Fish & Shellfish Immunology 33 (2012) 514–521

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Edwardsiella tarda sialidase: Pathogenicity involvement and vaccine potential

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ARTICLE INFO

Article history:
Received 25 January 2012
Received in revised form
4 June 2012
Accepted 4 June 2012
Available online 13 June 2012

Keywords:
Neuraminidase
Edwardsiella tarda
Virulence
Vaccine

ABSTRACT

Bacterial sialidases are a group of glycohydrolases that are known to play an important role in invasion of host cells and tissues. In this study, we examined in a model of Japanese flounder (*Paralichthys olivaceus*) the potential function of NanA, a sialidase from the fish pathogen *Edwardsiella tarda*. NanA is composed of 670 residues and shares low sequence identities with known bacterial sialidases. In silico analysis indicated that NanA possesses a sialidase domain and an autotransporter domain, the former containing five Asp-boxes, a RIP motif, and the conserved catalytic site of bacterial sialidases. Purified recombinant NanA (rNanA) corresponding to the sialidase domain exhibited glycohydrolase activity against sialic acid substrate in a manner that is pH and temperature dependent. Immunofluorescence microscopy showed binding of anti-rNanA antibodies to *E. tarda*, suggesting that NanA was localized on cell surface. Mutation of *nanA* caused drastic attenuation in the ability of *E. tarda* to disseminate into and colonize fish tissues and to induce mortality in infected fish. Likewise, cellular study showed that the *nanA* mutant was significantly impaired in the infectivity against cultured flounder cells. Immunoprotective analysis showed that rNanA in the form of a subunit vaccine conferred effective protection upon flounder against lethal *E. tarda* challenge. rNanA vaccination induced the production of specific serum antibodies, which enhanced complement-mediated bactericidal activity and reduced infection of *E. tarda* into flounder cells. Together these results indicate that NanA plays an important role in the pathogenesis of *E. tarda* and may be exploited for the control of *E. tarda* infection in aquaculture.

1. Introduction

Sialic acids are a diverse family of nine-carbon sugars derived from neuraminic acid [1]. They usually occur at the terminal positions of secreted and cell surface glycoconjugates such as oligosaccharides, polysaccharides, glycoproteins, gangliosides, and lipopolysaccharides [2]. Sialic acids play essential biological roles by mediating cellular adhesion, receptor-ligand binding, and cell–cell and cell–molecule interactions [3,4]. Sialic acids can be released from the linked macromolecules by sialidases or neuraminidases (E.C. 3.2.1.18), which are glycohydrolases that cleave the α-linkage of the terminal sialic acid residue from various sialoglycoconjugates. Sialidases have been found to exist in a wide range of organisms including viruses, bacteria, and vertebrates [5]. In bacteria, sialidases play important roles in both pathogenicity and nutrition [6,7]. It is known that bacterial sialidase can scavenge sialic acids as a carbon source for growth [8–11], regulate biofilm production [12,13], promote infection by exposing receptors on host cells [14,15], and modulate host immune response [16–18].

Structurally, bacterial sialidases contain a conserved sialidase domain and variable extra domains, the latter include membrane-anchoring domains and carbohydrate binding domains such as lectins [2,19,20]. In some sialidases, the carbohydrate binding domains are absent. The sialidase domain contains 2–5 Asp-box motifs (Ser/Thr-X-Asp-[X]-Gly-X-Thr-Trp/Phe), a RIP (Arg-Ile/Leu-Pro) motif, and a catalytic site composed of highly conserved residues which include a tyrosine residue that serves as a catalytic nucleophile, an arginine triad that interacts with sialic acid, a glutamate residue, and an aspartic acid residue [21–24].

Edwardsiella tarda is a Gram-negative bacterium and a serious pathogen of farmed fish. It is the etiological agent of a systematic disease called edwardsiellosis, which has been reported to affect a wide range of freshwater and marine fish [25,26]. In addition to fish, *E. tarda* is also an occasional human pathogen and known to cause both gastroenteritis and extraintestinal infections in humans [27,28]. A number of virulence-associated systems and factors, such as the type III and type VI secretion systems, the LuxS/AI-2 quorum sensing system, and hemolysin systems, have been identified in *E. tarda* [29]. However, the fundamental virulence mechanism

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http://dx.doi.org/10.1016/j.fsi.2012.06.002

of *E. tarda* remains elusive. In this study, we characterized an *E. tarda* sialidase, NanA, and examined its activity and potential function in a model of Japanese flounder (*Paralichthys olivaceus*). Our results indicate that NanA is a functional sialidase and a protective immunogen that is required for optimal bacterial infection.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli BL21(DE3) and DH5α were purchased from Tiangen (Beijing, China). *E. coli* S17-1λpir was purchased from Biomedal (Sevilla, Spain). *E. tarda* TX01 was isolated from diseased flounder [30]. All strains were grown in Luria-Bertani broth (LB) [31] at 37 °C (for *E. coli*) or 28 °C (for *E. tarda*). Where indicated, appropriate antibiotics were supplemented as follows: chloramphenicol, 30 μg/ml; polymyxin B, 100 μg/ml; rifampicin, 20 μg/ml.

2.2. Fish

Japanese flounder (*P. olivaceus*) (average 11 g) were purchased from a local fish farm and maintained at 22 °C in aerated seawater. Fish were acclimatized in the laboratory for two weeks before experimental manipulation. Fish were fed daily with commercial dry pellets (purchased from Shandong Sheng-suo Fish Feed Research Center, Shandong, China). Before each experiment, fish were randomly sampled for the examination of bacterial recovery from blood, liver, kidney, and spleen. Fish were considered healthy and used for experiment only when no bacteria could be detected from any of the examined fish. Fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, MO, USA) prior to experiments involving tissue collection.

2.3. Sequence analysis of nanA

The open reading frame of *nanA* was cloned from TX01 by PCR amplification using primers NanAF1 (5′-GCGCATATG CTGATTTTTGCCGAAC-3′, underlined sequence, NdeI site) and NanAR1 (5′-CTCGAGAAAGGTGTAGGTGAAGCTG-3′, underlined sequence, XhoI site), which were designed based on the sialidase sequence of *E. tarda* FL6-60 (GenBank accession no. CP002154). The DNA sequence of *nanA* has been submitted to GenBank (accession no. JX122859). NanA was analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. Domain search was performed with the conserved domain search program of NCBI. The calculated molecular mass and theoretical isoelectric point were predicted using EditSeq in the DNASTAR software package (Madison, WI, USA). Signal peptide search was performed with SignalP 3.0. Subcellular localization prediction was performed with the PSORTb v.3.0 server. Sequence alignment was created with the ClustalX program.

2.4. Plasmid and strain construction

To construct pEtNanA, which expresses NanA corresponding to the sialidase domain, PCR was carried out with primers NanAF1 and NanAR2 (5′-CTCGAGGGCATAGTCTCGCGCG-3′, underlined sequence, XhoI site); the PCR products were ligated with the T-A cloning vector pBS-T (Tiangen, Beijing, China), resulting in pBSNanA, which was digested with NdeI/XhoI, and the fragment containing *nanA* was retrieved and inserted into pET258 [32] between NdeI and XhoI sites. To construct pEtCol, which expresses a recombinant *E. tarda* collagenase (GenBank accession no. ACY83263.1) rCol, PCR was performed with primers ColF1 (5′-GCGCATATGCTGATTTTTGCCGAAC-3′, underlined sequence, NdeI site) and ColR1 (5′-CTCGAGGGCATAGT CTCGCGCG-3′, underlined sequence, XhoI site); the PCR products were ligated with pBS-T, resulting in pBSCol, which was digested with NdeI/XhoI, and the fragment containing the collagenase gene was retrieved and inserted into pET258 as described above. To construct the mutant strain TXnanA, in which the *nanA* gene was inactivated by insertion mutation, an internal fragment of *nanA* (position 127–667) was generated by PCR with primers KOF (5′-GGATCCGGTCAGAATCAGGATGTGC-3′, underlined sequence, BamHI site) and KOR (5′-GGATCCTGCTCTCGGAGGTGATGT-3′, underlined sequence, BamHI site). The PCR products were inserted into the suicide plasmid pDM4 [33] at the compatible BglII site, resulting in pDMNanA. S17-1λpir was transformed with pDMNanA, and the transformants were conjugated with TX01 as described previously [34]. The transconjugants were selected on LB agar plates supplemented with polymyxin B and chloramphenicol. One of the selected mutants was named TXnanA. Mutation of *nanA* in TXnanA was confirmed by PCR analysis and subsequent sequencing of the PCR products. To construct TXnanAC, pBSNanA was digested with NdeI/XhoI, and the fragment containing *nanA* was retrieved and inserted into the expression plasmid pBT3 [32] between NdeI and XhoI sites, resulting in pBT3NanA. TXnanA was transformed with pBT3NanA via electroporation, and the transformants were selected on LB agar plates supplemented with ampicillin (resistance marker of pBT3NanA). One of the transformants was named TXnanAC.

2.5. Purification of recombinant protein and preparation of antiserum

Recombinant NanA (rNanA) and rCol, which was used in subsequent activity analysis as a control protein, were purified as follows. *E. coli* BL21(DE3) was transformed with pEtNanA or pEtCol; the transformant were cultured in LB medium at 37 °C to mid-logarithmic phase, and expression of the exogenous genes was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.1 mM. After growing at 30 °C for an additional 5 h, the cells were harvested by centrifugation, and His-tagged rNanA and rCol were purified under denaturing conditions using nickel-nitrilotriacetic acid columns (GE Healthcare, Piscataway, NJ, USA) as recommended by the manufacturer. The purified proteins were reconstituted as described previously [35]. Both reconstituted and un-reconstituted proteins were dialyzed in phosphate-buffered saline (PBS). The proteins were concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The concentrated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250. The concentrations of the proteins were determined using the Bradford method [36] with bovine serum albumin as the standard. Preparation of rat anti-rNanA serum and determination of the titer and specificity of anti-rNanA antibodies were performed as described previously [37].

2.6. Sialidase activity assay

The activity of rNanA was determined according to Warwas et al. [38]. Briefly, reconstituted and rNanA and rCol in different concentrations were added to a 96-well plate containing 200 μM 4-methylumbelliferyl α-D-N-acetylneuraminic acid (MUN) (Sigma, St. Louis, MO, USA) and 40 mM sodium formate in a total volume of 100 μl. The plate was incubated at 37 °C for 15 min and added with 200 μl stop solution (0.1 M glycine, 0.014 M NaCl, 25% ethanol, pH 10.7). The amount of 4-methylumbelliferone released from MUN was determined using a fluorescence spectrophotometer at