A Single Aromatic Amino Acid at the Carboxyl Terminus of Helicobacter pylori α1,3/4 Fucosyltransferase Determines Substrate Specificity*

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Fucosyltransferases (FucT) from different Helicobacter pylori strains display distinct Type I (Galβ1,3GlcNAc) or Type II (Galβ1,4GlcNAc) substrate specificity. FucT from strain UA948 can transfer fucose to the OH-3 of Type II acceptors as well as to the OH-4 of Type I acceptors on the GlcNAc moiety, so it has both α1,3 and α1,4 activities. In contrast, FucT from strain NCTC11639 has exclusive α1,3 activity. Our domain swapping study (Ma, B., Wang, G., Palcic, M. M., Hazes, B., and Taylor, D. E. (2003) J. Biol. Chem. 278, 21893–21900) demonstrated that exchange of the hypervariable loops, 345CNDAHYSALH354 in UA948FucT, were sufficient to either confer or abolish α1,4 activity. Here we performed alanine scanning site-directed mutagenesis to identify which amino acids within 345CNDAHYSALH354 of UA948FucT confer Type I substrate specificity. The Tyr350 → Ala mutation dramatically reduced α1,4 activity without lowering α1,3 activity. None of the other alanine substitutions selectively eliminated α1,4 activity. To elucidate how Tyr350 determines α1,4 specificity, mutants Tyr350 → Phe, Tyr350 → Trp, and Tyr350 → Gly were constructed in UA948FucT. These mutations did not decrease α1,3 activity but reduced the α1,4 activity to 61.8%, 78.5%, and 4% of wild type level, respectively. Apparently the aromatic nature, but not the hydroxyl group of Tyr350, is essential for α1,4 activity. Our data demonstrate that a single amino acid (Tyr350) in the C-terminal hypervariable region of UA948FucT determines Type I acceptor specificity. Notably, a single aromatic residue (Trp) has also been implicated in controlling Type I acceptor preference for human FucT III, but it is located in an N-terminal hypervariable stem domain.

Helicobacter pylori is associated with gastritis and peptic ulcer formation and is a risk factor for the development of gastric cancer and mucosa-associated lymphoid tissue lymphoma. One of the virulence factors of H. pylori is the lipopolysaccharide, which contains lipid A, core oligosaccharide and O-antigens. The O-antigens of H. pylori lipopolysaccharide contain fucosylated oligosaccharides, predominantly the Type II blood group antigens, Lewis X (Galβ1,4(Fucα1,3)GlcNAc) and Lewis Y (Fucα1,2Galβ1,4(Fucα1,3)GlcNAc) (1), but a small number of H. pylori strains also express the Type I blood group antigens, Lewis A (Galβ1,3(Fucα1,4)GlcNAc), and Lewis B (Fucα1,2Galβ1,3(Fucα1,4)-GlcNAc) (2).

The role of Lewis antigens in H. pylori pathogenesis is still ambiguous. It has been suggested that Lewis antigens play a role in H. pylori adhesion to (3, 4) or internalization by (5) the gastric epithelial cells. Nevertheless, conflicting evidence argues that Lewis X and Lewis Y are not required for colonization of human gastric epithelium (6) or mouse stomach (7, 8). Lewis antigens may also play an important role in the persistence of H. pylori infection by molecular mimicry, helping the bacteria to evade the host immune response (2, 9, 10). Environmental changes such as pH influence the expression of H. pylori O-antigens, particularly Lewis X and Lewis Y. This may aid in adaptation of the bacterium to its niche in the stomach (10).

Fucosyltransferases (FucTs)3 are enzymes responsible for the last steps in the synthesis of Lewis antigens in H. pylori (11, 12). α1,2 and α1,3 or α1,3/4 FucTs have been identified and characterized in H. pylori (13–18). These FucTs catalyze the transfer of the L-fucose moiety from guanosine diphosphate β-L-fucose (GDP-Fuc) to the OH-2 of the galactose moiety and the OH-3 or the OH-3 and the OH-4 positions of the GlcNAc moiety in glycoconjugate acceptors, respectively. The H. pylori genome contains two homologous α1,3 or α1,3/4 FucT genes, futA and futB (19, 20), but they do not always encode functional proteins. For instance, the futA gene encodes an active FucT in H. pylori strains NCTC11639 and UA948 (13, 17).

Bacterial α1,3/4 FucTs are functionally equivalent to the mammalian α1,3/4 FucTs, which have been well characterized. Mammalian FucTs are Type II membrane proteins with a short N-terminal cytoplasmic tail, transmembrane domain, stem region, and C-terminal catalytic domain. H. pylori FucTs share weak homology with their mammalian counterparts in two small segments within the catalytic domain, called α1,3 FucT motifs (14, 21). H. pylori α1,3/4 FucTs lack the N-terminal domain that attaches the mammalian enzyme to the membrane. Instead, they contain 2–10 heptad repeats that connect the N-terminal catalytic domain with two amphipathic helices at the C terminus (17). These C-terminal heptad repeats and amphipathic helices are believed to be functionally equivalent to the N-terminal stem and transmembrane regions of mammalian FucTs, respectively (22).

Mammalian α1,3/4 FucTs exhibit distinct Type I and Type II acceptor preferences. Human FucT III, V, VI, and bovine FucT share consid-

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3 The abbreviations used are: FucT, fucosyltransferase; GDP-Fuc, guanosine diphosphate β-L-fucose; Type I, Galβ1,3GlcNAc; Type II, Galβ1,4GlcNAc; Type I-R, Galβ1,3GlcNAc-O(CH2)3CO2CH3; Type II-R, Galβ1,4GlcNAc-O(CH2)3CO2CH3; TMR, tetramethylrhodamine; CE-LIF, capillary electrophoresis with laser-induced fluorescence detection; WT, wild type.
eral protein sequence identity (>70%) (23, 24), but they display different acceptor specificity patterns. FucT VI and bovine FucT possess exclusively α1,3 activity, whereas FucT III and V have both α1,3 and α1,4 activity. FucT III favors type I acceptors, therefore, it is predominantly an α1,4 FucT; whereas FucT V prefers type II acceptors, hence it is primarily an α1,3 FucT (25). Domain swapping experiments performed between FucT III and VI (26) or between FucT III and V (27) demonstrated that the N-terminal hypervariable stem region in human FucTs determines acceptor specificity. In particular, Trp111 in human FucT III and VI (26) or between FucT III and V (27) have an Arg at the corresponding position.

Similar to mammalian FucTs, H. pylori α1,3/4 FucTs also exhibit Type I and Type II acceptor specificity. Radiochemical assays showed that 11639FucT is an α1,3 FucT (13), whereas UA948FucT displays both α1,3 and α1,4 activity with a preference for Type II acceptor (17, 22). In an effort to identify the region that determines acceptor specificity, we constructed 12 chimeric FucTs by domain swapping between 11639FucT and UA948FucT in our previous study. In contrast to mammalian FucTs, our data demonstrated that exchange of a small hypervariable region near the C terminus, specifically 347DNPFIFC353 in 11639FucT and 345CNDAHYSALH354 in UA948FucT, was sufficient to either confer or abolish α1,4 activity (22). The goal of our current investigation is to identify specific amino acids within 345CNDAHYSALH354 of UA948FucT that control Type I acceptor recognition.

**EXPERIMENTAL PROCEDURES**

**Materials**—Primers for site-directed mutagenesis were synthesized by Invirogen. Pfu turbo DNA polymerase was purchased from Stratagene. pGEM-T vector was obtained from Promega Co. (Madison, WI). Type II-R (Galβ1,4GlcNAc-O-(CH2)8CO-NHCH2CH2NH-TMR), Type I-R (Galβ1,3GlcNAc-O-(CH2)8CO-NHCH2CH2NH-TMR), and Type I-tetramethylrhodamine (Galβ1,3GlcNAc-O-(CH2)8CO-NHCH2CH2NH-TMR) were kindly provided by Dr. Ole Hindsgaul. C18 Sep-Pak cartridge was obtained from Waters (Milford, MA). α1,3/4-Fucosidase was purchased from Calbiochem-Novabiochem (La Jolla, CA). Anti-pentahistidyl monoclonal antibody, plasmid mini-preparation, and midi-preparation kits were purchased from Qiagen. GDP-Fuc and horseradish peroxidase-conjugated goat anti-mouse IgG were from Sigma. GDP-[3H]Fuc (0.1 mCi ml⁻¹, 17.3 Ci mmol⁻¹) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). The BCA protein assay kit was purchased from Pierce Biotech Inc. (Rockford, IL). Nitrocellulose membrane was obtained from Micron Separation Inc. (Westboro, MA). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Biosciences. BioMax MR film was obtained from Eastman Kodak Co. (Rochester, NY).

**Mutant Construction**—The wild-type (WT) futA gene of 11639FucT or UA948FucT was cloned into pGEM-T vector with a HindIII tag fused at the C-terminal end (22). Using plasmid DNA harboring the futA gene as DNA template, alanine substitutions were created by PCR following the QuickChange site-directed mutagenesis protocol from Stratagene. DNA manipulations were performed in Escherichia coli XL1-blue (Stratagene). The sequences of the primers used for constructing the mutants are shown in TABLE ONE. Each mutant was sequenced to confirm the desired single amino acid mutation. No unexpected mutations occurred during their construction.

**Induction and Expression of H. pylori FucT Genes**—WT and mutant FucT proteins were expressed in E. coli HMS174DE3 cells as described previously (22).

**Standard Fucosyltransferase Assay**—FucT enzyme activities were assayed in reactions containing donor GDP-[3H]Fuc at 200 µM, GDP-[3H]Fuc (60,000 dpm), and Type II-R or Type I-R acceptors at concentrations of 1.8 or 7.5 mM, respectively. The reversed-phase Sep-Pak cartridge was used to isolate the products (containing the hydrophobic aglycone and the acquired radiolabeled fucose moiety) from the unreacted GDP-[3H]Fuc as described previously (30). One milliunit represents the amount of enzyme that converts 1 nmol of acceptor substrate to product per min. The specific activity (milliunits µg⁻¹) was obtained by dividing the enzyme activity (milliunits) by the amount of total protein that was determined by BCA protein assay kit. Specific enzyme activity below 0.01 milliunit µg⁻¹ was considered undetectable.

**Immunoblot Analysis of WT and Mutant FucT Protein Expression**—Cell extracts containing equal amounts of total protein (7.35 µg) were separated by SDS-PAGE, and the proteins were transferred to nitrocellulose membrane (pore size, 0.22 µm). Nitrocellulose blots were probed with mouse anti-pentahistidyl monoclonal primary antibody (1:1000) and goat anti-mouse secondary antibody conjugated with horseradish peroxidase (1:2000). Blots were developed using an enhanced chemiluminescence (ECL) kit, and the images were visualized on BioMax MR films. A standard curve plotting the band density versus the amount of protein loaded for immunoblot analysis was carried out in our previous study, and 7.35 µg of total protein was shown in the middle of the linear range of standard curve (22). The density of each FucT band was quantified using Alpha Ease CF software (Alpha Innotech Corp., San Leandro, CA), and the expression level of WT UA948FucT was set to 1. Subsequently, the expression level of each FucT mutant was determined relative to that of UA948FucT and used to normalize the enzyme specific activity, by dividing the enzyme-specific activity (milliunits µg⁻¹) by the relative FucT expression level.

**Capillary Electrophoresis Assay**—To detect very low levels of α1,4 activity in FucT enzymes, capillary electrophoresis (CE) was used in our current study. The incubation mixtures contained 6.3 µl of the cell crude extract, 10 µM Type I-TMR, 200 µM GDP-Fuc in a total volume of 10 µl, including 20 mM HEPEs (pH 7.0), 100 mM NaCl, 35 mM MgCl2, 1 mM ATP, 5 mg ml⁻¹ bovine serum albumin, and 20 mM MnCl2. The reaction mixtures were incubated at 37 °C for 30 min, 1 h, 2 h, or 5 h. Samples were removed and diluted (1:25) with CE running buffer (10 mM phosphate, 10 mM borate, 10 mM SDS, and 10 mM phenylboronic acid, pH 9.0) to quench the reaction. Samples were subsequently electrokinetically injected onto the electrophoresis capillary (40-cm length and 9-µm internal diameter, Polymeric Technologies, Phoenix, AZ) at 4 kV for 4 s. Products were analyzed by capillary electrophoresis at 450 V cm⁻¹ as described previously (13, 31). The area of the product and substrate peaks were integrated by Igor Pro software (Lake Oswego, OR) and compared with each other to give a quantitative analysis of each reaction. The percentage of Type I-TMR conversion to Lewis A-TMR in each reaction was standardized with the total protein concentration as well as the FucT expression level that was detected by Western blot. The total protein concentration and the FucT expression from WT UA948FucT was set as 1; the relative levels of total protein concentration and FucT expression of each mutant were determined relative to those of WT UA948FucT.

The α1,3/4-fucosidase treatment was performed by removing 4 µl of 5-h reaction mixtures and heating up at 90 °C for 10 min to deactivate residual FucT activity. The mixtures were then incubated with 20 micromolar of α1,3/4-fucosidase in a total volume of 40 µl of 50 mM sodium phosphate buffer (pH 5.0) at 37 °C for 6 h. Products were analyzed by capillary electrophoresis. The percentage of Lewis A-TMR
Accpetor Specificity of H. pylori FucTs

**TABLE ONE**

Sequences of primers used for site-directed mutagenesis

| Primer name       | Primer sequence |
|-------------------|-----------------|
| UA948C345AF       | 5’ GAA AAC GAT ACG ATT TAT CAT gcC AAT GAT GCC CAT TAT TCT GC 3’ |
| UA948C345AR       | 5’ GC AGA ATA ATG Ggc ATG ATT GGC ATG AAT CTG ATC TTT TCC 3’ |
| UA948N346AF       | 5’ GC ACG ATG TAT CAT TGC gcT GAT GCC CAT TAT TCT GCT TCT C 3’ |
| UA948N346AR       | 5’ G AAG AGC AGA ATA ATG GGC ATC Agc GCA ATG AAT CTG ACG 3’ |
| UA948D347AF       | 5’ GAT CGC AAT GAT GCC CAT gcT TCT GCT CCT CAT GC G 3’ |
| UA948D347AR       | 5’ G AAG AGC AGA ATA ATG GGC Agc ATT GCA ATG AAT CTG ACG 3’ |
| UA948H349AF       | 5’ CG ATT TAT CAT TGC AAT GAT GCC gcT TAT GCT CCT CAT CG C 3’ |
| UA948H349AR       | 5’ CG ATG AAG AGC AGA ATA Agc GCC ATC ATT GCA ATG AAT CG 3’ |
| UA948Y350AF       | 5’ CAT TGC AAT GAT GCC CAT gcT TCT GCT CCT CAT GC G 3’ |
| UA948Y350AR       | 5’ CA CAG ATG AAG AGC AGc ATA ATG GGC ATC ATT GA 3’ |
| UA948S351AF       | 5’ GC AAT GAT GCC CAT TAT gcT GCT CCT CAT CG TAT TG 3’ |
| UA948S351AR       | 5’ CA AAT CAC ATG AAG AGC Agc ATA ATG GCC ATC ATT GC 3’ |
| UA948L353AF       | 5’ CA TAT GCT CAT TCT GCT GC TAT GCT CGT TAT TGG AAT GAG CC 3’ |
| UA948L353AR       | 5’ GG CTC ATTCAA ATC ACG ATG Agc AGC AGA ATA ATG GCC ATC ATT G 3’ |
| UA948H354AF       | 5’ GCC CAT TAT TCT GCT gcT GCT GAT TGG AAT GAG CC 3’ |
| UA948H354AR       | 5’ CGG CTC ATTCAA ATC ACG Agc AAG AGC AGA ATA ATG GCC 3’ |
| UA948Y350FF       | 5’ CAT TGC AAT GAC GCC CAT TAT gcT GCT CCT CAT CG G 3’ |
| UA948Y350FR       | 5’ GC AAT GAT GCC CAT TAT gcT GCT CCT CAT CG G 3’ |
| UA948Y350GR       | 5’ CAT TGC AAT GAT GCC CAT ggT TCT GCT CCT CAT CG G 3’ |
| UA948Y350GW       | 5’ CA CAG ATG AAG AGC AGc ATA ATG GGC ATC ATT GA 3’ |
| UA948Y350GW2       | 5’ GC AAT GAT GCC CAT TAT gcT GCT CCT CAT CG G 3’ |
| UA948Y350GW2        | 5’ CA CAG ATG AAG AGC AGc ATA ATG GGC ATC ATT GA 3’ |
| 11639F350Y       | 5’ CG ATT TAT CAC GAT AAC CCT TaC ATT TTC TGT GCT GAT GT 3’ |
| 11639F350Y2       | 5’ CAG ATC ACG ACG ACG AAG AAT GAA GAG GTC ATT GTG AAT CG 3’ |
| 11639F352Y       | 5’ CAG ATC ACG ACG ACG AAG AAT GAA GAG GTC ATT GTG AAT CG 3’ |
| 11639F352Y2       | 5’ CAG ATC ACG ACG ACG AAG AAT GAA GAG GTC ATT GTG AAT CG 3’ |
| 11639F352AR2        | 5’ CAG ATC ACG ACG ACG AAG AAT GAA GAG GTC ATT GTG AAT CG 3’ |

present in each reaction was quantified and standardized with total protein concentration and FucT expression level as described above.

Determination of Kinetic Parameters—Acceptor kinetics were performed using 0.03–2 mM Type II-R, or 0.4–25 mM Type I-R, with GDP-Fuc at 200 μM, including GDP-[3H]Fuc at 0.2 μM. 2 mM Type II-R was the highest concentration that can be reached in our study due to its low solubility. Due to the limited supply of the acceptors, donor kinetics were determined using 3–200 μM GDP-Fuc with Type II-R at 2 mM or Type I-R at 15 mM, including GDP-[3H]Fuc at 0.8 μM. Kinetic parameters were obtained by fitting the initial rate data to the Michaelis-Menten equation using non-linear regression analysis with Prism 4.0 software (GraphPad, San Diego, CA).

RESULTS

Substrate Specificity of UA948FucT and 11639FucT Tyrosine and Phenylalanine Mutants—The Tyr → Ala mutation removes both the aromatic ring and the hydroxyl group of Tyr. To determine the contribution of each group to Type I acceptor specificity in UA948FucT, Tyr350 was mutated to Phe, Trp, and Gly. These mutations did not cause any decrease in 1,4 activity. In addition, to determine the contribution of each group to Type I acceptor specificity in UA948FucT, Tyr350 was mutated to Ala, Gly, and Trp but absent in Ala and Gly is essential for α1,4 activity in UA948FucT, whereas the hydroxyl group in Tyr350 is optimal but not absolutely required.

The hypervariable region 347DNPFIIFC353 in 11639FucT contains two Phe residues, but it shares virtually no sequence homology with the hypervariable loop of UA948FucT. It is not clear which Phe, if any, could be aligned with Tyr350 in UA948FucT. Mutants 11639FucT Phe350 → Tyr and Phe352 → Tyr were constructed to determine if the addition of a hydroxyl group would improve α1,4 activity. In addition, to determine whether or not the presence of an extra bulky aromatic residue in the
variable loop of 11639FucT (instead of a single aromatic amino acid in UA948FucT) is responsible for the poor $\alpha_{1,4}$ activity in 11639FucT, mutants 11639FucT Phe$^{350}$ → Ala and Phe$^{352}$ → Ala were also made.

In comparison to WT 11639FucT, mutants Phe$^{350}$ → Tyr, Phe$^{352}$ → Tyr, and Phe$^{350}$ → Ala possessed a similar level of $\alpha_{1,3}$ activity, whereas Phe$^{352}$ → Ala displayed a reduced $\alpha_{1,3}$ activity at ~25% of WT level. None of the mutants Phe$^{350}$ → Tyr, Phe$^{352}$ → Tyr, Phe$^{350}$ → Ala, or Phe$^{352}$ → Ala displayed any $\alpha_{1,4}$ activity in our standard radiochemical assay. However, when the cell lysates were concentrated 4~5 times and the Type I acceptor concentration in radiochemical assays was raised from 7.5 mM to 25 mM, trace amounts of $\alpha_{1,4}$ activity were detected from all four mutants as well as from WT 11639FucT at 0.01~0.16 milliunit mg$^{-1}$. Again, mutant Phe$^{352}$ → Ala displayed the lowest $\alpha_{1,4}$ activity compared with WT and the other three mutants. Although $\alpha_{1,4}$ activity was just above the background level, it was detectable yet too weak for accurate quantification.

Capillary Electrophoresis Analysis—To measure the low level of $\alpha_{1,4}$ activity more precisely in WT 11639FucT and in its mutants Phe$^{350}$ → Tyr, Phe$^{352}$ → Tyr, Phe$^{350}$ → Ala, and Phe$^{352}$ → Ala, capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) was utilized with tetramethylrhodamine (TMR)-labeled Type I acceptor (Type I-TMR). CE-LIF is an ultra-sensitive analytical technique and can quantitate as few as 60 molecules of enzyme reaction product (32, 33). The WT UA948FucT and its mutants were included as positive controls, and the pGEM vector without a futA insert was taken as a negative control.

When the cell lysate of WT UA948FucT was incubated with 10 $\mu$M Type I-TMR for 5 h, a new peak was produced as shown in the electropherogram (peak 2 in Fig. 2a). This new peak had the same migration time as a synthetic Lewis A-TMR used as running oligosaccharide standard (Fig. 2a, peak 2). The Lewis A-TMR production by WT UA948FucT and its mutants Tyr$^{350}$ → Phe and Tyr$^{352}$ → Trp was rapid with up to 58%~69.5% of Type I-TMR being converted at 30 min, in contrast to 5.1%~7.2% of Type I-TMR being converted by mutants Tyr$^{350}$ → Ala and Tyr$^{352}$ → Gly at 30 min (Fig. 2b). These CE results independently verified our radioactive assay data in Fig. 1. It is notable that a very small peak with a migration time similar to Lewis A-TMR was also observed in the pGEM vector control (Fig. 2a), but the $\alpha_{1,3/4}$-fucosidase treatment data clarified that this small peak did not represent Lewis A-TMR (see below).

The Lewis A peak was also detected in WT 11639FucT and its mutants Phe$^{350}$ → Tyr, Phe$^{352}$ → Tyr, Phe$^{350}$ → Ala, and Phe$^{352}$ → Ala after 5-h incubation at 37 °C, albeit at a very much lower level (Fig. 2a). Compared with UA948FucT, WT 11639FucT and its mutants catalyzed the fucose-transfer reaction very slowly. Only 3.9%~12.7% of Type I-TMR was converted to Lewis A-TMR by 5 h (Fig. 2a). Although this low activity may not play a role in vivo, it is of interest from a mechanistic perspective to see if either Phe residue mimics Tyr$^{350}$ of UA948FucT.
Compared with WT 11639FucT, both Phe350 Tyr and Phe352 Tyr mutations caused an \( \frac{1}{2} \) increase in 1,4 activity. Mutant Phe350 Ala exhibited a marginal increase in 1,4 activity, whereas Phe352 Ala displayed a greater than 2-fold drop. Therefore, unlike Tyr 350 in UA948FucT, neither Phe350 nor Phe352 in 11639FucT seems to be selectively associated with the 1,4 activity. The presence of two bulky aromatic residues in the hypervariable loop of 11639FucT is very unlikely the reason for poor 1,4 activity. It is noted that mutation Phe352 \( \rightarrow \) Ala, but not Phe352 \( \rightarrow \) Tyr, caused a reduction in both 1,3 and 1,4 activity, indicating that the aromatic nature of Phe352 may play an important role in fucose transfer to both Type I and Type II acceptors.

The synthesis of Lewis A-TMR by WT FucTs and the mutants was verified by subsequent 1,3/4-fucosidase treatment of the 5-h reaction mixture. 1,3/4-Fucosidase specifically removes the fucose group at the 1,3 or 1,4 linkage. The quantitative analysis of 1,3/4-fucosidase hydrolysis of Lewis A-TMR from WT FucTs, the representative mutants, and the pGEM control reactions are shown in Fig. 2d. The degradation of Lewis A-TMR to Type I-TMR was observed to various extents in WT FucT enzymes and their mutants but not for the pGEM vector control (Fig. 2d). We also incubated the 5-h reaction mixture of mutant UA948FucT Tyr350 \( \rightarrow \) Trp in sodium phosphate buffer without 1,3/4-fucosidase. After 6 h, the Lewis A-TMR product remained at the same level (Fig. 2d), indicating that Lewis A-TMR is stable if not treated by 1,3/4-fucosidase. This confirms that the newly formed product (peak 2 in Fig. 2a) in WT FucTs and their mutants is indeed the fucosylated structure of Type I-TMR, whereas the small peak for the pGEM vector control (Fig. 2a) is due to an unrelated co-migrating substance of unknown identity.

**Expression of WT and Mutants** — The protein expression of WT and each mutant with a His\(_6\) tag at the C-terminus was determined by immunoblotting using an anti-pentahistidine monoclonal antibody. The expected molecular masses of 11639FucT and UA948FucT are 56.0 kDa and 54.6 kDa, respectively. Every UA948FucT mutant with either Ala or Phe substitution was expressed at a similar level to that of the WT (Fig. 3), and the same results were obtained with Trp and Gly substitution mutants (data not shown). Mutants 11639FucT Phe350 \( \rightarrow \) Tyr, Phe352 \( \rightarrow \) Tyr, Phe350 \( \rightarrow \) Ala, and Phe352 \( \rightarrow \) Ala were also expressed at a similar level to that of 11639FucT (data not shown). These results indicate that point mutations did not cause significant changes in FucT enzyme expression levels.

**Kinetic Parameters of Mutants** — Kinetic parameters of WT FucTs and mutants from UA948FucT are shown in TABLE TWO. Efforts were
made to determine the kinetic parameters of WT 11639FucT and its mutants Phe350 → Tyr, Phe352 → Tyr, Phe350 → Ala, and Phe352 → Ala for using Type I acceptor. Unfortunately, the $K_m$ was very high, so that the kinetic curves were still linear when up to 25 mM Type I acceptor was used (data not shown). As a result it was not possible to determine the kinetic parameters for these enzymes with confidence.

The acceptor kinetic data showed that the substitution of Tyr350 with Phe, Ala, Trp, or Gly in UA948FucT did not modify the $K_m$, to the Type II acceptor, whereas the $V_{\text{max}}$ was either increased or retained at WT level (TABLE TWO), suggesting that Tyr350 is not critical for Type II acceptor recognition. With Type I acceptor, mutant Tyr350 → Phe had similar $K_m$ and $V_{\text{max}}$ values when compared with WT UA948FucT. The $K_m$ and $V_{\text{max}}$ of mutant Tyr350 → Trp is half and two-thirds, respectively, of the WT level, and its $V_{\text{max}}/K_m$ is slightly higher than that of WT (TABLE TWO). Thus the replacement of Tyr350 with Phe or Trp does not significantly affect $\alpha_1\beta_4$ activity. In contrast, Tyr350 → Ala and Tyr350 → Gly showed a dramatic decrease in $V_{\text{max}}$ with a moderate increase in $K_m$ (TABLE TWO), which resulted in a 17-fold decrease of $V_{\text{max}}/K_m$, indicating that the Ala and Gly substitutions not only severely impaired the catalytic rate but also decreased the Type I acceptor binding affinity. The kinetic data confirm that the aromatic nature of Tyr350 in UA948FucT is indispensable for $\alpha_1\beta_4$ specificity.

In addition to the effects on acceptor binding, the $K_m$ for the donor substrate GDP-Fuc was modified by mutations of Tyr350 → Ala, Phe, Trp, or Gly. All mutants displayed tighter binding to the donor than WT, with the apparent $K_m$ value decreasing 2-fold with Type II acceptor, and 2-fold (Tyr350 → Phe and Tyr350 → Trp) or 4-fold (Tyr350 → Ala and Tyr350 → Gly) with Type I acceptor. Such modification of donor binding caused by a single amino acid mutation is not totally unexpected (see below).

**DISCUSSION**

Our previous domain swapping studies showed that the $\alpha_1\beta_4$ specificity of UA948FucT is determined by the C-terminal hypervariable loop (22). Our current data demonstrate that, within the loop, Tyr350 is the only residue that, if mutated to Ala, converts UA948FucT into an enzyme with predominantly $\alpha_1\beta_4$ activity. In contrast, when Tyr350 is mutated to Phe or Trp, a significant level of $\alpha_1\beta_4$ activity is retained, suggesting that the aromatic nature of residue 350 is required for $\alpha_1\beta_4$ activity. Other residues in the loop, particularly Ser351 and Leu353 may play a role in catalysis as mutating them to Ala affects both $\alpha_1\beta_3$ and $\alpha_1\beta_4$ activity. A very similar hypervariable loop sequence (343CDAH-NYSALH)352) has recently been reported for the newly characterized *H. pylori* $\alpha_1\beta_3/4$FucT from strain DSM6709. The underlined Tyr, Ser, and Leu residues correspond to the critical residues of UA948FucT. Indeed, DSM6709FucT is primarily an $\alpha_1\beta_4$ FucT with little $\alpha_1\beta_3$ activity (34).

In our previous work, a substantial level of $\alpha_1\beta_4$ activity (11% of WT UA948FucT) was introduced into 11639FucT when its hypervariable loop was replaced with that of UA948FucT (Chimera 11639FucT (CDAH-NYSALH)352) (22). This is in contrast to the marginal augmentation of $\alpha_1\beta_4$ activity we currently observed when Phe350 or Phe352 in the loop of 11639FucT was mutated to Tyr. Apparently, the Tyr residue itself in the hypervariable loop of 11639FucT is not sufficient for conferring significant $\alpha_1\beta_4$ activity. Some other features, as yet not recognized, within the loop in UA948FucT are certainly needed. Although we have highlighted the vital role of the C-terminal hypervariable loop, particularly the aromatic nature of Tyr350, in controlling the $\alpha_1\beta_4$ specificity in UA948FucT, one should not neglect the fact that other residues also contribute to conferring the $\alpha_1\beta_4$ activity, largely the 110 residues at the N terminus, as we concluded from our previous domain swapping study (22).

The hypervariable loop of 11639FucT contains two aromatic residues (Phe350 and Phe352). The CE-LIF data show that neither Phe350 nor Phe352 is specifically associated with the $\alpha_1\beta_4$ activity, but Phe352 seems to be crucial for both $\alpha_1\beta_3$ and $\alpha_1\beta_4$ activity. No matter if one or two aromatic residues (Phe or Tyr) is in the hypervariable loop, 11639FucT lacks a substantial level of $\alpha_1\beta_4$ activity. This indicates that, unlike Tyr350 in UA948FucT, the aromatic residue at neither 350 nor 352 in 11639FucT seems to be located at the favorable position for fucose transfer to the Type I acceptor. In addition, 11639FucT also lacks the other determinants that contribute to a significant level of $\alpha_1\beta_4$ activity in UA948FucT. As we discussed above, such determinants may include the unique features in the hypervariable loop and the N-terminal 110 amino acids. Similarly, no $\alpha_1\beta_4$ activity was obtained in bovine FucT when its Arg residue was replaced by Trp, which has been shown to be the single residue that confers the $\alpha_1\beta_4$ specificity in human FucT III (28).

UA948FucT is to date the only *H. pylori* enzyme that has been reported to possess substantial $\alpha_1\beta_4$ activity. Low $\alpha_1\beta_4$ activity has been demonstrated for both UA1111FucTa and UA1111FucTb (18). 11637FucTb was shown to only use Type II but not Type I as an acceptor thus it is an $\alpha_1\beta_3$ FucT, even though it can transfer fucose to Type I tetrasaccharide (Galβ1,3GlcNAcβ1,3Galβ1,4Glc), but the fucose was predominantly transferred to the glucose residue (14). FucTs from strains 26695, J99, Sydney SS1, UA1182, and UA802 are predicted to have exclusive $\alpha_1\beta_3$ activity, because Type II Lewis antigens, but not Type I Lewis antigens, were detected on the lipopolysaccharide of these strains by enzyme-linked immunosorbent assay, immunoblot, NMR spectroscopy, or fast atom bombardment-mass spectrometry (15, 35). The sequence alignment of the C-terminal segment of these *H. pylori* FucTs (36) shows that the hypervariable loop region of UA948FucT differs considerably from all other *H. pylori* FucTs (Fig. 4). Nine *H. pylori* FucTs, including 11639FucT and UA1111FucTa, possess an almost identical hypervariable loop. Austra244 FucT contains a similar loop but with three distinctive amino acids. The loop of 26695FucTa and UA1111FucTb is two amino acids longer, and six residues are divergent. In our attempt to measure the $\alpha_1\beta_4$ activity in 11639FucT mutants, we noted that even 11639FucT, which was previously considered as an $\alpha_1\beta_3$ FucT (13, 22), displays a very weak but detectable $\alpha_1\beta_4$ activity when the sensitivity of the assay is increased. As many *H. pylori* FucT enzymes contain a loop sequence that is identical to that of either 11639FucT-Ta, UA1111FucTa, or UA1111FucTb, it is possible that weak $\alpha_1\beta_4$ activity is present in all these *H. pylori* FucTs, but the activity level is too low to be detected by less-sensitive assays. The Lewis A structure generated on the lipopolysaccharide in these strains most likely is too scarce to be biologically significant. Nevertheless, the observation would be of inter-

**FIGURE 3.** Immunoblot of wild type UA948FucT and nine single amino acid mutants from UA948FucT. Wild-type and mutated FucTs with His6-tag were expressed in *E. coli* HMS174DE3 cells with induction using 1 mm isopropyl-β-D-thiogalactopyranoside and growth at 30 °C for 4 h. The FucT proteins were detected with mouse anti-pentahtidistine monoclonal antibody. Lane 1, UA948FucT; lane 2, pGEM vector alone; lane 3, UA948FucT Cys345→ Ala; lane 4, UA948FucT Asn346→ Ala; lane 5, UA948FucT Asp347→ Ala; lane 6, UA948FucT His349→ Ala; lane 7, UA948FucT Tyr350→ Ala; lane 8, UA948FucT Ser351→ Ala; lane 9, UA948FucT Leu353→ Ala; lane 10, UA948FucT His354→ Ala; lane 11, UA948FucT Tyr350→ Phe.
est for the evolution of the FucT family. We speculate that the acquisition of a significant α1,4 activity in some H. pylori FucTs would have been facilitated by the presence of an intrinsically weak α1,4 activity in this family.

The promiscuity of FucT enzymes to act on both Type I and Type II acceptors might result from the stereochemical similarity of these two isomers. The structural differences between Type I and Type II are largely confined to the orientation of the N-acetamido and 6-CH₂OH groups in the GlcNAc moiety. In Type II structures, the N-acetamido group is on the same side as the fucose addition site (OH-3 of GlcNAc), whereas in Type I this position is occupied by 6-CH₂OH (37–39) (Fig. 5). When oligosaccharide (including donor and acceptor substrate) binds to a glycosyltransferase during the catalytic reaction, the oligosaccharide can be distorted (40–42). This was confirmed in several glycosyltransferases with resolved crystal structures as reviewed previously (43). Using a panel of monodeoxynated Type I and Type II acceptor substrates, the 6-OH of the galactose moiety in both Type I and Type II acceptors and the reactive hydroxyl group (the OH-4 and OH-3 of GlcNAc in Type I and Type II, respectively) were found to be essential for the recognition by human FucT III, IV, V (44), VI (45), and human milk α1,3 and α1,3/4 FucTs (46, 47). This suggests that Type I and Type II acceptors, when bound to human α1,3 and α1,3/4 FucTs, most likely adopt a conformation so that the OH-4 of GlcNAc in Type I acceptor is placed in the same position relative to the 6-OH of the galactose moiety as is the OH-3 of GlcNAc in Type II acceptor. To achieve this, the orientation of the GlcNAc in Type I structures needs to be rotated by 180° relative to the galactose moiety, with the nonpolar side of the pyranose ring facing the opposite direction (Fig. 5). This hypothesis is strengthened by NMR studies of Lewis X and Lewis A trisaccharides, where the fucose and galactose in both compounds occupy very similar relative positions with the major difference being the 180° flip of the GlcNAc moiety (48). Thus, to be able to use Type I as an acceptor, FucT enzymes need to support the binding of the GlcNAc in the inverted orientation and to accommodate the reversed orientation of the N-acetamido and 6-CH₂OH groups with the former being more hydrophobic. The same situation may apply to H. pylori FucTs as we have found that the 6-OH of galactose is also critical for the binding of Type I and Type II acceptors to 11639FucT and UA948FucT, one cannot assume that either of the two Phe residues in the 11639FucT loop is aligned with Tyr³⁵⁰ of UA948FucT.

### Table Two

| WT/Mutants | Type II-R | Type I-R | Km for GDP-Fuc |
|-----------|-----------|----------|----------------|
|           | Kₘ  | Vₘₕ | Km/Vₘₕ | Kₘ  | Vₘₕ | Km/Vₘₕ | Kₘ  | Vₘₕ | Km/Vₘₕ |
| 11639     | 0.37 ± 0.03 | 11.8 ± 0.3 | 31.9 | ND' | ND' | ND' | 44.7 ± 5.5 | ND' |
| UA948     | 1.5 ± 0.2 | 29.0 ± 1.8 | 19.3 | 13.4 ± 1.4 | 11.7 ± 0.6 | 0.87 | 56.0 ± 10.1 | 33.7 ± 7.8 |
| UA948 Tyr³⁵⁰→Phe | 1.5 ± 0.2 | 45.0 ± 3.3 | 30 | 16.7 ± 1.4 | 12.1 ± 0.5 | 0.72 | 26.0 ± 2.2 | 15.2 ± 2.8 |
| UA948 Tyr³⁰→Ala | 1.7 ± 0.3 | 48.6 ± 4.9 | 28.5 | 24.4 ± 3.3 | 1.1 ± 0.1 | 0.05 | 26.4 ± 2.9 | 7.2 ± 2.0 |
| UA948 Tyr³⁰→Trp | 1.1 ± 0.1 | 29.0 ± 0.6 | 26.4 | 6.2 ± 0.4 | 7.7 ± 0.2 | 1.2 | 18.6 ± 2.5 | 11.8 ± 1.8 |
| UA948 Tyr³⁰→Gly | 1.8 ± 0.1 | 61.0 ± 1.9 | 33.9 | 19.6 ± 1.2 | 0.9 ± 0.03 | 0.05 | 21.1 ± 1.0 | 9.5 ± 2.5 |

### Notes

1. All FucTs possessed a His tag at the C terminus.
2. Type II-R: Galβ1,4GlcNAc-O(CH₂)₈CO₂CH₃.
3. Type I-R: Galβ1,3GlcNAc-O(CH₂)₈CO₂CH₃.
4. Vₘₕ (milliunits mg⁻¹ min⁻¹) was standardized based on FucT expression quantified by immunoblotting (Fig. 3).
5. Km/Kₘ, milliunits mg⁻¹ min⁻¹.
6. ND, not determined due to the very low level of α1,4 activity, thus the kinetic parameters could not be obtained with confidence.

**FIGURE 4. Alignment of C-terminal hypervariable loop region of 13 H. pylori α1,3/4 FucT enzymes.** Sequence alignments were performed using ClustalW from the LaserGene99 software DNASTAR. Highly conserved residues have a dark background, while partially conserved residues are shown on a gray-shaded background. Numbering at the end of each line refers to the position in each sequence. The α1,3 or α1,4 activity of each FucT is shown in the right column and has either been determined by radiochemical assays, or predicted based on the Lewis antigen expression pattern, or is unknown. The framed amino acids are the residues that have been identified as being responsible for acceptor specificity in 11639FucT and UA948FucT (22). Of note, due to the highly divergent sequence of the hypervariable loops of 11639FucT and UA948FucT, one cannot assume that either of the two Phe residues in the 11639FucT loop is aligned with Tyr³⁰ of UA948FucT.
Acceptor Specificity of H. pylori FucTs

To gain greater insight in the potential mechanism of substrate specificity, the kinetic parameters for four Tyr$^{350}$ mutants of UA948FucT were characterized (Table Two). We noted that mutations of Tyr$^{350}$ to non-aromatic residues generate a moderate increase of $K_m$ value but a rather dramatic reduction of $V_{max}$, implying that the aromatic side chain of residue 350 in UA948FucT is possibly more related to catalytic efficiency than acceptor affinity. All mutations, on the other hand, also caused a 2- to 4-fold decrease of donor $K_m$ values. The finding that a mutation causes modifications of both acceptor and donor binding affinity is not entirely unexpected. The analysis of several glycosyltransferase crystal structures has revealed a common theme for their kinetic mechanisms, with the sugar-nucleotide donor binding to the enzyme first followed by the acceptor (43). The donor binding event induces a marked conformational change of the enzyme in one or two flexible loops from an open to a closed conformation. Such change allows the loops to act as a lid to cover the bound donor and to create the binding sites for the acceptor. Thus the non-reducing end of the acceptor is held in position close to the sugar donor in the active sites prior to the start of the transfer reaction. After the formation of the new glycosidic bond, the oligosaccharide product is released first followed by the release of the nucleotide portion of the donor and eventually the flexible loops return to their original conformation to start a new catalytic cycle (43). Product inhibition studies for human FucT V suggest that this enzyme also follows an ordered sequential bi-bi mechanism (49), and it is likely that the H. pylori FucTs do the same. It is tempting to speculate that the hypervariable loops of H. pylori FucTs corresponds to a similar flexible active site loop that interacts with both donor and acceptor substrates. However, alternative mechanisms are possible and a crystal structure is ultimately needed to reveal the structural basis for enzyme activity and specificity. A better understanding of substrate specificity of H. pylori FucTs may enable us to engineer new enzymes with improved specificity for either Type I or Type II substrates. Such enzymes could be used to synthesize biologically important carbohydrates that contain Lewis structures and might be potential pharmaceuticals in the prevention of bacterial or viral infection, in the neutralization of toxins, and in immunotherapy for cancer (50–54).

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