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Molecular cloning and functional expression of a novel *Helicobacter pylori* α-1,4 fucosyltransferase

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Helicobacter pylori **is an important human pathogen which causes both gastric and duodenal ulcers and is associated with gastric cancer and lymphoma. This microorganism synthesizes fucosylated oligosaccharides, predominantly the Galβ-1,4GlcNAc (Type II) blood group antigens Lewis X and Y, whereas a small population also expresses the Galβ-1,3GlcNAc (Type I) blood group antigens Lewis A and B. These carbohydrate structures are known to mimic host cell antigens and permit the bacteria to escape from the host immune response. Here, we report the cloning and characterization of a novel *H. pylori* α-1,4 fucosyltransferase (FucT). In contrast to the family members characterized to date, this enzyme shows exclusively Type I acceptor substrate specificity. The enzyme consisting of 432 amino acids (MW 50,502 Da) was cloned using a polymerase chain reaction (PCR)-based approach. It exhibits a high degree of identity (75–87%) and similar structural features, for example, in the heptamer repeat pattern, with other *H. pylori* FucTs. The kinetic characterization revealed a very efficient transferase (k_{cat}/K_m = 229 $mM^{-1}s^{-1}$) for the Type I acceptor substrate (Gal)-1,3 GlcNAc-Lem (1). Additionally, the enzyme possesses a broad tolerance toward nonnatural Type I acceptor substrate analogs and therefore represents a valuable tool for the chemoenzymatic synthesis of Lewis A, sialyl Lewis A as well as mimetics thereof.**

Key words: enzymatic synthesis/*Helicobacter pylori*/sialy Lewis[a/x] and mimetics thereof/α-1,4 fucosyltransferase

Introduction

Fucosyltransferases (FucTs) are the enzymes responsible for the last step in the synthesis of Lewis antigens. They catalyze the transfer of fucose (Fuc) from guanidine 5′-diphospho-β-L-fucose (GDP-fucose) to oligosaccharide acceptors to form α1,2 (Larsen *et al.*, 1990), α1,3 (Weston *et al.*, 1992), and α1,4 linkages (Kukowska-Latallo *et al.*, 1990).

Although the chemical synthesis of carbohydrates is well developed (for reviews, see Ernst *et al.*, 2000a), the preparation of a particular oligosaccharide still remains a costly and cumbersome challenge. A complementary approach to the chemical synthesis of physiologically important oligosaccharides is the combined use of chemical and enzymatic methods. It has also been shown that glycosyltransferases can be successfully employed for the synthesis of a wide range of nonnatural oligosaccharides as well (Palcic and Hindsgaul, 1991; Baisch *et al.*, 1996a,b,c; Gijsen *et al.*, 1996; Ernst and Oehrlein, 1999; Ernst *et al.*, 2000b; Seto *et al.*, 2000; Blixt *et al.*, 2002; Bintein *et al.*, 2003). For their heterologous production mammalian (Larsen *et al.*, 1990; Kitagawa and Paulson, 1994) and baculovirus expression systems (Morais *et al.*, 2001; Kim *et al.*, 2003) as well as yeast (Malissard *et al.*, 2000) have been used.

Recently, several reports demonstrated the ability of human pathogenic bacteria such as *Helicobacter pylori* (Chan *et al.*, 1995; Martin *et al.*, 1997; Monteiro *et al.*, 1998; Wang *et al.*, 2000), *Campylobacter jejunii* (Gilbert *et al.*, 2000), *Neisseria gonorrhoeae,* and *Neisseria meningitis* (Gilbert *et al.*, 1996) to synthesize Lewis A and B as well as Lewis X and Y blood group antigens. As constituents of membrane lipopolysaccharides, these oligosaccharides mimic the host cell antigens and permit the bacterium to escape from the immune response (Appelmelk *et al.*, 1996; Wirth *et al.*, 1996; Gilbert *et al.*, 2000). The sequenced genome of two strains of the gastric pathogenic bacterium *H. pylori* revealed many putative FucT sequences (Tomb *et al.*, 1997; Alm *et al.*, 1999). As a consequence, the cloning and expression of *H. pylori* α-1,2 (Wang *et al.*, 1999), α-1,3 (Ge *et al.*, 1997; Martin *et al.*, 1997) and α-1,3/4 FucTs (Rasko *et al.*, 2000) has been reported.

Our interest in glycosyltransferases for the preparative chemoenzymatic synthesis of carbohydrates prompted us to search for cost effective sources. In this study, we report the cloning, functional expression, and characterization of a novel *H. pylori* α-1,4 FucT in *Escherichia coli* and its preliminary use for the chemoenzymatic synthesis of Lewis A, Galβ-1,3(Fucα-1,4)GlcNAc (Le[a]), sialyl Lewis A, NeuNAcα-2,3Galβ-1,3(Fucα-1,4)GlcNAc (sLe[a]), and mimetics thereof.

Results

Cloning of a H. pylori *fucosyltransferase*

In order to clone a gene encoding an α-1,3 or α-1,3/4 FucT in *H. pylori* strain DMS 6709, primers derived from the highly homologous *H. pylori* FucT sequences available in

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NCBI Genbank (AF194963, AAD06573 and AAD06169) were designed and used to amplify a PCR product of 1375 bp. The fragment was double-digested by EcoRI/BamHI and cloned into the expression vector pEZZ18. This vector offers the advantage of generating a recombinant protein containing two synthetic IgG-binding domains (ZZ) of *Staphylococcus aureus* protein A and an export signal for protein secretion in the culture medium. After transformation in *E. coli*, five positive clones were selected according to PCR and restriction enzyme control. The sequence of their first 400–500 bp from the N-terminus showed 100% identity at the amino acid level. Protein expression was carried out with three clones resulting in a secreted fusion ZZ-FucT polypeptide with a calculated molecular mass of 64,502 Da. FucT without ZZ-fusion domain encodes a 436-amino acid polypeptide with a predicted molecular mass of 50,502 Da. The full nucleotide sequence for one clone was confirmed by the double-stranded sequencing strategy and registered at the GenBank™/EBI Data Bank (AY450598).

Features of the novel H. pylori *fucosyltransferase sequence*

The nucleotide and the deduced amino acid sequence of the cloned FucT are shown in Figure 1A. The Kyte–Doolittle plot analysis shows a very hydrophilic protein without any transmembrane segments (Figure 1B). A sequence blast of our new FucT against the *H. pylori* genomes of J99 and 26695 strains demonstrates a high degree of similarity to two FucTs in strain J99 (NP223719.1 and NP223314.1) and two α-1,3 FucTs in strain 26695 (NP207177.1 and NP207445.1). The alignment of the amino acid sequence revealed a high degree of similarity to other *H. pylori* FucTs, for example 87% identity to the α-1,3 FucT (Martin *et al.*, 1997) and 75% identity to the α-1,3/4 FucT (Rasko *et al.*, 2000). This is in agreement with human FucTs sharing a high level of amino acid sequence similarity even when they exhibit distinct acceptor substrate specificities (Kukowska-Latallo *et al.*, 1990; Weston *et al.*, 1992; Xu *et al.*, 1996). In analogy to other *H. pylori* FucT sequences, the new FucT also contains repetitive sequences of the heptapeptide DDLRINY close to the C-terminus (Figure 1A). However, there are only four instead of seven, eight or ten repeats observed in *H. pylori* α-1,3 FucT (strain NCTC11637, Chan *et al.*, 1995), *H. pylori* α-1,3/4 Fuc (strain UA948, Rasko *et al.*, 2000), and *H. pylori* α-1,3 FucT (strain NCTC11639, Ge *et al.*, 1997), respectively. The intervals between the leucines show a high regularity similar to the eukaryotic leucine zipper motif, which is believed to be involved in protein–protein and protein–DNA interactions (Landschulz *et al.*, 1988). The peptide motif YxFxLxFENSxxxxYxTEK found in *H. pylori* α-1,3/4 FucT (Rasko *et al.*, 2000) as well as in the bovine FucT III (Oulmouden *et al.*, 1997), the panther FucT III (Costache *et al.*, 1997), human α-1,3/4 FucT (FucT III, Kukowska-Latallo *et al.*, 1990), and human α-1,3 FucT (FucT V, Weston *et al.*, 1992) is also conserved in the novel *H. pylori* FucT sequence (Figure 1A and C). Finally, in the reported *H. pylori* FucT a peptide sequence (343–352 : CDAHNYSALH) similar to *H. pylori* α-1,3/4 FucT (345–354: CNDAHYSALH) is present (Ma *et al.*, 2003) (Figure 1A). It seems to be related to the acceptor specificity.

(A)

```
atgttccagcccttactagacgcttatatagacagcacccgtttagatgaaaccgattat
M  F  Q  P  L  L  D  A  Y  I  D  S  T  R  L  D  E  T  D  Y
aagccccccctaaaaatcgctgtggcgaattggtggggaggcgttgaagaatttaaaaag
K  P  P  L  K  I  A  V  A  N  W  W  G  G  V  E  E  F  K  K
agcactctttatttcatcttaagccaacgctacacaatcactttacaccgaaaccctgat
S  T  L  Y  F  I  L  S  Q  R  Y  T  I  T  L  H  R  N  P  D
aaacctgcggacatcgttttttggtaaccctcttggatcggctagaaaaatcttatcttat
K  P  A  D  I  V  F  G  N  P  L  G  S  A  R  K  I  L  S  Y
caaaacgcaaaaagagtgttttacacgggtgaaaatgaagtccctaacttcaacctcttt
Q  N  A  K  R  V  F  Y  T  G  E  N  E  V  P  N  F  N  L  F
gattacgccataggctttgatgaattggattttaatgatcgttatttgagaatgcctttg
D  Y  A  I  G  F  D  E  L  D  F  N  D  R  Y  L  R  M  P  L
tattacgcccatctgcactatgaagctgagcttgttaatgacaccacttcaccttacaag
Y  Y  A  H  L  H  Y  E  A  E  L  V  N  D  T  T  S  P  Y  K
attaaagacaacagcctttatgctttaaaaaaaccttcccatcattttaaagaaaaccac
I  K  D  N  S  L  Y  A  L  K  K  P  S  H  H  F  K  E  N  H
cctaatttgtgcgcagtagtgaataatgagagtgatcctttgaaaagagggtttgcgagt
P  N  L  C  A  V  V  N  N  E  S  D  P  L  K  R  G  F  A  S
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F  V  A  S  N  P  N  A  P  K  R  N  A  F  Y  D  A  L  N  S
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G  Y  V  T  E  K  I  L  D  A  Y  F  S  H  T  I  P  I  Y  W
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G  S  P  S  V  A  K  D  F  N  P  K  S  F  V  N  V  H  D  F
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N  N  F  D  E  A  I  D  H  V  R  Y  L  H  T  H  P  N  A  Y
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L  D  M  L  Y  E  N  P  L  N  T  L  D  G  K  A  Y  F  Y  Q
aatttgagttttaaaaaaatcctagatttttttaaaacgattttagaaaacgatacgatt
N  L  S  F  K  K  I  L  D  F  F  K  T  I  L  E  N  D  T  I
tatcattgcgatgcccataattattctgctcttcatcgtgatttgaatgagccgttagtg
Y  H  C  D  A  H  N  Y  S  A  L  H  R  D  L  N  E  P  L  V
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S  I  D  D  L  R  I  N  Y  D  D  L  R  I  N  Y  D  D  L  R
atcaattatgatgatttgagaatcaattatgagcgcctttttgcaaaacgcttcgccttta
I  N  Y  D  D  L  R  I  N  Y  E  R  L  L  Q  N  A  S  P  L
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L  E  L  S  Q  N  T  S  F  K  I  Y  R  K  A  Y  Q  K  S  L
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P  L  L  R  A  I  R  R  W  V  K  K  -  V  C  K  T  N  P  S
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N  P  L  R  Y  H  R  R  R  Y  F
```

(B)

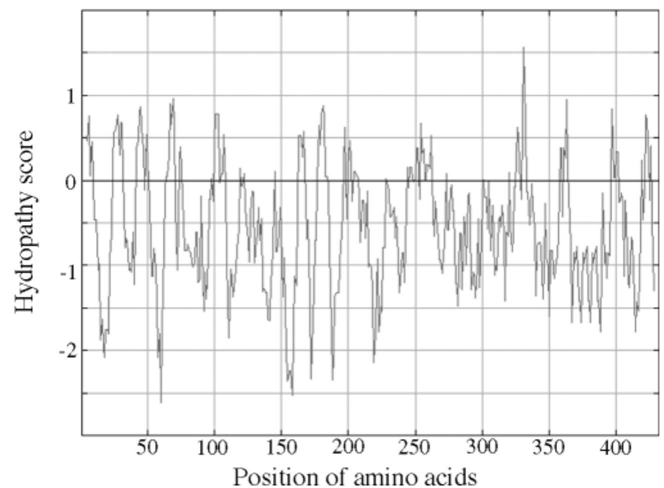

Fig. 1. (**A**) Nucleotide sequence and deduced amino acids sequence of the novel *Helicobacter pylori* α-1,4 fucosyltransferase (FucT). The heptamer-repeat motifs are underlined and in boldface type. Residues denoted by boldface type represent the conserved α-1,3 (Martin *et al.*, 1997) and α-1,3/4 FucT (Rasko *et al.*, 2000) motifs. Residues denoted in italic and boldface type seem to govern the Galβ-1,3GlcNAc (Type I) acceptor substrate specificity (Ma *et al.*, 2003). (**B**) Kyte–Doolittle hydropathy profile of the novel *H. pylori* α-1,4 FucT based on the DNA sequence. The enzyme shows a very hydrophilic profile without any transmembrane segments. (**C**) Amino acid sequence alignment of the bovine (Q11126), panther (O19058), human FucT III (P21217), human FucT V (Q11128), and *H. pylori* α-1,3/4 FucT (Q9L8S4) with the novel *H. pylori* α-1,4 FucT. The conserved α-1,3/4 FucTs peptide motif is indicated in boldface type.

(C)

Bovine FucT III	QLSQYKFYLA FENSLHPDYI TEKLWKNALQ AWAVPVVLGP SRVNYEQFLP
Panther FucT III	TLSRYKFYLA FENSLHPDYI TEKLWRNALE AWAVPVVLGP SRSNYERFLP
Human FucT III	TLSRYKFYLA FENSLHPDYI TEKLWRNALE AWAVPVVLGP SRSNYERFLP
Human FucT V	TLSRYKFYLA FENSLHPDYI TEKLWRNALE AWAVPVVLGP SRSNYERFLP
H. pylori α-1,3/4 FucT	FLSQYKFNLC FENSQGYGYV TEKIL.DAYF SHTIPIYWGS PSVAKDFNPK
H. pylori α-1,4 FucT	FLSQYKFNLC FENSQGYGYV TEKIL.DAYF SHTIPIYWGS PSVAKDFNPK
Consensus	Y.F.L. FENS....Y. TEK....A.. ....P...G

Fig. 1. continued

Expression and affinity purification of the recombinant soluble H. pylori *fucosyltransferase*

The vector containing the recombinant enzyme was transformed into the *E. coli* strains Top10, DHα5, and AD494. The AD494 strain cultivated in TB medium showed the highest level of enzyme expression (5 mg/L and 5U/mg) and therefore was used for further enzyme production. *E. coli* AD494 strain is a thioredoxin reductase mutant that enables disulfide bond formation and thus provides the potential to produce properly folded proteins (Derman *et al.*, 1993). The soluble enzyme secreted in the culture medium was recovered in a one step procedure by affinity chromatography using a GDP-hexanolamine column leading to much higher yields of active FucT than affinity purification with IgG sepharose (data not shown). The recombinant enzyme was stable for several months at −70°C in cacodylate buffer containing 10% glycerol.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis

To verify the degree of purification, the novel FucT was analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by silver staining showing a single band at the expected molecular mass of approximately 64.50 kDa (Figure 2). In addition, Figure 2 also shows the difference in the level of FucT expression in *E. coli* AD494 (clone 1) as compared to other strains (DHα5 and Top10).

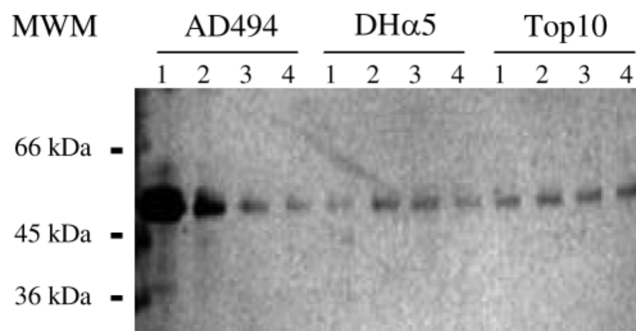

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of affinity purified *H. pylori* α-1,4 fucosyltransferase. The enzyme was expressed in AD494, DHα5, and Top10 *Escherichia coli* cells. Single colonies from each clone (1, 2, 3, and 4) were grown overnight at 37°C and by vigorous shaking (300 rpm) in Terrific Broth medium (TB). The protein purification was performed as described in *Materials and methods*. Equal volumes (10 μL) from the eluted fractions were separated on 10% SDS–PAGE followed by silver staining. The molecular weight marker (MWM) is indicated on the left.

Acceptor specificity of the recombinant soluble H. pylori *FucT*

To determine the specificity of the novel FucT, Galβ-1,3GlcNAc (Type I) and Galβ-1,4GlcNAc (Type II) disaccharides as well as their α-2,3 sialylated forms were used as acceptors and GDP-fucose as donor (Figure 3). All acceptor substrates contain the lipophilic Lemieux substituent (Lem : $O(CH_2)_8COOMe$) at the reducing end to facilitate the purification of the fucosylated products on Sep–Pak-C_{18} cartridges (Hindsgaul *et al.*, 1991). The kinetic parameters K_m and k_{cat} obtained are summarized in Table I. Under standard conditions, the novel transferase showed excellent activity with the Type I acceptor substrates **1** and **2**, whereas the Type II acceptor substrates **3** and **4** were only moderately fucosylated even at acceptor substrate concentrations of 2 mM. Thus, the novel bacterial enzyme showed the properties of an α-1,4 FucT, although the primers used for the sequence generation were derived from α-1,3 and α-1,3/4 FucT sequences. This is, however, not astonishing, because high homologies of *H. pylori* FucT sequences are known for the human enzymes. Similar to the human α-1,3/4 FucT (Kukowska-Latallo *et al.*, 1990), *H. pylori* α-1,4 FucT accepted 3′-sialyl-lacto-N-biose (**2**) slightly better than lacto-N-biose (**1**). The affinity of the enzyme for the GDP-fucose was very high (K_m = 5.73 μM). In general, depending on the strains (UA 861, UA 802, and UA 1182 [Chan *et al.*, 1995] and NCTC 11637 [Martin *et al.*, 1997]), the affinity of bacterial enzymes for GDP-fucose varies from 17 to 140 μM (Chan *et al.*, 1995; Ma *et al.*, 2003).

To study the applicability of the novel α-1,4 FucT to the preparative enzymatic syntheses of sLe^a mimetics, modified Type I oligosaccharides containing replacements of the GlcNAc and/or *N*-acetylneuraminic acid (NeuNAc) moiety by noncarbohydrate structures were evaluated. The enzymatic activity was quantified as the amount of radioactivity transferred from GDP-L-[^{14}C]Fuc to the corresponding acceptor (in % relative to **1**, Table II). The pseudotrisaccharide **5**, in which GlcNAc is substituted by (*R*,*R*)-cyclohexan-1,2-diol and NeuNAc by (*R*)-cyclohexyllactic acid showed only minimal acceptor properties (5.1%). The (*S*)-lactic acid derivative **6**, however, proved to be an excellent acceptor substrate (100%). The activity was practically abolished when compound **8** [GlcNAc moiety replaced by (*R*,*R*)-butan-2,3-diol and NeuNAc by (*S*)-3-phenyllactic acid] was used as acceptor substrate. Finally, reduced activity

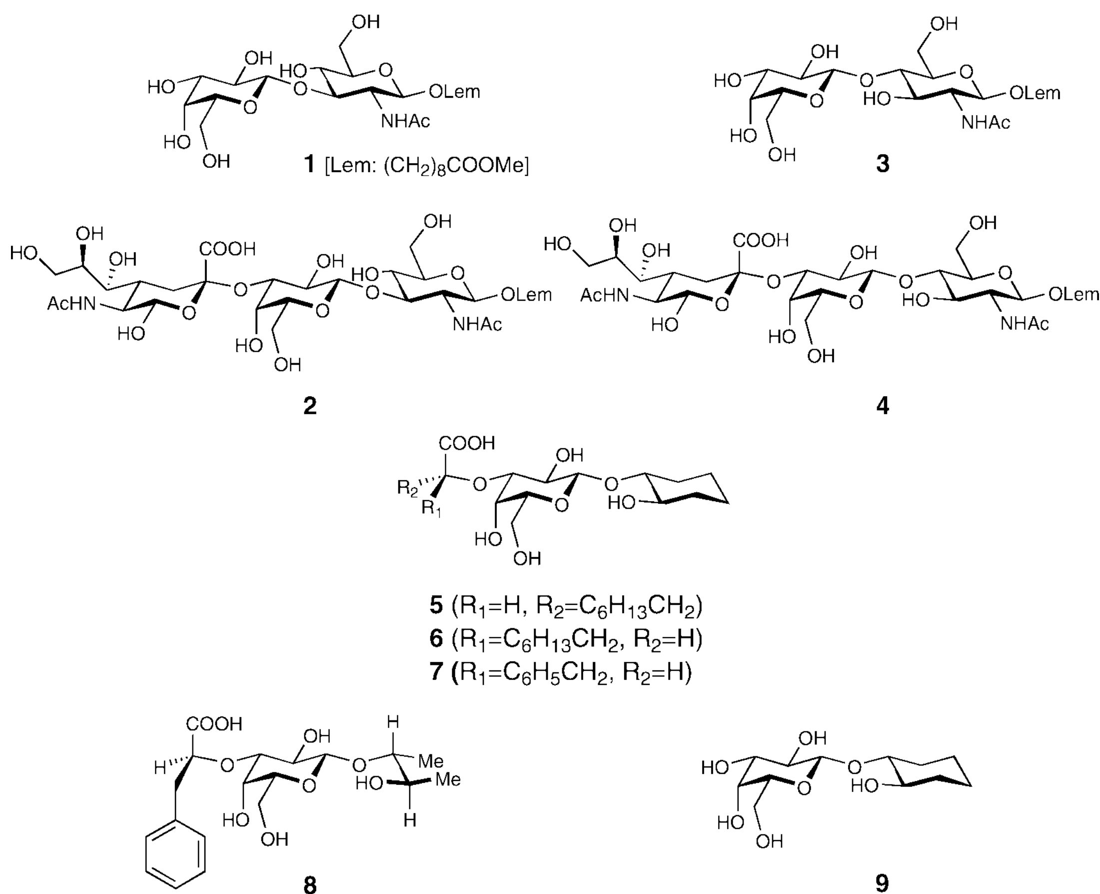

Fig. 3. Structures of natural (**1** and **2**) and nonnatural substrates (**3**–**9**).

Table I. Acceptor substrate specificity of the novel *Helicobacter pylori* α-1,4 fucosyltransferase: relative enzyme activity (100% correspond to the transfer of 3.76 μmol fucose/mg protein/min)

Acceptors	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Galβ-1,3GlcNAc-R (**1**)	114	26.12	229
Siaα-2,3Galβ-1,3GlcNAc-R (**2**)	111	34.33	310
Galβ-1,4GlcNAc-R (**3**)	313	0.067	0.21
Siaα-2,3Galβ-1,4GlcNAc-R (**4**)	432	0.114	0.26
GDP-fucose	5.73	3.947	689

The assay was conducted as described in *Materials and methods.*

Table II. Enzyme activity of the novel *Helicobacter pylori* α-1,4 fucosyltransferase with Galβ-1,3GlcNAc (Type I) and Galβ-1,4GlcNAc (Type II) acceptor

Acceptors	Relative activity (%)
1	100
2	133
3	0.3
4	2.7
5	5.1
6	100
7	8.6
8	0.9
9	2.8

The fucosyltransferase assay was run in duplicates using Type I or Type II acceptors and 0.150 mM 5′-diphospho-β-L-fucose (GDP-fucose). For the determination of the K_m for GDP-fucose, 2 mM of Type I acceptor substrate **1** was used. The reactions were quantified as described in *Materials and methods.*

was also observed with the acceptor substrates **7** (8.6%) and **9** (2.8%).

N-*ethylmaleimide inhibition assays and thermal stability*

FucTs have been discriminated according to their sensitivity to the sulfhydryl-binding reagent *N*-ethylmaleimide, NEM (Chou *et al.*, 1977; Campbell and Stanley, 1984). The human Lewis (FucT III) and the plasma-type (FucT VI) enzymes are inhibited by NEM, whereas others such as the myeloid-type enzyme (FucT IV) are insensitive to NEM treatment (Holmes *et al.*, 1995). The sensitivity of the novel bacterial α-1,4 FucT to NEM was very high, as 90% of the enzyme activity was abolished in the presence of 10 mM NEM (Figure 4). In contrast, it has been shown that

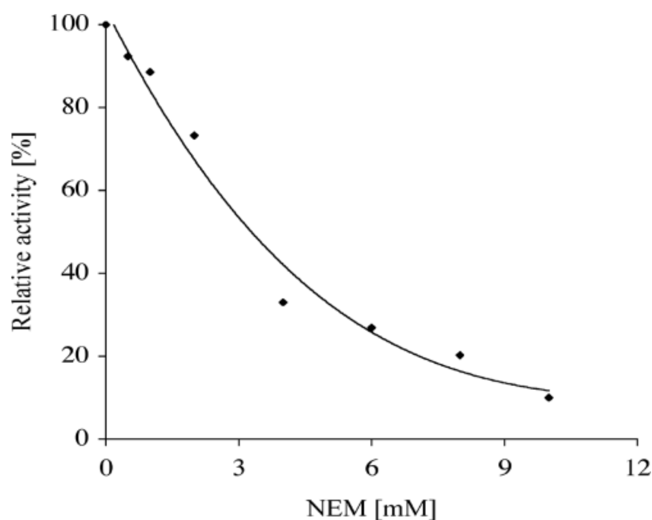

Fig. 4. *N*-ethylmaleimide (NEM) inhibition of the novel *Helicobacter pylori* α-1,4 fucosyltransferase. NEM was added to the ice-cooled reaction 30 min before incubation with Galβ-1,3GlcNAcβLem (**1**) to give final concentrations of 0.5–10 mM. The enzymatic reaction was quantified as described in *Materials and methods*.

H. pylori α-1,3 FucT is only minimally inhibited by NEM (Martin *et al.*, 1997).

As next, the applicability of the novel FucT at elevated temperature was tested. The recombinant α-1,4 FucT shows maximal activity between 37 and 42°C, below (25°C, 70%) and above (50°C, 60%) the catalytic activity was slightly reduced. This is in contrast to human FucT VII (α-1,4 FucT) expressed in *Sf*9 insect cells (Shinkai *et al.*, 1997), which shows a more pronounced deactivation at elevated temperatures (50°C, 35%).

Discussion

Recently, several putative FucT sequences have been identified in the genome of *H. pylori* (Tomb *et al.*, 1997; Alm *et al.*, 1999). Consequently, considerable attention has been devoted to this bacterium to understand the molecular mechanism of pathogenicity but also to use it as a cost effective source of glycosyltransferases. *Helicobacter pylori* strains express on their surface predominantly lipopolysaccharides containing Type II Lewis antigens (Wirth *et al.*, 1996; Simoons-Smit *et al.*, 1996). Type I Lewis antigens, however, are found only in a limited number of strains (Wirth *et al.*, 1996; Monteiro *et al.*, 1998).

In this study, we report the molecular cloning, functional expression and characterization of a novel *H. pylori* α-1,4 FucT. Analysis of the predicted protein sequence revealed a high degree of identity to other *H. pylori*, for example, α-1,3 (Martin *et al.*, 1997; Ge *et al.*, 1997) and α-1,3/4 FucTs (Rasko *et al.*, 2000). Within the FucT family the high level of sequence homology does not correlate with their substrate specificities (Kukowska-Latallo *et al.*, 1990; Weston *et al.*, 1992). Similar to other *H. pylori* FucT sequences, there is a direct repeat of heptamers close to the C-terminus. However, the number of these repeats is limited to four instead of seven, eight or ten found in other *H. pylori* FucTs (Ge *et al.*, 1997; Martin *et al.*, 1997; Rasko *et al.*, 2000). The influence of the number of heptameric repeats on the substrate specificity has not been investigated yet. However, the deletion of the ten repeat motifs in *H. pylori* strain NCTC11639 (Ge *et al.*, 1997) generates an inactive transferase, suggesting a role of these motifs in the enzyme function.

Recently, Ma *et al.* (2003) reported the construction of enzyme chimeras by subdomain swapping between α-1,3 and α-1,3/4 *H. pylori* FucTs. The result indicates that Type I acceptor specificity seems to be governed by ten amino acids (CNDAHYSALH) corresponding to residues 345–354 in α-1,3/4 FucT. In the reported *H. pylori* α-1,4 FucT a similar peptide sequence (343–352) is present (Figure 1A).

The expression of the novel *H. pylori* FucT in *E. coli* AD494 cells yields an enzyme, which fucosylates Galβ-1,3GlcNAcβ-Lem (**1**) and its α-2,3 sialylated form **2** with high efficiency (Table I). However, even at acceptor substrate concentrations up to 2 mM, the activity with the *N*-acetyllactosamine (LacNAc) derivatives **3** and **4** was not significant (0.3–2.7%). In contrast to the activity of the *H. pylori* α-1,3/4 FucT, which is 5 to 20-fold higher with LacNAc compared to the Type I acceptor substrate lacto-N-biose (Rasko *et al.*, 2000), the novel *H. pylori* FucT shows an almost exclusive acceptance for Type I substrates. In preliminary experiments for the preparative chemoenzymatic synthesis of $sLe^{a/x}$ mimetics as potential E-selectin antagonists, the novel *H. pylori* FucT showed a remarkable tolerance for a variety of nonnatural acceptor substrates (Figure 3, Table II). Although some of the activities were relatively weak, they are still sufficient for a preparative enzymatic approach. Furthermore, these results suggest that NeuNAc can be substituted by analogs with an appropriate stereochemistry, for example [*S*]-lactic acid derivatives. In addition, these replacements are also tolerated in combination with GlcNAc mimetics such as (*R,R*)-butan-2,3-diol or (*R,R*)-cyclohexan-1,2-diol.

Besides their acceptor substrate specificity, FucTs have been classified according to their sensitivity toward NEM, which is known to modify proteins by forming a covalent linkage with the sulfhydryl group of cysteine residues (Chou *et al.*, 1977; Campbell and Stanley, 1984). Our bacterial α-1,4 FucT was inhibited to 90% with 10 mM NEM, whereas the activity of the *H. pylori* α-1,3 FucT was reduced by only 35% in the presence of 15 mM NEM (Martin *et al.*, 1997). This is in accordance with the data obtained for the corresponding human FucTs (Holmes *et al.*, 1995). Examination of the cysteine distribution within the two highly homologous *H. pylori* enzymes (87% identity) shows two conserved cysteines (Cys164 and Cys233 in α-1,4 FucT and Cys168 and Cys237 in α-1,3 FucT) as well as a third cysteine, which is located differently in the two sequences (α-1,4 FucT : Cys343; α-1,3 FucT : Cys282). Site-directed mutagenesis analysis should provide the information on the cysteine residue responsible for sensitivity toward NEM.

Finally, it has been shown that differences in *N*-glycosylation affect the thermal stability of FucTs. Therefore, depending on the expression system, FucTs show different thermal profiles, for example *Sf*9 verus Namalwa KJM-1 cells (Shinkai *et al.*, 1997). Interestingly, the catalytic activity

of *H. pylori* α-1,4 FucT expressed in *E. coli* is only slightly affected by elevated temperatures.

In summary, this study reports the complete gene sequence of a novel α-1,4 FucT. Sequence comparison indicates high similarity with other *H. pylori* FucTs and to a lower extent with eukaryotic FucTs. The enzyme catalyzes almost exclusively the fucosylation of Type I acceptor substrate and mimetics thereof. Whereas active mammalian FucTs cannot be expressed in *E. coli* because of the lack of glycosylation (Kukowska-Latallo *et al.*, 1990; Larsen *et al.*, 1990), the expression of active *H. pylori* α-1,4 FucT is possible. This allows the cost-effective bacterial production of FucTs in preparative amounts and their use in large-scale enzymatic syntheses of oligosaccharides.

Materials and methods

Materials

The *H. pylori* strain was isolated from endoscopic biopsies at the University Hospital, Gävle, Sweden and deposited by Hoffmann-La Roche, Basel, Switzerland, under the number DMS6709 at the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany. *Escherichia coli* JM109, DHα5, Top10, and AD494 strains have been purchased from Novagen (Lucerne, Switzerland). Ampicillin, kanamycin and protein standard bovine serum albumin (BSA) were obtained from Sigma (Buchs SG, Switzerland). IgG sepharose column material and GDP-L-[^{14}C]fucose (287 mCi/mmol) were purchased from Amersham Biosciences (Otelfingen, Switzerland). GDP-hexanolamine was synthesized as described (Bamford *et al.*, 1996) and was coupled to cyanogen bromide-activated sepharose 4B. Type I and Type II acceptor substrates and analogs thereof have been chemically synthesized as described (Hindsgaul *et al.*, 1991; Ernst and Oehrlein, 1999; Ernst *et al.*, 2000b).

Cloning and expression of H. pylori *α-1,4 fucosyltransferase*

Helicobacter pylori genomic DNA was prepared using the genomic DNA extraction kit from Wizard (Promega, Wallisellen, Switzerland). The α-1,4 FucT gene was amplified by PCR with the high fidelity *pfu* polymerase (Stratagene, Amsterdam, The Netherlands). The forward and reverse primers were designed on the basis of the published *H. pylori* α-1,3 and α-1,3/4 FucT sequences (Accession numbers: AF194963, AAD06573, and AAD06169). 5′-EcoRI forward primer (5′-gcgaattcgatgttccagcccttactagacgctt-3′) and 5′-BamHI reverse primer (5′-gccggatccctattaggaaagtagcgtctgcgat-3′) were synthesized at Microsynth (Balgach, Switzerland). The PCR-generated fragment (1375 bp) was treated with EcoRI and BamHI and ligated (T4 DNA-ligase, Promega) into the appropriate site of pEZZ18 cloning vector (Pharmacia-Biotech, Otelfingen, Switzerland). Chemocompetent *E. coli* JM109 cells were transformed with the ligation product, plated on selective Luria-Betani-agar plates and incubated overnight at 37°C. Single colonies were then inoculated in 2 mL LB-medium supplemented with 70 μg/mL ampicillin. Plasmid DNA was isolated by minipreparation according to the instructions of the supplier (GFX™ Micro Plasmid Prep Kit, Amersham Biosciences). The constructs containing two ZZ domains of protein A and the coding sequence for *H. pylori* FucT were verified by PCR and restriction enzyme digestion. DNA sequence analysis was performed by double-stranded sequence reactions by Microsynth. The recombinant vector was then transformed into the *E. coli* strains DHα5, Top10, and AD494.

Bacterial culture and protein production

The bacterial clones containing the pEZZ18/FucT construct were grown overnight with vigorous shaking (300 rpm) at 37°C and in 50 mL Terrific Broth medium (TB, Becton Dickinson, Basel, Switzerland) containing ampicillin (70 μg/mL) and additionally kanamycin (25 μg/mL) for AD494 cells. After 16 h the cells were collected by centrifugation for 20 min at 5000 rpm and 4°C. Aliquots (5 mL) from supernatants and cell lysates were concentrated by ultrafiltration to 50 μL volume (Amicon YM 50) and tested for enzyme activity.

Purification of H. pylori *α-1,4 fucosyltransferase*

The supernatant of the overnight bacterial culture was collected by centrifugation and filtration (0.2 μm, Millipore, Volketswil, Switzerland). The filtrate was adjusted to pH 7.6 with 2.5 M Tris–HCl solution. After loading the supernatant on the IgG-sepharose–6 column (Pharmacia-Biotech) attached to the BioLogic fast protein liquid chromatography system (BioRad, Reinach BL, Switzerland) two washing steps were performed, the first with 10 bed volumes of buffer containing 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, and 0.05% Tween 20 and the second with 2 bed volumes of 5 mM NH_4OAc (pH 5.0). The enzyme was eluted with 10 mL of 500 mM NH_4OAc (pH 3.4). The collected fractions were immediately neutralized with 2.5 M Tris–HCl solution and concentrated by ultrafiltration (Amicon, YM 50) to a volume of 50 μL. In a second ultrafiltration step, the buffer was exchanged by cacodylate buffer (pH 6.5).

In a second purification strategy, the supernatant of a bacterial overnight culture was conditioned with 20 mM $MnCl_2$ and loaded on a GDP-hexanolamine column according to the procedure described (De Vries *et al.*, 2001). The protein concentration of pooled active fractions was determined by the Bradford assay using bovine serum albumin (BSA, Sigma) as a standard.

SDS–PAGE analysis

Protein electrophoresis was performed on 10% polyacrylamide gel according to the method of Laemmli (1970). The purity of eluted fractions was verified by silver-staining (Silver staining Kit, Pharmacia-Biotech) using low molecular weight marker (Sigma).

Fucosyltransferase assay

The reactions, run in duplicates, were conducted in a total volume of 30 μL containing 50 mM cacodylate (pH 6.5), 20 mM $MnCl_2$, 0.150 mM GDP-fucose, 55,000 cpm GDP-[^{14}C]fucose (287 mCi/mmol, Amersham Pharmacia), 3 μg BSA (Sigma), 0.01 mM acceptor substrate, and 15 mU of *H. pylori* α-1,4 FucT. After 30 min of incubation at 37°C,

the reaction was stopped by the addition of 1 mL of water and loaded onto a Sep–Pak-C_{18} cartridge (Waters, Rupperswil, Switzerland). The cartridge was washed twice with 5 mL bidistilled water and eluted with 5 mL methanol. The transfer rate of [^{14}C]Fuc onto the acceptor substrate was determined by liquid scintillation counting. The activity of the enzyme was calculated as a percentage of radioactivity counted in the methanol fraction.

Determination of kinetic parameters

The kinetic parameters were determined using 0.150 mM of GDP-fucose and concentrations of the acceptor substrates ranging from 0.05 to 2 mM. For the determination of the K_m for GDP-fucose, the concentration of the Type I acceptor substrate **1** was kept at 2 mM. Reaction mixtures were prepared as described above.

Thermal stability of the recombinant H. pylori *α-1,4 fucosyltransferase*

The temperature stability of the enzyme was measured in cacodylate buffer at pH 6.5 by preincubating the enzyme at different temperatures ranging from 25 to 55°C for 5 min. The reaction mixtures were then incubated for further 30 min at the indicated temperatures. The activity of the enzyme was quantified as described above.

Treatment with NEM

To verify the susceptibility of the novel *H. pylori* α-1,4 FucT to the sulfhydryl-binding reagent, various amounts of NEM were added to the assay reaction to give final concentrations of 0.5–10 mM. The reaction was cooled for 30 min on ice before incubation with **1** and quantified as described above.

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Abbreviations

BSA, bovine serum albumin; FucT, fucosyltransferase; Gal, galactose; GDP-fucose, guanidine 5′-diphospho-β-L-fucose; LacNAc, *N*-acetyllactosamine; Le^a, Lewis A, Galβ-1,3(Fucα-1,4)GlcNAc; Lem, Lemieux spacer, $(CH_2)_8COOMe$; NEM, *N*-ethylmaleimide; NeuNAc, *N*-acetylneuraminic acid; sLe^a, sialyl Lewis A, NeuNAcα-2,3Galβ-1,3(Fucα-1,4)GlcNAc; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Type I, Galβ-1,3GlcNAc; Type II, Galβ-1,4GlcNAc.

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