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Recombinant sialidase NanA (rNanA) cleaves α2-3 linked sialic acid of host cell surface N-linked glycoprotein to promote *Edwardsiella tarda* infection

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ABSTRACT

Edwardsiella tarda is one of the major pathogenic bacteria affecting both marine and freshwater fish species. Sialidase NanA expressed endogenously in *E. tarda* is glycosidase removing sialic acids from glycoconjugates. Recently, the relationship of NanA sialidase activity to *E. tarda* infection has been reported, however, the mechanism with which sialidase NanA aids the pathogenicity of *E. tarda* remained unclear. Here, we comprehensively determined the biochemical properties of NanA towards various substrates *in vitro* to provide novel insights on the potential NanA target molecule at the host cell. GAKS cell pretreated with recombinant NanA showed increased susceptibility to *E. tarda* infection. Moreover, sialidase inhibitor treated *E. tarda* showed a significantly reduced ability to infect GAKS cells. These results indicate that NanA-induced desialylation of cell surface glycoconjugates is essential for the initial step of *E. tarda* infection. Among the natural substrates, NanA exhibited the highest activity towards 3-sialyllactose, α2-3 linked sialic acid carrying sialoglycoconjugates. Supporting this finding, intact GAKS cell membrane exposed to recombinant NanA showed changes of glycoconjugates only in α2-3 sialo-linked glycoproteins, but not in glycolipids and α2-6 sialo-linked glycoproteins. Lectin staining of cell surface glycoprotein provided further evidence that α2-3 sialo-linkage of the N-linked glycoproteins was the most plausible target of NanA sialidase. To confirm the significance of α2-3 sialo-linkage desialylation for *E. tarda* infection, HeLa cells which possessed lower amount of α2-3 sialo-linkage glycoprotein were used for infection experiment along with GAKS cells. As a result, infection of HeLa cells by *E. tarda* was significantly reduced when compared to GAKS cells. Furthermore, *E. tarda* infection was significantly inhibited by mannose pretreatment suggesting that the bacterium potentially recognizes and binds to mannose or mannose containing chains following desialylation. Together, these results suggest that *E. tarda* may employ endogenous NanA to desialylate α2-3 glycoproteins on host cells, thus revealing one of the potential binding molecules during infection.

1. Introduction

In cultured fish, bacterial diseases pose great threat to both marine and freshwater species. It has previously been reported that attachment of bacteria to the epithelial cells of host animals is the initial step in the infection. The attachment process facilitates bacterial colonization and penetration of the cells [1]. *Edwardsiella tarda*, a Gram-negative bacterium belonging to the family Enterobacteriaceae, is one of the most well characterized species in the

Abbreviations: MU-NANA, 4-methylumbelliferyl-N-acetyl-α-D-neuraminic acid; DANA, 2-deoxy-2,3-didehydro-N-acetylneuraminic acid; DMEM, Dulbecco modified eagle medium; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; GD1a, NeuAcα2 → 3Galβ1 → 3GalNAcβ1 → 4[NeuAcα2 → 3]Galβ1 → 4Glcβ1 → Cer; GM1, Gal β1 → 3GalNAcβ1 → 4[NeuAcα2 → 3]Galβ1 → 4Glcβ1 → Cer; GM3, NeuAcα2 → 3Galβ1 → 4Glcβ1 → Cer; GST, Glutathione-S-transferase; MALII, *Maackia amurensis* lectin II; MAM, *Maackia amurens* lectin; PCR, polymerase chain reaction; SSA, *Sambucus sieboldiana* agglutinin.

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family. The bacterium causes Edwardsiellosis, a disease reported in both freshwater and marine fish species [2,3]. Cases of *E. tarda* have been reported in tilapia (*Oreochromis niloticus*) [4,5], channel catfish (*Ictalurus punctatus*) [6,7], eel (*Anguilla japonica*) [8], seabass (*Lates calcarifer*) [9], Japanese flounder (*Paralichthys olivaceus*) [7] and turbot (*Scophthalmus maximus*) [10]. A number of virulence factors have been described to-date in various *E. tarda* pathogenic strains including flagellar genes [11], lysozyme inhibitor [12], type III secretion system (T3SS) [13] and NanA sialidase [14].

Sialidases (Neuraminadases; EC 3.2.1.18) are key enzymes in the removal of sialic acid in glycoconjugates [15]. They cleave N-acetylneuraminic acid from carbohydrate chains of glycolipids, glycoproteins and other glycoconjugates [16,17]. Sialidase are widely distributed in nature, from mammals to micro-organisms including bacteria. In bacteria, endogenously expressed sialidases have been shown to enhance their virulence advantages by promoting biofilm formation [18], inducing chemokine release from epithelial cells [19] and promoting bacterial adhesion to sialic acid and/or glycoconjugate epitopes present at the plasma membrane of host cells [20,21]. In general, sialic acids are present at the reducing ends of sugar chains, where they cap glycoconjugates at the cell surface and the modulation of their content has been implicated in different bacterial binding abilities during infection [22]. Furthermore, bacterial binding affinity to the host cells is also dependent on the structure of glycoconjugates [23], which in-turn determines the content and ease with which sialidases liberate sialic acid. For instance, different bacterial sialidase have been reported to show varying sialic acid cleavage rate depending on linkage pattern [24,25]. Therefore, glycoconjugates desialylation by sialidases seemed plausible as one of the critical factors in bacterial infection. Direct evidence of the involvement of glycoconjugates and desialylation during pathogenic bacterial infection common to fish is limited. Available results suggest that some pathogenic bacteria in fish target and bind to glycoconjugates during infection. For instance, Vibrio has been shown to adhere to gangliosides GM3 and GM4 in intestinal tract of fish [26,27].

E. tarda has received significant attention recently and a great deal of vaccines candidates has been developed. Unfortunately, the bacterium still poses significant threat to fish production due to the ineffectiveness of these vaccines and of other remedial mechanisms. Recent research advances have shown that an endogenous *E. tarda* sialidase NanA is involved in its ability to infect and colonize fish tissues after a mutant of NanA showed a reduction in colonization and infectivity in Japanese flounder [14], although the actual mechanism still remains elusive. According to previous studies in other types of bacteria [28,18], we hypothesize that *E. tarda* NanA is required to liberate sialic acid thereby exposing the binding epitopes on the host cell surface, crucial for *E. tarda* recognition and adhesion. More recently varying sialidase activities and biochemical properties have been observed in bacterial sialidases according to sialic acid linkage type in glycoconjugates residues [29]. Indeed, different cell types possess different sugar linkage types depending on their glycoconjugates compositions. In *E. tarda* data ascertaining glycoconjugate family targeted by sialidase NanA has been lacking. Furthermore, the molecular basis of sialidase NanA involvement in pathogenicity has not been characterized in details. Therefore, it is crucial to further explore sialidase NanA properties, nature of preferred glycoconjugates substrates and linkage patterns. To better define the importance of *E. tarda* NanA, we prepared recombinant NanA and analyzed its activity towards various substrates. Furthermore, we demonstrated the infection ability of *E. tarda* in human and fish cell lines after exposure to sialidase NanA and after inhibition of endogenous NanA.

2. Materials and methods

2.1. Bacteria strain and culture conditions

The *E. tarda* used in this study was type FPC498 strain, isolated from the ascites of Japanese flounder (*P. olivaceus*) in Nagasaki, Japan [30]. The bacterial strain was anaerobically grown on Tryptic Soy Agar (TSA, Nissui Pharmaceutical Co., Japan) and maintained at 28 °C.

2.2. Cell culture

Goldfish scale fibroblast GAKS cells and Human cervical carcinoma HeLa cells were obtained from RIKEN CELLBank (Japan). The cells were cultured in Dulbecco modified eagle medium (DMEM) containing 10% (v/v) fetal bovine serum at 37 °C in a 5% CO_2 incubator.

2.3. Cloning of E. tarda sialidase nanA

Genomic DNA from *E. tarda* was extracted from the cell suspension by boiling the sample for 3 min, followed by centrifugation at 12,000 × g. The supernatant was used as template for PCR reaction. For amplification of full length *nanA* open reading frame (ORF), PCR was carried out using sequence specific primers as follows; *nanA*-5′HindIII (5′-GTAAGCTTCCACCATGCTGATTTTTGCCGA-3′) (forward) and *nanA*-3′BamHI (5′-GTGGATCCCTAAAAGGTG-TAGGTGAAGCTG-3′) (reverse). These primers were designed according to *nanA* nucleotide sequence of TX01 strain (accession No: JX122859). The ORF of *nanA* were amplified using KOD-Plus-Neo DNA polymerase (TOYOBO, Japan) with the following conditions; an initial denaturation of 94 °C for 2 min followed by 30 cycles of two-step cycle denaturation at 94 °C for 10 s, annealing and extension for 2 min at 68 °C. The PCR products were digested with appropriate restriction enzymes, cloned into pBluescript SK (+) vector (TAKARA) and sequenced using ABI 3130xl Genetic Analyzer (Applied Biosystems).

2.4. Preparation and expression of recombinant NanA polypeptide

For the expression of recombinant NanA protein (rNanA), plasmid construct for the glutathione-S-transferase (GST)-fused NanA was prepared. DNA fragments coding for full length of *nanA* ORF were constructed by PCR using pBluescript subcloned transcripts by the following primers; 5′-GTGGATCCCTGATTTTTGCC-GAACACGATC-3′ (forward) and 5′-GTCTCGAGCTAAAAGGTGTAG GTGAAGCTG-3′ (reverse) under the following PCR conditions; an initial denaturation at 98 °C for 2 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 15 s, and extension at 68 °C for 5 min using KOD-Plus-Neo DNA polymerase. The PCR products were sequenced and subsequently sub-cloned into the BamHI - XhoI sites of the pGEX-6P vector (GE Healthcare). GST fusion proteins were expressed in *Escherichia coli* BL21 (Nippon Gene, Japan). Briefly, transformed competent cells were cultured at 23 °C until mid-log phase (OD_{600} = 0.55), at which isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.1 mM. After induction, the bacteria were cultured for an additional 3 h after which the bacteria were harvested by centrifugation at 1,450 × g for 10 min and pellets were suspended in sonication buffer (1% Triton X-100/PBS). The suspension was sonicated for 30 s and centrifuged at 20,630 × g for 10 min at 4 °C. The supernatant (soluble fraction) and pellet (insoluble fraction) were resolved on 10% SDS-PAGE. Gel was stained using coomassie brilliant blue (CBB) to confirm the expression and solubilization of rNanA. To solubilize the rNanA