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Targeted Fucosylation of Glycans with Engineered Bacterial Fucosyltransferase Variants

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Fucosyltransferases (FucTs) are crucial for the synthesis of Lewis-type glycan epitopes. The synthetic capacity of efficient bacterial enzymes and their variants has not yet been fully exploited. In the present work, we investigated two previously described variants of α1,3FucT from *Helicobacter pylori* strains for their flexibility in substrate utilization and their applicability in the enzymatic synthesis of Lewis epitopes. We used the truncated enzyme variant of FutA from *H. pylori* 26695 (FucTΔ52A128N/H129E/Y132I/S46F, FucTΔ52-4M) and the truncated α3FucT from *H. pylori* NCTC11639 (FucTΔ66). *N*-Acetyllactosamine type 1 and type 2 as well as *N′,N″*-diacetyllactosamine were investigated as substrates. Both FucT variants exhibit α1,3/4FucT activity. Novel glycan structures were obtained displaying Lewis blood group antigens in a site-specific sequence. Fucosylated *N′,N″*-diacetyllactosamine was synthesized for the first time with FucTΔ52-4M. Our work paves the way for targeted fucosylation patterns that can be tested for lectin binding.

Introduction

Fucosylated oligosaccharides play an important role in immunity. As a part of human milk oligosaccharides (HMOs), they support the development of the infant's immune system; as Lewis type and ABO blood group antigens, they are involved in the recognition processes of pathogens and cancer.[1] These processes can be studied in more detail by producing the glycan structures involved. For a large-scale enzymatic synthesis, fucosyltransferases (FucTs) are crucial. FucTs from glycosyltransferase (GT) families 10 and 11 from different microbial organisms have already been described in literature for the production of HMOs and Lewis blood group epitopes.[2] Bacterial α2FucTs (GT 11) attach fucose to terminal *N*-acetyllactosamine (LN) structures, such as type 1 (Galβ1,3GlcNAc, LN1) and type 2 (Galβ1.4GlcNAc, LN2),[3] producing H-LN1 and H-LN2 antigens. Other FucTs are more complex in terms of their regioselectivity. For the synthesis of the Lewis epitopes from LN1 and LN2, α3/4FucTs and α3FucTs are required for fucosylation at the *N*-acetylglucosamine (GlcNAc) moiety of the glycan. While α3FucTs glycosylate only lactose and LN2,[4] α3/4FucTs can also modify LN1.[5] Both FucTs are able to fucosylate internal LN2 subunits of human milk oligosaccharides and poly-LN.[4b,6] Strains of the genus *Helicobacter pylori* are known for their repertoire of different FucTs. In *H. pylori* 26695, the genes *futA* and *futB* encode for α3FucTs and the *futC* gene for an α2FucT.[7] Other *H. pylori* strains possess α3/4FucTs and exhibit both α3- and α4FucT activities.[3c] The genetic composition of these enzymes influences the fucosylation pattern of glycan structures. α3- and α3/4-FucTs comprise two to ten C-terminal heptad repeats and a basic, hydrophobic tail.[3c,8] Depending on the number of heptad repeats, the FutA and FutB monomers form homo- and heterodimers, which determine the fucosylation pattern (Galβ1,4[Fucα1,3]GlcNAc, Le^x) on a poly-LN glycan.[9] Lin and coworkers found that truncation of the heptad repeats increased the catalytic efficiency (V_{max}/K_M).[10] This is due to fact that the acceptor-binding domain and heptad repeats are located in close vicinity. Based on these results the group speculated that the length of truncation influences the fucosylation pattern and location, depending on the way the acceptor binds to the enzyme. Hence, FutB (with 10 heptad repeats) fucosylates terminal LN2, while FutA (with 2 heptad repeats) prefers internal LN2 glycan subunits of poly-LN glycans.[6,11] Lin et al. produced several truncated variants of α3FucT from *H. pylori* (NCTC 11639). FucTΔ66 comprises seven heptad repeats and shows the highest specific activity with LN2 as an acceptor substrate.[10] Recently, directed evolution of FucTΔ57 from *H. pylori* (NCTC 11639) resulted in the variant M32 with a 4.7-fold improved activity toward LN2 compared to the wild-type FutA.[12] The truncated variant FucTΔ52 of FutA from *H. pylori* 26695 contains one heptad repeat and catalyzes the formation of 3-fucosyllactose and Le^x.[13] Subsequent iterative saturation mutagenesis of FucTΔ52 gave the variant FucTΔ52A128N/H129E/Y132I/S46F (FucTΔ52-4M) with nearly 8-fold higher activity for the fucosylation of LN2 to produce Le^x.[12]

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These and other *H. pylori* FucTs were used for the synthesis of fucosylated HMOs with lactose-based oligo- and polysaccharides as substrates.[4b,14] The truncated FucTs FucTΔ66 from *H. pylori* NCTC11639[10] and FucTΔ52 from *H. pylori* 26695 were previously reported to lack α4FucT activity with LN1 as a substrate.[15]

For this study, we selected FucTΔ66 from *H. pylori* NCTC11639[10] and FucTΔ52-4M from *H. pylori* 26695.[13] These enzymes were tested with a library of LN1- and LN2-based acceptor glycans. In addition to their ability to target the fucosylation site in the glycan acceptor, we demonstrate for the first time an α1,4 activity for both FucT variants, leading to a range of Le^a/Le^b and Le^x/Le^y glycans.

Results and Discussion

Production of glycan acceptors – LN1 and LN2 substrates and their H-antigen derivatives

We synthesized a variety of LN1 and LN2 and the corresponding H-antigen glycan epitopes (Table 1) to test them as acceptor substrates for α3FucTs (FucTΔ52-4M, FucTΔ66). FucTΔ66 from *H. pylori* NCTC11639 was previously shown to synthesize a series of fucosylated HMOs containing LN2, lacto-*N*-*neo*tetraose (LN*n*T), and lacto-*N*-*neo*hexaose.[4b] LN2-LN2, LN1-LN1, and mixed-type LNs (Table 1) have never been tested yet.

The LN substrates were prepared as described in our previous studies.[16] Analytical data (HPLC and MS analyses) are shown in the Supporting information (Schemes S1–S3; Figures S1–S8). The H-antigen derivatives were produced with α2FucT (FutC) from *H. pylori*,[3b,17] which carries an N-terminal propeptide fusion to increase solubility, as previously demonstrated for the α2FucT WbgL.[3a] FutC (*H. pylori* 26695) is known to produce 2′-fucosyllactose and H-antigens, preferring LN1 type substrates,[3b,18] which is confirmed by our results with LN disaccharides and Lewis structures (Figure S9, Figure S10). Fucosylation of LN-based tetrasaccharides showed interesting results (Figure 1). While the LN1 subunit at the non-reducing end increases the activity of FutC with the glycan acceptor (LN2-LN2: 20.7% yield, LN1-LN2: 89.7%; Figure 1, entry 1 and 3), LN1 at the reducing end leads to barely detectable activity (LN2-LN1: 1.7% yield, LN1-LN1: 4.8% yield; Figure 1 entries 2 and 4). Regarding the fucosylation of HMOs in previous studies,[18–19] the fucosylation of LN2-based tetrasaccharides (LN2-LN2, LN1-LN2) is comparable.

Lacto-*N*-tetraose (LNT, LN1-Lac) is converted faster, while fucosylation of lacto-*N*-neotetraose (LN*n*T, LN2-Lac) required longer incubation times.[18,19b] The LN1-based tetrasaccharides (LN2-LN1, LN1-LN1) are not constituents of HMOs.[20] However, the oligosaccharide LN1-LN1-Lac was an excellent substrate for FutC, with H-LN1-LN1-Lac as a product.[18] We conclude that our results show an altered FutC acceptance for LN-based oligosaccharides when compared with literature data.

Table 1. Substrates for FucTΔ52-4M and FucTΔ66.[a]

Lactosaminyl substrates	H-antigen derivatives
LN2	H-LN2
LN1	H-LN1
GlcNAc-LN2	
GlcNAc-LN1	
LN2-LN2	H-LN2-LN2
LN2-LN1	H-LN2-LN1
LN1-LN2	H-LN1-LN2
LN1-LN1	H-LN1-LN1
LDN	

R = ▼ = Fuc ■ = GlcNAc ○ = Gal □ = GalNAc

[a] Structures are assigned with their glycan name. Conditions for enzyme production and glycan synthesis are listed in the Supporting information (Table S1, Table S2). Abbreviations: Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; LN, *N*-acetyllactosamine; H-LN, H-antigen derivative of *N*-acetyllactosamine; LDN, *N*′,*N*″-diacetyllactosamine; 1, type 1; 2, type 2.

Fucosylation of LN disaccharides, H-LN-antigen derivatives, LN-based trisaccharides, and LDN

FucTΔ52-4M and FucTΔ66 have been used in various studies for the production of HMOs[4b,17,21] or analyses of enzyme structure and mechanism.[8] Both enzymes are described as α3FucTs and showed no α4-fucosylation activity with LN1 acceptor substrates in previous studies.[10,13,15a] Both enzymes carry modifications that increase their solubility and activity towards lactose and LN2. Due to the C-terminal truncation, FucTΔ66 has seven heptad repeats (wildtype: ten repeats) and FucTΔ52-4M has one heptad repeat (wildtype: two repeats). Furthermore, FucTΔ52-4M carries four point mutations in the lactose-binding site. In our study, we produced both enzymes from codon-optimized genes for the fucosylation of LN structures (Supporting information). Enzymes and substrates were co-incubated for 60 min and the reactions were analyzed by HPLC. Product peaks were purified and measured by ESI-MS. Further confirmation of the structure was obtained by treating the products with a commercial α3/4-fucosidase (Table S3). Additionally, we determined the approximate specific activity of all FucTs for the corresponding substrates (Table S4).

In the first step to fucosylation of complex LN structures, LN2 and LN1 were used as substrates (Figure 2A, Figure S11). Surprisingly, both FucTs were active with LN1 as substrate and produced Le^a. While FucTΔ52-4M fucosylated both LN types in comparable amounts (LN2: 45.5%; LN1: 37.4%), FucTΔ66 had a clear preference for LN2 (LN2: 64.0%; LN1: 15.9%). Fucosidase treatment confirmed the formation of Le^x and Le^a, respectively (Figure S12). The trends of LN2 and LN1 as substrates are

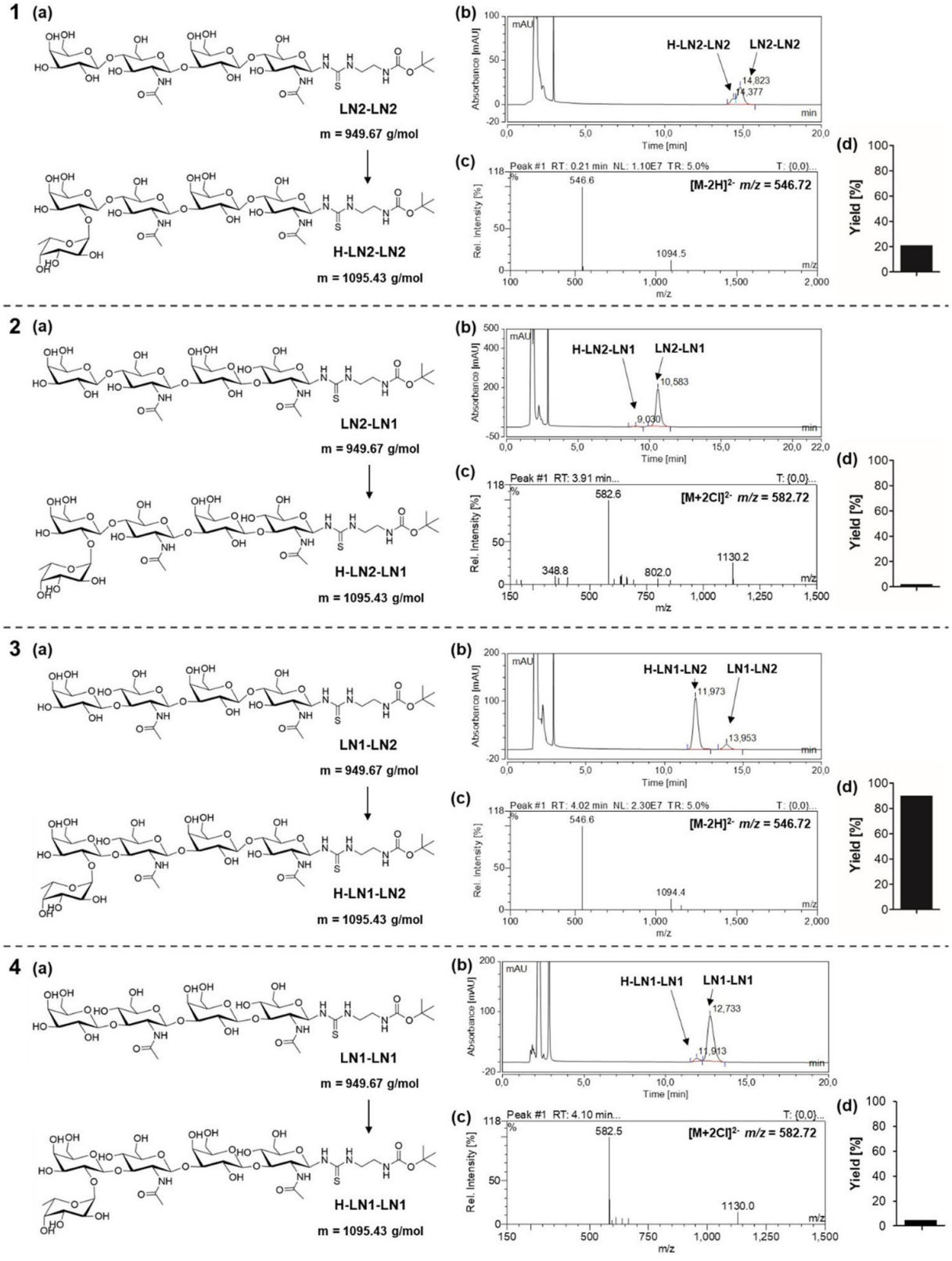

Figure 1. FutC with LN tetrasaccharides as substrates. (a) Reaction scheme with exact masses of compounds. (b) HPLC chromatogram of the reaction after 60 min. (c) MS spectrum of the reaction product. Measurements were performed in the negative mode. Expected *m/z* ratios ($[M\text{-}2H]^{2-}$, $[M+2Cl]^{2-}$ for chloride adducts) are given in the spectrum and correspond to the detected masses. 1) LN2-LN2; 2) LN2-LN1; 3) LN1-LN2; 4) LN1-LN1.

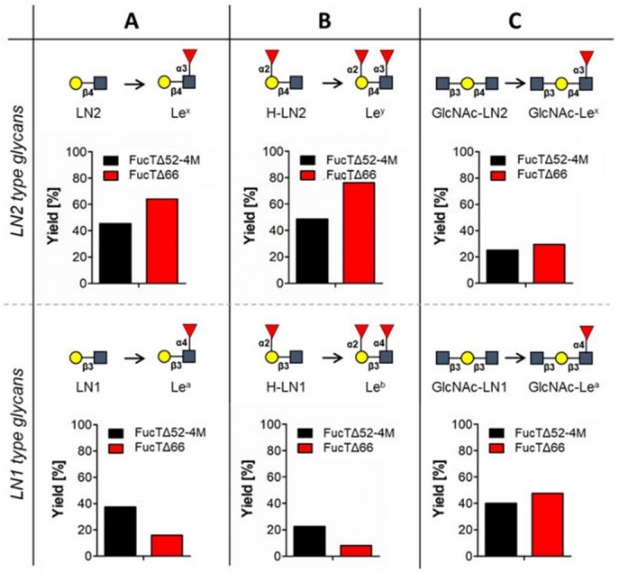

Figure 2. Yields of FucTΔ52-4M and FucTΔ66 with LN disaccharides (A), H-antigens (B), and LN-based trisaccharides (C) as substrates. Samples were incubated for 60 min and yields were detected by HPLC measurements. Products were confirmed by ESI-MS and fucosidase-catalyzed cleavage. Yields are shown in bar plots; reactions are shown above.

enhanced by terminal α2-fucosylation. Using the H-antigens as substrates higher conversion of the type 2 glycan (FucTΔ52-4M: 48.6%; FucTΔ66: 75%) and lower conversion for the type 1 glycan (FucTΔ52-4M: 25.4%; FucTΔ66: 8.2%) (Figure 2B, Figure S13) was detected and confirmed (Figure S14). The acceptance of LN1 and H-LN1 indicates α4FucT activity, which was not previously observed for any of the enzymes. Ma et al.[15a] found no conversion of LN1 with the wildtype (not truncated) form of FucTΔ66 from *H. pylori* NCTC11639. For FucTΔ66, the increased activity with the H-antigen derivative of LN2 has already been observed.[10] For the α3/4FucT from *H. pylori* strain UA948, the residues ^{345}CNDAHYSALH^{354} in the C-terminal domain are essential for α4FucT activity. Substitutions of the amino acid Y350 led to a decreased α4-activity.[15c] In the sequences of both FucTΔ66 and FucTΔ52-4M, phenylalanine substitutes the position of tyrosine (Figure 3, red box; Supporting information, amino acid sequences of FucTs). The increased α4-activity of FucTΔ52-4M (compared with FucTΔ66) may also be due to the point mutations of this FucT variant. Serine at position 46 in the N-terminal domain is exchanged for phenylalanine, as is the case with the *H. pylori* UA948 α3/4FucT (Figure 3, purple box). Serine is conserved in α3FucTs throughout various *H. pylori* strains except for the UA948 strain.[15c] A similar substitution S45F in α3FucT from *H. pylori* NCTC11639 was rationalized by docking experiments to create a new clamp-like structure with W33 and W34 in the substrate-binding pocket, improving activity towards LN2.[12] Although LN1 was not examined in this study, the observed α4FucT activity of FucTΔ52-4M may also be due to improved binding of LN1. We conclude that substitutions in the N-terminal domain and reported amino acid sequences in the C-terminal domain may extend the selectivity of FucTΔ52-4M with respect to its α3- and α4-activity. Another reason for an α3/α4 FucT activity might be the type of aglycon. While we exclusively used an N-linked thiourea linker in our acceptors, Choi et al. used no linker at the reducing end for their study with FucTΔ52-4M.[13] Huang et al. found an altered α3/α4 activity of the *B. fragilis* FucT with O- and N-linked modifications at the reducing end of the acceptor sugar.[19a]

Figure 3. Alignment of the FucTs used in this study with an α3/4FucT from *H. pylori* strain UA948. Marked amino acids might be responsible for α4FucT-activity.

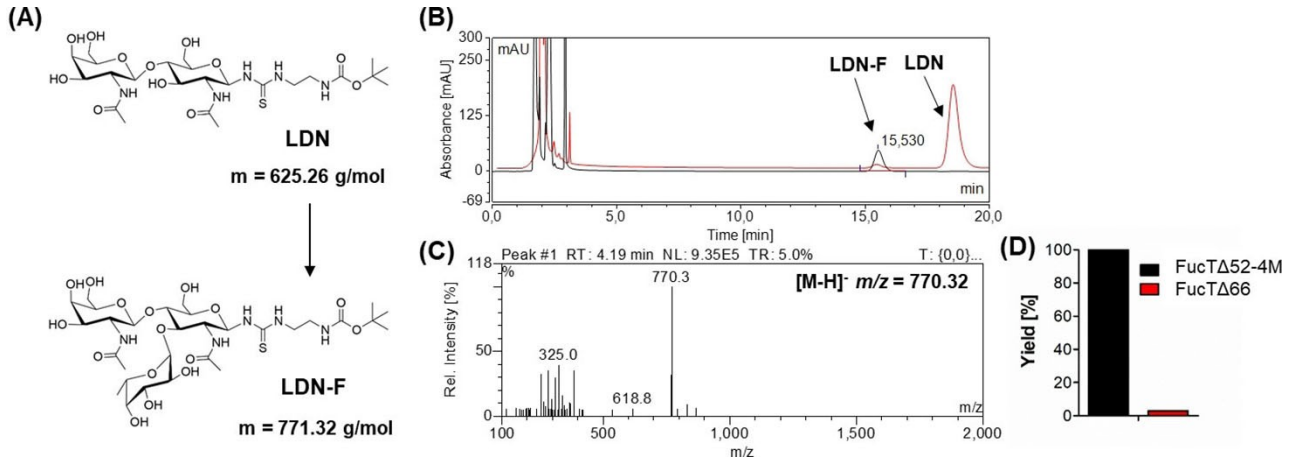

Figure 4. FucTΔ52-4M and FucTΔ66 with LDN as substrate. (A) Reaction scheme with exact masses of compounds. (B) HPLC chromatogram of the FucTΔ52 (black) and FucTΔ66 (red) reaction after 60 min. (C) MS spectrum of the reaction product. Measurements were performed in the negative mode. Expected *m/z* ratios ($[M\text{-}H]^-$) are given in the spectrum and correspond to the detected masses. (D) Relative yields after 60 min.

Bai et al.[4b] used the FucTΔ66 for fucosylation of LNT II (GlcNAcβ1,3Lac). GlcNAc-terminated saccharides are interesting substrates for targeted fucosylation of the LN subunit at the reducing end because a single terminal GlcNAc does not function as an acceptor for fucosylation. The α3/4FucT from *H. pylori* DSM 6709 can fucosylate LNT II as well as the LN-based trisaccharides (GlcNAc-LN2, GlcNAc-LN1).[5a] In the present study, both FucTs fucosylated the reducing GlcNAc of the trisaccharides (Figure 2C, Figure S15).

This was confirmed by using an α3/4-fucosidase. The fucosidase hydrolyzes only terminal fucosyl residues attached to the non-reducing end of the glycan epitope. HPLC analysis of the fucosidase-treated glycan with the untreated glycan showed no shift in the retention time of the compound (Figure S16). In contrast to the α3/4FucT from *H. pylori* DSM 6709,[5a] FucTΔ66 and FucTΔ52-4M showed a higher conversion for the glycan GlcNAcβ,3LN1. However, both enzymes showed unexpected α4FucT activity.

Fucosylated LDN glycans are found in schistosomes[22] and fucosyltransferases from *Schistosoma mansoni* were used for the synthesis of fucosylated LDN (F-LDN, LDN-F, and F-LDN-F).[22c] However, LDN was so far not investigated for fucosylation with bacterial FucTs. In our recent study, we presented fucosylated LDN as a possible scavenger for *Clostridium difficile* toxin A.[23] Hence, fucosylation of LDN is of particular interest for the production of larger amounts of fucosylated LDN glycans. Most importantly, FucTΔ52-4M converted LDN with a yield of 100% in contrast to FucTΔ66, which showed negligible conversion (Figure 4). ESI-MS analysis revealed a mono-fucosylated product (F-LDN or LDN-F) (Figure 4C). Treatment with α3/α4 fucosidase gives LDN as a product and suggests that LDN-F was formed (Figure S12). Therefore, this enzyme is suitable for the large-scale production of the LDN-F glycan epitope. The product could be used to study disease processes (e.g., bacterial infections).

Fucosylation of LN tetrasaccharides (LN-LN) and their H-antigen derivatives (H-LN-LN)

Targeted fucosylation of more complex LN oligosaccharides and HMOs is an area of research with many different approaches. Ye et al.[24] used α2,6-sialylation to 'protect' potential fucosylation sites in poly-LN glycans. Fucosylation reactions were performed with α3FucT from *H. pylori* strain NCTC11639 and *H. pylori* strain UA948 α3/4FucT. Sialic acid moieties were enzymatically removed after fucosylation. Yu and coworkers used the same enzyme to produce a library of Lewis structures and HMOs in a one-pot multienzyme approach.[5b] Tsai et al.[5a] applied different ratios of fucose acceptor and donor to achieve mono- or difucosylation with α3/4FucT from *H. pylori* DSM 6709. To investigate the structure and reaction mechanism of an exemplary FucT (*H. pylori* NCTC11639 α3FucT), Sun et al. provided a model for *H. pylori* NCTC11639 α3FucT (wildtype version of FucTΔ66) to rationalize the accommodation of poly-LN oligosaccharides and their fucosylation.[8] We investigated FucTΔ52-4M and FucTΔ66 for the targeted fucosylation of LN-based glycans.

Experiments using LN2-LN2, LN2-LN1, LN1-LN2, and LN1-LN1 (Figure 5) as substrates for FucTΔ52-4M and FucTΔ66 showed an interesting evolution of product peaks revealing different fucosylation sites.

With LN2-LN2 as substrate (Figure 5A, Figure S17), FTΔ52-4M preferentially fucosylates the LN2 subunit at the non-reducing end (Le^x-LN2, 49.2% after 30 min), but also fucosylates the LN2 subunit at the reducing end (LN2-Le^x, 19.5%). At longer incubation times, the ratio between the products shifts to the difucosylated product Le^x-Le^x (30 min: 18.1%; 60 min: 25.8%) as confirmed by NMR analysis (Table S5, Figures S32–S35).

In contrast, FucTΔ66 prefers the inner LN subunit (37.9% after 30 min for LN2-Le^x) and fucosylates the terminal LN moiety, although to a lesser extent (17.7% for Le^x-LN2). The fully fucosylated glycan is formed with a similar yield compared to FucTΔ52-4 M. In other studies,[4b,14] FucTΔ66 was found to

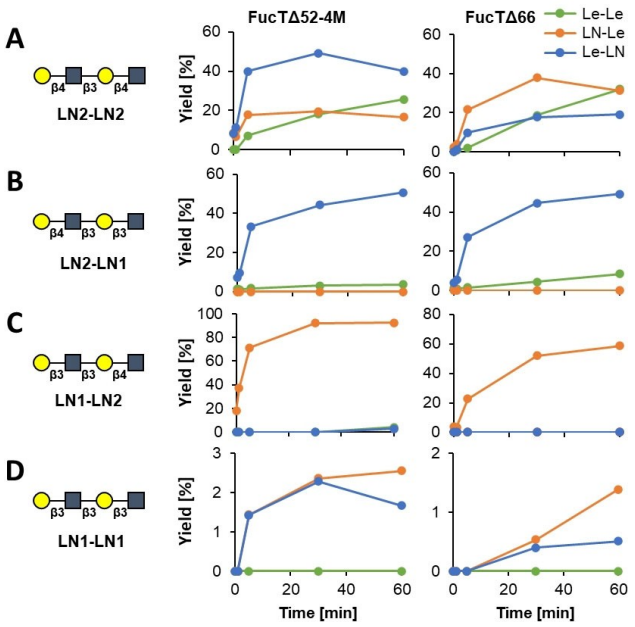

Figure 5. Time-yield curves of reactions catalyzed by FucTΔ52-4M and FucTΔ66 using LN-LN tetrasaccharides as substrates. (a) LN2-LN2-based; (b) LN2-LN1-based; (c) LN1-LN2-based; (d) LN1-LN1-based. Samples were incubated for 60 min and yields were detected by HPLC measurements. Products were confirmed *via* ESI-MS and by fucosidase-catalyzed cleavage. Yields were calculated from the combined peak areas of products and substrate (100%). Abbreviations: LN, *N*-acetyllactosamine; Le, Lewis epitope.

prefer the terminal LN subunit of LN*n*T, which is expected since LN2 is the preferred substrate for FucTΔ66 over lactose.[4b] With the introduction of an LN moiety at the reducing end, we provide a different range of fucosylated structures in terms of targeted fucosylation by FucTΔ66.

To investigate whether the presence of LN1 subunits affects the activities and fucosylation patterns of the enzymes, LN2 at the reducing end of the tetrasaccharide was replaced with an LN1 subunit (LN2-LN1, Figure 5B). Both enzymes preferentially fucosylate the external LN2 subunit of the LN2-LN1 tetrasaccharide and produce Le^x-LN1 (FucTΔ52-4M: 50.7%; FucTΔ66: 49.2%) (Figure 5B, Figure S19, Figure S20). While the double fucosylated product Le^x-Le^a was produced only at low yields (FucTΔ52-4M: 3.6%; FucTΔ66: 8.6%), LN2-Le^a was undetectable. Le^x-LN1 as the major product was confirmed by NMR spectroscopy (Table S6, Figure S36–S38).

When LN1 is at the non-reducing end and LN2 at the reducing end of the tetrasaccharide, the fucosylation pattern changes (Figure 5C, Figure S21, Figure S22). LN1-Le^x is the main product for both enzymes as found by NMR (Table S7, Figure S39–S41). The α3/4FucT from *H. pylori* DSM 6709 showed comparable behavior. Regardless of its position in the tetrasaccharide, the enzyme preferentially fucosylates LN2 and lactose subunits.[5a] When the tetrasaccharide consists of only LN1 subunits, none of the FucTs investigated in this study exhibited high product yields (Figure 5D, Figure S23). Both monofucosylated structures, Le^a-LN1 and LN1-Le^a, are produced with very low yields of up to 2.6% with FucTΔ52-4M and 1.4% with FucTΔ66.

We conclude that both FucTs prefer LN2 units for α3-fucosylation. The formation of internal Le^x can be controlled by terminal LN1 (LN1-LN2 substrate), which gives the highest yield for the synthesis of LN1-Le^x with FucTΔ52-4M. With LN2-LN1, both enzymes synthesize Le^x-LN1 with comparable yields. The α4-activity appears only at very low conversion for the LN1-LN1 substrate. However, the extent of fucosylation is tunable by the concentration of GDP-Fuc, as described in previous studies.[4b,23] When an excess of GDP-Fuc (2.4- to 4-fold compared to the acceptor) is used in the reactions, fucosylation is targeted at more than one site, with the reducing end being the least preferred fucosylation site.[4b] In this study, the acceptor:donor ratio was 1:1.3 to force targeted fucosylation.

The behavior of both enzymes changes significantly with H-antigen derivatives (Figure 6). With terminally attached α2Fuc (H-LN2-LN2), FucTΔ52-4M fucosylates only the terminal H-antigen glycan subunit (68.1% yield for Le^y-LN after 60 min), whereas FucTΔ66 produces all three types of products (Figure 6A, Figure S25, Figure S26). Le^y-LN2 is the main product after 60 min (31.6%), followed by H-LN2-Le^x (18.7%) and Le^y-Le^x (10.3%). Both Le^y-LN2 and Le^y-Le^x (excess of GDP-Fuc) were produced in our previous study with the FucTΔ52-4M and confirmed by NMR.[23]

The H-LN2-LN1 substrate leads to the epitope Le^y-LN1 with lower yields (FucTΔ52-4M: 13.4%; FucTΔ66: 13.9%; after 30 min), with any intermediates completely absent (Figure 6B,

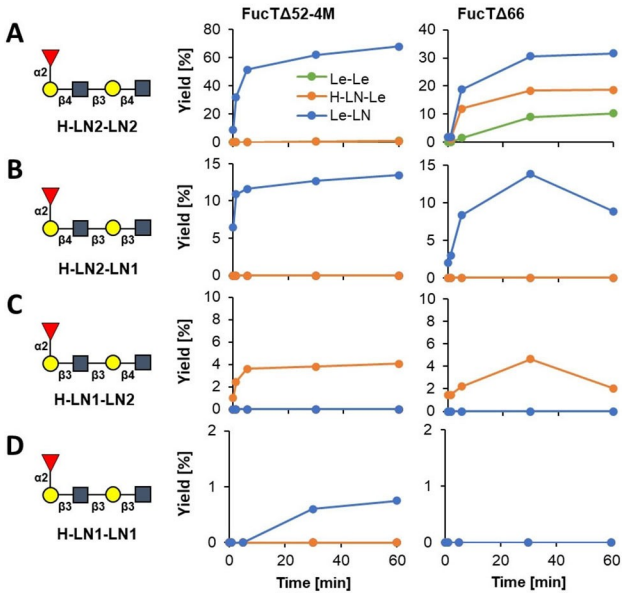

Figure 6. Time-yield curves of reactions catalyzed by FucTΔ52-4M and FucTΔ66 using the respective H-LN-LN pentasaccharides as substrates. (a) LN2-LN2-based; (b) LN2-LN1-based; (c) LN1-LN2-based; (d) LN1-LN1-based. Samples were incubated for 60 min and yields were detected *via* HPLC measurements. Products were confirmed by ESI-MS and fucosidase-catalyzed cleavage. Yields were calculated from the combined peak areas of products and substrate (100%). Abbreviations: LN, *N*-acetyllactosamine; H, H-antigen derivative; Le, Lewis epitope.

Figure S27, Figure S28). For H-LN1-LN2, the activity of both enzymes is barely detectable (Figure 6C, Figure S29, Figure S30). H-LN1-Le^x is the only product with yields of only 2% (FucTΔ66) and 4% (FucTΔ52-4M). Thus, although the fucosylation site remains the same, H-LN1-LN2 appears not to act as a substrate for either enzyme. The low activity with this oligosaccharide can be explained in two different ways. One possibility is that the preferences work against each other: Fucosylation is pulled to the subunit at the non-reducing end by the terminal α2Fuc and to the reducing end by the LN2 subunit. The other possibility is that both subunits are non-preferred substrates: Neither terminal LN1-based structures nor internal subunits are favored. As expected, the H-LN1-LN1 is not a substrate for FucTΔ66 and is poorly converted by FucTΔ52-4M (0.8% yield) (Figure 6D, Figure S31).

In summary, LN2 subunits in tetrasaccharide structures are favored by both enzymes – FucTΔ52-4M and FucTΔ66 – due to the β4-linkage between galactose and *N*-acetylglucosamine.[8] In contrast, LN1 subunits are barely converted and direct fucosylation towards LN2 subunits. The terminal α2-fucosylated LN2 is the preferred substrate for FucTΔ52-4M for efficient synthesis of Le^y-LN, whereas FucTΔ66 is less selective.

FucTΔ66 has been described as an α3FucT that is unable to fucosylate β3-galactosides.[5b,10,13,15a]

The FucTΔ52-4M variant has never been studied with LN1 substrates. In our study, both enzymes show α3/4FucT activity with LN2 and LN1, respectively. With mixed LN2 and LN1 combinations, the preference is for the LN2 subunits. Most importantly, distinct fucosylated products (Le^x-LN1, LN1-Le^x, Le^y-LN2) are accessible with FucTΔ52-4M. Fucosylation is forced by an excess of GDP-Fuc and should lead to higher product yields and to the conversion of less preferred substrates as demonstrated for other fucosyltransferases.[4b,5b,14] The use of truncated bacterial FucTs may affect the acceptability in terms of poly-LN glycans. Studies by Nilsson et al. demonstrate that the heptad repeat regions of FutA and FutB from *H. pylori* act as a molecular ruler for the fucosylation of individual LN2 units – one heptad repeat per LN2 fucosylation.[11,25] FucTΔ66 and FucTΔ52-4M comprise seven and one heptad repeat, respectively, which may influence their activity and capacity for fucosylation of LN-based glycans.

Conclusion

Targeted fucosylation of complex oligo- and polysaccharides is a challenging field. Several approaches have been pursued, taking advantage of different methods such as protection of putative fucosylation sites or sequential assembly of glycans. The resulting products could be used to study biological processes related to immunity, cancer, and infection. Here, we present new insights into the substrate spectrum of two α3FucT variants giving rise to a library of distinct fucosylated LN-based glycans, which are valuable products for testing lectin or toxin binding. We demonstrate the influence of parameters like the type of the glycosidic bond within the LN subunits, the location of the LN subunits, and additional α2-fucosylation at the terminal galactose on the behavior and fucosylation patterns of the enzymes. We discovered interesting fucosylation patterns of the α3FucTs, and, moreover, we also observed a never-seen α4FucT activity. In addition, LDN was identified as a new substrate for FucTΔ52-4M. We demonstrate for the first time the synthesis of fucosylated LDN with FucTΔ52-4M, a bacterial FucT. Investigation of the complete substrate spectrum of bacterial FucT variants will expand the possibilities of targeted fucosylation of glycans.

Experimental Section

Production and Purification of Recombinant Proteins

For the synthesis of different glycans and investigation of enzymes, proteins were expressed according to the standard procedure. To increase solubility and activity, selected proteins were modified with the propeptide from *Staphylococcus hyicus*,[26] which was inserted between the purification tag and the gene sequences of the enzymes (Table S1, Table S2). Expression strains were cultivated in an LB preculture, then in the main culture of Terrific Broth (TB) at 37 °C. At an OD600 between 0.5 and 0.8, protein production was induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG). The temperature was decreased to 25 °C and cultures were incubated for 24 h. Cells were harvested and stored at −20 °C until further processing. Enzyme purification was performed by affinity chromatography with a HisTrap or MBPTrap column using an Äkta Purifier.[16,23] Cells were suspended in lysis/equilibration buffer and sonicated (6.5 min, 15 s pulse, 60 s pause). After centrifugation, crude extracts were filtered and loaded onto the purification columns. Buffers were prepared according to the manufacturer's or literature's instructions (Table S1, Table S2). For the FKP enzyme, the buffer was replaced with phosphate-buffered saline (PBS) and the enzyme was stored in 50% glycerol at −20 °C.

Synthesis of LN Oligosaccharides

Glycan production was performed with 5 mM acceptor (tBoc sugar) and 6.5 mM donor sugar (nucleotide sugar) (acceptor:donor ratio 1:1.3). For optimization of the reaction, 20 U/mL FastAP were added to the GT reactions. The precursor GlcNAc linker-NH_2-*t*Boc (GlcNAc-*t*Boc) was produced chemically.[27] Sequential application of the glycosyltransferases β4GalT (50 μg/mL), β3GalT (500 μg/mL), β3GlcNAcT (50 μg/mL), and β4GalTY284L (50 μg/mL) as described before[16] resulted in the precursors LN2, GlcNAc-LN2, LN2-LN2, LN1-LN2, LN1, GlcNAc-LN1, LN2-LN1, LN1-LN1, and LDN for the FucT reactions.

FucT Assays and Fucosylated Compounds

The acceptance of FucTs for the glycan substrates was investigated with fixed enzyme concentrations (FucTΔ52-4M, 1.7 mg/mL; FucTΔ66, 1.7 mg/mL; FutC, 1.5 mg/mL) (Table S2) at 37 °C for FucTΔ52-4M and FutC and at 25 °C for FucTΔ66. GDP-Fuc was used as the glycan donor, which was prepared before use[3a] (Table S1) (6 h, 37 °C). The respective glycan acceptor (5 mM) was incubated with 6.5 mM GDP-Fuc and the enzyme. To analyze the behavior of the enzymes with the di- and trisaccharides, the reactions were stopped after 60 min. Reactions with tetrasaccharides were stopped after 0, 1, 5, 30, and 60 min to obtain time-yield curves. Samples were analyzed by RP-HPLC.

High-Performance Liquid Chromatography

Glycans were investigated by RP-HPLC and ESI-MS.[23] For the HPLC measurements, an isocratic eluent of 15% acetonitrile and 85% water was used. Reactions were stopped at 95 °C. Samples were analyzed on a Dionex RP-HPLC system and a Shimadzu Prominence LC analytical system (selected fucosidase samples) with an analytic MultoKrom 100-5C18 column (250 mm × 4 mm) at 254 nm and a flow rate of 1 mL/min. Fucosylated reaction products were collected for further investigations (MS, fucosidase assays, NMR).

Fucosidase Assays

Fucosylated products were incubated with a commercial α3/4-fucosidase[28] to determine the site of fucosylation in complex oligosaccharides (1 mM, 24 h, 37 °C), according to the manufacturer's instructions. The fucosidase was inactivated at 95 °C and hydrolysis was analyzed using RP-HPLC. The fucosidase products were collected and analyzed by ESI-MS to confirm the molecular mass.

Electrospray Ionization-Mass Spectrometry

Purified reaction products were analyzed by ESI-MS[23] with a Multospher 120 RP 18 HP-3 μ column, an eluent of 50% acetonitrile, and a flow rate of 0.2 mL/min (needle voltage = 4 kV, temperature = 400 °C, cone voltage = 100 V, negative mode). Masses were analyzed as the mass/charge ratio (*m/z*).

Nuclear Magnetic Resonance Spectroscopy

Selected structures were analyzed by NMR spectroscopy.[16b,23] A Bruker Avance III 600 Hz spectrometer was used to record spectra in D_2O (99.96 atom% deuterium (D)) at 30 °C. The residual signal of D_2O (δ_H 4.732 ppm) was used as a reference for the proton spectra; the signal of acetone (δ_C 30.50 ppm) was applied as a reference for the carbon spectra. ^{1}H NMR, ^{13}C NMR, gradient correlation spectroscopy (gCOSY), ^{1}H-^{13}C gradient heteronuclear single quantum coherence (gHSQC), ^{1}H-^{13}C gradient heteronuclear multiple bond coherence (gHMBC), one-dimensional total correlation spectroscopy (1D-TOCSY), and HSQC-TOCSY were done to analyze the glycan structures using the manufacturer's software.

Supporting Information contains detailed glycosyltransferase reaction conditions, HPLC, MS, and NMR analyzes of structures of the produced compounds.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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