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Enzymatic production of HMO mimics by the sialylation of galacto-oligosaccharides

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ABSTRACT

Human milk oligosaccharides (HMOs) are a family of structurally diverse unconjugated glycans that exhibit a wide range of biological activities. In this report, we describe an efficient, Multi-Enzyme One-Pot strategy to produce HMO mimics by the sialylation of galacto-oligosaccharides (GOSs), which are often added to infant formula as an inexpensive alternative to HMOs. In this system, the sialyltransferase donor, cytidine-5′-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac), was generated *in situ* using a CMP-sialic acid synthetase. The sialylated GOSs were obtained by one-step purification after digesting CMP using the alkaline phosphatase PhoA to cytidine and inorganic phosphate. Although the synthesized α2,3-, α2,6- and α2,3/8-sialyl-GOSs exhibit different sialylation levels and patterns, all of these mixtures can be fermented by *Bifidobacterium longum* subsp. *infantis* ATCC 15697 but not by *Bifidobacterium adolescentis* ATCC 15703. The sialidase NanH2, which is unique to the former strain, hydrolyzed all of the synthesized HMO mimics.

1. Introduction

Human milk contains more than one hundred structurally distinct oligosaccharides (human milk oligosaccharides, HMOs), in addition to glycoproteins, glycopeptides and glycolipids. HMOs are unique to human milk in terms of their high abundance and structural diversity and are believed to have a wide range of biological activities beyond providing nutrition to the infant.

HMOs comprise neutral and acidic species constructed from 5 monosaccharides: glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid (Sia), with N-acetylneuraminic acid (Neu5Ac) as the predominant, if not only, form of Sia. HMOs are built on a lactose core at the reducing end, which can be elongated by N-acetyllactosamine units. The lactose or polylactosamine backbone is further fucosylated in α1,2-, α1,3- or α1,4-linkages and/or is sialylated in α2,3- or α2,6-linkages with greater structural diversity produced. HMOs have primarily been recognized for their prebiotic effects. However, since the early 1990s, accumulating evidence has suggested that HMOs protect the breastfed infant against infections and diarrhea by serving as antiadhesive antimicrobials. Many viral, bacterial and protozoan pathogens use lectin-glycan interactions to initiate infection, and HMOs have been shown to block this type of attachment. For example, the Sia components of HMOs can prevent or at least reduce the *in vitro* adhesion of pathogenic bacteria, such as *Escherichia coli* with S-fimbriae, *Salmonella*, *Vibrio cholerae*, *Helicobacter pylori*, *Campylobacter jejuni*, and viruses. (Coppa et al., 2006; Kunz, Rudloff, Baier, Klein, & Strobel, 2000; Ten-Bruggencate, Bovee-Oudenhoven, Feitsma, van-Hoffen, & Schoterman, 2014; Varki, 2008; Yu et al., 2012). In addition, sialyllactose prevents cholera toxin binding (Idota, Kawakami, Murakami, & Sugawara, 1995).

HMOs also function as immune modulators that protect breastfed infants from excessive immune responses. Direct immunological effects of sialylated HMOs have been observed in a recent study (Eiwegger et al., 2010). In addition, new *in vitro* data suggest that HMOs might exhibit glycome-modifying effects. In 2005, Angeloni et al. for the first time demonstrated that the surface expression of α2,3- and α2,6-linked Sia residues on Caco-2 cells is significantly reduced on exposure to 3′-sialyllactose; this change caused a 90% reduction in the adhesion of enteropathogenic *E. coli* (EPEC) compared to control cells (Angeloni et al., 2005).

Although HMOs have potential applications in infants and adults alike, this potential is limited by the difficulty of manufacturing HMOs. No natural sources other than human milk, including

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the milk of farm animals, provide sufficient quantities of the structurally complex HMOs. As an alternative and in an attempt to mimic the prebiotic effects of HMOs, two major classes of non-HMOs, namely galacto-oligosaccharides (GOSs) and fructo-oligosaccharides (FOSs), are currently added to infant formula. GOSs are Gal oligomers with a degree of polymerization (DP) between 3 and 10 and different glycosidic linkages which lead to a variety of different structural isomers (Yanahira et al., 1995). GOSs are synthesized from lactose by a transgalactosylation reaction that is catalyzed by β-galactosidases from bacterium or yeast (Macfarlane, Steed, & Macfarlane, 2008; Park & Oh, 2010). Although both GOSs and HMOs have a lactose core, GOSs are neither sialylated nor fucosylated. However, the carboxyl group of Sia in acidic oligosaccharides (such as sialyllactose) introduces a negative charge that is crucially important for some of the benefits of HMOs.

More recently, several enzymatic approaches have been developed to synthesize sialylated GOSs that are more HMO-like. For example, trans-sialidases from various *Trypanosoma* species were used to produce α2,3-sialylated GOSs (Jers et al., 2014; Sallomons et al., 2013). PmST, a sialyltransferase (SiaT) that is derived from *Pasteurella multocida* subsp. multocida str. Pm70, transfers a Sia moiety from sialoside to GOSs (Guo et al., 2014). However, according to previously reported kinetic data, the trans-sialylation reaction catalyzed by trans-sialidase is less efficient than sialylation catalyzed by sialyltransferase (Table S2). In the PmST-catalyzed trans-sialylation reaction for 6′-sialyllactose production, a mixture of 3′-sialyllactose and 6′-sialyllactose is generated in the early stage, whereas the 3′-sialyllactose can be partially converted to 6′-sialyllactose by prolonging the reaction time.

In this study, we enzymatically and efficiently produced various sialylated products from GOSs using various SiaTs. The sialylation patterns and levels of these HMO mimics were systematically assessed and evaluated. The fermentation of α2,3-, α2,6- and α2,3/8-sialyl-GOSs by various *Bifidobacteria* was also investigated. The specificity of NanH2 (a sialidase that is recruited by *Bifidobacterium longum* subsp. *infantis* ATCC 15697 to metabolize HMOs (Sela et al., 2011)) toward synthesized HMO mimics was also examined *in vitro*.

2. Materials and methods

2.1. Materials

Bifidobacterium adolescentis ATCC 15703 and *B. longum* subsp. *infantis* ATCC 15697 were obtained from the American Type Culture Collection. The Histrap FFTM column was purchased from Qiagen (Valencia, CA). The BCA protein assay kit and 96-well culture plates were purchased from Pierce Biotechnology. Neu5Ac and CTP were purchased from Sigma–Aldrich. Pure GOSs ($_p$GOSs) in powder form (GOS-1000-P, purity > 99%) was kindly provided by New Francisco Biotechnology Corporation (NFBC, Guangdong Province, China).

2.2. Expression and purification

nmCSS, *pmST1*, *pd2,6ST*, *cstII* and *pmPpA* were kind gifts from Dr. Chen (University of California-Davis). All enzymes, including CMP-sialic acid synthetase from *Neisseria meningitidis* group B strain MC58 (NmCSS), α2,3-SiaT from *P. multocida* strain Pm70 (PmST1), α2,6-SiaT from *Photobacterium damsela* ATCC 33539 (Pd2,6ST), α2,3/8-SiaT from *C. jejuni* strain OH4384 (CstII) and inorganic pyrophosphatase from *P. multocida* strain P-1059 (PmPpA), were expressed and purified as previously reported (Cheng et al., 2008, 2010; Lau et al., 2010; Muthana et al., 2012; Yu et al., 2005). The full-length codon-optimized (for the *E. coli* expression system) *nanH2* gene was synthesized by GENEWIZ (Suzhou, China) and was inserted in the pET22b(+) vector. *phoA* was cloned from *E. coli* K12 using the primer pair pF1 (5′-AAATCATATGAAACAAA GCACTATTGCACTGGCA) and pR1 (5′-GCGCTCGAGTTTCAGCCCC AGAGCGGCTTTCATG) and inserted into the pET22b(+) vector. The plasmids containing the target genes were then transformed into *E. coli* BL21(DE3) chemically competent cells. The plasmid-bearing strains were cultured in Luria Bertani broth (LB) medium containing ampicillin (100 μg mL^{-1}).

Overexpression of the target proteins was achieved by inducing the *E. coli* culture with 0.1 mM of IPTG at 16 °C for 24 h (180 rpm) when the OD (600 nm) of the culture reached 0.8 with vigorous shaking at 37 °C (250 rpm). The cells were then harvested by centrifugation at 8000 rpm for 10 min, resuspended in lysis buffer (100 mM Tris–HCl pH 8.0 containing 0.1% Triton X-100), and lysed by sonication. After centrifugation (12,000 rpm for 20 min) of the cell lysate, the supernatant was applied onto a Ni^{2+}-NTA affinity column that had been pre-equilibrated with 5 column volumes of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 8.0). Followed by washing with 8 column volumes of washing buffer (40 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 8.0), the target proteins were eluted with elution buffer (200 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 8.0).

2.3. General procedures for the Multi-Enzyme One-Pot sialylation of $_p$GOSs

Enzymatic sialylation of $_p$GOSs was performed in a Multi-Enzyme One-Pot (MEOP) system containing PmPpA, NmCSS and one of the three SiaTs (PmST1; Pd2,6ST; or CstII) with Neu5Ac, CTP and $_p$GOSs (approximately 9.1 mg/mL) as the starting materials (Fig. 1). To optimize the efficiency of the sialylation reaction, various ratios of Neu5Ac and CTP versus $_p$GOSs were tested. In this study, the average molecular weight of $_p$GOSs was estimated to be 479.2 Dalton after the components of DP2 up 6 were separated on a Thin-Layer Chromatography (TLC) plate, which was developed with EtOAc/MeOH/H_2O/HOAc = 4:2:1:0.2 (by volume) and stained with ρ-anisaldehyde sugar stain, and were analyzed by the software Quantity One. The reaction was carried out in 100 mM Tris–HCl buffer (pH 8.0) and incubated at 37 °C for 2 h. In small-scale reactions, 8 μg PmPpA, 14.6 μg NmCSS and either 17.2 μg PmST1; 27.2 μg Pd2,6ST; or 61.6 μg CstII were used with precursor of Neu5Ac and CTP (1, 2.5 or 5 equivalents) and 10 mg $_p$GOSs (approximate 20 mM) as the starting materials in a total volume of 1.1 mL. In large-scale reactions, tenfold enzymes were used with precursor of Neu5Ac and CTP (2.5 equivalents in PmST1 and Pd2,6ST catalyzed reactions, and 5 equivalents in CstII catalyzed reaction) and 100 mg $_p$GOSs (approximate 20 mM) as the starting materials in a total volume of 11 mL. Product formation was monitored using TLC (EtOAc/MeOH/H_2O/HOAc = 4:2:1:0.2). When two of the most abundant components, di- and tri-$_p$GOSs, had reacted completely, the reaction was first heated at 100 °C for 5 min to inactivate the enzymes and then treated with alkaline phosphatase

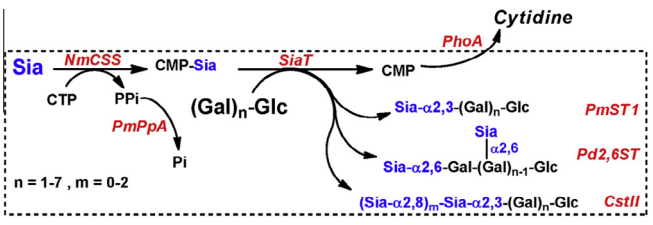

Fig. 1. Schematic representation of sialylation reactions catalyzed by various SiaTs.

PhoA (440.6 μg) to hydrolyze the CMP byproduct and residual CTP and CMP-Neu5Ac. The reaction was monitored using an ion-pair reverse-phase HPLC system equipped with an SPD-20A detector (Shimadzu) and a C18 column (250 × 4.6 mm I.D., 5 μm particle size, Phenomenex). The products were eluted using a 15–90% linear gradient of solvents A (10 mM phosphate buffer pH 7.0 containing 2.5 mM tetrabutylammonium bromide (TEABr) as the ion-pair reagent) and B (100% acetonitrile). The reaction mixture was then boiled again and centrifuged to remove PhoA. The supernatant was concentrated, passed through a filtration column (100 cm × 2.5 cm) packed with BioGel P-2 gel (Bio-Rad), and eluted with water to obtain the sialoside mixture. The final product was lyophilized as a white powder.

2.4. Mass spectrometry

The samples were analyzed using MALDI-TOF mass spectrometry (Bruker, model Ultraflex III TOFTOF) with 2,5-dihydroxybenzoic acid as matrix, and the positive reflectron mode was used.

2.5. Growth assays of HMO mimics

The cultures were aerobically propagated at 37 °C in a semisynthetic medium (Barrangou, Altermann, Hutkins, Cano, & Klaenhammer, 2003). $_p$GOSs, α2,3-sialylated $_p$GOSs, α2,6-sialylated $_p$GOSs or α2,3/8-sialylated $_p$GOSs were added as carbohydrate sources. All media were supplemented with 0.2% (wt/vol) L-cysteine. Cell growth was monitored by recording the OD at 600 nm using a Thermo Varioskan Flash spectral scanning multimode reader. The cultures were grown in triplicate, and representative data are presented.

2.6. Sialidase assays

Sialidase assays were buffered with 50 mM MES (pH 5.5) and performed at 37 °C in a total volume of 100 μL containing 32 μg NanH2 and 0.8 mg purified α2,3-, α2,6- or α2,3/8-sialyl-$_p$GOSs.

3. Results and discussion

A characteristic of GOSs is that the set of structures present depends on the enzyme used for synthesis. For example, β-galactosidase from *Bacillus circulans* primarily catalyzes the formation of β1,4-linkages, and to a lesser extent β1,6-, β1,3- and β1,2-linkages (Rodriguez-Colinas, Poveda, Jimenez-Barbero, Ballesteros, & Plou, 2012; Van-Leeuwen, Kuipers, Dijkhuizen, & Kamerling, 2014; Yanahira et al., 1995), whereas *Kluyveromyces lactis* β-galactosidase predominantly produces β1,6-linkages (Rodriguez-Colinas, Fernandez-Arrojo, Ballesteros, & Plou, 2014; Rodriguez-Colinas et al., 2011). In this study, the GOSs ($_p$GOSs) used for further sialylation was produced by a *B. circulans* β-galactosidase. To determine the DP of $_p$GOSs, the sample was diluted and analyzed using MALDI-TOF mass spectrometry. The resulting spectrum contained signals at *m*/*z* 365.1, 527.2, 689.2, 851.3, 1013.3, 1175.4 and 1337.5, indicating that $_p$GOSs contain oligosaccharides with DPs ranging from 2 to 8.

Compared to neutral GOSs, the carboxyl-group of Sia introduces a negative charge that is crucial for some HMO effects. We herein employed three SiaTs to produce HMO mimics by sialylation of $_p$-GOSs. Although only 10–20% of the HMOs in mature human milk are sialylated, sialyl-oligosaccharides are much more abundant in colostrum, and overall decreases of nearly 60% and 80% were observed in mature milk over the following one and three months, respectively. The reason for this change is unknown (Wang, Brand-Miller, McVeagh, & Petocz, 2001). Therefore, the Sia component might play a more important role in the health of neonates than previously thought.

In addition to α2,3- and α2,6-sialosides that are rich in HMOs, α2,8-sialosides, such as ganglioside GD3, were also detected (Lee, An, Lerno, German, & Lebrilla, 2011; Newburg, 1996). GD3 was recently found to inhibit the adherence of *H. pylori* to MKN-45 cells to a greater extent than the gangliosides GM1a and GM3 (Lee et al., 2011). Therefore, various SiaTs, including PmST1 (α2,3-SiaT); Pd2,6ST (α2,6-SiaT); and CstII (a bifunctional SiaT that transfers Sia to the 3-hydroxyl group of Gal and to the 8-hydroxyl of a terminal Sia (Li, Zhang, Zuccolo, Zheng, & Ling, 2011; Yu et al., 2009)) were used to evaluate the sialylation of $_p$GOSs in a recently developed MEOP system (Fig. 1). To optimize the sialylation reaction, 1:1, 2.5:1 and 5:1 molar ratios of Neu5Ac and CTP precursors:$_p$GOSs were tested in small-scale reactions. Affording excess Neu5Ac and CTP can push the reaction forward and facilitate the formation of di- or multisialylated products when more than one terminal Gal moiety is available. As expected based on the mass spectral data (Fig. S-1), only monosialylated $_p$GOSs were observed in the α2,3-SiaT (PmST1)-, α2,6-SiaT (Pd2,6ST)- or α2,3/8-SiaT (CstII)-catalyzed reactions with 1 equivalent of Neu5Ac and CTP afforded. Unreacted $_p$GOSs were observed in all three reactions. Based on TLC and MS analyses (data not shown), the sialylation efficiency and patterns for the PmST1- and Pd2,6ST-catalyzed reactions did not obviously differ when either 2.5 or 5 equivalents of Neu5Ac and CTP were added. Unexpectedly, in the CstII-catalyzed reaction, a Sia dimer (probably with an α2,8-linkage) was observed, even in the trials with only 1 equivalent of Neu5Ac and CTP afforded. Therefore, 2.5 equivalents of Neu5Ac and CTP were used in the preparative syntheses of sialylated $_p$GOSs that were catalyzed by PmST1 and Pd2,6ST; however, 5 equivalents of these precursors were used in the CstII-catalyzed reaction because some of the precursors will be hijacked to produce the Sia dimer side-product. All reactions were carried out using 100 mg $_p$GOSs as the starting material.

The reaction included NmCSS, which converts Neu5Ac and CTP to CMP-Neu5Ac *in situ*, and one of three SiaTs; an inorganic pyrophosphatase (PmPpA) was also added to degrade the pyrophosphate (PPi) byproduct, thus driving the reaction toward the formation of CMP-Neu5Ac. Large amounts of the byproduct CMP were formed during the sialylation reaction; to facilitate purification, this CMP and residual CTP and possibly CMP-Neu5Ac were hydrolyzed using the alkaline phosphatase PhoA (Fig. S-3). The Neu5Ac and resulting cytidine impurities were easily removed by passing the reaction mixture through a BioGel P-2 gel filtration column. After purification, 142.1, 143.8 and 237.2 mg of α2,3-, α2,6- and α2,3/8-sialylated $_p$GOSs were obtained, respectively. The sialylated products were characterized by ^{1}H nuclear magnetic resonance (NMR) and MALDI-TOF mass spectrometry (Figs. S-4 and 2). As shown in Fig. S-2A, the R_f values of the purified sialylation products indicated that different sialylation levels might have been achieved.

Compared to PmST1 and Pd2,6ST, CstII, which is bifunctional and capable of transferring more than one Sia residue to the initially formed α2,3-linked Sia in an α2,8-linkage, produced the most heavily sialylated products. The DP2 components of GOSs (di-$_p$-GOSs) appeared to be sialylated as monomeric products by PmST1 and Pd2,6ST; however, most of these products were disialylated by CstII into analogs of GD3. Trisaccharides of $_p$GOSs (tri-$_p$GOSs) were introduced one or two Sia residues by PmST1 and Pd2,6ST and up to three Sia residues by CstII, probably due to the presence of both the linear and branched forms. The Gal moieties within the branched tri-$_p$GOSs could be easily accessible and represent good targets for various SiaTs. Tetra- and penta-$_p$GOSs, which have more complex structures (Van-Leeuwen et al., 2014), are suitable sub-

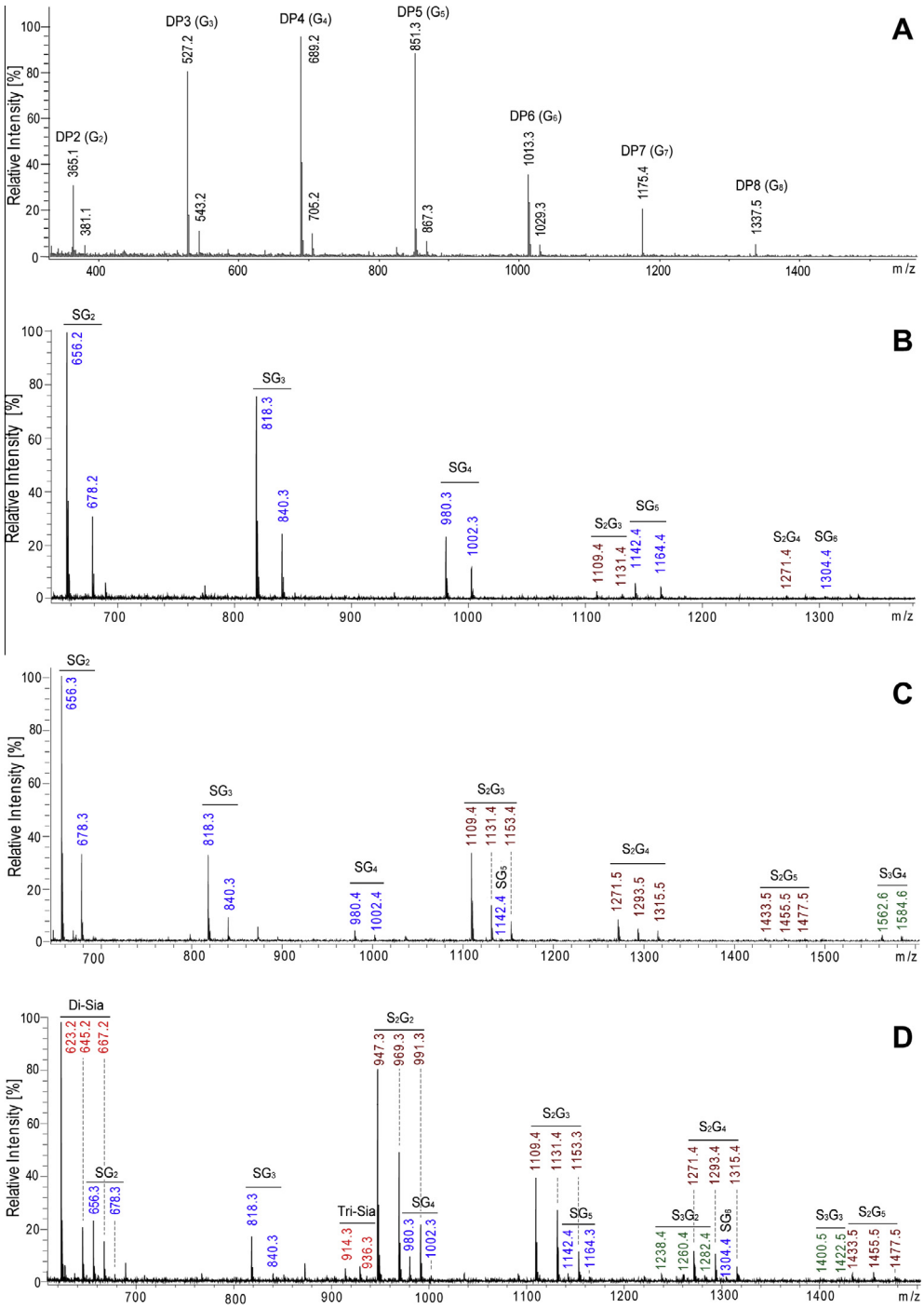

Fig. 2. MALDI-TOF spectra of $_{p}$GOSs (A) and various sialylated $_{p}$GOSs formed by PmST1 (B), Pd2,6ST (C) and CstII (D). Mono-(SG_n), di-(S_2G_n) and trisialylated (S_3G_n) products are highlighted in blue, dark red and green, respectively. The $_{p}$GOSs were detected as both sodiated and potassiated forms. The monosialyl-$_{p}$GOSs appeared as both the $[M+Na]^+$ and $[M-H+2Na]^+$ cationized species. The di- and trisialyl-$_{p}$GOSs appeared as multiple cationized species including $[M+Na]^+$, ($[M-H+2Na]^+$) and ($[M-2H+3Na]^+$) forms.

strates for all three enzymes and were sialylated to monomeric and dimeric products (Table S1 and Fig. 2B–D). Unlike Pd2,6ST and CstII, PmST1 only produced trace amounts of disialyl-$_{p}$GOSs and no multisialyl-$_{p}$GOSs (Fig. 2B). If the minor dimeric products are neglected, the yield of α2,3-sialyl-$_{p}$GOSs is approximately 86.4%.

Of these HMO mimics, α2,6-sialyl-$_{p}$GOSs with long chain might be capable to bind to influenza A virus hemagglutinin. Previously, an integrated biochemical, analytical and data mining approach demonstrated that human-adapted H1N1 and H3N2 viruses specifically bind to long α2,6-sialylated glycans with an umbrella-like topology that is unique to Sias with an α2,6-linkage and chains of sufficient length (Chandrasekaran et al., 2008).

In addition to the sialylation products of $_{p}$GOSs, Sia-Sia and very small amounts of the tri-Sia oligomer were found in the CstII-catalyzed product (Fig. 2D). Therefore, oligo-Sia structures could be synthesized by CstII, either bound to $_{p}$GOSs via an α2,3-linkage or in a free form (Fig. 2D). Poly-Sia structures with DPs ranging from 2 to 18 have been detected in glycoproteins from human

milk. One of these glycoproteins was further characterized as CD36, which contains di-Sia (Neu5Acα2,8Neu5Acα2,3Gal) and poly-Sia (exhibiting a DP of 11 or greater). In contrast, platelet CD36 was not polysialylated, suggesting that the multiple Sia residues in CD36 are specific for CD36 in milk and may be a natural trap for various pathogenic viruses and bacteria to prevent neonate infection (Yabe, Sato, Matsuda, & Kitajima, 2003).

According the relative abundance determined using mass spectrometry, more of the dimeric sialylation product of tri-$_p$GOSs was formed in the reaction catalyzed by Pd2,6ST than in the reaction catalyzed by PmST1. Although Sia is typically found on the exposed termini of various glycoconjugates, Pd2,6ST was recently found to be able to introduce Sia to both the terminal and internal Gal residues of galacto-N-biose and poly-LacNAc extended glycans (Meng et al., 2014; Nycholat et al., 2013). Thus, linear tri-$_p$GOSs, which account for more than 59.2% of all trisaccharides and contain the Galβ1,4-Gal motif (Yanahira et al., 1995), might be sialylated to dimeric products. To examine this possibility, the tri-$_p$GOSs were isolated by gel filtration chromatography on BioGel P-2.

The efficiencies of tri-$_p$GOSs sialylation by PmST1 and Pd2,6ST were compared under two conditions (tri-$_p$GOSs, Neu5Ac and CTP were added at either a 1:1:1 ratio or a 1:10:10 ratio). The sialylation of tri-$_p$GOSs using only Pd2,6ST and 10 equivalents of excess CTP and Neu5Ac yielded products that primarily contained two Sia residues, suggesting that Pd2,6ST, but not PmST1, can react with the internal Gal of the Galβ1,4-Gal motif (Figs. 3 and S-5). Therefore, observing trisialylated tetra-$_p$GOSs in Pd2,6ST-catalyzed sialyl-$_p$GOSs can be explained (Fig. 2C).

Although the consumption of α2,3-sialylated GOSs by several probiotic strains has been reported (Jers et al., 2014), the prebiotic effect of α2,6- and α2,8-sialylated GOSs has never been tested. Herein, *B. longum* subsp. *infantis* strain ATCC15697 (the archetypical HMO-utilizing bacterium) and *B. adolescentis* ATCC 15703 (which consumes GOSs, but not HMOs) were used to test the prebiotic effect of our synthesized sialyl-$_p$GOSs. As expected, the *B. longum* subsp. *infantis* strain ATCC15697 was able to grow on $_p$GOSs and all three sialylated products of $_p$GOSs, although lower cell density was obtained when CstII-catalyzed sialyl-$_p$GOSs (which contain more Sia components than other sialyl-$_p$GOSs) was used as the sole carbon source. However, *B. adolescentis* ATCC 15703 metabolized $_p$GOSs but none of the sialylated $_p$GOSs (Fig. 4).

More recently, *B. longum* subsp. *infantis* ATCC 15697 was demonstrated to recruit the sialidase NanH2 to cleave α2,3- and α2,6-linked Sia residues. During HMO fermentation, *nanH2* was upregulated approximately 4-fold relative to its expression level when lactose was used as the sole fermentation substrate. NanH2 is active *in vitro* on several HMO substrates, such as SLNT and DSLNT (Sela et al., 2011). We herein investigated whether the structurally complex products made by the various SiaTs can be hydrolyzed by NanH2. Interestingly, both TLC (Fig. S-2B) and mass spectrometry (Fig. S-6) analyses demonstrated that NanH2 is active toward all synthesized sialyl-$_p$GOSs as well as oligo-Sias, which are exclusively present in the CstII-catalyzed product, independent of the linkages (α2,3-, α2,6- or α2,8-) of the sialyl moieties present. Thus, the substrate specificity of NanH2 is very flexible. It is worth mentioning that studying glycoside hydrolases from *bifidobacteria* is not only important for understanding the molecular mechanisms of prebiotics utilization, but also from a perspective of discovering novel selective prebiotics. In earlier studies, a β-fructofuranosidase recruited by *B. longum* KN29.1 to degradate fructo-oligosaccharides has been aerobically expressed in *E. coli* BL21 (DE3) and demonstrated to have identical physical and catalytic properties to the native enzyme (Jedrzejczak-Krzepkowska, Tkaczuk, & Bielecki, 2011). Another recombinant β-fructofuranosidase from *B. adolescentis* G1 shows similar substrate specificity and kinetic parameters to its native counterpart despite the slightly difference of pH-stability profile (Omori et al., 2010). Recently, a panel of glycosyl hydrolases including an α-sialidase, two α-fucosidases, two β-galactosidases and three β-N-acetylglucosaminidases from the *B. longum* subsp. *infantis* ATCC 15697 were heterologously expressed in *E. coli*. Functional studies on the enzymic properties of these recombinant hydrolases have greatly advanced our

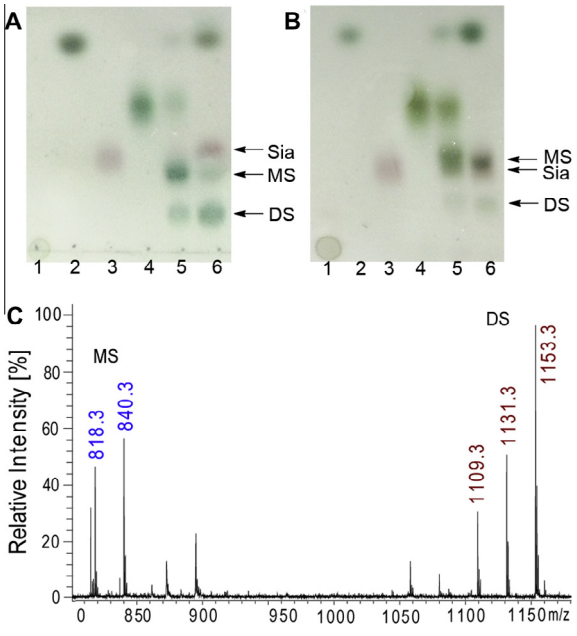

Fig. 3. TLC and mass spectrometry analyses of tri-$_p$GOSs sialylation reactions catalyzed by Pd2,6ST (A and C) and PmST1 (B). MS, the monosialylated product; DS, the disialylated product. Lane 1, CTP; Lane 2, cytidine; Lane 3, Neu5Ac; Lane 4, tri-$_p$GOSs; Lanes 5 and 6, reactions in which tri-$_p$GOSs, Neu5Ac and CTP were afforded at 1:1:1 and 1:10:10 ratios, respectively. The reaction mixtures were applied to TLC plates after treatment with PhoA.

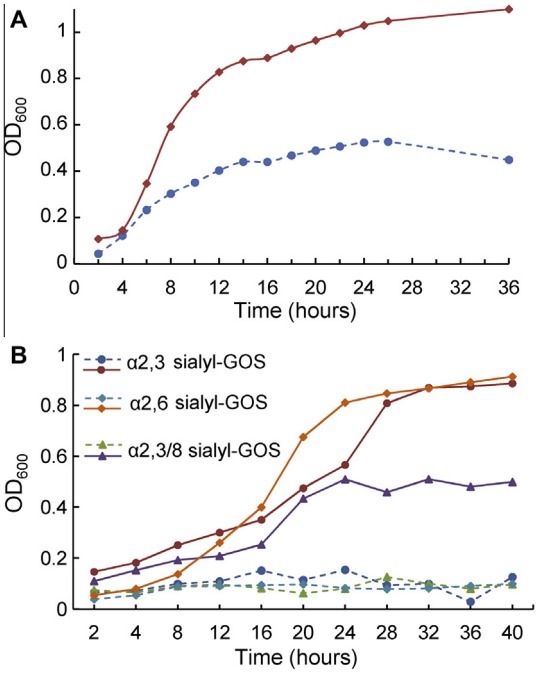

Fig. 4. Growth curves of *B. longum* subsp. *infantis* ATCC 15697 (solid line) and *B. adolescentis* ATCC 15703 (dot line) on modified semisynthetic medium containing 2% (wt/vol) $_p$GOSs (A) and synthesized sialyl-$_p$GOSs (B). Growth studies were independently carried out in triplicate, and a representative data set is shown.

understanding of HMO consumption by *B. infantis* (Garrido, Dallas, & Mills, 2013).

Because Sia has important biological functions, sialylated oligosaccharides might be useful for the fortification of infant formula. Moreover, the advantages presented by HMOs are likely related to the structural and functional diversity of multiple components that act synergistically. In this study, we successfully produced α2,3-, α2,6- and α2,3/8-sialyl-$_p$GOSs with even more structurally complex than $_p$GOSs using three SiaTs in a MEOP system. With the availability of large amounts of these compounds, studies on their biological activities, as well as supplementation of infant formula, might become feasible. Meanwhile, this study expands our knowledge regarding the substrate specificities of various SiaTs, including PmST1, Pd2,6ST and CstII, as well as the sialidase NanH2 recruited by *B. longum* subsp. *infantis* ATCC 15697 to ferment HMOs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.02.064.

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