wells.

Fluorescence activated cell sorting (FACS)

The ability of anti-fusion protein antisera to interact with the surface-exposed epitopes of the OmpC of *A. hydrophila* was analyzed using FACS. *A. hydrophila* cells (log phase, 1×10^6 colony forming units) washed with 1× PBS were incubated with pre-immune sera/anti-fusion protein antisera (1:200) at 4 °C for 1 h. Bacterial cells were then washed with 1× PBS and incubated with FITC-conjugated anti-Fc antimouse IgG secondary antibody (1:200) at 4 °C for 1 h. Cells were harvested, washed, and resuspended in 1× PBS. Fluorescence intensity was determined using FACS Calibur™ (Becton Dickinson Immuno Cytometry System, San Jose, CA) and evaluated using FCS Express 4 flow cytometry analysis software. Single-cell events were gated using forward scatter and side scattering. An identical experiment performed with *E. coli* cells was also included as a negative control.

Statistical analysis

Data represent mean ± standard deviation of three independent experiments, performed in triplicates. Student's two-tailed *t* test was used for evaluating significance of the experiments. p values ≤ 0.05 were considered statistically significant.

Results

Cloning of immunodominant B-cell epitopes of *Ah*OmpC in fusion with LTB

Three aa stretches (193–217, 280–293, and 323–336) representing immunodominant B-cell epitopes of *A. hydrophila* OmpC were identified using different softwares. Oligonucleotide sequences and aa residues corresponding to the predicted epitopes are listed in Table 1. These oligonucleotides were cloned in translational fusion with LTB. Initially, the epitope-gene fragments were cloned as a C-terminal fusion to LTB in pQE.LTB [27] and expressed in *E. coli* M15 cells. However, these constructs gave minimal expression, only detected by Western blot analysis. Therefore, different *ltb.OmpC*$_{epitope}$ gene fragments were subcloned into pET22b+ under the control of T7 promoter (Supplementary Fig. 1). This resulted in the generation of recombinant clones, namely pET.*LTB.ompC*$_{193–217}$, pET.*LTB.ompC*$_{280–293}$, and pET.*LTB.ompC*$_{323–336}$, harboring the three B-cell epitopes comprising aa residues 193–217, 280–295, and 323–336, respectively.

Expression, localization, and purification of the recombinant fusion proteins

Transformation of different plasmids harboring the '*ltb-epitope*' fusion gene fragments into *E. coli* BL21(λDE3)pLysS cells, followed by induction with IPTG, resulted in the expression of recombinant fusion proteins at approximately ~ 13 kDa (Fig. 1a). Distinct bands representing recombinant fusion proteins, namely rLTB.OmpC$_{193–217}$, rLTB.OmpC$_{280–293}$, and rLTB.OmpC$_{323–336}$ (Fig. 1a, lanes 2, 4, 6), in the induced cell lysate lanes only indicated tight control of recombinant protein expression. Detection of a single sharp immunoreactive band in Western blot analysis using anti-His antibody authenticated the expressed proteins to be histidine-tagged recombinant proteins (Fig. 1b, rLTB.OmpC$_{193–217}$, lane 2; rLTB.OmpC$_{280–293}$, lane 4; rLTB.OmpC$_{323–336}$, lane 6). All the recombinant proteins were expressed as inclusion bodies (data not shown).

SDS-PAGE analysis of the recombinant fusion proteins purified from the solubilized inclusion bodies, using Ni^{2+}-NTA affinity chromatography and subsequently refolded using gradient dialysis, is shown in Fig. 1c (rLTB.OmpC$_{193–217}$, lane 1; rLTB.OmpC$_{280–293}$, lane 2; rLTB.OmpC$_{323–336}$, lane 3). As evident, purification to near homogeneity could be achieved with protein yields ranging between ~ 210 and 280 mg/l, at the shake-flask level.

Specificity and cross-reactivity analysis of the antifusion proteins' antisera

The anti-fusion protein antisera were analyzed for the presence of antibodies against the respective fusion protein as well as the parent protein. Immunization with the three fusion proteins generated high end point titer (> 1:80,000) antisera for the respective fusion proteins as compared to the pre-immune and anti-PBS antisera (data not shown). Analysis of comparative antibody titers specific to the parent protein rOmpC in the three anti-fusion protein antisera revealed significantly higher rOmpC-specific antibody titers (~ 80% with respect to anti-rOmpC antisera, included as a positive control) in the anti-rLTB.OmpC$_{323–336}$ antisera. The anti-rLTB.OmpC$_{193–217}$

Table 1 Sequences of complementary oligonucleotides corresponding to the in silico identified epitopes of OmpC of *A. hydrophila*, with nucleotides constituting the restriction enzyme sites (bold fonts overhang) are depicted

Epitope-based fusion protein	Amino acid residue position	Complementary annealed oligonucleotides	Amino acid sequence comprising the epitope (N- to C-termini)
rLTB.OmpC$_{193–217}$	193–217	5′ **C**CCAGGCGGGCAAAACCGATGAACAGGCGGCGAACGCGACGCTTTGGCGGCGATAGCGATGCGAAACTGTATGGCGTGA**A** 3′ 3′ **CATGG**GGTCCGCCCGTTTTGGCTACTTGTCCGCC GCTTGCGCTCGAAACCGCCGCTATCGCTACGCTTTGACATACCGCACT**TTCGA** 5′	QAGKTDEQAANASFGGDSDAKLYGV
rLTB.OmpC$_{280–293}$	280–293	5′ **C**CGCGCGCGGCATTGATGATACCGCGACCGAATATGTGAGCATTA**A** 3′ 3′ **CATGG**GCGCGCCGCTAACTATGGCGCTGGCTTATACACTCGTAATT**TTCGA** 5′	ARGIDDTATEYVSI
rLTB.OmpC$_{323–336}$	323–336	5′ **C**CAACAGCCTGGGCAAAAACACCGATGATGTGGTGGCGGTGGCGA 3′ 3′ **CATGG**GTTGTCGGACCCGTTTTTGTGGCTACTACACCACCGCCACCGCT**TTCGA** 5′	NSLGKNTDDVVAVA

and anti-rLTB.OmpC$_{280–293}$ antisera gave moderate (~ 56%) to significantly lower titers (~ 11%), respectively (Fig. 2a).

Due to generation of maximum OmpC-specific antibody titers by rLTB.OmpC$_{323–336}$ immunization, further analyses were carried out with the rLTB.OmpC$_{323–336}$ and anti-rLTB.OmpC$_{323–336}$ antisera. The antibodies present in the anti-rLTB.OmpC$_{323–336}$ antisera were able to recognize both the fusion partners (lanes 1 and 3) as evident by the presence of distinct immune-reactive bands as well as the fusion protein (lane 2) (Fig. 2b). ELISA performed for assessing the GM_1 ganglioside binding ability of the LTB moiety present in rLTB.OmpC$_{323–336}$ fusion protein revealed that the fusion protein exhibited significant binding to the GM_1 receptor (~ 94% as compared to native LTB; Fig. 2c), while negligible binding was obtained with BSA (included as a negative control).

Humoral and cellular immune response analysis against the recombinant fusion protein rLTB.OmpC$_{323–336}$

The anti-rLTB.OmpC$_{323–336}$ antisera were examined for the presence of different IgG antibody isotypes (IgA, IgG1, IgG2a, and IgG2b), indicating the type of T-helper response development. A significant increase in all the isotypes IgA, IgG1 > IgG2b > IgG2a indicated mucosal and systemic immune response activation (Fig. 3). IgG1/IgG2a and IgG1/IgG2b ratios (> 1) 7 weeks post-immunization hinted toward a Th2-biased immune response. T-cell proliferation assay validated the enhanced T-helper cell activity, indicated by increased levels of IgG isotypes' levels (Fig. 4a). Greater stimulation indices in rLTB.OmpC$_{323–336}$-stimulated splenocytes were observed when compared to PBS-stimulated splenocytes (both isolated from rLTB.OmpC$_{323–336}$ and PBS-immunized mice) at all the study intervals (~ 2.33; $p < 0.005$; 72 h post-stimulation). T-helper cell polarization (assessed by antibody isotyping) was further verified by the IFN-γ and IL-4 levels (Th1 and Th2 markers, respectively) in the splenocyte culture supernatants. A significant increase in the levels of both cytokines (p value ≤ 0.005–0.01) was observed in the culture supernatants of rLTB.OmpC$_{323–336}$-stimulated splenocytes isolated from the fusion protein-immunized mice as compared to the respective controls. Although an increase in IFN-γ secretion was approximately three times greater than that in the IL-4 (Fig. 4b, c; ~ 586 and 179 pg/ml, respectively), a significant increase in the levels of both these cytokines indicated the generation of a mixed immune response.

Besides IFN-γ and IL-4, relative levels of other chemokines/cytokines were also determined in the culture supernatant collected from rLTB.OmpC$_{323–336}$-stimulated splenocytes. Cytokine array analysis revealed a significant increase in a number of cytokines and chemokines, namely CXCL2, CCL5, IL-1F3, IL-3, IL-4, IL-5, IL-6, GM-CSF, (p value ≤ 0.05), CXCL10, CCL3, CCL1 (p value ≤ 0.02), and IL-2 (p value ≤ 0.005) (Supplementary Fig. 2, Fig. 5).

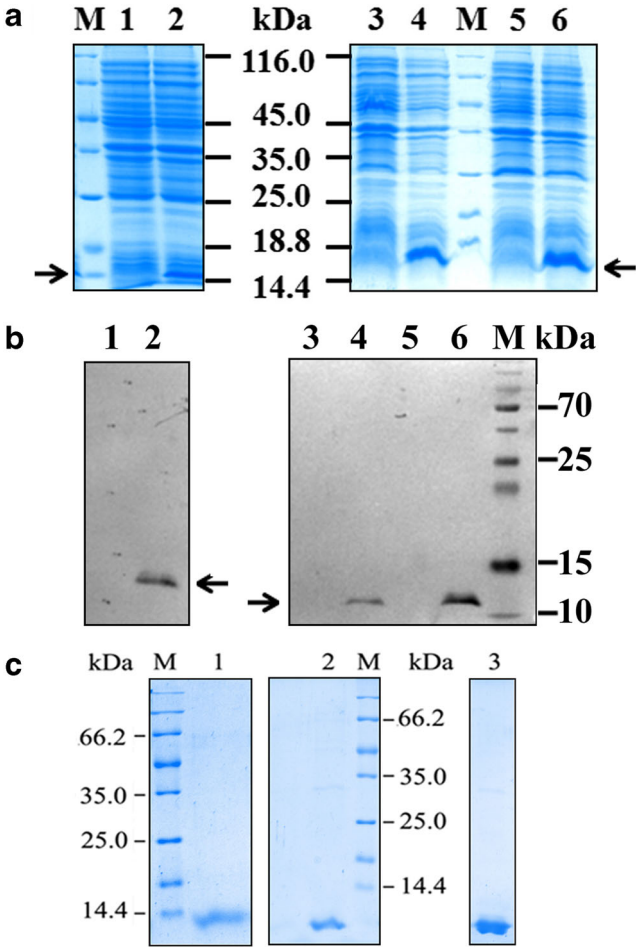

Fig. 1 Expression and purification of recombinant fusion proteins. **a** SDS-PAGE analysis of *E. coli* BL21(λDE3)pLysS cells harboring p*ET.LTB.ompC*$_{epitopes}$ for expression analysis. Lanes 1, 3, and 5 depict the cell lysates prepared from the uninduced *E. coli* BL21(λDE3)pLysS cells, while lanes 2, 4, and 6 indicate the cell lysates prepared from induced *E. coli* BL21(λDE3)pLysS cells harboring rLTB.OmpC$_{192–217}$, rLTB.OmpC$_{280–293}$, and rLTB.OmpF$_{323–336}$, respectively. Arrow points to the expressed recombinant fusion proteins as indicated. **b** Western blot analysis of the recombinant fusion proteins. Lanes 1, 3, and 5 show cell lysates prepared from the uninduced *E. coli* BL21(λDE3) pLysS cells, while lanes 2, 4, and 6 show cell lysates prepared from induced *E. coli* BL21(λDE3) pLysS cells expressing rLTB.OmpC$_{192–217}$, rLTB.OmpC$_{280–293}$, and rLTB.OmpC$_{323–336}$, respectively. Arrow points to immunoreactive bands at ~13 kDa detected using AP-conjugated monoclonal anti-polyhistidine-tagged antibodies, seen only in the induced cell lysates. **c** Purification of rLTB.OmpC$_{epitope}$ fusion proteins using immobilized Ni^{2+}-NTA affinity chromatography. Lanes 1–3 show purified rLTB.OmpC$_{192–217}$, rLTB.OmpC$_{280–293}$, and rLTB.OmpC$_{323–336}$, respectively. Lane M in all the panels indicates the migration of protein molecular weight marker (kDa)

rLTB.OmpC$_{323–336}$ generates bacterial agglutinating antibodies

Since *A. hydrophila* interacts with the host cell via its outer membrane's epitope, investigating anti-rLTB.OmpC$_{323–336}$ antisera's ability to recognize and interact with *Aeromonas* membrane was imperative for establishing its protective potential. Preliminary interaction between the OmpC on *Aeromonas* cell membrane and anti-rLTB.OmpC$_{323–336}$ antisera was analyzed using bacterial agglutination assay. Anti-rLTB.OmpC$_{323–336}$ antisera gave positive agglutination with *A. hydrophila* cells (Fig. 6), while no detectable agglutination was observed with the pre-immune sera, included as a negative control. Anti-rLTB.OmpC$_{323–336}$ antisera also agglutinated other *Aeromonas* species namely *A. culicicola*, *A. liquefaciens*, and *A. sobria*, exhibiting a broad range agglutination potential against different species of *Aeromonas*. Quantitative analysis revealed bacterial agglutination titer of > 1:700 for the anti-rLTB.OmpC$_{323–336}$ antisera, while no lysis was seen in the wells containing dilutions of anti-PBS antisera.

The interaction between linear B-cell epitope$_{323–336}$ in *A. hydrophila* outer membrane and the anti-rLTB.OmpC$_{323–336}$ antisera was validated using flow cytometry (Fig. 7). Observed increase in fluorescent cell population was directly correlated with the positive interaction between anti-rLTB.OmpC$_{323–336}$ antisera and OmpC in *A. hydrophila* outer membrane (Supplementary Fig. 3; parent protein OmpC antisera included as a positive control). Fluorescent cell population obtained with pre-immune sera (6.94 ± 0.57, Supplementary Fig. 3a) was taken as the negative control. Approximately four- to five-fold increase in fluorescence intensity upon incubation with anti-rLTB.OmpC$_{323–336}$ antisera (34.94 ± 0.68, Supplementary Fig. 3b) and anti-rOmpC antisera (38.51 ± 0.96, Supplementary Fig. 3c, positive control), as compared to pre-immune sera, confirmed specific interaction with OmpC epitope$_{323–336}$ in *A. hydrophila* outer membrane. Interaction of anti-rLTB.OmpC$_{323–336}$ antisera with *E. coli* cells (included as a negative control for the secondary antibody) also yielded minimal fluorescent cell population (6.087 ± 1.2; Supplementary Fig. 3d), highlighting the specificity of the outer membrane B-cell epitopes with *A. hydrophila* membrane.

Humoral immune response investigation in *L. rohita*

Immunization with the fusion protein rLTB.OmpC$_{323–336}$ was able to generate highly specific titers ~ 1:2000 till the 4th week, which were maintained at 1:1000 till week 17 (Supplementary Fig. 4a), indicating efficient presentation and processing of the fusion protein, facilitated by the GM_1 receptors in the mucosa-associated lymphoid tissues (MALTs). Immunoblot analysis of the antifusion protein antisera raised in fish revealed distinct immunoreactive bands recognizing the fusion partner rLTB, fusion protein rEpi3, and parent protein rOmpC (Supplementary Fig. 4b; lanes 1, 2, and 3, respectively).

Discussion

A number of attempts have been made toward the development of a vaccine against *A. hydrophila* targeting different cellular

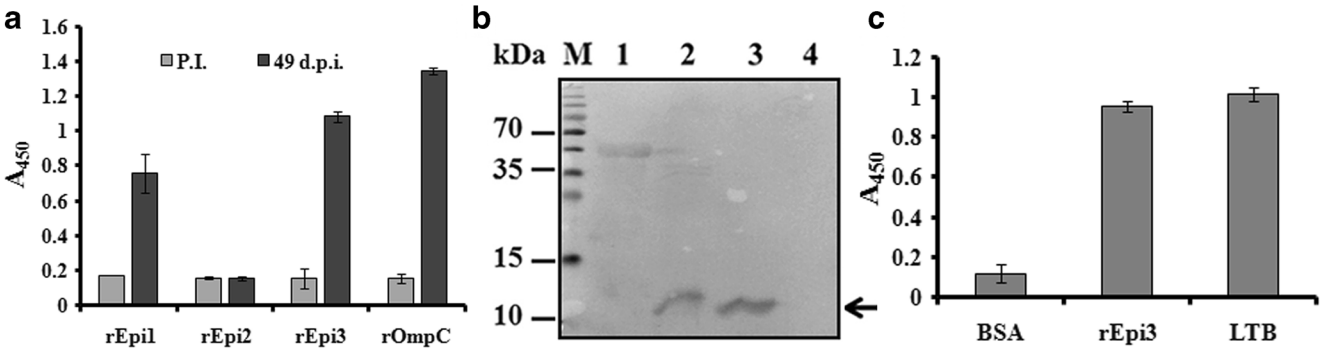

Fig. 2 Recognition of parent proteins by the anti-fusion protein antisera. **a** Ability of anti-rLTB.OmpC$_{192–217}$ (anti-rEpi1), anti-rLTB.OmpC$_{280–293}$ (anti-rEpi2), and anti-rLTB.OmpC$_{323–336}$ (anti-rEpi3) antisera (drawn at 49 days post-immunization) to recognize the parent protein rOmpC (target protein) was demonstrated by ELISA. Absorbance at 450 nm indicated relative cross-reactivity of the anti-fusion protein antisera with the rOmpC. Anti-rOmpC antisera and PI (pre-immune antisera) were included as the positive and negative controls, respectively. **b** Specificity determination of anti-rLTB.OmpC$_{323–336}$ (anti-rEpi3) antisera by immunoblot analysis. Lanes 1, 2, 3, and 4 represent cell lysates of induced *E. coli* BL21(λDE3)pLysS cells expressing rOmpC, rEpi3, and rLTB, respectively, and purified BSA. Lane M indicates the migration of the protein molecular marker in kilodaltons. **c** Determination of GM_1 ganglioside receptor binding activity. Interaction between GM_1 receptor moiety present in rEpi3 was determined by sandwich ELISA using anti-rLTB antisera (1:5000) followed by AP-conjugated secondary antibody (1:10,000). rLTB and BSA were included as positive and negative controls, respectively

components. Bacterial outer membrane proteins have been successfully evaluated as effective vaccine candidates against *A. hydrophila* [8]. Earlier studies have reported the immunogenic and protective potential of outer membrane proteins OmpF, OmpC, and OmpR [10, 14, 30]. While OmpF and OmpR generated a Th1-biased mixed immune response, OmpC elicited a Th2-biased mixed immune response against *A. hydrophila.*

To focus the immune response stimulated by full-length protein OmpF to specific aa stretches, its continuous B-cell epitopes have been evaluated for their immunodominant character [17]. The fusion protein comprising OmpF B-cell epitope$_{66–80}$ and carrier protein LTB triggered a robust immune response against *A. hydrophila*. In the earlier study [28], putative epitopes in the N-terminal of OmpC were evaluated and analyzed for their immunodominant character. Further in silico and in vitro studies were carried out to determine the presence of any other immunodominant stretches, if any in the C-terminal of the OmpC. Epitope-based fusion protein eliciting maximum immunodominant character could then be processed for future immunization trials in *L. rohita*, a primary model for *A. hydrophila* pathogen. In silico prediction using multiple softwares, previously validated for reliable epitope prediction [17, 28, 31, 32], was carried out, so as to involve maximum characteristics for determining immunodominance of the epitopes. Three more aa residue stretches of *A. hydrophila* OmpC were predicted as immunodominant B-cell epitopes.

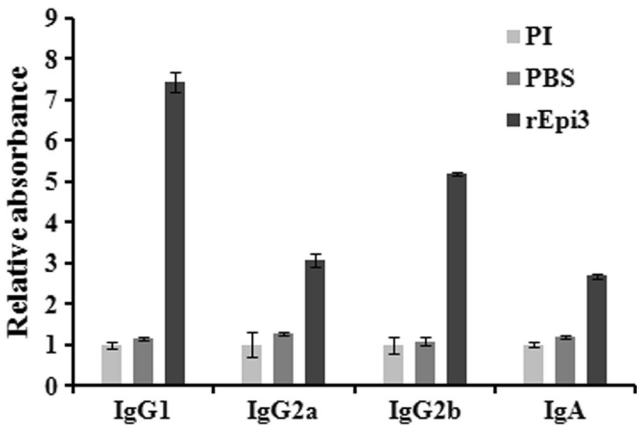

Fig. 3 Antibody isotype determination in the anti-rLTB.OmpC$_{323–336}$ (anti-rEpi3) antisera. Antisera of mice immunized (i.p.) with rEpi3 (collected at day 49) were examined for the presence of different IgG isotypes by ELISA. Absorbance obtained for different isotypes in the experimental group is expressed relative to that obtained with pre-immune sera, wherein A_{405} nm for pre-immune sera is normalized to 1. Data indicate mean ± standard deviation (SD) of three independent experiments, each performed in triplicates

Because of the small size of these antigenic epitopes, they were cloned in fusion with LTB, a well-established mucosal and parenteral adjuvant [33, 34], thus eliminating the risks of degradation by serum proteases and poor immune response [23]. The presence of a penta-glycine linker between the epitope and LTB could assist in efficient protein folding and interaction [35–37]. Unlike wild-type LTB and LTB fusion proteins that have been expressed in the periplasmic and secretory fractions with lower yields in both prokaryotic and eukaryotic expression systems [38–40], the recombinant LTB.OmpC$_{epitope}$ fusion proteins were expressed exclusively as insoluble inclusion bodies. The yields of different rLTB.OmpC$_{epitope}$ fusion proteins were comparable to those obtained for other LTB-based fusion proteins reported earlier by Sharma and Dixit and significantly higher than those obtained for insoluble expression of other LTB fusion proteins [17, 28, 41, 42].

Since the rLTB.OmpC$_{epitope}$ fusion proteins were produced with a primary goal to assess their potential as a vaccine candidate against *A. hydrophila*, it was imperative to establish and

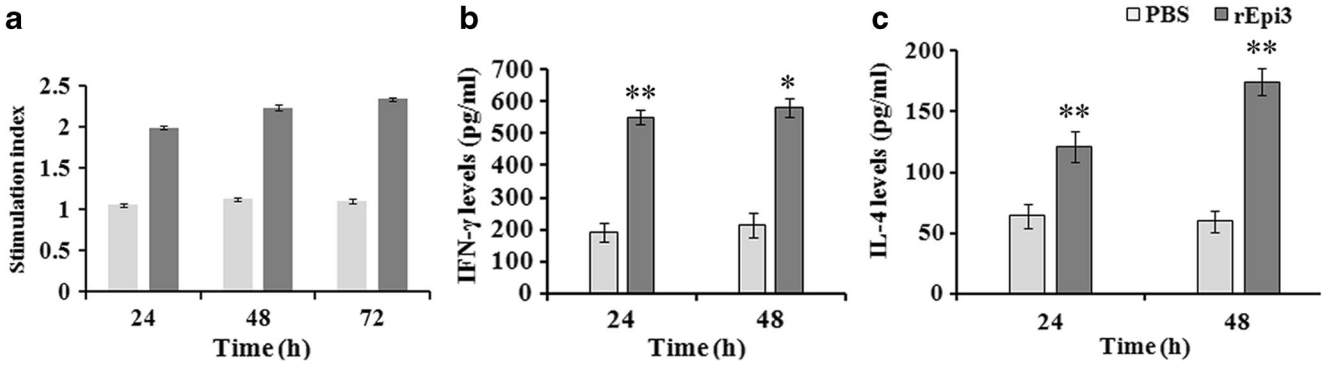

Fig. 4 Analysis of T-cell response. **a** In vitro T cell proliferation assay. Splenocytes isolated from Balb/c mice immunized (i.p.; 15 μg/mice) with rLTB.OmpC$_{323–336}$ (rEpi3) and 1 × PBS (with or without stimulation with rEpi3) were cultured at 37 °C under 5% CO_2 conditions and subjected to XTT assay till 72 h post-stimulation. **b**, **c** Cytokine ELISA to detect IFN-γ and IL-4, respectively, in the culture supernatants from the PBS- and fusion protein-immunized mice, collected at 24 and 48 h post-stimulation with the fusion protein. In both panels, $*p \leq 0.01$; $**p \leq 0.005$. Data indicate mean ± standard deviation (SD) of three independent experiments, each performed in triplicates

compare the immunogenic potential of these fusion proteins together with the already characterized OmpC B-cell epitope$_{143–175}$ [28]. Initial studies for detailed immune response characterization of the three B-cell epitopes were carried out using intraperitoneal immunization of the fusion protein and PBS (negative control) in mice, with appropriate delivery vehicles. Parenteral immunization with LTB provides consistent antibody responses as compared to oral routes [43]. After evaluating the differential immunogenic potential of the predicted B-cell epitopes via parenteral route, oral immunization studies were initiated in *L. rohita*, the host organism for *Aeromonas* infection. Despite being much smaller in size (14 aa residues) when compared to that of the rLTB.OmpC$_{193–217}$ (25 aa residues) as well as rLTB.OmpC$_{143–175}$ (33 aa residues)

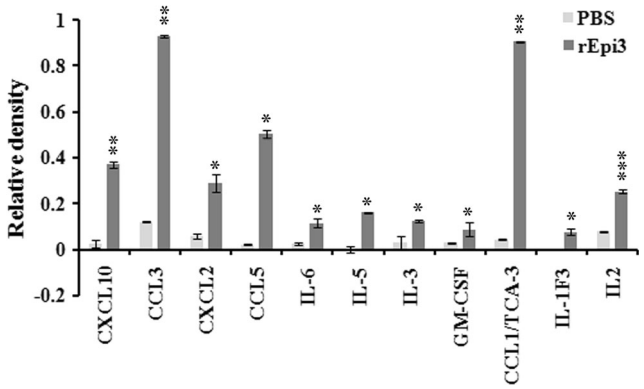

Fig. 5 Cytokine-chemokine profile determination by cytokine array analysis. Culture supernatants from rLTB.OmpC$_{323–336}$ (rEpi3; 15 μg/ml) and PBS-sensitized splenocytes were collected 72 h post-stimulation and subjected to cytokine analysis using mouse cytokine array kit from R&D Systems. Data are expressed as density of immunoreactive spots obtained with the culture supernatants of the fusion protein-immunized mice, relative to the respective immunoreactive spots obtained with the PBS-immunized mice. Cytokines showing significantly increased levels are depicted in the bar diagram with $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.005$

[28], the rLTB.OmpC$_{323–336}$ generated stronger antibody response specific to OmpC. Maximum parent protein OmpC-specific antibodies were detected in anti-rLTB.OmpC$_{323–336}$ antisera ~80% (rLTB.OmpC$_{143–175}$ ~62% [28]), establishing rLTB.OmpC$_{323–336}$ as the fusion protein harboring the most immunodominant epitope of *A. hydrophila* OmpC. Detection of both the fusion partners by anti-rLTB.OmpC$_{323–336}$ antisera established that the fusion protein retained the antigenicity of the carrier protein rLTB as well as the predicted OmpC B-cell epitope$_{323–336}$. Significant immune molecules like macrophages and B cells have been reported to express GM_1 receptors, improving uptake of the associated antigen by antigen-presenting cells (APCs) [25]. The observed GM_1 receptor binding activity of the fusion protein was equivalent to or slightly greater than that observed with other fusion proteins using LTB as a carrier protein for B-cell epitopes of both gram-positive and gram-negative bacteria [17, 27, 38]. Highly significant GM_1 receptor binding (~94%) not only indicates proper B subunit pentamer assembly [44] but also points to effective mucosal adjuvant activity. Efficient B subunit protein refolding and effective monosialoganglioside receptor binding and generation of heightened fusion protein- and parent protein-specific titers by rLTB.OmpC$_{323–336}$ further demonstrate its immunogenic potential and are in agreement with the established adjuvancy of LTB [25].

Both humoral and cell-mediated immunity have their respective roles at different stages of immune response development. A complex participation of both these arms of immune response is critical for developing effective resistance against virulent pathogens. Antibody isotyping data revealed the presence of both complement-fixing and noncomplement-fixing antibodies. The antibody isotype profiles (IgG1 > Ig2b > IgG2a) observed in the present study are similar to that obtained post B-cell epitope-based vaccination against mastitis, anthrax, *A. hydrophila* outer membrane protein B-cell epitope-based fusion proteins as well as with the

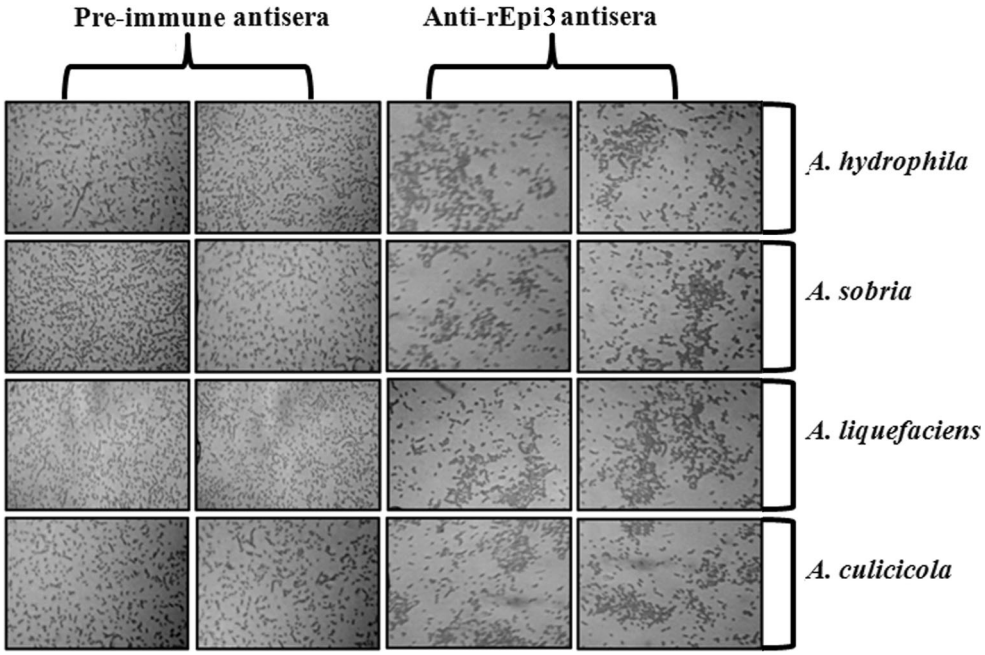

Fig. 6 Bacterial agglutination assay using anti-rLTB.OmpC$_{323–336}$ (anti-rEpi3) antisera. Different *Aeromonas* spp. as indicated (log phase cells; 1 × 10^8 cfu) were incubated with pre-immune antisera (1:320; left panel) and anti-rEpi3 antisera (1:320; right panel) in 1 × PBS at 37 °C for 1 h. Anti-rEpi3 antisera gave visible agglutination with all the *Aeromonas* species tested. Images are taken at ×40 magnification

parent protein OmpC [14, 17, 28, 45, 46]. The presence of IgG1, the prominent isotype response observed against membrane proteins, has been associated with the generation of neutralizing antibodies post-multiepitope (both T and B cell)-based vaccination [47, 48], highlighting Th2-biased immunodominance of this single epitope-based fusion protein. A significant increase in IgA titers ($p < 0.0001$) suggests that the fusion protein could be used for mucosal immunization either through oral or nasal route.

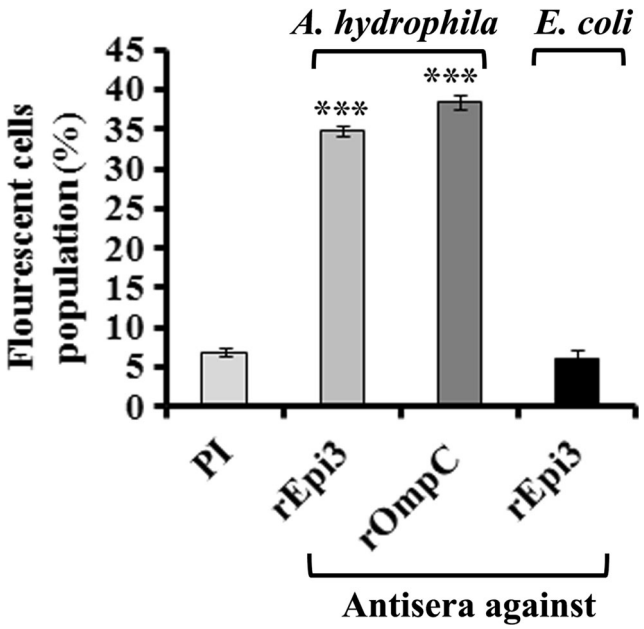

Fig. 7 Flow cytometry analysis to investigate interaction of anti-rLTB.OmpC$_{323–336}$ (anti-rEpi3) antisera with OmpC on *A. hydrophila*. Fluorescent cell population (%) of live *A. hydrophila* cells (log phase culture; 1 ×10^6 cfu) incubated with pre-immune (PI) antisera, anti-rEpi3 antisera, and anti-rOmpC antisera can be directly correlated to their respective interaction with *A. hydrophila* outer membrane. Data represents mean ± SD of three independent experiments. ***$p \leq 0.001$

Stimulation indices obtained with the rLTB.OmpC$_{323–336}$ were slightly higher than those obtained with the parent protein (~ 2.33 and ~ 2.1; $p < 0.005$; 72 h post-stimulation [14]), accentuating superior T-cell activation by the epitope in comparison to the full-length protein, upon stimulation with the respective recombinant proteins. T-cell stimulation index obtained with the rLTB.OmpC$_{323–336}$ was greater than those obtained post-immunization with both B- and T-cell-based subunit vaccine candidates (~ 0.5–2.0) [48]. A prominent increase in IFN-γ and IL-4 levels observed in the present study is in agreement with the earlier reports on B-cell peptides and individual B/T-cell peptides against both gram-positive and gram-negative bacteria, respectively [46, 49]. A concurrent increase in IFN-γ levels along with IL-4 secretion indicates active participation of the plasmacytoid dendritic cells, linking the innate and adaptive immune response generation [50]. Interestingly, IFN-γ cytokine levels observed for rLTB.OmpC$_{323–336}$-stimulated splenocytes surpassed the IFN-γ levels obtained upon immunization with a T-cell epitope of influenza virus in fusion with flagellin as the carrier [51], signifying the use of LTB as a carrier molecule. Antibody isotyping and cytokine ELISA results in the present study are in line with the established role of LTB in generating a Th2-biased immune response [25].

Cytokine profile pattern obtained postarray analysis highlighted the role of individual cytokines in protective immune response development. Increased levels of GM-CSF, an

important differentiation factor responsible for maintaining monocytic and granulocytic cell lineages, indicated elevated innate immune response activation [52]. High levels of CXCL10 and CCL5 proinflammatory cytokines point toward the activation of $CD4^+$ T-helper cell activation [53, 54]. A concurrent increase in IL-2 levels, a principal Th1 cytokine active in T-cell proliferation and B-cell differentiation, clearly establishes stimulation of Th1-mediated cellular immunity [55]. A parallel increase in the levels of CCL3 (Th1-biased) and CCL1 (Th2-biased) along with CXCL2 indicates development of a mixed immune response [56]. IL-1F3/IL-1ra is instrumental in blocking IL-1 receptor binding, keeping a check on the inflammatory damage resulting from acute phase response reactions [57]. IL-3, IL-5, and IL-6 are the characteristic cytokines representing a protective Th2-biased immune response. IL-3 has been associated with shifting monocyte differentiation into dendritic cells with a Th2-skewed response [58, 59]. Increased levels of all these interleukins suggest an expansion of the Th2 cell population, along with the established Th1 activity. Altogether, the cytokine array data reveals generation of a balanced mixed immune response with a Th2 bias, justifying the immunostimulatory effect imparted by the LTB moiety to the OmpC B-cell epitope. Cytokine profiles from array analysis with the OmpF epitope$_{66–80}$, OmpC epitope$_{143–175}$, and OmpC epitope$_{323–336}$-based fusion proteins revealed the presence of some cytokines which were common (CCL3, CXCL2, IL-4, IFN-γ, CCL1) [17, 28], while the others were exclusively increased upon stimulation with the respective fusion proteins, generating a differentially balanced immune response specific for individual epitope.

Elicitation of protective immune response is based on the formation of an epitope-antibody complex, responsible for intercepting the invading pathogen and impeding pathogen adhesion. Antibody-mediated bacterial agglutination has been associated with providing protection against mucosal invasion of the pathogen [60]. Mucosal route is the primary route of invasion for fish pathogens like *A. hydrophila*. Thus, the immunogenicity of putative surface exposed B-cell epitopes could be utilized only if the anti-rLTB.OmpC$_{323–336}$ antisera is capable of epitope recognition on pathogen's surface. While anti-rLTB.OmpF$_{66–80}$ antisera could partially agglutinate *A. hydrophila* [17], anti-rLTB.OmpC$_{323–336}$ antisera exhibited extensive agglutination of *A. hydrophila*. The extent of agglutination by the anti-rLTB.OmpC$_{323–336}$ antisera was comparable to that observed with the anti-rOmpC, i.e., parent protein antisera [14], suggesting that the OmpC epitope$_{323–336}$ significantly retained the antigenicity of the parent protein. The antigenic potential was perhaps augmented by the LTB used as a carrier. A broader range of agglutination potential of anti-rLTB.OmpC$_{323–336}$ antisera as compared to anti-rLTB.OmpC$_{143–175}$ antisera [28] with different *Aeromonas* species makes it an attractive candidate in vaccine design, capable of detecting heterogeneous strains of this bacteria. Flow cytometry analysis using a tagged antibody or antibody raised against the outer membrane protein can establish the insertion of an epitope or a loop into the outer membrane [17, 61]. It has been used previously to analyze the surface exposure of different OMP epitopes in whole-cell membranes for gram-negative bacteria [62, 63]. The results obtained from FACS analysis were in tune with the cross-reactivity observed between anti-rLTB.OmpC$_{323–336}$ antisera with rOmpC in ELISA as well as that of anti-rOmpC antisera with live *A. hydrophila* cells [14].

Though enzymes of the Ig gene rearrangement molecular machinery for fishes and mammals have been shown to be remarkably conserved [64], investigation into the fish immune system using *L. rohita* was carried out to assess the suitability of these peptides in fish vaccine formulations. High GM_1 receptor binding activity of the fusion protein and detected increase in IgA levels, indicative of humoral mucosal immune response, in mouse antifusion protein antisera prompted us to carry out a preliminary investigation assessing the oral immunization potential of the fusion protein in *L. rohita*. After screening the immunogenic potential of different immunodominant B-cell epitopes, an initial antigenicity analysis was carried out with rLTB.OmpC$_{323–336}$ in *L. rohita.* Oral immunization resulted in long-lasting immune response. Differences observed between fishes exposed to fusion protein (vaccinated) and PBS-containing (unvaccinated) feed pellets were within statistically significant limits at 99% confidence intervals ($p \leq 0.005$). These preliminary results obtained indicated the generation of a highly specific B-cell-mediated immune response in comparison to that obtained via full antigenic load of combined inactivated bacterin vaccine preparation [65].

The present study thus reports a comprehensive analysis of the immunodominance exhibited by *A. hydrophila* OmpC linear B-cell epitope$_{323–336}$, in fusion with LTB. Earlier, Sharma and Dixit have reported a Th1-biased mixed immune response for the OmpF B-cell epitope$_{66–80}$ and a Th1/Th17-biased mixed immune response for the OmpC B-cell epitope$_{143–175}$ of *A. hydrophila* [17, 28]. On the other hand, cytokine profile analysis of the supernatants of rLTB.OmpC$_{323–336}$-stimulated splenocytes revealed a Th2-biased mixed immune response. The release of different cytokines highlights distinct immunoreactive pattern generation owing to the incorporation of separate B-cell epitopes, while generation of common cytokines can be possibly attributed to the use of the same carrier protein LTB. Identification of this immunodominant OmpC epitope$_{323–336}$ can be exploited in oral vaccine design by itself, or in combination with the previously identified immunodominant OmpF epitope$_{66–80}$ and OmpC epitope$_{143–175}$ as a multi-epitope vaccine, offering novel insights into treatment strategies against the bacterial pathogen *A. hydrophila*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval For this type of study, formal consent from human participants is not required.

Statement on the welfare of animals All procedures performed in studies involving animals were in accordance with the ethical standards of the institution as per guidelines recommended by the Animal Care and Use Committee (ACUC), under Institutional animal Ethic Committee (IAEC code 12/2014), JNU, Delhi, India.

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