Biochemical characterization of *Helicobacter pylori* α-1,4 fucosyltransferase: metal ion requirement, donor substrate specificity and organic solvent stability

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Abstract The effect of metal ions on the activity, the donor substrate specificity, and the stability in organic solvents of *Helicobacter pylori* α-1,4 fucosyltransferase were studied. The recombinant enzyme was expressed as soluble form in *E. coli* strain AD494 and purified in a one step affinity chromatography. Its activity was highest in cacodylate buffer at pH 6.5 in the presence of 20 mM Mn^{2+} ions at 37°C. Mn^{2+} ions could be substituted by other metal ions. In all cases, Mn^{2+} ions proofed to be the most effective ($Mn^{2+} > Co^{2+} > Ca^{2+} > Mg^{2+} > Cu^{2+} > Ni^{2+} > EDTA$). The enzyme shows substrate specificity for Type I disaccharide (**1**) with a K_M of 114 μM. In addition, the *H. pylori* α-1,4 fucosyltransferase efficiently transfers GDP-activated L-fucose derivatives to Galβ1-3GlcNAc-OR (**1**). Interestingly, the presence of organic solvents such as DMSO and methanol up to 20% in the reaction medium does not affect significantly the enzyme activity. However, at the same concentration of dioxane, activity is totally abolished.

Keywords *Helicobacter pylori* · α-1,4 Fucosyltransferase · Metal ions · Donor substrate · Organic solvents

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Abbreviations

BSA	Bovine serum albumin
FucT	Fucosyltransferase
GDP-L-fucose	Guanosine 5′-diphospho-β-L-fucose
GDP-2-fluoro-L-fucose	Guanosine 5′-diphospho-2-deoxy-fluoro-β-L-fucose
GDP-D-glucose	Guanosine 5′-diphospho-β-D-glucose
GDP-L-arabinose	Guanosine 5′-diphospho-β-L-arabinose
DMSO	Dimethyl sulfoxide
Lem	Lemieux spacer, $(CH_2)_8COOMe$
Type I	Galβ-1,3GlcNAc-OR

Introduction

Mammalian glycosyltransferases catalyze the transfer of glycosyl-residues from their activated glycosyl-donors, usually sugar nucleotides, to OH-groups of acceptor molecules. Hundreds of glycosyltransferases are known to be involved in the biosynthesis of the oligosaccharide moieties of mammalian glycoproteins and glycolipids (Davies et al. 2005). These transferases are broadly classified into two main families, termed either 'retaining' or 'inverting', depending on whether the anomeric configuration of the donor is retained or inverted in the product glycoside.

Fucosyltransferases (FucTs, EC 2.4.1.xy) belong to the family of inverting enzymes transferring L-fucose from guanosine 5′-diphospho-β-L-fucose (GDP-L-fucose) to oligosaccharide acceptors. They are present in both prokaryotic and eukaryotic organisms (for a recent review see Ma et al. 2006). In bacteria, FucTs were identified in *Salmonella* (McClelland et al. 2001), *E. coli* (Shao et al. 2003) and *Yersinia* (Chain et al. 2004). In addition, the genome of the pathogenic bacterium *Helicobacter pylori* has been shown to encode several FucTs (Tomb et al. 1997; Alm et al. 1999). In this organism FucTs are involved in the synthesis of Lewis A, B, X and Y blood group antigens (Chan et al. 1995; Martin et al. 1997; Monteiro et al. 1998; Wang et al. 2000). As constituents of membrane lipopolysaccharides, these Lewis structures have been suggested to mimic the antigens of the host cell and therefore permit the bacterium to escape from the immune response (Appelmelk et al. 1996; Monteiro et al. 1998).

Despite their involvement into the synthesis of identical oligosaccharide structures, the alignments of *H. pylori* FucTs and mammalian α-1,4 and α-1,3/4 FucT show only low sequence homology, which in addition is restricted to short regions. One example is the peptide motif YxFxLxFENSxxxxYxTEK which is conserved in bovine (Oulmouden et al. 1997), chimpanzee (Costache et al. 1997), and human α-1,3/4 FucTs (Kukowska-Latallo et al. 1990) as well as in α-1,4 *H. pylori* FucT (Rabbani et al. 2005a). The short conserved motif Asp-X-Asp (DXD) or Glu-X-Asp (EXD) present in various mammalian glycosyltransferase families (Breton et al. 1998; Wiggins and Munro 1998), was also found in *H. pylori* α-1,4 FucT (Rabbani et al. 2005a) and in *H. pylori* α-1,3/4 FucT (Horton et al. 2004). The DXD motif located in the catalytic site is essential for the enzyme activity, because it can stabilize a divalent metal ion, which on its part interacts with the diphosphate in GDP-L-fucose (Ünligil et al. 2000; Gastinel et al. 2001). This is in contrast to *H. pylori* α-1,3 FucT, where the interaction with the phosphates of GDP-fucose is established by a direct contact with positively charged amino acid side chains of Arg195 and Lys250 (Sun et al. 2007), redundantizing the DXD or EXD motives.

Human FucTIII and FucTVI have been shown to also accept non-natural donors allowing the synthesis of oligosaccharide libraries with various acceptor substrates as well as modified fucose-donors (Oehrlein 1999 and references therein). In the past, we have already reported the acceptor substrate specificity of *H. pylori* α-1,4 FucT (Rabbani et al. 2005a). In this contribution, we investigated: (1) the requirements of *H. pylori* α-1,4 FucT for metal ions, (2) its specificity for various donor substrates and (3) the influence of different organic solvents on its activity.

Experimental procedures

Materials

The *H. pylori* strain DMS6709 was isolated from endoscopic biopsies at the University Hospital, Gävle, Sweden and deposited by Hoffmann-La Roche, Basel, Switzerland at the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany. *E. coli* AD494 strain has been purchased from Novagen. Ampicillin, kanamycin and protein standard bovine serum albumin (BSA) were obtained from Sigma. Type I acceptor substrates (**1**) and (**2**) (Fig. 4) have been chemically synthesized as described (Lemieux et al. 1975; Rabbani et al. 2005b). GDP-L-fucose (**3**) was a gift from Yamasa Corp., GDP-2-deoxy-2-fluoro-L-fucose (**4**), GDP-L-glucose (**5**) and GDP-D-arabinose (**6**) (Fig. 4) were synthesized as described (Baisch and Ohrlein 1997). GDP-L-[^{14}C]fucose (287 mCi/mmol) was purchased from Amersham Biosciences.

Expression, purification and activity assay

Expression and purification of *H. pylori* α-1,4 FucT were performed as described previously (Rabbani et al. 2005a). For the evaluation of metal ions effect, the effect of pH and organic solvents, GDP-L-[^{14}C]fucose was used as donor substrate. After incubation, the reaction mixture was loaded onto a sep-Pak-C_{18} cartridge (Waters). The cartridge was washed twice with 5 ml bidistilled water and eluted with 5 ml methanol. The enzyme activity was calculated as percentage of [^{14}C]fucose transferred onto the acceptor substrate **1** as determined by liquid scintillation counting. For the evaluation of the donor substrate specificity, the fluorescent-labeled Type I acceptor substrate **2** was employed and the fucosylated product was quantified by RP-HPLC analysis (see below).

HPLC analysis

For the determination of the transfer efficiency of GDP-L-fucose (**3**), GDP-2-deoxy-2-fluoro-L-fucose (**4**), GDP-L-glucose (**5**) and GDP-D-arabinose (**6**) the fluorescent-labeled Type I acceptor substrate **2** was used. The isolation of the product was performed on a C_{18}-RP column (Nova-Pak C_{18}, 60 Å, 3.9 × 150 mm) attached to a Spectra Physics HPLC system (Waters) and the signal was monitored at 245 nm. A linear gradient using water (A) and methanol (B) at a flow rate of 1 ml/min and at RT was run as follow: 0–5 min, 100% A; 5–10 min, 50% A; 10–25 min 25% A; 25–30 min, 100% B.

Effect of metal ions on *H. pylori* α-1,4 FucT

The effect of metal ions was investigated by using $MgCl_2$, $CaCl_2$, $MnCl_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$ and $ZnCl_2$ at a final concentration of 20 mM in cacodylate buffer pH 6.5. The enzyme activity was assayed in the presence of 0.150 mM GDP-L-fucose (**3**) and 2 mM Type I acceptor **1** (saturating donor and acceptor substrate concentrations). For the measurement of the enzyme activity in the absence of metal ions, EDTA was added at 2 mM final concentration.

Determination of kinetic parameters

The K_M values for Type I acceptor **1** in the presence of different metal ions were determined using 0.150 mM of GDP-L-fucose (**3**) and different concentrations of the acceptor substrate **1** ranging from 0.05 to 2 mM. The K_M values were determined from Lineweaver-Burk plots.

pH stability of *H. pylori* α-1,4 FucT

The pH stability of the enzyme was measured in cacodylate buffer at pH values ranging from 5 to 7.5 in the absence and in the presence of 20 mM $MnCl_2$. The enzyme activity assay was carried out as described above using the Type I acceptor substrate **1** and GDP-L-fucose (**3**) as donor substrate.

Organic solvent stability of H. pylori α-1,4 FucT

The enzymatic activity was evaluated in cacodylate buffer pH 6.5 containing 0.150 mM GDP-L-fucose (**3**), 2 mM Type I acceptor **1** and various amounts of

DMSO, methanol or dioxane ranging from 0 to 60% (V/V). The reaction mixtures were then incubated for 30 min at 37°C and the enzyme activity quantified by liquid scintillation counting as described above.

Results and discussion

Mammalian FucTs have been shown to be valuable tools for the chemo-enzymatic synthesis of natural and non-natural oligosaccharides (for reviews see Oehrlein 1999; Koeller and Wong 2000; Ernst et al. 2000). The *H. pylori* FucTs so far identified (Ge et al. 1997; Wang et al. 1999; Rasko et al. 2000; Rabbani et al. 2005a) exhibit similar catalytic properties and therefore represent a competitive alternative to the mammalian enzymes. For an efficient application in the chemo-enzymatic synthesis of oligosaccharides, a detailed characterization of the catalytic properties of our *H. pylori* α-1,4 FucT would be highly beneficial. We therefore investigated the effect of metal ions and different organic solvents on its activity. Furthermore, the pH optimum and the specificity for various unnatural donor substrates were determined.

Effect of manganese ions (Fig. 1)

The enzymatic activity of *H. pylori* α-1,4 FucT was measured in the absence and in the presence of Mn^{2+}
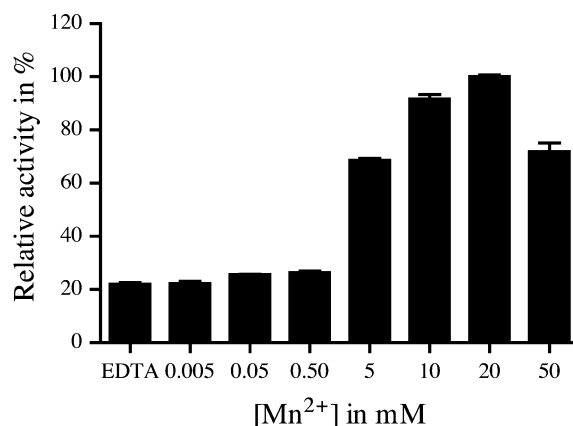

Fig. 1 Effect of manganese ions on *H. pylori* α-1,4 FucT. The activity of the enzyme was assayed in the presence of Type I acceptor substrate **1** (2 mM) and GDP-L-fucose **3** (0.150 mM) and different Mn^{2+} concentrations ranging from 5 μM to 50 mM. EDTA was used at 2 mM concentration. The relative enzyme activity was calculated as a percentage of radioactivity counted in the methanol fraction

ions at concentrations ranging from 5 μM to 50 mM. The in vitro enzyme activity was 5-fold increased at the optimal Mn^{2+} concentration of 20 mM. This is in agreement with the results obtained for human FucTs. Thus, human FucT III was reported to be activated 6-fold in the presence of 10 mM Mn^{2+} (Palma et al. 2004), whereas the activity of human FucT VII was increased even 12-fold in the presence of 25 mM Mn^{2+} (Shinoda et al. 1997). Interestingly, *H. pylori* α-1,4 FucT retains a residual activity of approximately 20% after the addition of EDTA, indicating that manganese ions are not an absolute requirement for activity. This may be the result of a different binding mode of the sugar nucleotide. Whereas in human FucT V (α-1,3 FucT) Mn^{2+} ions have been shown to mediate the complex formation with GDP-L-fucose (**3**) (Murray et al. 1996), *H. pylori* α-1,3 FucT binds the sugar nucleotide directly and without participation of Mn^{2+} ions (Sun et al. 2007). It has been proposed that in this case the role of Mn^{2+} ions is to induce conformational changes leading to an optimal positioning of donor and acceptor substrates in the catalytic site (Qasba et al. 2005).

Effect of alkaline ions, earth and transition metal ions (Fig. 2, Table 1)

The effect of alkaline earth ions and transition metal ions on the activity of *H. pylori* α-1,4 FucT was studied in the presence of constant donor (0.150 mM)
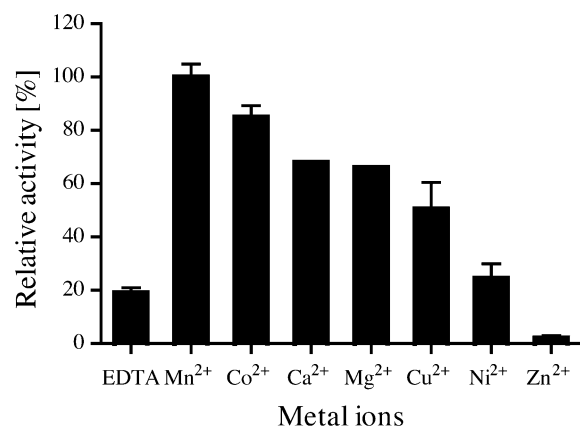

Fig. 2 Effect of alkaline earth and transition metal ions on *H. pylori* α-1,4 FucT. The enzyme activity was assayed in the presence of the indicated metal ions at 20 mM concentration. EDTA was used at 2 mM concentration. The relative enzyme activity was calculated as a percentage of radioactivity counted in the methanol fraction

Table 1 Enzyme kinetic of the *H. pylori* α-1,4 FucT: The K_M for Type I acceptor substrate **1** in the presence of different metal ions was determined using 0.150 mM of GDP-fucose and Type I acceptor concentrations ranging from 0.05 to 2 mM

Metal ions	K_M [μM]
Mn^{2+}	114
Co^{2+}	169.3
Mg^{2+}	126
Ca^{2+}	116.5

The K_M values were determined from Lineweaver-Burk plots

and acceptor substrate (2 mM) concentrations. The results indicate that *H. pylori* α-1,4 FucT can be activated by several divalent ions in the order $Mn^{2+} > Co^{2+} > Ca^{2+} > Mg^{2+} > Cu^{2+} > Ni^{2+} > > Zn^{2+}$. Similarly to the homologous human FucT III (Palma et al. 2004), FucT V (Murray et al. 1996), and FucT VII (Shinoda et al. 1997), *H. pylori* α-1,4 FucT shows a preference for metal ions that mostly adopt octahedral coordination geometries. With Zn^{2+}, which prefers tetrahedral or quadratic pyramidal coordinations, the activity of *H. pylori* α-1,4 FucT disappeared. Furthermore, the K_M for the substrate acceptor Type I (**1**) was only slightly decreased when Mn^{2+} was substituted with Ca^{2+} or Mg^{2+}, however, a substantial increase was observed with Co^{2+}. In summary, these results demonstrate the influence of metal ions on the activity of *H. pylori* α-1,4 FucT and that the substitution of paramagnetic Mn^{2+} by other metal ions such as Mg^{2+} or Ca^{2+} necessary for the study of the enzymatic mechanism by NMR is possible.

pH effect on FucT activity (Fig. 3)

In the presence of Mn^{2+}, the *H. pylori* α-1,4 FucT showed the highest activity at pH 6.5, whereas a slight reduction was observed above and below this pH value. Without Mn^{2+} ions, the enzymatic activity decreased by 70% at pH 5, by 90% at pH 6.5 and was completely abolished at pH 7.5. Since GDP-L-fucose (**3**) is stable between pH 5 and 8 (Nunez et al. 1981), the observed reduction of product formation is not a consequence of the hydrolysis of the fucosyl donor. The observed pH dependence is comparable to human FucTs as documented for example for FucT III. In the presence of Mn^{2+}, the highest activity was observed between pH 6 and 8 and in the absence of
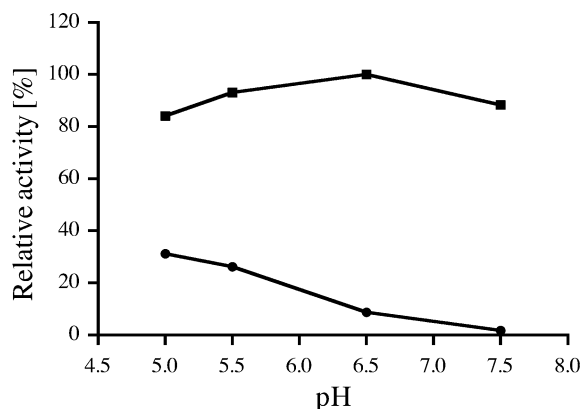
Fig. 3 Effect of pH on the enzyme activity in the presence and absence of Mn^{2+} ions. The enzyme activity was evaluated at different pH values in cacodylate buffer in the presence (■) of 20 mM Mn^{2+} and in the absence (●) of Mn^{2+}. The activity of the enzyme was calculated as a percentage of radioactivity counted in the methanol fraction

Mn^{2+}, the activity is significantly reduced at pH 5 and practically not existing at neutral and alkaline pH values (Palma et al. 2004).

Donor substrate specificity (Fig. 4; Table 2)

For the examination of the donor substrate specificity of *H. pylori* α-1,4 FucT, the sugar nucleotides GDP-2-

Table 2 Donor substrate specificity of *Helicobacter pylori* α-1,4 FucT: The fucosyltransferase assay was run in duplicate (average values are shown) using the dansyl-Type I acceptor substrate **2** at 2 mM concentration

Sugar nucleotides	Relative activity [%]
GDP-L-fucose (**3**)	100
GDP-2-deoxy-2-fluoro-L-fucose (**4**)	79.8
GDP-L-glucose (**5**)	42.5
GDP-D-arabinose (**6**)	19.2

The product of the reaction was analyzed by RP-HPLC as described in material and methods

deoxy-2-fluoro-L-fucose (**4**), GDP-L-glucose (**5**), and GDP-D-arabinose (**6**) were compared with GDP-L-fucose (**3**). With the dansyl-derivative of Galβ(1-3)GlcNAc (**2**), which was used as acceptor substrate, the quantification of the reaction products could be conducted by RP-HPLC. The results indicate a remarkable tolerance of the enzyme toward the different sugar donors. GDP-2-deoxy-2-fluoro-L-fucose (**4**), which has been shown to be a potent competitive inhibitor of the human FucT V (Murray et al. 1996), was transferred efficiently (79%) onto the disaccharide acceptor (**2**). Less effective, but still sufficient for a preparative application, GDP-L-glucose (**5**) and GDP-D-arabinose (**6**) were transferred by 42 and 19%, respectively. Surprisingly, similar results

Fig. 4 Structure of acceptor **1**, **2** and donor substrates **3**, **4**, **5** and **6**

3 (GDP-L-Fuc)

4 (GDP-2-deoxy-2-fluoro-L-Fuc)

5 (GDP-L-Glc)

6 (GDP-D-Ara)
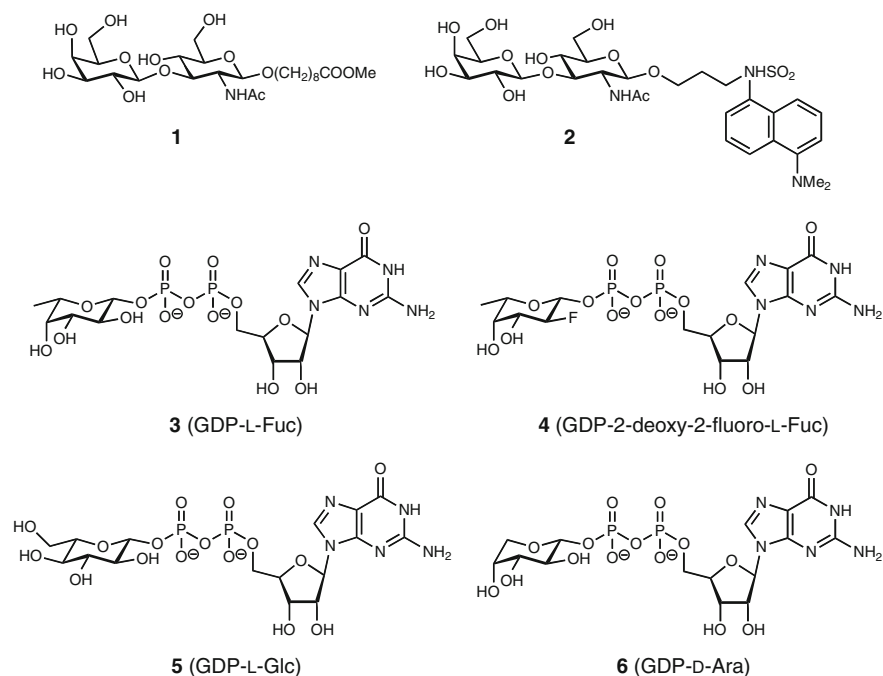
have been reported for the human FucT III (Baisch et al. 1997), since the two enzymes show a very low sequence similarity. The tolerance of *H. pylori* α-1,4 FucT in respect to substrate donors qualifies this bacterial enzyme for the preparation of non-natural oligosaccharides.

Enzyme stability in organic solvents (Table 3)

For the evaluation of the stability of the enzyme toward water miscible organic solvents the enzyme activity was tested in aqueous dimethylsulfoxide (DMSO), methanol and dioxane. With up to 20% DMSO, the activity of the enzyme was only slightly reduced, whereas higher DMSO proportions led to enzyme inactivation. With a content of 20 and 30% methanol, the enzyme activity was only slightly reduced by 10 and 30%, respectively. Interestingly, even at a content of 50% methanol, 11% of the original activity was still sustained. In contrast, the enzyme revealed a much higher sensitivity towards dioxane. Already at 10% dioxane content the activity was reduced by 50%. At 20%, it was practically abolished. The reduced or abolished catalytic activity could be caused by the loss of critical water residues from the surface of the enzyme leading to a drastic decrease in the polarity of the enzymes microenvironment.

In summary, we report further biochemical characterizations of *H. pylori* α-1,4 FucT. In the presence of Mn^{2+} ions, the enzyme activity is increased 5-fold.

Table 3 Reactivity of *H. pylori* α-1,4 FucT in buffer/organic solvent mixtures: The enzyme activity was assayed in the presence of different proportions of methanol, DMSO and dioxane in cacodylate buffer

Buffer/organic solvent (v/v)	Relative activity [%]		
	DMSO	Methanol	Dioxane
100/0	100	100	100
90/10	95	93	48
80/20	70	90	3.8
70/30	26	71	1.3
50/50	5	11	0.7
40/60	3.4	3.5	0.8

The activity of the enzyme was calculated as a percentage of radioactivity counted in the methanol fraction. 100% represents the enzymatic activity in the absence of any organic solvent

Activation, however to a smaller extent, could also be observed with other metal ions as Co^{2+}, Ca^{2+}, Cu^{2+}. Furthermore, the enzyme exhibits a remarkable flexibility towards GDP-L-fucose derivatives. A surprising tolerance towards organic solvents such as DMSO and methanol could be demonstrated, whereas already small amounts of dioxane abolish enzyme activity. Finally, the properties of *H. pylori* α-1,4 FucT recommend its application for the preparative synthesis of oligosaccharides and mimetics thereof.

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