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In vivo fucosylation of lacto-*N*-neotetraose and lacto-*N*-neohexaose by heterologous expression of *Helicobacter pylori* α-1,3 fucosyltransferase in engineered *Escherichia coli*

Claire Dumon, Bernard Priem*, Steve L. Martin[1], Alain Heyraud, Claude Bosso and Eric Samain

Centre de Recherches sur les Macromolécules Végétales, 601 rue de la Chimie, BP53X, 38041 Grenoble cedex 09, France, *[1]GlaxoSmithKline Medicines Research Centre, Stevenage SG1 2NY, UK*

We report here the *in vivo* production of type 2 fucosylated-*N*-acetyllactosamine oligosaccharides in *Escherichia coli*. Lacto-*N*-neofucopentaose Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc, lacto-*N*-neodifucohexaose Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc, and lacto-*N*-neodifucooctaose Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc were produced from lactose added in the culture medium. Two of them carry the Lewis X human antigen. High cell density cultivation allowed obtaining several grams of fucosylated oligosaccharides per liter of culture. The fucosylation reaction was catalyzed by an α-1,3 fucosyltransferase of *Helicobacter pylori* overexpressed in *E. coli* with the genes *lgtAB* of *N. meningitidis*. The strain was genetically engineered in order to provide GDP-fucose to the system, by genomic inactivation of gene *wcaJ* involved in colanic acid synthesis and overexpression of RcsA, positive regulator of the colanic acid operon.

To prevent fucosylation at the glucosyl residue, lactulose Galβ1-4Fru was assayed in replacement of lactose. Lactulose-derived oligosaccharides carrying fucose were synthesized and characterized. Fucosylation of the fructosyl residue was observed, indicating a poor acceptor specificity of the fucosyltransferase of *H. pylori*.

Keywords: *Escherichia coli*, fucosylation, Lewis X, metabolic engineering, oligosaccharides

Abbreviations: CO5, chitopentaose; Fuc, fucose; Fru, fructose; Gal, galactose; Glc, glucose; Glc*N*Ac, *N*-acetyl glucosamine; HMO, Human Milk Oligosaccharide; Le^X, Lewis X; LNnT, Lacto-*N*-neotetraose; LNnFP, lacto-*N*-neo-fucopentaose; LNnDFH, lacto-*N*-neo-di fuco-hexaose; $LNnT^U$, lacto-*N*-neo-tetraose analog in which fructose replaces glucose; $LNnH^U$, lacto-*N*-neo-hexaose analog in which fructose replaces glucose.

Introduction

L-fucose is commonly found in complex carbohydrates and plays a major role in many processes of biological recognition. The availability of large amounts of fucosylated oligosaccharides would make them useful as precursors or ready-to-use drugs for fundamental investigative research as well as for therapeutic trials. The chemical synthesis of fucose-containing oligosaccharides such as ABH and lewis antigens has long been achieved [1], but this technology is expensive and time-consuming. Enzymatic synthesis offers a seductive alternative way to the synthesis of oligosaccharides; and indeed the *in vitro* enzymic synthesis of Le^X has been reported [2]. Nevertheless, the cost of GDP-β-L-fucose, which is the activated sugar nucleoside-sugar precursor used by fucosyltransferase, is a limiting factor for large scale application.

In both prokaryotes and eukaryotes, GDP-L-fucose is biosynthesized *de novo* from mannose-6-phosphate and GTP by the successive action of four enzymes [3,4]. In *E. coli* these four enzymes are encoded by the *manB, manC, gmd* and *wcaG* genes which are part of the colanic acid gene cluster. Several processes have been designed to produce GDP-L-fucose by recombinant *E. coli* enzymes. The *in vitro* conversion of GDP-mannose to GDP-L-fucose requires an NADPH supplementation and can be achieved using purified recombinant Gmd and WcaG [5]. Taking advantage of the high cytoplasmic pool of

*To whom correspondence should be addressed:
E-mail: priem@cermav.cnrs.fr
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GDP-mannose in *Saccharomyces cerevisiae*, Mattila and collaborators have obtained a significant production of GDP-L-fucose by a recombinant yeast strain expressing the *E. coli gmd* and *wcaG* genes [6].

A large scale system to produce GDP-L-fucose from GMP and mannose was also established by coupling permeabilized resting cells of *Corynebacterium ammoniagenes* and recombinant *E. coli* expressing GDP-L-fucose biosynthetic genes: GTP was produced from GMP by *C. ammoniagenes* cells and used by *E. coli* cells to convert mannose into GDP-L-fucose [7].

One of the limitation of these systems is the strong inhibition of the Gmd enzyme by the end product GDP-L-fucose [8]. This problem can be overcome by carrying out separately the last step catalyzed by the GDP-L-fucose synthase WcaG. One alternative is to couple the GDP-L-fucose synthesis with oligosaccharide fucosylation. In this respect, the living factory approach is particularly attractive. This approach consists in producing oligosaccharides in a single growing living bacteria that overexpresses recombinant glycosyltransferase genes and provides the energetic requirement for maintaining the sugar-nucleotide pool level [9].

The living factory approach has been initially developed with the chitooligosaccharide synthase NodC which has the unique property of producing chitooligosaccharides without requiring an exogenous acceptor [10]. The method has been recently extended to the synthesis of lactose-derived oligosaccharides by using β-galactoside permease to internalized lactose [11]. By this way more than $5\,g\,l^{-1}$ of a mixture of lacto-*N*-neotetraose (LNnT) and lacto-*N*-neohexaose (LNnH) were obtained by cultivating at high cell density an *E. coli* strain overexpressing the *Neisseria meningitidis* genes encoding the β1,3 *N*-acetyl glucosaminyltransferase (LgtA) and the β1,4 galactosyltransferase (LgtB).

The α-1,3 fucosyltransferase encoded by the *fucT* gene of *Helicobacter pylori* has been successfully expressed in *E. coli* [12]. This enzyme uses *N*-acetyllactosamine and LNnT as acceptors but not lactose. An *E. coli* strain that coexpresses *fucT* and the *lgtAB* of *N. meningitidis* should thus be able to produce fucosylated derivatives of LNnT from lactose (Figure 1). We show in this paper that this production requires a metabolic engineering of the GDP-L-fucose biosynthesis pathway to increase the availability of GDP-L-fucose, which is not sufficiently produced in our fermentation conditions by regular *E. coli* strains. In addition we report the structural characterization of the different fucosylated oligosaccharides that were produced from lactose and its β-galactoside analog lactulose (4-O-β-D-galactopyranosyl-D-fructofuranose).

Results

In vivo fucosylation of lacto-*N*-neotetraose

The first attempts to detect a production of fucosylated oligosaccharides in lactose supplemented cultures of *E. coli* strain JM107 overexpressing the *lgtAB* and *fucT* genes were unsuccessful. In vitro assays of fucosyltransferase activity indicated that *fucT* was correctly expressed in *E. coli*, though the activity was considerably higher when *fucT* was under the control of the PT7 promoter ($5100\,pmol.mg^{-1}.min^{-1}$) than of the Plac promoter ($100\,pmol.mg^{-1}.min^{-1}$).

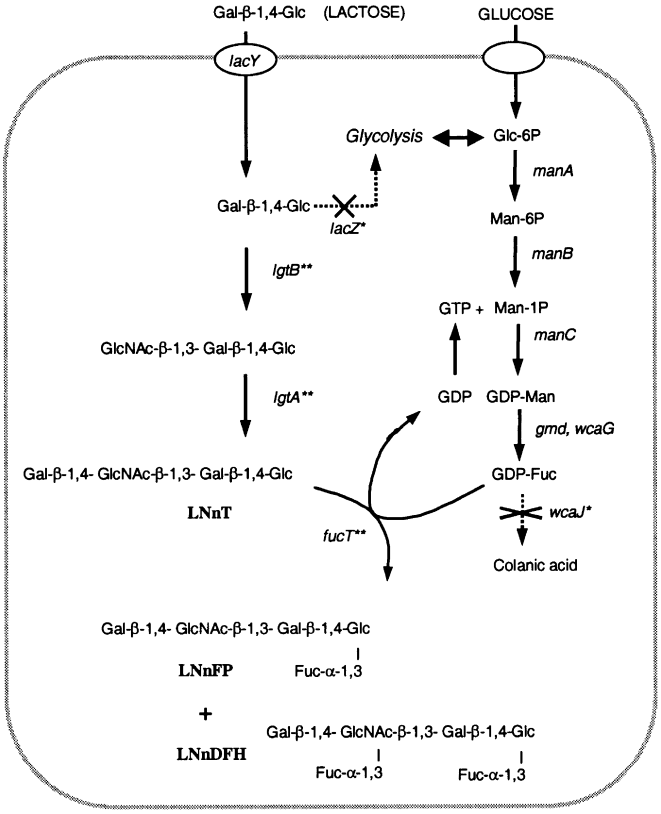

Figure 1. *In vivo* biosynthesis pathway of fucosylated lacto-*N*-neotetraose in engineered *Escherichia coli* cells. Genes encoding the enzymes involved in the different reactions are shown in italic characters. Glycosyl residues which have been underlined are potential fucosylation sites of the fucosyltransferase according to its *in vitro* activity. Lactose is internalized by specific permease LacY and cannot be degraded due to *lacZ* inactivation. It is glycosylated by GlcNAc-T and Gal-T encoded by genes *lgtAB* of *N. meningitidis*. Main final product LNnT is a possible acceptor for fucosyltransferase encoded by gene *fucT* of *H. pylori*. *Inactivated endogenous genes; **heterologous genes carried on expression vectors.

As the lack of fucosylation could be due to an insufficient level of GDP-L-fucose in the cells, we tried to increase the GDP-L-fucose availability by metabolic engineering. We used a two level strategy focused on the regulation of colanic acid biosynthesis: (i) the gene *rcsA* which encodes a positive regulator of the colanic acid operon [13] was cloned by PCR and overexpressed in a plasmid under the control of the Plac promoter. By this way the induction of rcsA expression by IPTG indirectly induced the expression of the GDP-L-fucose biosynthesis genes located in the colanic acid cluster- (ii) the production of colanic acid was suppressed by inactivating the

wcaJ gene, located downstream of the GDP-L-fucose biosynthesis genes in the colanic acid cluster and believed to encode the initiating glucose-1-phosphate transferase that attaches the first glucose residue on the lipid carrier intermediate [3]. This suppression allows the GDP-L-fucose biosynthesis flux to be diverted completely from colanic acid production to the synthesis of fucosylated oligosaccharide.

The efficiency of fucosylation was compared with different strains and plasmid combinations, data are summarized in Table 1. Highest fucose production was obtained with the strain col1K,DE3 (pLNTR1T, pSM9710) called Le^x cells. The absence of fucosylation in the control strain col1 (pLNTR1T alone) indicated that the fucosyltransferase encoded by *fucT* is essential for fucosylation. In addition, the very low fucosylation in the control strain col1 (pLNT1T, pSM9711), which do not overexpress *rcsA,* confirmed that an induction of the GDP-L-fucose biosynthesis genes is required to achieve an efficient fucosylation.

No significant fucosylation was observed with the strain col1 (pLNTR1T, pFUTA1) which has the *fucT* gene under the control of the Plac promoter. This result correlates the *in vitro* enzymatic assays.

Purification and analysis of fucosylated oligosaccharides produced from lactose

Seventy percent of fucose and GlcNAc containing oligosaccharides produced by Le^X cells (see Table 1) was recovered in the intracellular fraction, which was first purified by charcoal adsorption. The separation of the intracellular oligosaccharide content was then performed by gel permeation on Biogel P2. Three main oligosaccharides (compounds **1**, **2**, and **3**) were detected and purified, besides unreacted LNnT, LNnH and lactose (Figure 2).

Compound **1**— FAB-MS analysis showed a molecular ion $[M+H]^+$ of 854 compatible with composition $Fuc_1Gal_2GlcNAcGlc$. The ^{1}H-NMR spectrum by comparison with the literature (Table 2) was consistent with the proposed structure Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc.

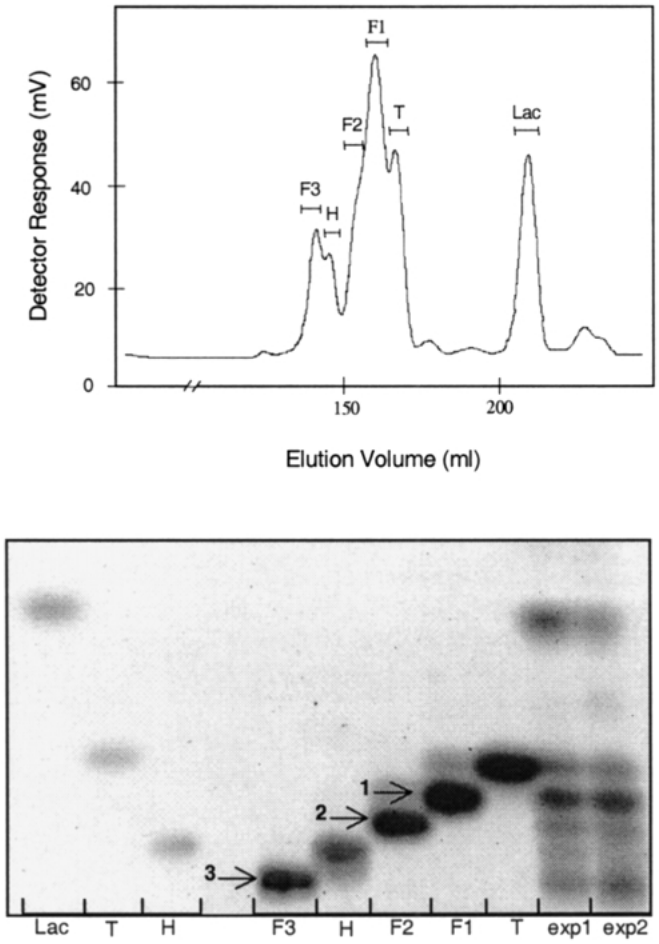

Figure 2. Separation of oligosaccharides produced by Le^X cells. Upper panel: Bio-Gel P-2 chromatography. Extracted oligosaccharides from activated charcoal where analysed on a calibrated Bio-Gel P-2 column and monitored with an in-line differential refractometer. Lower panel: TLC plate analysis of Bio-Gel P-2 fractions. Legend: T, Lacto-*N*-neotetraose; H, Lacto-*N*-neohexaose; exp1-2, intracellular cells contents from two independent experiments. Compounds **1**, **2**, and **3** (black arrows) were recovered from corresponding F1, F2 and F3 fractions and subsequently purified as described in Materials and Methods.

Table 1. Rate of fucosylation in *coli* strains with different expression vectors

Strain	*Plasmids*	*Genes*	*Intracellular fucosylation (Fuc g/l)*
col1K	pLNTR1T	*lgtA, lgtB, rcsA*	–
col1K	pLNTR1T pFUTA1	*lgtA, lgtB, rcsA* *fucT (lac promoter)*	0.01
col1K, DE3*	pLNT1T pSM9711	*lgtA, lgtB,* *fucT (T7 promoter)*	0.05
col1K, DE3*	pLNTR1T pSM9710	*lgtA, lgtB, rcsA* *fucT (T7 promoter)*	0.70

*Genomic insertion of T7 polymerase cassette.

Compound **2**—FAB-MS analysis showed a molecular ion $[M+H]^+$ of 1000 compatible with composition $Fuc_2Gal_2GlcNAcGlc$. The ^{1}H-NMR spectrum by comparison with the literature was consistent with the proposed structure Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc.

Compound **3**—FAB-MS analysis showed a molecular ion $[M+H]^+$ of 1365 compatible with composition $Fuc_2Gal_3GlcNAc_2Glc$, suggesting a LNnH skeleton substituted with two Fuc residues. The ^{1}H-NMR spectrum confirmed the substitution of reducing Glc with one Fuc residue, but did not allow the location of the second Fuc residue to be determined. On the other hand, the FAB-MS fragmentation pattern of permethylated compound **3** indicated that the second Fuc

Table 2. ^{1}H-NMR analysis of oligosaccharides 1-3 produced from lactose. The legend of symbols is △, fucose; ●, glucose; ■, galactose; ○, *N*-acetylglucosamine. Referenced compound data are from *[23]; **[24]; ***[14]

Residues	*Reporter groups*	*Oligosaccharides*						
		*	**	*	***	1	2	3
		ppm						
α-L-Fuc (Glc)	H-1α	5.389	5.371	5.372		5.36	5.36	5.37
	H-1β	5.446	5.427	5.428		5.42	5.41	5.41
	H-6α	1.193	1.167	1.163		α.β 1.16	–	α.β 1.16
	H-6β	1.187	1.161	1.157				
α-L-Fuc^{II} (GlcNAc^{III})	H-1			5.135	5.111		5.12	
	H-6			1.174	1.147		–	
α-L-Fuc^{II} (GlcNAc^{V})	H-1				5.132			5.13
	H-6				1.175			1.17
β-D-Gal^{II}	H-1	4.437	4.415	4.414	4.432	4.41	4.4	4.41
	H-4	3.898	4.096	4.466	4.154	4.09	4.08	4.1
β-D-Gal^{IV}	H-1		4.48	4.096	4.442	4.47	4.43	4.45
	H-4		–	3.782	4.097	3.96		4.15
β-D-Gal^{VI}	H-1				4.464			4.48
	H-4				3.897			3.96
D-Glc	H-1α	5.187	5.18	5.180	5.218	5.17	5.17	5.18
	H-1β	4.658	4.65	4.651	4.66	4.64	4.64	4.65
β-D-GlcNAc^{III}	H-1		–	4.7	4.714	4.69	4.69	4.7
	NAc-CH_3		2.028	2.018	2.020	2.02	2.02	2.03
β-D-GlcNAc^{V}	H-1				4.701			4.7
	NAc-CH_3				2.016			2.018

Numeration of residues is: Gal^{VI}-β-1 → 4-GlcNac^{V}-β-1 → 3-Gal^{IV}-β-1 → 4-GlcNac^{III}-β-1 → 3-Gal^{II}-β-1 → 4-Glc

	3		3
	↑		↑
	1		1
	|		|
	α		α
	Fuc^{II}		Fuc

was attached to the proximal GlcNAc (III) (Figure 3). The presence of m/z fragments 464 and 668 is typical of terminal non-reducing LacNAc residue, indicating substitution on the internal GlcNAc. The proposed structure of compound **3** is therefore Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc.

Assays with lactulose as acceptor

Due to the preferential fucosylation on Glc of LNnT, lactulose (Gal-β-1,4-Fru) was assayed instead of lactose in order to favor the fucosylation of LN groups i.e. the synthesis of Le^{X}. Without Fuc-T overexpression, incorporation of lactulose into oligosaccharides occurred at a similar rate to that of lactose (data not shown). In fucosylating strains, two major fucosylated oligosaccharides (compounds **4** and **5**) were produced.

Compound **4**—FAB-MS analysis showed a molecular ion $[M+H]^+$ of 854 compatible with composition $FucGal_2GlcNAcFru$, suggesting a LNnT^{U} skeleton substituted with one Fuc residue. The linkage position of the sugar residues was determined by methylation analysis (Table 3). Surprisingly, the Glc*N* residue is 4-substituted, and therefore cannot be fucosylated. On the other hand, the Fru derivative is 3-substituted, implying that Fuc is linked to Fru (Figure 4). The FAB-MS spectrum of permethylated oligosaccharidiotol (Figure 5) showed fragments at m/z 464, 668, and 859, consistent with a lack of fucosylation on GlcNAc. The proposed structure for compound **4** is Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)Fru.

Compound **5**—FAB-MS analysis showed a molecular ion $[M+H]^+$ of 1000 compatible with composition $Fuc_2Gal_2GlcNAcFru$, suggesting a LNnT^{U} skeleton substituted with two Fuc residues. The linkage position of the sugar residues was determined by methylation analysis. The Glc*N* residue is 3,4-substituted, in agreement with a Le^{X} motif. The Fru derivative is 3-substituted as for compound 4, and the following structure

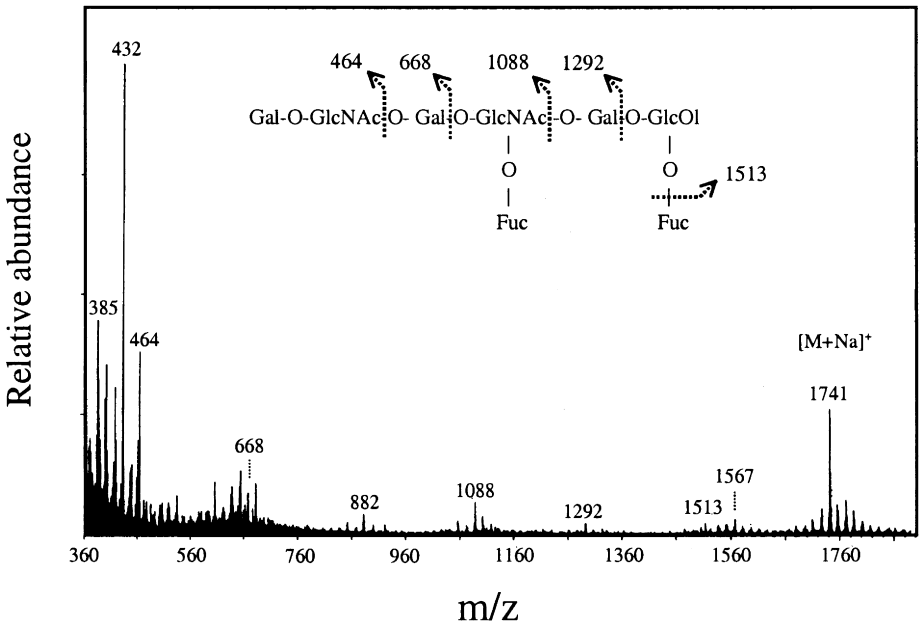

Figure 3. FAB-MS analysis of permethylated and reduced compound **3**.

Table 3. Methylation analysis of the fucosylated oligosaccharides **4** and **5** produced from lactulose

		*Relative amount**	
Residue	*Linkage*	**4**	**5**
Fru	3-linked	1	1
Gal	terminal	1.3	1.4
	3-linked	1.2	1.3
GlcN**	4-linked	+++	+++
	3,4-linked	–	+++
Fuc	terminal	0.8	1.7

*Calculated on the base of the total ion response in GC-MS analysis and relative to the Fru residue.
**Detected on a separate column and not quantified.

for compound **5** can be proposed: Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)Fru.

Compounds **4** and **5** are structural analogs of compounds **1** and **2** produced from lactose.

Discussion

We report here the *in vivo* fucosylation of oligosaccharides in *Escherichia coli*. The substrates LNnT and LNnH were produced endogenously by overexpression of genes *lgtAB* as previously described [11]. The key point of our strategy has been to engineer an *E. coli* strain capable of furnishing GDP-fucose to the system, despite the fact it is not constitutively produced in *E. coli*, unlike the UDP-galactose and UDP-*N*-acetylglucosamine precursors of LNnT and LNnH.

The major oligosaccharide (compound **1**) obtained from lactose is a lacto-*N*-neo-fucopentaose. It represents about 80% of the total fucosylated fraction, and its production can be approximated to 3 g/L of culture. This oligosaccharide does not carry the Le^X determinant. This result was in agreement with the known specificity of the FucT of *H. pylori*, which fucosylates the reducing glucosyl residue of LNnT more efficiently than GlcNAc [12].

Two oligosaccharides (compounds **2** and **3**) obtained from lactose do carry Le^X motifs. Compound **2** is a lacto-*N*-neo-difucohexaose. It likely originates from subsequent fucosylation of compound **1** by Fuc-T. Its presence was reported in human milk, it thus belongs to the "HMO" family [14].

We attempted to improve the yield of Le^X motif production by using another β-galactoside precursor lacking glucose reducing end. Lactulose (Galβ1,4Fru) was chosen for its low cost and its resemblance to lactose. As expected, action of LgtA and LgtB led to the endogenous synthesis of LNnT and LNnH analogs (*i.e.* $LNnT^U$ and $LNnH^U$). Our wish was that only Glc*N*Ac residue could be fucosylated, giving Le^X products. Quite unexpectedly, a fucosylation of the $OH(C_3)$ of fructose was observed. This suggests that the position of this residue among the tetraose chain is at least as important as the nature of the residue itself in the enzyme recognition. Indeed, it suggests that the distal LN motif (in LN-Lac or LN-Lac^U) probably plays an important role in Fuc-T recognition. Fucosylation of the internal LN motif of LNnH in compound **3** argues for this hypothesis.

We describe here for the first time heterologous expression of an α-fucosyltransferase coupled to its *in vivo* catalysis in *E.*

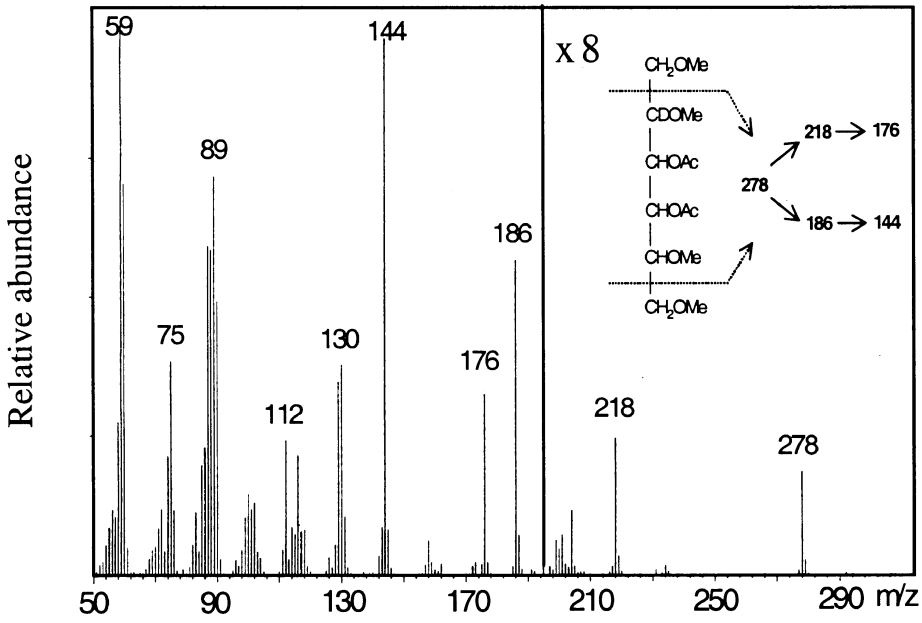

Figure 4. Electron impact fragmentation profile of partially methylated alditol acetate derivative of fructose from compounds 4 and 5. Compounds **4** and **5** were reduced with $NaBD_4$ before methylation. Acetylation on carbon 3 indicates substitution (i.e. fucosylation) prior to hydrolysis and acetylation.

coli cells. The *N*-acetyllactosaminyl skeleton that was used as acceptor could be modified by many other fucosyltransferases. For instance, the biosynthesis of the human H determinant Fuc-α-1,2-Lac*N*Ac- could be attempted by the same approach. The limitation for this and other determinants would be the availability of enzymes with the desired specificity which can be expressed in *E. coli*. At present, the only available bacterial α-1,2-fucosyltransferase is that of *H. pylori*, but this enzyme is almost inactive on type 2 *N*-acetyllactosaminyl acceptors *in vitro* [15].

The Le^X motifs that were synthesized are potential precursors for the synthesis of sialyl Le^X. Heterologous expression of sialyltransferase such as Nst01 of *Neisseria meningitidis*, which has been used to produce sialyllactose in *E. coli* [11], should allow the production of SLe^X motifs by a similar approach in a near future.

Materials and methods

Construction of expression plasmids

pLNT1C and pLNT1T were both plasmids providing the capability of synthesizing the type 2 poly-*N*-acetyllactosamine skeleton. They contain two genes of *N. meningitidis* MC58, namely *lgtA* encoding an β-1,3 *N*-acetylglucosaminyltransferase (genbank # 25839) and *lgtB* encoding a β-1,4 galactosyltransferase (genbank # 25839). The plasmids were constructed as follows: the gene *lgtA* together with its upstream promoter tac-tac was amplified by PCR from the expression vector pCWlgtA [16] using a pair of primers containing *Hind*III sites, *i.e.* 5′-CTTT*AAGCTT*CCGGCTCGTATAA (sense) and 5′-GACAGCTTATCATCGAT*AAGCTT* (antisense). After intermediate subcloning in a pPCR 2.1-TOPO vector (Invitrogen PCR kit), the *Hin*dIII fragment was ligated into the *Hin*dIII site of pBBlgtB, expression vector carrying the gene *lgtB* expressed under control of a lac promoter [11] to generate the expression vector pLNT1C, a low copy vector harboring a broad host range *ori* compatible with Col1 *ori* expression vectors, carrying *lgtA* and *lgtB* both inducible by IPTG but differentially controlled by tac-tac and lac promoters. A related expression vector pLNT1T, differing only in its antibiotic resistance gene (tetracycline instead of chloramphenicol) was obtained by subcloning the XhoI-XbaI *lgtAB* expression cassette fragment released from pLNT1C into XhoI-XbaI polylinker sites of pBBR-MCS3 [17].

pLNTR1T was made by subcloning *E. coli* gene *rcsa* in pLNT1T as follows: *rcsa* was obtained by PCR amplification from genomic DNA of strain JM109 with primers 5′-AG*GGTACC*CATGTTGTTCCGTTTAG (*Kpn*I, sense) and 5′AA*TCTAGA*GTAATCTTATTCAGCCTG (*Xba*I, antisense). After intermediate subcloning in a pPCR 2.1-TOPO vector (Invitrogen PCR kit), the 0.8 kb *Kpn*I-*Xba*I fragment was inserted between the *Kpn*I-*Xba*I sites of pBBR-MCS1 to generate pRC1C. pRC1C was linearised with *Kpn*I, blunt-ended and digested with *Xba*I in order to be inserted between the *Sma*I-*Xba*I sites of pLNT1T dowstream *lgtA*. The resulting construction was named pLNTR1T.

pSM9711 contained the α1,3-fucosyltransferase gene *fucT* (HP0379) of *Helicobacter pylori.* The *fucT* gene was amplified from *H. pylori* 26695 genomic DNA using the PCR primers

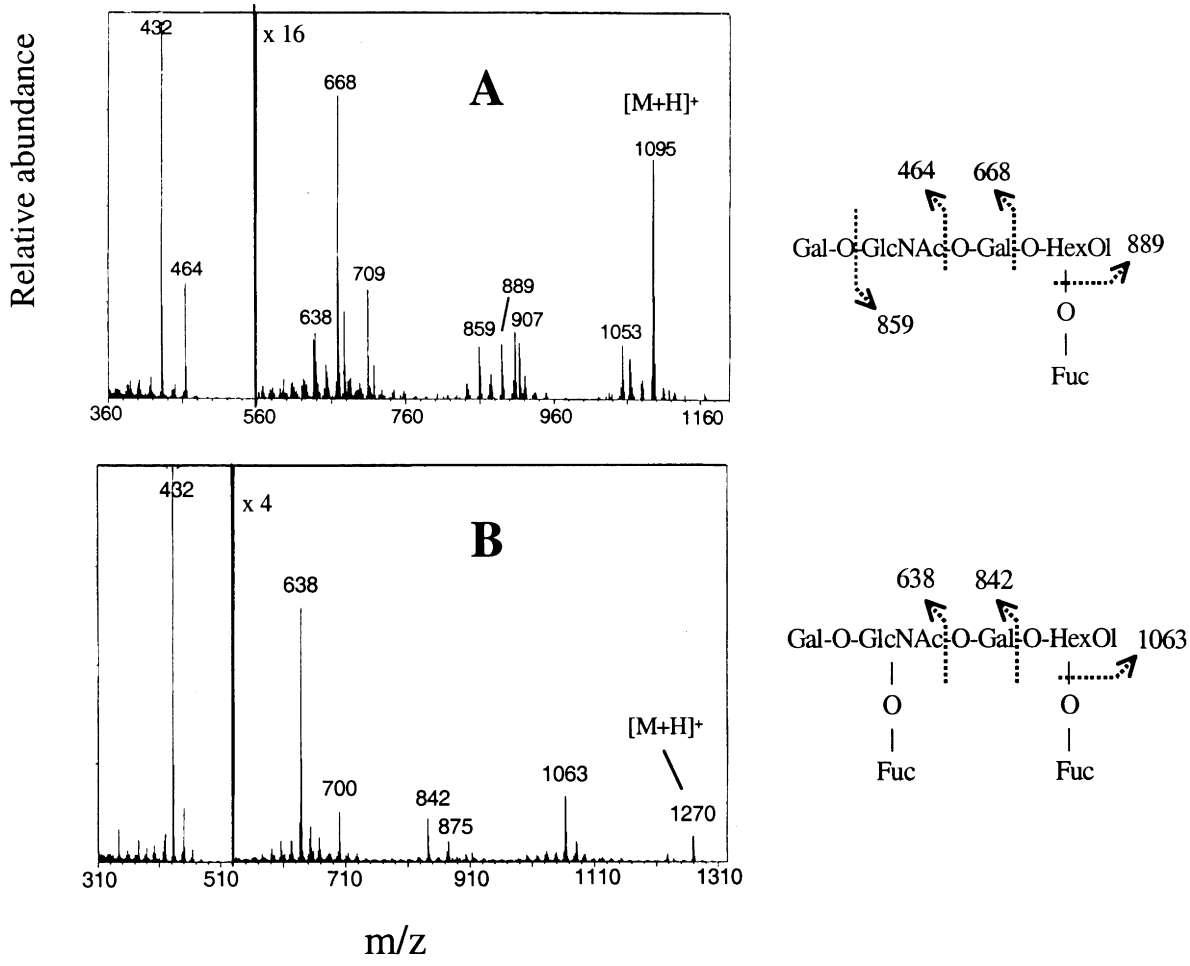

Figure 5. FAB-MS analysis of permethylated and reduced compounds **4** and **5**.

5′-TGTGCGGAGTTTGGATCC*CATATG*TTCCAACCCCTA-TTA (*Nde*I, sense) and 5′-GCGATGATGCGCAAGGGGTTT-GA (antisense). After intermediate cloning into pGEM-T Easy (Promega), a *Nde*I-*Eco*RI fragment containing *fucT* was excised and inserted into pET-21a (Novagen) between the *Nde*I and *Eco*RI sites, to generate pSM9710. Another construction pFUCT1 was done with a vector carrying a promoter lac by insertion of the Xba1-Sac1 fragment of pSM9711 containing *fucT* in pUC19.

Bacterial strains

E. coli JM107 and JM109 were obtained from the Deutsche Sammlung von Mikroorganismen. Due to the Δ(lacZ)M15 deletion in the *lacZ* gene, these strains are unable to produce a complete active β-galactosidase.

Strain deficient in colanic acid synthesis (col1K) was obtained by knock-out of *wcaJ* encoding for a protein involved in the last steps of colanic acid synthesis [3]. This was carried out by insertional inactivation of *wcaJ* with a kanamycin resistance cassette (pUC4K, Pharmacia). A DNA sequence containing *wcaJ* and some flanking DNA was amplified by PCR from *E. coli* DNA of JM109 using primers 5′-AAGCTCATATCAATATGCCGCT and 5′-CCACGATC-CACGTCTCTCC. The resulting 1.8 kb fragment was cloned pTOPO2.1 (Invitrogen), released with *Eco*RI and subcloned into the *Eco*RI site of pUC19. *Eco*RI sites were subsequently methylated by treatment with *Eco*RI methylase. The vector was linearised at the ApoI site within *wcaJ*, into which was inserted a kanamycin resistance cassette excised from pUC4K (Pharmacia). Finally, the XbaI-XmnI *wcaJ::KanR* fragment was moved into XbaI-SmaI polylinker sites of the suicide vector pCVD442, and subsequently used to obtain mutants from a JM107 *E. coli* strain (recA+) by homologous recombination as previously described [18]. Recombining mutants col1K were recovered on selective plates containing kanamycin.

Strain col1K, DE3 was prepared by genomic insertion of a DE3 cassette (T7 pol gene under control of lacUV5 promoter) using the DE3 lysogen kit from Novagen.

E. coli strain "Le^X" was made by transformation of strain col1K,DE3 with plasmids pLNTR1T and pSM9711.

Plasmids and strains are summarized in Table 4.

High cell density cultivation

High-density cell culture was carried out in 2 liter reactors containing one liter of mineral culture medium as previously described [19]. Except where otherwise indicated the culture temperature was 28°C and the pH was 6.8. The cultivation strategy included 3 phases: a first exponential growth phase which started at the inoculation of the fermentor and lasted until exhaustion of the carbon substrate (glucose) initially added to the medium at a concentration of $17\,g\,l^{-1}$. Then, the acceptor lactose (4 g) was added together with the inducer (IPTG $50\,mg\,l^{-1}$). Medium growth rate was maintained during 5 hours by glucose supplementation at feeding rate of $3.6\,g\,l^{-1}\,h^{-1}$. Finally, glucose supplementation was decreased to $1.8\,g\,l^{-1}\,h^{-1}$ and a lactose supplementation of $0.45\,g\,l^{-1}\,h^{-1}$ was also provided until the end of the fermentation (10–15 hours).

Table 4. *E. coli* strains and plasmids

Strains or plasmids	*Description*	*Source*
Escherichia coli		
JM 107	*SupE* Δ(*lac-proAB*) *hsdR*4 F′ *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15	
M 109	JM 107 *recA*	
col1K	JM 107 *wcaJ* ::Kan^r	this work
col 1K, DE3	col1K, DE3	this work
Plasmids		
pSM9711	plasmid pET-11a *fucT, Amp*^{r}	this work
pUC19	*Amp*^{r}	Biolabs
pFUCT1	pUC19 *fucT, Amp*^{r}	this work
pBBR	plasmid pBBR-MCS1, *Cm*^{r} plasmid pBBR-MCS3, *Tet*^{r}	
pBBlgtB	plasmid pBBR-MCS1 *lgtB*, *Cm*^{r}	
pCW lgtA	plasmid pCW ori$^+$ *lgtA*, *Amp*^{r}	
pLNT1T	plasmid pBBR-MCS3 *lgtA, lgtB, Tet*^{r}	this work
pRC1C	pBBR-MCS1 *rcsa, Cm*^{r}	this work
pLNTR1C	plasmid pBBR-MCS1 *lgtA, lgtB, rcsA, Cm*^{r}	this work
pLNTR1T	plasmid pBBR-MCS3 *lgtA, lgtB, rcsA, Tet*^{r}	this work
Le^X cells	strain col 1K, DE3 pLNTR1T, pSM9711	this work

Fucosyltransferase assay

Cells were suspended in 50 mM Tris-HCl buffer (pH 8.0) and disrupted with a French Press. Soluble extract (5 µL) was incubated with 20 µM GDP-fucose, 833 Bq ^{14}C-fucose (Amersham), 5 mM LNT, 5 mM $MnCl_2$, 1 mM ATP, 50 mM Tris-HCl buffer (pH 7.2) in a final volume of 25 µL. The reaction mixture was incubated at 37°C for up to 2 hours. Reaction was stopped by addition of 500 µl of mixed bed resin (AG 1×8 in water, 1 : 4 p/v). The mixture was spun briefly, and incorporated fucose in the supernatant was quantified. Protein concentration was determined using the *Protein assay* kit (Biorad).

Quantification of oligosaccharides during fermentation

Fresh culture samples (1 ml) were centrifuged in microfuge tubes (2 min, $10{,}000 \times g$). The supernatants were saved for quantification of extracellular oligosaccharides. The pellets were resuspended in 1 ml of distilled water, boiled for 10 min and centrifuged as above. The second supernatant was kept to quantify the intracellular oligosaccharides.

After acid hydrolysis *N*-acetylglucosamine (Hex*N*) content was quantified colorometrically using the Ehrlich reagent [20]. Total fucose was quantified colorimetrically after acid hydrolysis by the cystein chloride reagent method [21]. Lactose concentration was quantified using the kit from Roche Diagnostic for the enzymatically determination of lactose and glucose.

Purification of oligosaccharides

At the end of the fermentation time course, the bacterial cells were recovered by centrifugation (20 min at 7000 g). The supernatants were saved for purification of extracellular oligosaccharides. The pellets were resuspended in a volume of distilled water equal to that of the original culture medium and the cells were permeabilized autoclaving at 100°C for 30 min. After another centrifugation (30 min at 7000 g) the cells debris were discarded and the supernatant was retained to purify the intracellular oligosaccharides.

Oligosaccharides were adsorbed on activated charcoal and, after thorough washing with distilled water, eluted as previously described with 50% (v/v) aqueous ethanol.

Oligosaccharides were further purified by size exclusion chromatography (SEC) on a Biogel P2 (4.5×95 cm) column, with distilled water as mobile phase, at a flow rate of $40\,ml\,h^{-1}$.

Oligosaccharides **1** and **2** were purified from enriched SEC fractions by a second chromatography on Biogel P2.

Oligosaccharides **3**, **4**, and **5** were purified from enriched SEC fractions by HPLC using a preparative amino column (Hypersyl 8 µ, Interchrom, 250×27 mm) coupled to a refractive index detector and eluted with a mixture of acetonitrile/water (65 : 35 for compound **3**; 70 : 30 for **4** and **5**) at a flow rate of 9 ml/min.

The purity of oligosaccharides was estimated by TLC plate analysis on silica gel eluted with the mixture butanol/acetic acid/water (2 : 1 : 1, two runs). Sugars were detected by dipping in orcinol sulfuric acid reagent (sulfuric acid-/ethanol/water 3 : 70 : 20, orcinol 1‰) and heating.

Carbohydrate structural analyses

^{1}H-NMR spectra were recorded at 30°C with a 400 MHz Avance (Bruker) spectrometer using D_2O as solvent. Mass spectra in the FAB(+) mode were recorded with a Nermag R 10 10C spectrometer.

Oligosaccharides (5–10 mg) subjected to methylation (**3**, **4** and **5**) were first reduced with $NaBD_4$ (5 mg/500 µl water and 20 µl ammoniac 20%) overnight at room temperature. The reaction was stopped by addition of 100 µl of glacial acetic acid. Reduced compounds were desalted by gel permeation on a TKHW40F/50F (Interchrom) column (500 × 21.2 mm) coupled to a refractive index detector and eluted with water at a flow rate of 4 ml/min. Methylation was done according to Hakomori [22]. Part of the permethylated samples were kept for FAB-MS analysis. The rest was hydrolyzed in two steps, first in formic acid (90%, 1 h under reflux), then (after evaporation) in trifluoroacetic acid (2 M, 3 h under reflux). Partially methylated derivatives were reduced with $NaBD_4$ and then peracetylated with the mixture acetic anhydride/pyridine (1:1, 100°C, 1 h). After evaporation, partially methylated alditol acetates (PMAA) were dissolved in chloroform and analyzed in GC-MS on a capillary column SP 2380 (Supelco) for neutral derivatives, or an OV1 column (Spikhl) for GlcNAc derivatives.

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