Characterization of *Helicobacter pylori* α1,2-Fucosyltransferase for Enzymatic Synthesis of Tumor-Associated Antigens

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Abstract: The α1,2-fucosyltransferase (α1,2-FucT) from *Helicobacter pylori* catalyzes the fucosylation of acceptor oligosaccharides at the C2-OH of terminal Galβ units. The enzyme from strain NCTC11639 was evaluated for its ability to synthesize cancer-associated antigens. The α1,2-FucT was determined to be active over a pH range between 4.0 and 8.0 with the optimum occurring at pH 5.0. Although a divalent metal ion cofactor was not required for catalysis, enhancement of the enzyme activity was detected upon supplement with Mn^{2+}. Detailed substrate specificity analysis revealed that α1,2-FucT can catalyze the fucosylation of a wide variety of oligosaccharide substrates. The α1,2-FucT preferentially fucosylated type 1 structure (Galβ1-3GlcNAc)-containing glycans over type 2 structure (Galβ1-4GlcNAc)-containing glycans. The Lewis^a trisaccharide [Galβ1-3-(Fucα1-4)GlcNAc] was found to be the best acceptor. The only exception was that the Lewis^x pentasaccharide LNFP III [Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc] was favored over the Lewis^a analogue LNFP II [Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc]. Furthermore, the enzyme exhibited high levels of fucosylation with the type 3/4 disaccharide (Galβ1-3GalNAc). Enzymatic hydrolysis of the donor substrate, guanosine-5′-diphospho-β-L-fucose (GDP-fucose), could be observed in the absence of oligosaccharide substrate. This hydrolysis was completely shifted to an efficient fucosyl transfer in the presence of a substrate sugar. Finally, the *H. pylori* α1,2-FucT was successfully used in combination with its α1,3-FucT counterpart to synthesize the Lewis^y tetrasaccharide [Fucα1-2Galβ1-4(Fucα1-3)GlcNAc] from LacNAc (Galβ1-4GlcNAc) in a milligram scale one-pot reaction.

Keywords: carbohydrates; enzyme catalysis; fucosyltransferase; glycosylation; oligosaccharides

Introduction

Fucosylated glycoconjugates bear an indispensable significance for mammalian physiology.[1] They serve as the determinants of human blood groups and mediate cell-cell interactions that trigger several important biological events such as fertilization,[2] embryogenesis,[3] neuronal development,[4] and immune response. On the other hand, it has been shown that fucose-containing carbohydrate structures displayed on the cell surface are also associated with the emergence and progression of chronic inflammation and cancer.[5] Furthermore, expression of fucosylated oligosaccharides has been observed in several pathogenic processes. For example, Lewis^y (Le^y) and Lewis^x (Le^x) epitopes that are produced in the O-antigenic regions of lipopolysaccharides (LPSs) of *Helicobacter pylori* are known to participate in adhesion and escape from the host immune surveillance, thus accounting for the long-term colonization of the human stomach.[6]

Owing to the pathological and pathogenic relevance, the broad availability of fucosylated sugar molecules is strongly desired not only for various biological investigations, but also for the discovery of novel avenues in diagnosis and therapy. The efficient extraction and purification of these glycans from natural sources is difficult due to their heterogeneity and low abundance. Intense efforts have thus been undertaken to find suitable routes for preparative access. Chemical synthesis of fucosylated glycans is often restricted by numerous reaction steps and tedious procedures such as protection and deprotection.[7] Fermentative procedures are not easily applicable, as these require the time-consuming engineering of the producing or-

ganism.[8] Therefore, the specificity and efficiency of enzymatic or chemoenzymatic alternatives likely harbor the highest potential for optimizing the large-scale production of fucosylated glycans.[9]

Fucosylation, often representing the terminal steps in the biosynthesis of functional carbohydrates, is catalyzed by fucosyltransferases (FucTs). These enzymes generate an α1,2-, α1,3-, α1,4-, or α1,6-glycosidic linkage between fucose (Fuc) from the donor substrate guanosine-5′-diphospho-β-L-fucose (GDP-fucose) and a saccharide substrate as acceptor. Prokaryotic organisms are a more promising resource of FucTs for synthetic purposes in consideration of the ability to produce the recombinant enzymes heterologously in a larger scale with high levels of activity.

α1,2-FucTs are of special interest for synthetic approaches due to their importance in the formation of H-type determinants (terminal Fucα1-2Gal motif) and cancer-associated antigens such as Le^y [Fucα1-2Galβ1-4(Fucα1-3)GlcNAc] and Globo H (Fucα1-2GalNAcβ1-3Galα1-4Galβ1-4Glcβ-R). The majority of α1,2-FucT genes have been cloned from eukaryotic species, among them invertebrates, plants, animals, and humans.[10] In contrast, only two bacterial α1,2-FucTs have been cloned; *wbsJ* from *E. coli*[9,11] and *futC* from *H. pylori*.[12,13] However, unlike the corresponding α1,3-FucT that has been studied extensively up to structural level,[14,15] *H. pylori* α1,2-FucT has not been investigated in detail. By preferring type 1 (Galβ1-3GlcNAc) over type 2 (Galβ1-4GlcNAc) acceptor glycans, *H. pylori* α1,2-FucT appears to exhibit the opposite substrate specificity of the α1,3-FucT.[13] Slight variations of this bias could be observed for certain *H. pylori* source strains.[16] *H. pylori* α1,2-FucT has been employed for the synthesis of 2′-fucosyllactose[17] and H-type 2[8] products *in vitro* and *in vivo*, respectively, without gaining further information about its selectivity and potency.

This study presents a detailed characterization of the *H. pylori* α1,2-FucT and evaluates its ability to function in the enzymatic synthesis of fucosylated oligosaccharides. The recombinant protein was overexpressed in *E. coli* under optimized conditions. After purification, the enzyme activity was evaluated for its dependence on pH and divalent metal cofactor. A comprehensive acceptor substrate specificity study was conducted to reveal additional insight into the enzyme selectivity. The results were corroborated by determining the enzyme kinetics with a subset of the oligosaccharide substrates. Finally, the α1,2-FucT was applied in combination with the α1,3-FucT for the enzymatic synthesis of the Le^y tetrasaccharide in a one-pot strategy.

Results

Generation and Purification of the Recombinant α1,2-FucT

Cloning of the *futC* gene (HP0094/HP0093) from *Helicobacter pylori* NCTC11639 yielded a construct containing a 13C region in HP0094 that disrupts the open reading frame and disables efficient expression.[12,18] A 12-nuleotide sequence was substituted for this tract according to an established approach[8] that was modified by using site-directed mutagenesis to restore the reading frame. Following this procedure, the recombinant protein was successfully overexpressed as a C-terminal His$_6$-tagged fusion in *E. coli* BL21 (DE3) cells and was purified to approximate homogeneity by single-step Ni^{2+} affinity chromatography, as confirmed by SDS-PAGE (see Supporting Information, Figure S2). The α1,2-FucT (36 kDa calculated mass) was obtained in an estimated yield of 1.5 mg L^{-1} bacterial culture with a specific activity of 90 mU mg^{-1} (determined with the type 1 disaccharide, see Table 1). Purified α1,2-FucT exhibited stability at -80 °C for several months, and endured at least three cycles of thawing and refreezing without significant loss in activity.

Effect of pH on FucT Activity

The effect of pH on the α1,2-FucT activity was evaluated using the type 1 disaccharide as the acceptor substrate (Figure 1). The profile revealed the highest rate at pH ~5.0 (set to 100%) with a sharp decline in activity between pH 4.0–5.0 and a gradual decrease at pH greater than 5.0. Subsequent assays and reactions were performed at pH 7.0 to ensure comparability of the results to related published work, in which FucTs were analyzed at pH 6.5–7.5,[11,14,19] and to evaluate the applicability of the α1,2-FucT in one-pot synthetic strategies with other proteins. Under these conditions, about 35% of the maximal FucT activity was retained. Crude kinetic analysis (data not shown) confirmed an approximately 4-fold higher k_{cat} at pH 5.0 in comparison to pH 7.0 but a similarly increased K_m implicated a marginal influence of pH on the catalytic efficiency.

Effect of Divalent Metal Ions on FucT Activity

The activity of several glycosyltransferases containing a DxD motif (GT-A superfamily) is dependent on divalent metal cofactors, mostly Mn^{2+}.[20] As the *H. pylori* NCTC11639 α1,2-FucT does not include these invariant amino acid residues, it did not appear that metal ions would be necessary for catalysis. However, when the enzyme was tested for its ability to fucosy-

Table 1. Panel of all acceptor substrates used in this study.

Entry	Substrate name	Abbreviation	Structure[a]
1	Type 1 disaccharide	Type 1	**Galβ1-3GlcNAc**
2	Type 2 disaccharide	Type 2	*Galβ1-4GlcNAc* (LacNAc)
3	Type 1-N$_3$		**Galβ1-3GlcNAc**β1-C$_6$H$_{12}$-N$_3$
4	Type 2-N$_3$		*Galβ1-4GlcNAc*β1-C$_6$H$_{12}$-N$_3$
5	Lacto-*N*-tetraose	LNT	**Galβ1-3GlcNAc**β1-3 Galβ1-4Glc
6	Lacto-*N*-hexaose	LNH	**Galβ1-3GlcNAc**β1-3(*Galβ1-4GlcNAc*β1-6)Galβ1-4Glc
7	Lacto-*N*-neohexaose	LNnH	*Galβ1-4GlcNAc*β1-3(*Galβ1-4GlcNAc*β1-6)Galβ1-4Glc
8	Lacto-*N*-fucopentaose II	LNFP II	**Galβ1-3**(Fucα1-4)**GlcNAc**β1-3Galβ1-4Glc
9	Lacto-*N*-fucopentaose III	LNFPIII	*Galβ1-4*(Fucα1-3)*GlcNAc*β1-3Galβ1-4Glc
10	Lewis^a trisaccharide	Le^a	**Galβ1-3**(Fucα1-4)**GlcNAc**
11	Lewis^x trisaccharide	Le^x	*Galβ1-4*(Fucα1-3)*GlcNAc*
12	Sialyl Lewis^a	SLe^a	Neu5Acα2-3**Galβ1-3**(Fucα1-4)**GlcNAc**
13	Sialyl Lewis^x	SLe^x	Neu5Acα2-3*Galβ1-4*(Fucα1-3)*GlcNAc*
14	LS-tetrasaccharide b	LSTb	**Galβ1–3**(Neu5Acα2-6)**GlcNAc**β1-3Galβ1-4Glc
15	Disialyl-lacto-*N*-tetraose	DSLNT	Neu5Acα2-3**Galβ1–3**(Neu5Acα2-6)**GlcNAc**β1-3Galβ1-4Glc
16	Galactose		Gal
17	Glucose		Glc
18	*N*-Acetyl-galactosamine		GalNAc
19	*N*-Acetyl-glucosamine		GlcNAc
20	Lactose	Lac	Galβ1-4Glc
21			Galα1-3Gal
22	Linear B-6 trisaccharide	B-6	Galα1-3Galβ1-4Glc
23	Type 3/4 disaccharide	Type 3/4	Galβ1-3GalNAc
24			Galβ1-6GalNAc
25			Galβ1-6GlcNAc
26	Gb5		Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ-C$_5$H$_{10}$-NH$_2$

[a] The type 1 and type 2 motifs are printed in bold and italic letters, respectively.

late the type 1 disaccharide in the presence of different Mn^{2+} concentrations (up to 20 mM), the enzyme activity was significantly elevated compared to the reaction in absence of the metal ion (Figure 2). The enhancement of fucosylation was found to be optimal at

5 mM (set to 100%) with a three-fold increase in comparison to the negative control. At concentrations higher than 5 mM the stimulation dropped slightly, still showing a 2.5-fold activation at 20 mM. Other selected divalent metal ions (Mg^{2+}, Co^{2+}, and Ni^{2+})
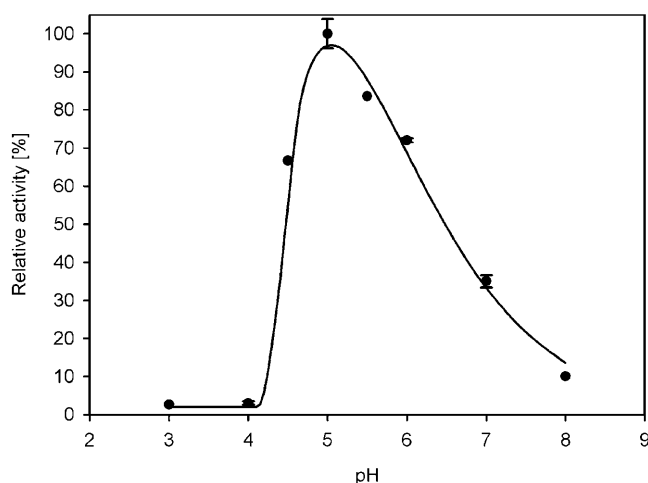

Figure 1. Effect of pH on the α1,2-FucT activity. Relative values were determined with the type 1 disaccharide and plotted as a function of the pH. Data was averaged from three independent experiments.
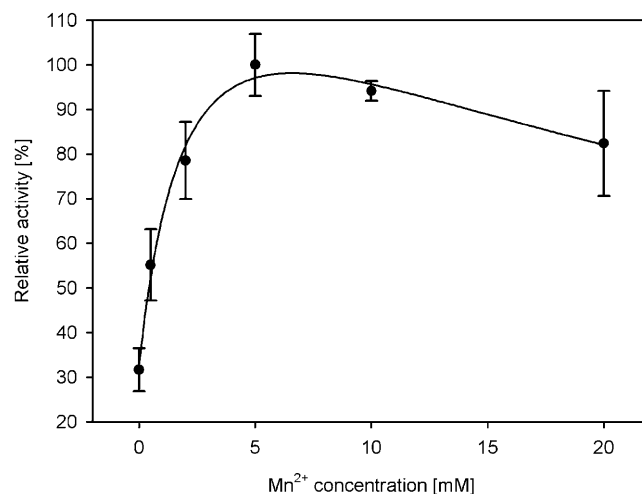

Figure 2. Effect of Mn^{2+} on the α1,2-FucT activity. Relative values were determined with the type 1 disaccharide and plotted as a function of the Mn^{2+} concentration. Data were averaged from three independent experiments.
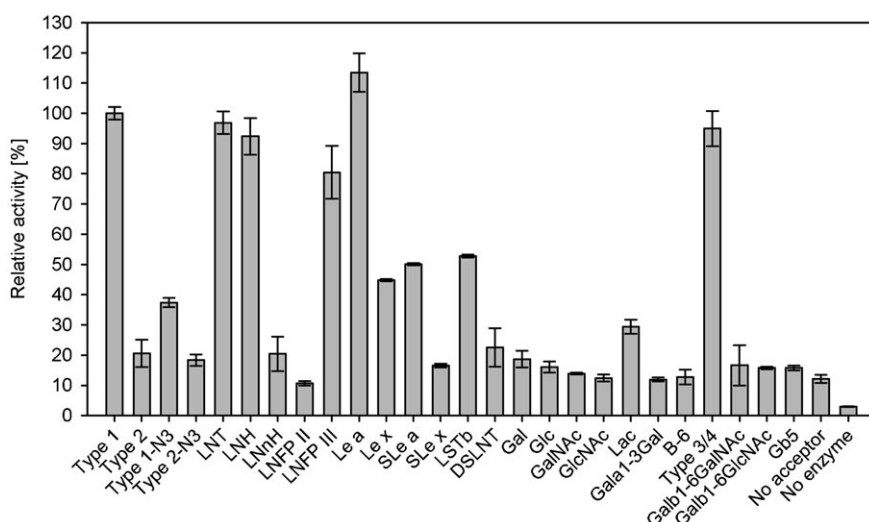
Figure 3. Substrate specificity of the α1,2-FucT. Relative values were determined with the indicated acceptor saccharides and illustrated in a vertical bar chart. Data were averaged from three independent experiments. For comprehensibility, the applied substrates are labeled by their common trivial names or abbreviations. The respective structures are assigned in Table 1.

were also tested (supplemented at 5 mM) and determined to be activators as well, with approximately 90, 80, and 60%, respectively, of the potency of Mn^{2+}. In reactions containing 5 mM EDTA and no additional metal ions, α1,2-FucT interestingly showed about 60% activity.

Acceptor Substrate Specificity

Previously reported studies have suggested that the substrate specificity of *H. pylori* α1,2-FucT (preference of type 1 over type 2 acceptors) is the opposite of its α1,3-counterpart but might vary among particular strains of origin.[13,16]

A variety of mono-, di-, tri-, and oligosaccharides (see Table 1) were utilized for a detailed analysis of the acceptor specificity. The relative activities are illustrated in Figure 3. Despite the ability of α1,2-FucT to fucosylate a wide range of oligosaccharides, the enzyme distinctly favored type 1 glycans (Galβ1-3GlcNAc-) over type 2 (Galβ1-4GlcNAc-) glycans. The value obtained for the reaction with the type 1 disaccharide was thus defined as 100%. In contrast, the type 2 disaccharide (LacNAc) was converted at 21%. Introduction of a 6-azidohexyl group ($-C_6H_{12}$-N_3) at the reducing end of both the type 1 and the type 2 disaccharide substrates (type 1-N_3, 37%; type 2-N_3, 18%) affected the ability of enzymatic α1,2-fucosylation. Fractionally lowered rates of fucosyltation were detected when substrates LNT (97%) and LNH (92%) were tested, indicating that elongation of the type 1 chain and the presence of an additional type 2 branch did not significantly affect the ability of α1,2-

fucosyl transfer. Fucosylation of the type 1 chain in LNH was corroborated, as a clearly decreased activity was observed with the analogue LNnH (20%), in which both branches are type 2. The specificity of α1,2-FucT to favor type 1 over type 2 substrates was supported by the data obtained for the α1,3/α1,4 fucosylated trisaccharides Le[x] and Le[a], respectively, with Le[a] demonstrating the highest level of fucosylation (113%) and Le[x] displaying modest fucosylation (45%). Surprisingly, the inverse situation was found using the corresponding derivatives LNFPII (containing the Le[a] structure in the non-reducing trisaccharide, 11%) and LNFPIII (containing the Le[x] structure in the non-reducing trisaccharide, 80%). The ability of α1,2-FucT to fucosylate the sialylated glycans SLe[x] (17%), SLe[a] (50%), LSTb (53%) and DSLNT (23%) was reduced but still took place with a significant extent, when compared to the results of Le[x], Le[a], and LNT, respectively. The general tolerance of sialylation by *H. pylori* α1,2-FucT is dissimilar to that of *H. pylori* α1,3-FucT, which was reported to accept 2,3-sialylation but not 2,6-sialylation.[14] Moreover, the monosaccharides Gal, Glc, GalNAc, and GlcNAc were determined to be poor substrates of α1,2-FucT (12–19%), while lactose was moderately fucosylated (29%). Low fucosylation activities were found with Galα1-3Gal and its derivative B-6 (Galα1-3Galβ1-4Glc, ~12%) as well as with Galβ1-6GalNAc and Galβ1-6GlcNAc (~16%). Interestingly, the type 3/4 disaccharide (Galβ1-3GalNAc) was determined to be one of the most potent substrates (95%), but the derived pentasaccharide Gb5 (Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ-C_5H_{10}-NH_2, containing the type 4 structure) was only weakly fucosylated (16%). Al-

though it could not be excluded that the extended length and the C_5-amine (-C_5H_{10}-NH_2) moiety linked to the Gb5 substrate played a secondary role in impairing the enzyme's tolerance, the result suggests a novel preference of the *H. pylori* α1,2-FucT for the type 3 (Galβ1-3GalNAcα-) over the type 4 (Galβ1-3GalNAc**β**-) glycans.

A strikingly high signal was measured for the negative control in the absence of a glycan substrate (12%), whereas a low background value (3%) was observed when omitting the enzyme in the reaction. Radioisotope-TLC analysis confirmed that GDP-fucose will be hydrolyzed steadily by the α1,2-FucT if no acceptor substrate is added to the mixture (see the section "Kinetics of α1,2-FucT activity").

Kinetics of α1,2-FucT activity

In order to gain profound insight into the selectivity of the α1,2-FucT and to evaluate its potential for the use in preparative enzymatic syntheses of fucosylated oligosaccharides, apparent kinetic parameters were determined for the best five of the previously selected sugars (type 1, LNT, LNH, Le^a, and type 3/4) as well as for type 2 and Le^x. Additionally, the characteristics of GDP-Fuc were investigated in the presence and absence of an acceptor. The resulting data (summarized in Table 2) verified the general bias reflected by the relative substrate specificity study. Specifically, highest affinities of the enzyme were found for Le^a (K_m ~130 μM) and type 1 (K_m ~130 μM). In contrast, the enzyme showed a slightly lower affinity for Le^x (K_m = 438 μM), while the affinity for the type 2 disaccharide was significantly lower (K_m ~12 mM). K_m values in the range of 300–400 μM were found for the other substrates. Virtually no variation occurred among the compounds regarding the turnover (k_{cat}) with assessed rates between 0.8 min^{-1} and 2.0 min^{-1}.

Table 2. Kinetic parameters of the recombinant *H. pylori* α1,2-FucT.

Substrate[a]	K_m [mM]	k_{cat} [min^{-1}]	k_{cat}/K_m [min^{-1} m$_M^{-1}$]
Type 1	0.137 ± 0.018	1.83 ± 0.068	13.36
Type 2	11.75 ± 0.624	1.04 ± 0.026	0.089
LNT	0.321 ± 0.034	2.00 ± 0.065	6.23
LNH	0.385 ± 0.013	1.77 ± 0.024	4.60
Lewis^a	0.129 ± 0.020	2.01 ± 0.072	15.58
Lewis^x	0.438 ± 0.088	0.798 ± 0.048	1.82
Type 3/4	0.342 ± 0.043	2.03 ± 0.080	5.94
GDP-Fuc (with type 1)	0.046 ± 0.009	1.80 ± 0.122	39.13
GDP-Fuc (no acceptor)	0.044 ± 0.009	0.267 ± 0.016	6.07

[a] Structures of acceptor substrates are shown in Table 1.

Therefore, the catalytic efficiencies (k_{cat}/K_m) precisely represented the preferences of the enzyme. In this context, it could be revealed that the α1,2-FucT discriminates the type 1 against the type 2 disaccharide by approximately 150-fold but Le^a against Le^x to a much lower degree (about 9-fold). The determined efficiency with the type 3/4 disaccharide was in the same range as that measured for LNT and LNH (both sugars containing the type 1 structure). Furthermore, the affinity of α1,2-FucT for the donor substrate (GDP-Fuc) was strong (K_m ~45 μM) and did not depend on the presence of an acceptor substrate. In comparison with the fucosyl transfer, the enzyme showed a more than 6-fold weaker GDP-Fuc hydrolyzing activity that was completely abolished as soon as an oligosaccharide had been added. Therefore, enzymatic GDP-Fuc hydrolysis will not interfere with the desired fucosylation, even if there is a weak acceptor substrate.

Preparative Enzymatic One-Pot Synthesis of Lewis^y

The *H. pylori* α1,2-FucT was applied to synthesize Le^y enzymatically from the type 2 disaccharide (LacNAc) as the substrate. In order to enable synthesis in a one-pot strategy for milligram scale preparation, the enzyme was combined with the α1,3-FucT (Δ45) from the same bacterial strain[14]. Because the two enzymes have different substrate specificities, it is interesting to know which fucosyl transfer occurs first. TLC analysis indicated that the α1,3-FucT initially catalyzed formation of Le^x, which was subsequently converted to Le^y by the α1,2-FucT. Precursors and by-products (GDP-Fuc, GDP, and traces of fucose) were removed by anion exchange chromatography and gel filtration to yield 4 mg (45%) of purified Le^y.

Discussion

Bacterial fucosyltransferases have been proved as useful tools for the synthesis of biorelevant and disease-associated oligosaccharides.[9] These enzymes can be overproduced in an easily manipulated heterologous organism like *E. coli*.[19] Although putative genes encoding FucTs have been identified in various bacteria, cloning and characterization of the corresponding proteins still represents a challenge.[10] This study presents the generation and an in-depth characterization of the recombinant *H. pylori* NCTC11639 α1,2-FucT and evaluates its utility for the synthesis of cancer-associated antigens.

The length of the polyC region contained in the encoding gene (*futC*) appears to determine whether the protein α1,2-FucT is functional, as in strain UA802, or non-funtional due to a frameshift that produces the

two fragments, HP0094 and HP0093, as in strain 26695.[18] Such varying compositions of *futC* prompt certain *H. pylori* strains to generate other carbohydrates than Le^y.[21] It has been reported that disrupted *futC* from strain 26695 can be restored utilizing gene splicing by overlap extension for the modification of the homopolymeric tract.[8] Similarly, *futC* cloned from strain NCTC11639 was successfully altered in this work by a single step of site-directed mutagenesis after plasmid integration.

It has been described that protein fusions can enhance the efficiency of heterologous *H. pylori* α1,2-FucT overproduction in *E. coli*.[17] In contrast, other studies have not been able to reproduce this effect.[8] Thus, in this study the enzyme was generated in stand-alone form and exhibited a specific activity comparable to the reported protein fusion.[17] Although the enzyme has been demonstrated to be soluble,[13] the expression level and specific activity found in this study were much lower compared to the values published for the α1,3-[14] and α1,4-counterparts.[19] However, the *H. pylori* α1,2-FucT appeared to be a more effective fucosyltransferase, as displayed by the kinetic results (Table 2), when compared to the recently investigated *E. coli* α1,2-FucT (WbsJ).[11] In contrast to earlier observations,[13] *H. pylori* α1,2-FucT showed stability enabling long-term storage in additive-free buffer at −80 °C without significant loss of activity.

The *H. pylori* α1,2-FucT was active in the range of pH 4.0–8.0 but demonstrated the highest rate of fucosylation at pH 5.0, which correlates with the optimal pH conditions referred for human FucT V[22] or the *E. coli* α1,2-FucT (WbsJ).[11] As it has been proposed that an acidic side chain is involved in the catalysis of the SN$_2$-like inverting reaction of vertebrate FucT III[23] and *H. pylori* α1,3-FucT,[15] the observed ideal pH environment appears reasonable.

Consistent with the absence of a DxD motif in the amino acid sequence, the activity of the *H. pylori* α1,2-FucT did not depend on the supplement of divalent metal ions. The binding of GDP-Fuc independent of a metal ion was demonstrated by the co-crystal structure of the corresponding α1,3-FucT.[15] Nevertheless, α1,2-FucT activity could be increased by a factor of three upon addition of Mn^{2+} at 5 mM, in contrast to the recently characterized *E. coli* analog that has not exhibited significant stimulation by metal ions.[11] Stabilization of the negatively charged sugar nucleotide phosphate groups by chelation of the metal ion[24] might enhance the transfer reaction for the *H. pylori* α1,2-FucT.

The substrate specificity of the α1,2-FucT has been previously analyzed in order to explain the biosynthetic routes to Lewis antigens displayed on the *H. pylori* LPS.[13,16] These studies have shown that the α1,2-FucT exhibits a preference for type 1 substrates over type 2 but that the selectivity of the α1,2-FucT from different strains might vary. Examination of the α1,2-FucT from strain NCTC11639 verified the previously shown preference and revealed distinctive features. The enzyme accepted various oligosaccharide substrates unlike the α1,3- and α1,4-counterparts from *H. pylori*, which show a strong selectivity for type 2 and type 1 sugars, respectively.[14,19] Certain bovine[25] and *C. elegans*[26] α1,2-FucTs, for instance, exhibit exclusive type 3/4 specificity, whereas the *H. pylori* α1,2-FucT fucosylated the type 3/4 structure almost as efficiently as the favored type 1 analogue. *H. pylori* α1,2-FucT also seems to be more permissive than the *E. coli* α1,2-FucT (WbsJ), which preferentially fucosylates type 3/4 structures and Galβ1-4Glc, -4Fru, and -4Man.[11] Mammalian α1,2-FucTs FUT1 and FUT2 typically act on type 1, type 2, and type 3/4 substrates rather than on Le^a or Le^x. In contrast to the mammalian counterparts, the *H. pylori* α1,2-FucT shows a high affinity for the subterminally fucosylated acceptors Le^a and Le^x. While the Le^a trisaccharide was determined to be a better acceptor than the Le^x substrate, interestingly, the inverse was found for the Le^a and Le^x pentasaccharides LNFP II and LNFP III, respectively. The observed variation in specificity for Le^a and Le^x-containing structures can be an additional explanation for the very low population of *H. pylori* strains expressing Le^b epitopes.[16] It was also shown that the high affinity of the enzyme for GDP-Fuc does not depend on the presence of an acceptor substrate, which supports the mechanism proposed for human FucT V where glycosidic cleavage occurs prior to nucleophilic attack.[27] A co-purified GDP-Fuc hydrolase activity has been mentioned for an α1,2-FucT purified from porcine submaxillary gland.[28] Purification and use of a recombinantly generated protein in the work presented here verified that both activities are present in one peptide. However, significant hydrolysis of GDP-Fuc by the *H. pylori* α1,2-FucT was only detectable in the absence of acceptor. A similar result was observed for the *H. pylori* α1,3-FucT.[15] Therefore, other FucTs might also exhibit GDP-Fuc hydrolase activities that cannot be detected in the presence of an acceptor substrate.

A one-pot strategy was developed to synthesize Le^y from LacNAc using both the α1,2- and the α1,3-FucT (Δ45)[14] from *H. pylori* NCTC11639. In fact, the chemical synthesis of Le^y is well established and offers more flexibility for the introduction of modifications, but the drawbacks include low overall yields and time-consuming multiple-step procedures such as protection group chemistry.[29] The approach presented here exploits the efficiency and specificity of the FucTs to yield the desired product, Le^y, after simultaneous addition of all components. However, alterations in reaction and work-up conditions are needed to optimize the yield. In order to advance or auto-

mate the synthesis of fucosylated oligosaccharides,[30] the identification of suitable enzyme sources, a thorough characterization, and a deliberate design of synthetic routes are requisite. These efforts will certainly help to overcome the general limitations of biocatalyzed reactions including protein production cost and substrate selectivity.

Conclusions

The *H. pylori* α1,2-FucT represents an effective enzyme that is active under various conditions and has the ability to fucosylate a broad range of acceptor sugars. The enzyme can be used in conjunction with α1,3-FucT for the milligram-scale synthesis of the tetrasaccharide Le^y in a one-pot manner. This study confirms that investigations of bacterial FucTs merit further attention to expand the inventory of biocatalysts for the synthesis of valuable glycans.

Experimental Section

Cloning and Modification of *futC*

All DNA manipulation and purification procedures were carried out by standard techniques.[31] *E. coli* DH5α (Yeastern Biotech, Taipei, Taiwan) was utilized for cloning and preparation of recombinant plasmids. Restriction enzymes were purchased from New England Biolabs (Ipswich, MA).

The *futC* gene (HP0094/HP0093) was amplified by PCR from chromosomal DNA of *Helicobacter pylori* NCTC11639. The oligonucleotides 5′-CAAAGGGATCA-TATGGCTTTTAAAGTGGTGCAA-3′ and 5′-CTTTTT-TTAAGCCCTCGAGAGCGTTATATTTTTG-3′ (incorporated terminal recognition sites of restriction enzymes are underlined) were designed in reference to the published sequence of *H. pylori* 26695.[32] After digestion with *Nde*I and *Xho*I, the amplificate was ligated with a similarly cut pET21b vector (Novagen, San Diego, CA). The resulting plasmid, pET21b-futC, served as a template for the modification of *futC* according to a previously described strategy.[8] To this end, the site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used with the primers 5′-CACTCTACCACCACCACCC*GAAAATAATAAGAAT-AATAATAAAAAAGAG-3′ and 5′-TAATTTTC*GGGT-GGTGGTGGTAGAGTGAAGGTTTGCTTGATTAAAG-G-3′ (exchanged bases are underlined, whereby "*" indicates deletion) following the guidelines of the manufacturer to yield pET21b-futC$_{restored}$. The desired recombinant constructs were verified by restriction analysis and DNA sequencing.

Overexpression and Protein Purification

The heterologous host *E. coli* BL21 (DE3) was transformed with the plasmid pET21b-futC$_{restored}$ for overproduction of the C-terminally hexahistidine (His$_6$)-tagged α1,2-fucosyltransferase. Cells were grown to a dense culture overnight (16 h) in ampicillin containing (100 μg mL^{-1}) LB medium at 16 °C with agitation (250 rpm). After induction with 0.2 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG), expression proceeded for 5–8 h under the same conditions. Subsequently, cells were harvested by centrifugation (6000 rpm, 15 min, 4 °C), resuspended in FPLC buffer A [50 mM HEPES (pH 8.0), 300 mM NaCl], and stored at −20 °C until further usage.

The thawed suspension was passed through a French press for cell disruption and the lysate was centrifuged (17000 rpm, 60 min, 4 °C). Protein purification was performed by single step Ni^{2+} affinity chromatography on an ÄKTA FPLC system (GE Healthcare Life Sciences, Uppsala, Sweden) equipped with a 5 mL HiTrap chelating HP column (GE Healthcare Life Sciences). For elution, the concentration of FPLC buffer B [50 mM HEPES (pH 8.0), 300 mM NaCl, 250 mM imidazole] was gradually increased in the flow system (FPLC buffer A, as aforementioned). After confirmation by SDS/PAGE,[33] desired fractions were pooled, and the solvent was exchanged for assay buffer [50 mM HEPES (pH 7.0), 100 mM NaCl] *via* HiPrep 26/10 Desalting (GE Healthcare Life Sciences). An Amicon Ultra-15 centrifugal filter unit (Millipore, Taipei, Taiwan, 10 kDa molecular weight cut-off) was used to concentrate the protein. The enzyme solution was shock-frozen in liquid nitrogen and stored at −80 °C. The α1,2-FucT concentration was determined photometrically using the calculated extinction coefficient for absorbance at 280 nm (54060 M^{-1} cm^{-1}).

Fucosyltransferase Activity Assay

The enzyme activity was detected by measuring the incorporation of radioactive label from GDP-L-[U-^{14}C]fucose (240 mCi mmol^{-1}, PerkinElmer Life and Analytical Sciences, Boston, MA) into reaction products. Mono- and oligosaccharides were acquired from Sigma (St. Louis, MO), Toronto Research Chemicals (North York, Canada), and Dextra Laboratories (Reading, UK). Type 1- and type 2-N$_3$ were synthesized complying with established practices[34–37] and Gb5 was provided by Dr. Chung-Yi Wu (Genomic Research Center, Academia Sinica). GDP-β-L-fucose was purchased from Sigma.

Reactions (see detailed composition in sections below) were conducted in assay buffer at ambient temperature (25 °C), initiated upon addition of GDP-fucose, and processed for analysis by either of the methods that are described in the following.

For liquid scintillation counting (LSC), fucosyl transfer was stopped at defined points in time by adding 300 μL of a Dowex 1×8 200–400 mesh chloride anion-exchange resin (Sigma/Fluka) suspension in water (250 mg mL^{-1}). After centrifugation (5000 rpm, 5 min), the supernatant (200 μL) was transferred into a 5 mL-vial containing 1 mL of Ultima-Flo™ M scintillation cocktail (Perkin–Elmer), and radioactivity was measured with a Beckman LS 6500 system (Beckman Coulter, Taipei, Taiwan).

For radio thin layer chromatography (radio-TLC), samples were taken at defined points in time and spotted directly onto a Silica Gel 60 F$_{254}$ TLC plate (Merck, Darmstadt, Germany). Following the development with a mixture of 2-propanol/acetic acid/water (7:1:3), radioactivity was detected by phosphoimaging with a BAS-MS 2040 imaging plate and

a BAS-1500 scanner (Fujifilm, Taipei, Taiwan). Signals could be quantified by means of the software Image Gauge V4.0 (Fujifilm).

Studies of pH-Profile, Metal Ion Effect, and Acceptor Substrate Specificity

Assays were performed as described above in a final volume of 10 μL containing 10 mM $MnCl_2$, 50 μM GDP-fucose, 5 μM GDP-[^{14}C]fucose, 2 mM acceptor substrate and 1.66 μM enzyme. A time course of the reaction with the type 1 disaccharide was tracked by applying the LSC method which revealed linear product formation for 20 min.

For investigating the influence of pH on the enzyme activity, similar mixtures were incubated at varied pH (3.0–8.0). The effect of divalent metal ions was examined at pH 7.0 by applying different $MnCl_2$ concentrations (0–20 mM). $MgCl_2$, $CoCl_2$ or $NiCl_2$ were added at 5 mM. A complementary control reaction contained 5 mM EDTA and no supplemented metal ions. In all cases, samples were analyzed after a reaction time of 20 min by LSC. The TLC method was used in separate experiments to confirm these results.

Several sugar molecules (2 mM) were tested as acceptor substrates for the α1,2-FucT. Reaction conditions and analytical method were the same as for the time course determination, except that 5 mM $MnCl_2$ was used in the mixture. Acceptor or enzyme was omitted in respective control reactions.

Kinetic Analysis

Enzyme kinetics were examined by performing reactions in a final volume of 5 μL containing 5 mM $MnCl_2$. Apparent K_m values for selected acceptors were assessed at a constant GDP-fucose concentration of 208 μM (8 μM GDP-[^{14}C]fucose and 200 μM unlabeled GDP-fucose). The K_m for GDP-fucose was ascertained by applying 10 μM GDP-[^{14}C]fucose and different concentrations of GDP-fucose (0–200 μM) at a type 1 disaccharide concentration of 2 mM. Similar experiments were carried out in the absence of acceptor with various amounts of unlabeled GDP-fucose (10–500 μM) to evaluate the enzymatic GDP-fucose hydrolysis. The final α1,2-FucT concentration was adjusted (0.67–3.99 μM) to deliver optimal linear product increase over the observed time range. Aliquots (0.5 μL) were taken at defined points in time to be analyzed by the radio-TLC method described above. Initial rates were plotted as a function of substrate concentration for nonlinear regression to the Michaelis–Menten equation using the software Sigma-Plot® 8.0 with the Enzyme Kinetics Module 1.1.

One-Pot Enzymatic Synthesis of Lewis^y

The α1,2-FucT was applied in combination with the α1,3-FucT (Δ45) from *H. pylori* NCTC11639[14] for one-pot enzymatic synthesis of Le^y on a milligram-scale, starting from the disaccharide LacNAc. A mixture (2 mL final volume) of 5 mM $MnCl_2$, 15 mM GDP-fucose, 6.5 mM LacNAc, and 400 μg of each enzyme was incubated at ambient temperature in assay buffer. The reaction progress was monitored by TLC with butanol/acetic acid/water (2:1:1) in two runs.[8] After complete formation of Le^y, the proteins were removed by short boiling and centrifugation (4500 rpm, 10 min). The

nucleotides were separated by two passages of anion exchange with HiTrap Q HP-columns (2×5 mL, GE Healthcare Life Sciences) and water as mobile phase. Subsequently, the product was purified by Bio-Gel P-2 (Bio-Rad, Hercules, CA) filtration (1×45 cm, water). Desired fractions were pooled, lyophilized, and stored at −20°C. The carbohydrate was analyzed by fast atom bombardment-sector-mass spectrometry (FAB-sector-MS) and NMR (see Supporting Information for spectra).

FAB-sector-MS analysis verified the identity of the product by detection of the $[M+H]^+$ species at $m/z = 676.2650$ in agreement with the calculated value ($[M+H]^+$, 676.2664; Δ [ppm], −2.1). The results of NMR analysis were in agreement with data reported for chemically synthesized Le^y oligosaccharides.[38,39] ^{1}H NMR (400 MHz, D_2O): $\delta = 5.33$ (d, $J = 3.7$ Hz, 1H, Fuc-H^1), 5.13 (d, $J = 3.4$ Hz, 1H, Fuc-H^1), 4.93–4.80 (m, 3H, including anomeric proton), 4.55 (dd, $J_1 = 3.0$ Hz, $J_2 = 7.7$ Hz, 1H), 4.34–4.26 (m, 1H, anomeric proton), 4.22–4.19 (m, 0.7H, anomeric proton), 4.04–3.62 (m, 18H), 3.53–3.49 (m, 0.7H), 3.21–3.17 (m, 0.5H), 2.07 (s, 3H, GlcNAc-CH$_3$), 1.30 (d, $J = 6.6$ Hz, 3H, Fuc-CH$_3$), 1.27 (d, $J = 6.8$ Hz, 3H, Fuc-CH$_3$); ^{13}C NMR (100 MHz, D_2O): $\delta = 174.35$ (GlcNAc-C=O), 174.14 (GlcNAc-C=O), 100.14 (anomeric center), 99.25 (anomeric center), 98.54 (anomeric center), 94.57 (anomeric center), 91.01 (anomeric center), 76.19, 75.52, 74.88, 74.74, 73.53, 73.28, 73.15, 72.79, 71.85, 71.58, 71.14, 69.62, 69.17, 69.09, 68.66, 68.14, 67.60, 66.81, 66.64, 22.15 (GlcNAc-CH$_3$), 21.88 (GlcNAc-CH$_3$), 15.33 (Fuc-CH$_3$), 15.27 (Fuc-CH$_3$).

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