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Synthesis of fucosylated lacto-*N*-tetraose using whole-cell biotransformation

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ABSTRACT

Fucosylated oligosaccharides present a predominant group of free oligosaccharides found in human milk. Here, a microbial conversion of lactose, D-glucose and L-fucose to fucosylated lacto-*N*-tetraose by growing *Escherichia coli* cultures is presented. The recombinant expression of genes encoding for the β1,3-*N*-acetylglucosaminyltransferase (LgtA) and the β1,3-galactosyltransferase (WbgO) enables the whole-cell biotransformation of lactose to lacto-*N*-tetraose. By the additional expression of a recombinant GDP-L-fucose salvage pathway together with a bacterial fucosyltransferase, lacto-*N*-tetraose is further converted into the respective fucosylated compounds. The expression of a gene encoding the α1,2-fucosyltransferase (FutC) in the lacto-*N*-tetraose producing *E. coli* strain led to the formation of lacto-*N*-fucopentaose I, whereas the expression of a gene encoding the α1,4-fucosyltransferase (FucT14) mainly led to the conversion of lacto-*N*-tetraose to lacto-*N*-difucohexaose II.

1. Introduction

Next to lactose and lipids, human milk oligosaccharides (HMOs) are the major solid components of breast milk. The possible functions of HMOs are in the focus of scientific investigations. As summarized in a recent review,[1] beneficial effects on the well-being of infants have been shown either for mixtures of HMOs, or for a couple of single HMO compounds. Therefore, HMOs have gained considerable interest in recent years.[1] HMOs are not (or only in traces) present in cow milk or in milk of other domesticated mammalians. A large variety of HMOs exists (>200), however, the laborious extraction of single compounds from human breast milk is the only source of HMOs so far.[2,3] Due to this and to obvious ethical reasonings ('withholding mother's milk from babies') isolation from human milk is not a favorable method for the supply of HMOs.

The chemical synthesis of HMOs affords the need of several and cumbersome protection and deprotection steps.[4] Enzymatic synthesis protocols are known, but relatively expensive donor substrates (a.o. nucleotide-activated sugars) or low yields,[4] leave many possible HMOs not available to further research at reasonable prices. As no natural microbial HMO producers exist, whole-cell biosynthesis with recombinant bacteria could be an attractive way for the production of HMOs from inexpensive carbon sources (glucose, glycerol, lactose), combining enzymatic regio- and stereoselectivity with the in vivo generation of donor substrates.

In general, HMO core structures display a lactose residue at their reducing end; this moiety can be elongated by addition of *N*-acetyl-lactosamine (LacNAc) or lacto-*N*-biose units, resulting in type II or type I oligosaccharides, respectively (Table 1). These core oligosaccharides can further be decorated by fucosyl- or sialyl-residues, leading to a large variety of structures.[5] A division into neutral and acidic HMOs, that is, with or without sialyl residues, respectively, has been made.[5] As summarized by Urashima et al.,[6,7] previous studies found that human milk contains higher amounts of neutral HMOs than of acidic HMOs. Furthermore, the total amounts and the structural diversity of oligosaccharides, and the quantitative dominance of type I over type II oligosaccharides also seem to be unique to human milk. The overall predominant oligosaccharides in human milk, found in previous studies which were dominated by milk from so-called secretor women are 2′-fucosyllactose (2′-FL), lacto-*N*-fucopentaose I (LNF I), lacto-*N*-difucohexaose I (LNDFH I), and lacto-*N*-tetraose (LNT).[7]

Focusing on neutral HMOs, previous studies described chemical, enzymatic or whole-cell syntheses of fucosyllactoses[8–14] and core structures such as lacto-*N*-neotetraose (LNnT) and LNT.[15–19] Fucosylated derivatives of the core structure LNnT were synthesized in whole-cell cultivations,[10,11,20] while syntheses of

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Table 1
Common neutral human milk oligosaccharides identified in human milk samples[7]

Name	Abbreviation	Core	Structure[a]
2′-Fucosyllactose	2′-FL	—	Fuc(α1,2)Gal(β1,4)Glc
3-Fucosyllactose	3-FL	—	Gal(β1,4)[Fuc(α1,3)]Glc
Lactodifucotetraose	LDFT	—	Fuc(α1,2)Gal(β1,4)[Fuc(α1,3)]Glc
Lacto-*N*-tetraose	LNT	Type I	Gal(β1,3)GlcNAc(β1,3)Gal(β1,4)Glc
Lacto-*N*-neotetraose	LNnT	Type II	Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)Glc
Lacto-*N*-fucopentaose I	LNF I	Type I	Fuc(α1,2)Gal(β1,3)GlcNAc(β1,3)Gal(β1,4)Glc
Lacto-*N*-fucopentaose II	LNF II	Type I	Gal(β1,3)[Fuc(α1,4)]GlcNAc(β1,3)Gal(β1,4)Glc
Lacto-*N*-fucopentaose III	LNF III	Type II	Gal(β1,4)[Fuc(α1,3)]GlcNAc(β1,3)Gal(β1,4)Glc
Lacto-*N*-difucohexaose I	LNDFH I	Type I	Fuc(α1,2)Gal(β1,3)[Fuc(α1,4)]GlcNAc(β1,3)Gal(β1,4)Glc
Lacto-*N*-difucohexaose II	LNDFH II	Type I	Gal(β1,3)[Fuc(α1,4)]GlcNAc(β1,3)Gal(β1,4)[Fuc(α1,3)]Glc
Lacto-*N*-neohexaose	LNH	Type I & II	Gal(β1,3)GlcNAc(β1,3)[Gal(β1,4)GlcNAc(β1,6)]Gal(β1,4)Glc

[a] Abbreviations of monomer units: Fuc, L-fucose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Glc, D-glucose.

fucosylated LNT structures so far have been only described from in vitro enzymatic conversions.[21–23] Enzymatic or whole-cell synthesis requires the availability of fucosyltransferases which can be used either as recombinant human genes, or from bacteria such as *Helicobacter pylori*.[23] Thus, fucosylation of LNT was carried out in vitro with *H. pylori* α1,2- or α1,3-fucosyltransferases, or with human α1,2- or α1,3/4-fucosyltransferases, whereas in vivo fucosylation reactions with LNnT were catalyzed by recombinant *H. pylori* fucosyltransferases in *Escherichia coli* cells.[10,11,20] These *H. pylori* fucosyltransferases, namely the α1,2-fucosyltransferase FutC and the α1,3-fucosyltransferase FutA, enabled the synthesis of H antigen and Lewis X structures, respectively. While the α1,2-fucosyltransferase showed higher in vitro activity for the acceptor substrate LNT than for LNnT,[22] the α1,3-fucosyltransferase showed lower in vitro activity for LNT, since the position 3 of the *N*-acetylglucosamine (GlcNAc) of LNT, one possible target for fucosylation, is already bound by the galactose residue. In this case, the fucosyl-residue is connected to the glucose unit of LNT, resulting in the product lacto-*N*-fucopentaose V.[21] Thus, for the in vivo synthesis of fucosylated LNT with a Lewis a structure, an α1,3/4- or α1,4-fucosyltransferase activity is needed. The molecular cloning and heterologous expression of an α1,4-fucosyltransferase from *H. pylori* was described earlier, including analysis of enzyme activity and specificity.[24,25] According to these in vitro experiments, the enzyme shows little activity with substrates carrying LacNAc residues, when compared to substrates with lacto-*N*-biose residues. As other Leloir fucosyltransferases with an Asp-X-Asp motif and dependency on divalent cations, it uses GDP-L-fucose as donor substrate, making it a plausible candidate for in vivo synthesis using GDP-L-fucose generated by the cell.

Previously, we demonstrated the enhanced in vivo fucosylation of lactose by recombinant expression of the salvage synthesis pathway of GDP-L-fucose (starting from L-fucose with the bifunctional enzyme Fkp with L-fucose kinase and L-fucose-1-phosphate guanylyltransferase activity from *Bacteroides fragilis*) in an *E. coli* strain, capable of 2′-fucosyllactose (2′-FL) synthesis.[13,26] As described therein, the addition of L-fucose together with the expression of the *fkp* gene, resulted in increased amounts of intracellular GDP-L-fucose. This GDP-L-fucose was thus available as donor substrate for a Leloir fucosyltransferase. Another recent publication[27] demonstrates the in vivo synthesis of LNT from lactose and glucose or glycerol, employing the enzymes of the two recombinant genes *lgtA* and *wbgO*, encoding for a β1,3-*N*-acetylglucosaminyltransferase (LgtA)[28] from *Neisseria meningitidis* and a β1,3-galactosyltransferase (WbgO)[29] from *E. coli* O55:H7, respectively. In the present work, we combined the capabilities of enhanced GDP-L-fucose synthesis and LNT formation and added α1,2- and α1,4-fucosyltransferase activity for the in vivo synthesis of fucosylated LNTs (Fig. 1).

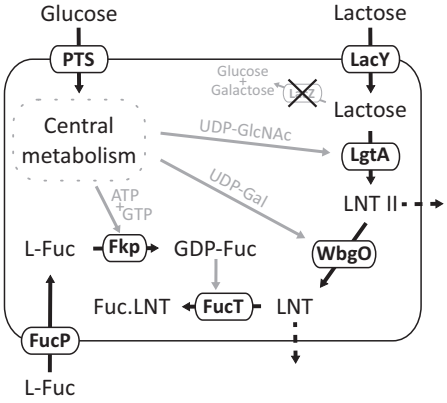

Figure 1. In vivo synthesis of fucosylated lacto-*n*-tetraoses using Leloir glycosyltransferases and intracellularly generated nucleotide-activated sugars. Utilization of different fucosyltransferases leads to differently fucosylated lacto-*N*-tetraose. Abbreviations are as follows: ATP, adenosine triphosphate, Fuc.LNT fucosylated lacto-*N*-tetraose, GDP guanosine diphosphate, GDP-Fuc guanosine 5′-diphospho-β-L-fucose, GTP guanosine triphosphate, L-Fuc L-fucose, LNT II lacto-*N*-triose II, LNT lacto-*N*-tetraose, UDP-Gal uridine 5′-diphosphogalactose, UDP-GlcNAc uridine 5′-diphospho-*N*-acetylglucosamine, Fkp bifunctional L-fucose kinase/L-fucose-1-phosphate guanylyltransferase, FucP L-fucose permease, FucT α1,2- or α1,4-fucosyltransferase, LacY lactose permease, LacZ β-galactosidase, LgtA β1,3-*N*-acetylglucosaminyltransferase, PTS phosphotransferase system, WbgO β1,3-galactosyltransferase.

2. Material and methods

2.1. Bacterial strains and chemicals

All strains constructed for syntheses of fucosylated oligosaccharides are based on the previously described *E. coli* strain LJ-AY (LJ110 *lacZYA*::*lgtA*-FRT *fucIK*::*lacY*-FRT) and are listed in Table 2. Briefly, this strain was constructed from *E. coli* LJ110[30] by deletion of the genes *lacZYA* and chromosomal integration of the expression cassettes of the *E. coli* K12 lactose permease (*lacY*) and the *Neisseria meningitidis* β1,3-*N*-acetylglucosaminyltransferase (*lgtA*),[28] both under the control of a P_{tac}-promoter. For plasmid constructions, unless stated otherwise, *E. coli* DH5α cells were grown on lysogeny broth (LB) agar plates or in LB liquid medium with the corresponding antibiotic (50 μg ml^{-1} of chloramphenicol or kanamycin or 100 μg ml^{-1} of ampicillin) at 37 °C. For in vitro demonstration of FucT14 activity, the β-galactosidase negative, but lactose-permease positive laboratory strain *E. coli* JM109 was utilized for overexpression of the fucosyltransferase. After verification of plasmid integrities and enzyme activities, plasmids or chromosomal gene-encoding expression cassettes were transferred to the synthesis strains for in vivo production of fucosylated oligosaccharides.