

Identification of Oligosaccharides in Human Milk Bound onto the Toxin A Carbohydrate Binding Site of *Clostridium difficile*^S

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The oligosaccharides in human milk constitute a major innate immunological mechanism by which breastfed infants gain protection against infectious diarrhea. *Clostridium difficile* is the most important cause of nosocomial diarrhea, and the C-terminus of toxin A with its carbohydrate binding site, TcdA-f2, demonstrates specific abolishment of cytotoxicity and receptor binding activity upon diethylpyrocarbonate modification of the histidine residues in TcdA. TcdA-f2 was cloned and expressed in *E. coli* BL21 (DE3). A human milk oligosaccharide (HMO) mixture displayed binding with TcdA-f2 at 38.2 respond units (RU) at the concentration of 20 µg/ml, whereas the eight purified HMOs showed binding with the carbohydrate binding site of TcdA-f2 at 3.3 to 14 RU depending on their structures via a surface plasma resonance biosensor. Among them, Lacto-N-fucopentaose V (LNFPV) and Lacto-N-neohexaose (LNnH) demonstrated tight binding to TcdA-f2 with docking energy of −9.48 kcal/mol and −12.81 kcal/mol, respectively. It displayed numerous hydrogen bonding and hydrophobic interactions with amino acid residues of TcdA-f2.

Keywords: *Clostridium difficile*, human milk oligosaccharides, molecular docking, surface plasmon resonance, toxin A

Introduction

Diarrhea disease is the second leading cause of death in children under five years of age. There are nearly 1.7 billion cases, responsible for the deaths of around 760 thousand children every year [2]. Breast feeding is the major strategy for the prevention of morbidity and mortality resulting from diarrhea in the first few years after birth. Studies have shown that human milk glycans, which include oligosaccharides in their free and conjugated forms, are part of a natural immunological mechanism that accounts for the protection against diarrheal disease observed in breastfed infants [21]. The third most abundant class of chemical components in human milk, after lactose and lipids, is human milk oligosaccharides (HMOs), present at

about 20–24 g/l in colostrum and 7–14 g/l in mature milk [6, 26, 33]. HMOs are structurally diverse, unconjugated glycans, which are non-nutritive and non-digestible [21]. Currently, through microfluidic Chip-TOF mass spectrometry, 200 HMOs have been detected [26]. HMOs play a key role in creating and maintaining a healthy infant gut microbiota through two established mechanisms: prebiotic effects (promoting growth of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*) [7, 18] and compete binding to the carbohydrate recognition domains of pathogen-generated proteins (such as surface lectins and toxins) [12]. HMOs, which are structurally similar to some intestinal mucosal cell surface glycans [16], could act as decoys and disrupt the binding of microbial lectins to host cell receptors, thus preventing infection of the host by these organisms [3, 14,

17, 22, 31].

Clostridium difficile, the bacterium responsible for antibiotic-associated diarrhea and colitis [19], is a gram-positive, spore-forming, strict anaerobe, responsible for a suite of human pathological conditions collectively referred to as *C. difficile*-associated disease (CDAD) [1]. It is estimated that the rates of CDAD have at least doubled for both adults [37] and children [9] in the last decade. Among 178 fecal samples taken from children aged 2 months to 2 years, *C. difficile* was found in 68.6% [36]. *C. difficile* produces intestinal inflammation and diarrhea through the action of two protein exotoxins, toxin A (TcdA) and toxin B [19]. TcdA, like other large clostridial toxins, is a high-molecular-weight single-subunit polypeptide consisting of at least four functional domains: an amino-terminal glucosyltransferase, followed by an autocatalytic cysteine protease domain, a hydrophobic membrane spanning sequence, and a highly repetitive carboxyl-terminal host cell binding domain (CBD) [8, 10]. The role of the CBD is to anchor the toxin to its host cell receptors on intestinal epithelial cells, which initiates the internalization process that ultimately delivers the amino-terminal enzymatic domain to the cytoplasmic compartment of the target cells [9, 14]. The C-terminus of toxin A consists of 255 amino acid residues, TcdA-f2, and has carbohydrate binding function: diethylpyrocarbonate modification of the histidine residues in TcdA was reported to specifically abolish the cytotoxicity and receptor binding activity [14, 25].

In this study, the binding effects of a mixture of HMOs and of eight purified HMO compounds on the carbohydrate binding domain (TcdA-f2) of toxin A from *C. difficile* were examined. Surface plasmon resonance (SPR) was used for analysis of the binding interactions.

Materials and Methods

Preparation of Recombinant Toxin A (TcdA-f2)

The TcdA-f2 gene [13] was synthesized (Bioneer, Daejeon, Korea) and cloned into the pET28a (Novagen, Darmstadt, Germany) vector (pET28a-TcdA-f2). The process of cloning and expression of pET28a-TcdA-f2 was carried out according to the methods in a previous report [32]. Competent *E. coli* BL21 (De3) (Carlsbad, CA, USA) cells transformed with pET28a-TcdA-f2 were grown in 1 L of L-Broth (LB) containing 30 µg kanamycin/ml at 37°C until the OD₆₀₀ reached 0.5. IPTG was then added to the final concentration of 1 mM, after which the cells were grown at 25°C for 20 h. The cells were harvested by centrifugation (8,000 ×g for 30 min at 4°C) and resuspended in 50 ml of lysis buffer (pH 7.5, 50 mM Tris). The suspension was then sonicated on ice to lyse the cells and centrifuged (12,000 ×g for 30 min at 4°C). After

centrifugation (12,000 ×g for 30 min), the clarified cell lysate was loaded onto 10 ml of Ni-Sepharose resin (GE Healthcare, Buckinghamshire, UK). Protein was eluted from the column with 20 mM Tris (pH 7.5), 0.3 M imidazole, and 0.2 M NaCl. Fractions (1 ml each) were collected, and 20 µl of each fraction was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 12% gels with molecular size markers (Bio-Rad, Hercules, CA, USA). The fractions containing the active enzyme were pooled and dialyzed against PBS buffer. The protein concentration was determined using the Bradford assay, with bovine serum albumin as the standard. The purified enzyme was stored at –80°C until further study.

Surface Plasmon Resonance

The human milk oligosaccharides in Table S1 were purchased from Elicityl with 95% purity by NMR and HPAEC (Crolles, France). The mixture of HMOs was prepared as follows. First, human milk samples were received from Chonnam National University Hospital (Gwangju, Korea: Approved by Chonnam National University Hospital Institutional Review Board, August 1, 2012; CNUH-2012-126). The milk oligosaccharides were isolated as described previously by Chaturvedi *et al.* [5]. Briefly, the milk samples were thawed immediately before use and centrifuged at 5,000 ×g for 30 min at 4°C. The solidified layer of fats and lipids was then removed from the lower, aqueous layer, and then the proteins and a portion of lactose were precipitated overnight at 4°C after the addition of ethanol to a final concentration of 66.7%. The precipitate was removed by centrifugation at 5,000 ×g for 30 min at 4°C, and the ethanol was removed by an evaporator. Finally, the clear supernatant containing the mixture of HMOs was lyophilized and stored at –20°C until further study.

SPR buffers and regeneration solutions were purchased from GE Healthcare. Sensor chips were purchased from Reichert Technologies (Depew, NY, USA). The interactions between TcdA-f2 and the HMO compounds were analyzed by SPR using the Reichert SR7500DC system (Reichert Technologies; Depew, NY, USA). The Reichert Autolink SR software (Reichert Technologies; Depew, NY, USA) was used for data collection. TcdA-f2 was directly immobilized on a CMDH chip (#13206066) using 1 mg/ml TcdA-f2 in 10 mM PBS. HMOs were dissolved in PBS buffer at 10% (w/v) or 10 mM as a stock. The mixture of HMOs was injected at concentrations from 0.02% (w/v) to 0.15% (w/v), and each pure HMO was injected at 1 mM onto the surface of the chip. Response units were recorded in real time before and during small-molecule injection (1 min) and washout (2 min). Recorded responses that included disturbances produced by air bubbles, aggregation, or precipitation of the analytes were discarded.

Molecular Docking Study of HMOs with TcdA-f2

The Autodock 4.0 docking software, using the Lamarckian genetic algorithm [20], was used for the automated molecular docking analysis between TcdA-f2 and LNFPV, LNnH 2-FL, and LNnDFH-II. The TcdA-f2 crystal structure (PDB code 2G7C

bound to α -Gal-(1,3)- β -Gal-(1,4)- β -GlcNAcO(CH₂)₈CO₂CH₃ (CD-grease) at 2.0 Å resolution) [13] was prepared for docking and post-docking refinement. The three-dimensional atomic coordinates of the HMOs were generated by the Corina program (Molecular Networks GmbH, Erlangen, Germany). Gasteiger charges were then added, and nonpolar hydrogen atoms were merged. For the docking experiment between HMOs and TcdA-f2, all water molecules and the inhibitor CD-grease located in the active site of 2G7C were removed, and only the structure information containing the amino acid residues of the TcdA-f2 enzyme was utilized. The redocking experiments between TcdA-f2 and its ligand (CD-grease) in two binding sites were performed and then the molecular docking between HMOs and TcdA-f2 was examined as described previously [23–25]. The hydrogen interaction between HMOs and the TcdA-f2 active-site pocket was visualized by Ligplot software [35].

Results

Preparation of Active Recombinant TcdA-f2

The C-terminus of TcdA (TcdA-f2) is composed of 255 residues (amino acid residues 2456–2710; GenBank Accession No. M30307), and binds to a synthetic derivative of a natural carbohydrate receptor, α -Gal-(1,3)- β -Gal-(1,4)- β -GlcNAcO(CH₂)₈CO₂CH₃ (CD-grease). The TcdA-f2 gene (0.78 kb) was synthesized using a custom synthesis service (Bioneer, Daejeon, Korea) and inserted into the pET28a vector between the EcoRI and XhoI sites. The recombinant protein was then expressed in *E. coli* BL21 (DE3) via induction with 1.0 mM IPTG at 25°C for 20 h, after which

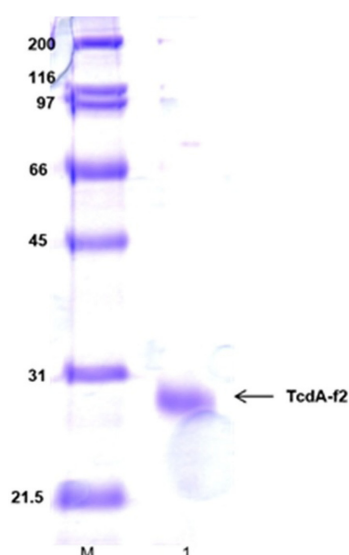


Fig. 1. Electrophoresis of the purified TcdA-f2.

Lane M, the molecular mass markers. Lane 1, the purified TcdA-f2 obtained after expression in *E. coli* BL21 (DE3) and purification via Ni-NTA column chromatography.

the active purified protein was obtained in a single Ni-Sepharose chromatographic step. SDS-PAGE analyses showed a protein band for a subunit of approximately 29.4 kDa (Fig. 1). The purified enzyme was immobilized on a carboxymethyl dextran sensor chip using the standard amine coupling methods. The ligand was bound at the density of about 2,560 resonance units (RUs) on the sensor surface, where 1 RU = 1 pg/mm².

TcdA-f2 and HMO Interaction

A mixture of the HMOs extracted from human milk, after removal of the lactose and lipids, was first investigated for binding to TcdA-f2 by the surface plasmon resonance method. Different concentrations of the mixture of HMOs (0.02% (w/v) to 0.15% (w/v)) were examined (Fig. 2), while the concentration of 20 µg/ml displayed TcdA-f2 binding. Eight purified HMO oligosaccharides, for which the chemical structures and properties are shown in Table S1, respectively, were further investigated via SPR analysis for binding with TcdA-f2. The binding study throughout of SPR response unit is shown in Fig. 3. They displayed weak and different binding with the carbohydrate binding site of TcdA-f2 with response unit from 3.0–14.0 RUs (Fig. 3).

Molecular Modeling Studies for Inhibitory Mechanism

The TcdA-f2 crystal structure (2G7C) contains two carbohydrate binding sites (BS1A and BS1B) and α -Gal-

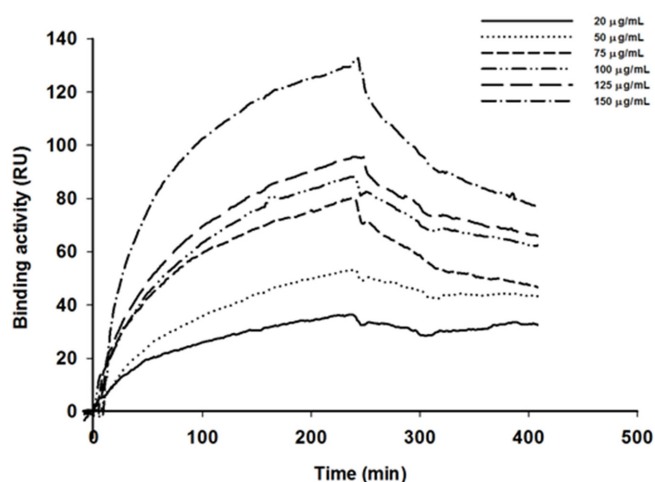


Fig. 2. Binding of a mixture of HMOs extracted from human milk, after removal of lipid, protein, and lactose, to TcdA-f2 as analyzed with SPR.

A CMDH chip was coupled with TcdA-f2 to 2560 response units. The flow cell was perfused with increasing concentrations (20–150 µg/ml) of HMOs. Results from a representative experiment are shown (*n* = 2). RU, response units.

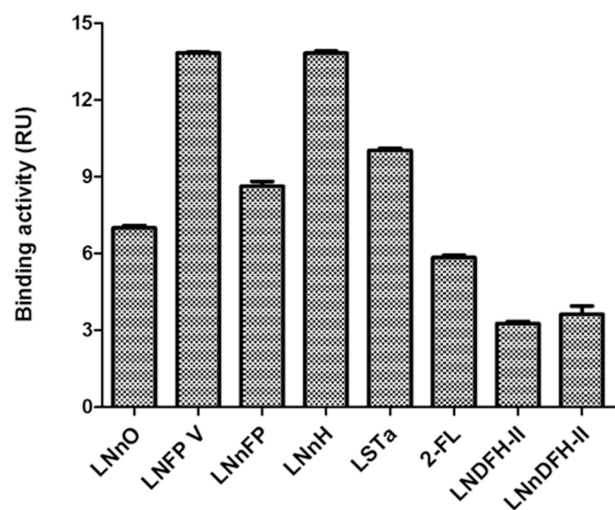


Fig. 3. SPR response units corresponding to the binding activity of the purified HMO compounds and TcdA-f2.

The errors bars represent standard deviation.

(1,3)- β -Gal-(1,4)- β -GlcNAc bound similarly to these binding sites [13]. The redocking experiments between α -Gal-(1,3)- β -Gal-(1,4)- β -GlcNAc and both carbohydrate binding sites were performed and showed similar docking energy (-6.73 kcal/mol). Thus, carbohydrate binding site 1 (BS1A) of 2G7C was selected to perform molecular docking simulations with the purified HMOs to understand the inhibitor binding mode with TcdA-f2. Among eight purified HMOs bound onto the carbohydrate binding site of TcdA-f2, LNFPV, LNnH, 2-FL, and LNnDFH-II were selected for docking experiments and the docking energy values were -9.48 kcal/mol -12.81 kcal/mol, -7.53 kcal/mol, and -7.51 kcal/mol, respectively. The chemical structure and binding of LNFPV and LNnH onto the carbohydrate binding site of TcdA-f2 are shown in Figs. 4A and 4B. LNFPV and LNnH showed binding onto the carbohydrate binding site of TcdA-f2 by numerous hydrogen bonding interactions (H-bonds) and hydrophobic interactions with various amino acid residues. Fig. 4C shows details of the specific interactions between LNFPV and TcdA-f2. LNFPV displayed numerous hydrophobic interactions in the carbohydrate binding site of TcdA-f2 with Lys154, Asn155, Ile192, and Arg193. The LNFPV formed five H-bonds with residues in the carbohydrate binding site of TcdA-f2 (Fig. 4C). The N of the amino group of Asn 153 underwent H-bonds with the O⁷ atom of 1-OH of the galactose group of LNFPV at a distance of 2.73 Å. The O²⁷ atom of 26-OH of the N-acetyl glucosamine group of LNFPV had H-bonds with the N atom of the side-chain amino group of Lys213 at a distance of 2.62 Å. Arg193 had three H-bonds with

LNFPV: one was between O⁴⁷ of C44-OH of the fucose group and the N atom of the main-chain amino group with distance of 2.45 Å and the others were two H-bonds between O⁴⁶ of C45-OH of the fucose group with two N atoms of the side-chain guanidine group with distance of 2.85 Å and 3.31 Å, respectively.

LNnH displayed numerous hydrophobic interactions in the carbohydrate binding site of TcdA-f2 with Ile150, Asn153, Lys154, Ile192, Arg193, and Ser212. The LNnH formed seven H-bonds interaction with carbohydrate binding site of TcdA-f2 (Fig. 4C). The N atom of the main-chain amino group of Asn153 formed two H-bonds with O⁹ and O¹¹ of 1-OH and 10-OH of the galactose group of LNnH at distance 3.11 Å and 3.20 Å, respectively. The N atom of the main-chain amino group of Asn155 underwent H-bond with O⁶⁹ of 68-OH of the N-acetyl glucosamine group of LNnH. The O⁷⁵ and O⁵³ atoms of 23-OH and 52-OH of the galactose of LNnH received H-bonds with the N atom of the side-chain amino group of Lys213 and Lys154 at 2.66 Å and 2.82 Å, respectively. The Arg193 had two H-bonds with LNnH: one was between the N atom of the guanidine side-chain group and O⁶⁶ of 58-OH of the glucose group at 3.13 Å and another one was between the N atom of the main-chain amino group and O⁴² atom of 41-OH of the second N-acetyl glucosamine group at 2.72 Å of LNnH.

Discussion

C. difficile is the bacterium responsible for antibiotic-associated diarrhea and colitis [19]. The intestines of newborn infants are sterile, but they come to contain flora similar to that of an adult by 12 months of age [15]. Among 178 fecal samples taken from children aged 2 months to 2 years, *C. difficile* was found in 68.6% [36]. Although the linear B type 2 trisaccharide α -Gal-(1,3)- β -Gal-(1,4)- β -GlcNAc binds specifically to TcdA [13], the binding of this trisaccharide to the TcdA-f2 fragment was weak. HMOs, which vary from 3 to 32 sugars in size, are composed of available combinations of five monosaccharides, including glucose, galactose, fucose, N-acetylglucosamine, and N-acetylneuraminic acid, which exhibit great structural diversity and serve several important biological functions to promote infant health. Although HMO was studied for over 60 years and 200 structures have been characterized to date, the exact quantitation of individual HMOs in milk or colostrum was not reported because of variations, such as milk collection depended on time, methods, and lacking suitable analytical and separation methods [34]. Expression of individual milk components differs among humans, and

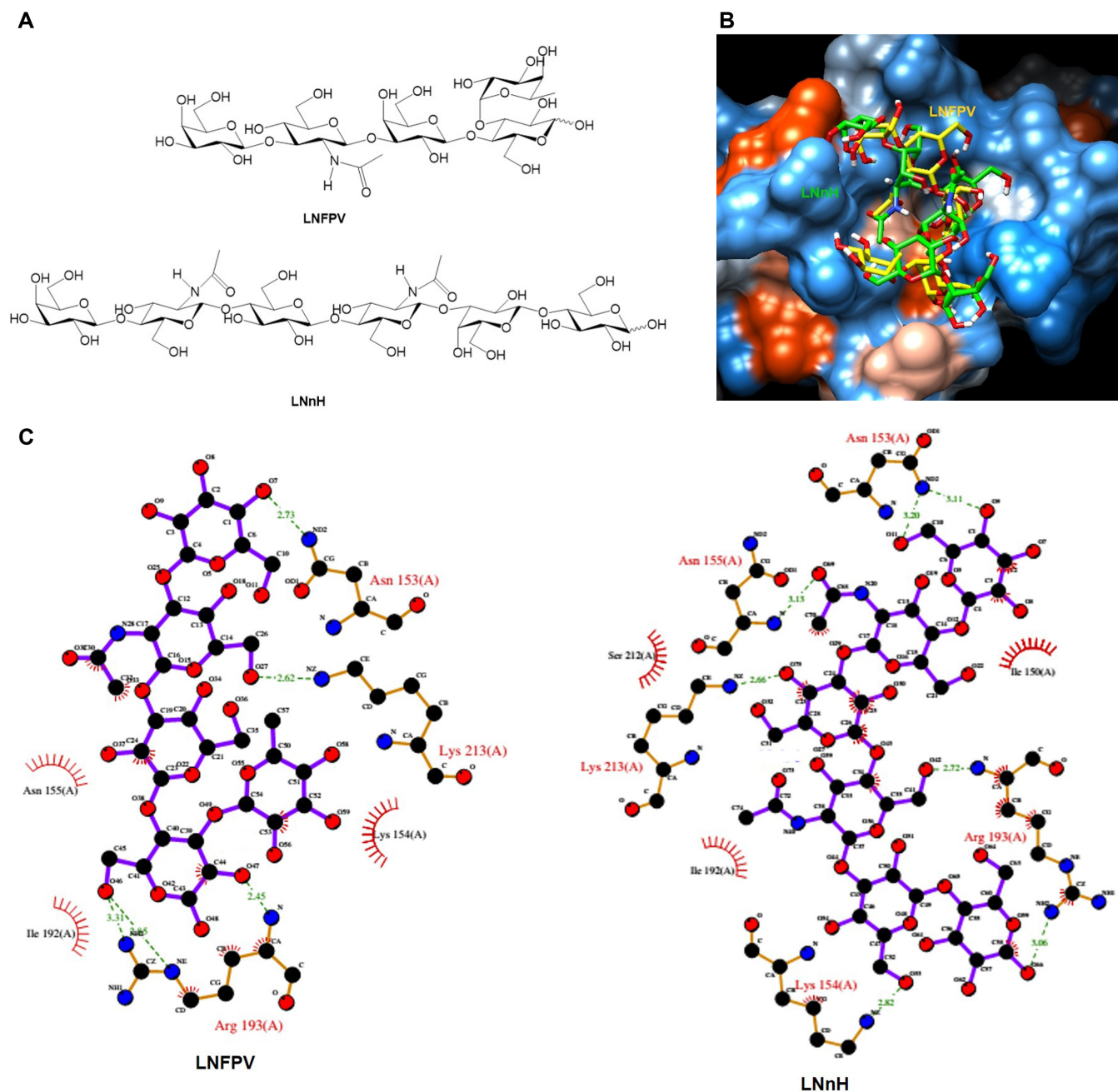


Fig. 4. Computational docking and hydrophobic and hydrogen bond interactions of Lacto-N-fucopentaose V (LNFVPV) and Lacto-N-neohexaose (LNnH) with amino acid residues in the carbohydrate binding site of TcdA-f2, determined with the TcdA-f2 crystal structure (PDB code 2G7C).

(A) Chemical structure of LNFVPV and LNnH. (B) Comparison of binding modes of LNFVPV (yellow) and LNnH (green) in the carbohydrate binding site of TcdA-f2. (C) Hydrophobic and H-bond interactions between LNFVPV and LNnH and amino acid residues in the carbohydrate binding site of TcdA-f2. H-bond interactions are represented by green dashed lines (red, oxygen; cornflower blue, nitrogen; black, carbon).

human milk varies with stage of lactation, diet, and other biological parameters. Published data on the total HMOs as well as the concentration of each purified HMO in the total HMOs vary because there are no routine methods available

for the qualification of HMO [34]. Until now, only 11 oligosaccharides from neutral HMO and 8 oligosaccharides from acidic HMO in total 200 oligosaccharides from HMO were identified for their concentrations, but the concentration

of each oligosaccharide was varied depending on different studies [26, 34] (Table S2). Thus, the mixture of HMOs from human milk was bound to TcdA-f2 at 20 µg/ml and it showed increased binding to TcdA-f2 when the mixture of HMOs concentration was increased. HMOs are present at about 20–24 g/l in colostrum and 7–14 g/l in mature milk [6, 26, 33]. The inhibition of carbohydrate binding on TcdA by its repeat domain is essential for obstructing TcdA binding to the receptor [29]. By binding to the carbohydrate binding site of TcdA-f2, the HMO mixture seemed to prevent the interactions with TcdA of *C. difficile* cellular receptor. Based on the results of mixture binding, eight purified HMO oligosaccharides that represent the main carbohydrate antigens of human milk, such as core structures (LNnO, LNnH), monofucosylated HMOs (LNFPV, LNnFP, 2-FL), difucosylated HMOs (LNDFH-II, LNnDFH-II), and sialylated HMO (LSTa) were further investigated via SPR analysis for binding with TcdA-f2. The eight compounds displayed different binding with TcdA-f2 (Fig. 3) as follows: LNFPV > LNnH > LSTa > LNnFP > LNnO > 2FL > LNnDFH-II > LNDFH-II. These differences in binding could reflect a different binding surface density or the preservation of the terminal glucose ring structure [30]. TcdA-F2 bound strongly to Lacto-N-fucopentaose (LNFP V) (Fig. 3) and it also bound to fucosyloligosaccharides (LNnFP, LNDFH-II, LNnDFH-II), demonstrating an affinity to GlcNAcβ-3Galβ-4(Fuca-3)Glc. Two compounds (LNDFH-II, LNnDFH-II) belonging to difucosylated HMOs displayed the weakest binding to TcdA-f2 and this result agreed with a previous report by El-Hawiet *et al.* [11] that increased complexity of the HMOs does not significantly increase in affinity. For more detailed binding information of the two strongest binding compounds (LNFPV and LNnH) to TcdA-f2, molecular docking was performed. Based on molecular docking, two compounds, LNFPV (docking energy: -9.48 kcal/mol) and LNnH (docking energy: -12.81 kcal/mol), showed stronger binding onto TcdA-f2 than that of α-Gal-(1,3)-β-Gal-(1,4)-β-GlcNAc, a control with docking energy of -6.73 kcal/mol.

Previous studies and our study showed that the individual purified HMO showed weak binding to TcdA-f2 [11, 12]; however, we found that the binding of the HMO mixture (20 µg/ml containing many different kinds of HMO) to TcdA-f2 was more efficient than that of single purified HMO (1 mM of purified HMO containing over 0.5 mg/ml) possibly due to synergistic action of the individual compounds [4, 27].

The mixture of HMOs and the eight purified HMOs tested demonstrated binding onto the carbohydrate binding site

of TcdA-f2 expressed in *E. coli* BL21 (De3), as revealed by SPR methods and molecular docking study. The Lacto-N-fucopentaose V and Lacto-N-neohexaose (LNFPV and LNnH) compounds demonstrated significantly higher binding to TcdA-f2 throughout with numerous hydrophobic and H-bond interactions with the amino acid residues in the carbohydrate binding site of TcdA-f2. The HMO mixture and purified HMO seemed to prevent the interactions with TcdA of the *C. difficile* cellular receptor.

Sample Availability

Samples of the purified HMO compounds are available from Elicityl (Crolles, France), and mixtures of HMOs are available from the authors.

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