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Synthesis of globopentaose using a novel β1,3-galactosyltransferase activity of the *Haemophilus influenzae* β1,3-*N*-acetylgalactosaminyltransferase LgtD

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Abstract **We have previously described a bacterial system for the conversion of globotriaose (Gb3) into globotetraose (Gb4) by a metabolically engineered *Escherichia coli* strain expressing the *Haemophilus influenzae* lgtD gene encoding β1,3-*N*-acetylgalactosaminyltransferase [Antoine, T., Bosso, C., Heyraud, A. Samain, E. (2005) Large scale in vivo synthesis of globotriose and globotetraose by high cell density culture of metabolically engineered *Escherichia coli*. Biochimie 87, 197–203]. Here, we found that LgtD has an additional β1,3-galactosyltransferase activity which allows our bacterial system to be extended to the synthesis of the carbohydrate portion of globopentaosylceramide (Galβ-3GalNAcβ-3Galα-4Galβ-4Glc) which reacts with the monoclonal antibody defining the stage-specific embryonic antigen-3. In vitro assays confirmed that LgtD had both β1,3-GalT and β1,3-GalNAcT activities and showed that differences in the affinity for Gb3 and Gb4 explain the specific and exclusive formation of globopentaose.**

Keywords: Globosides; Glycosyltransferases; Oligosaccharides; Metabolic engineering; Antigens

1. Introduction

The globopentaose Gb5 (Galβ-3GalNAcβ-3Galα-4Galβ-4Glc) is the carbohydrate moiety of the globopentaosylceramide [2] which has been first identified in tetracarcinoma cells as a structure reacting with the monoclonal antibody MC631 that defines the stage-specific embryonic antigen-3 (SSEA-3) and whose epitope is R-GalNAcβ-3Galα-4Gal. The pentasaccharide sequence is also found in other related glycolipids of the globo-series reacting with the monoclonal anti-SSEA-3. They carry an additional fucose (Globo-H) [3] or sialic acid (SSEA-4 antigen) [2] on the terminal galactosyl residue of Gb5. These embryonic epitopes have been identified on various tumor cells [4] and are promising targets for the development of anti-cancer vaccines [5].

Chemical synthesis of Gb5 was first achieved in 1988 [6] but the high potential interest of this molecule and of its possible derivatives for cancer immunotherapy has led several groups to investigate different strategies to improve the synthetic yields [7–9].

An alternative to these very demanding chemical syntheses is the enzymatic approach which has been successfully applied to the synthesis of globotriaose (Gb3) [10] and globotetraose (Gb4) [11] using *Neisseria meningitidis* α1,4-galactosyltransferase (LgtC) and β1,3-*N*-acetylgalactosaminyltransferase from *Haemophilus influenzae* strain Rd (LgtD) [12]. In addition, large-scale enzymatic synthesis of oligosaccharides can be conveniently achieved in whole bacterial cells [13] and we have recently designed a bacterial system for the production of Gb4 by taking advantage of the fact that living *Escherichia coli* cells can actively internalize Gb3 [1]. As shown in Fig. 1, this system could be extended to the synthesis of Gb5 if the suitable β1,3-galactosyltransferase could be found. Up to now the only enzyme reported to direct the synthesis of the globopentaosylceramide has been the human β1,3-galactosyltransferase V [14]. However, the use of this enzyme for the production of Gb5 in metabolically engineered *E. coli* cells could be seriously hampered by the fact that mammalian glycosyltransferase genes are generally badly expressed in bacteria. Another β1,3-galactosyltransferase candidate is the CgtB protein from *Campylobacter jejuni*, which add a galactosyl residue on a *N*-acetylgalactosaminyl β1,4-linked motif [15]. The initial goal of this study was thus to investigate the production of Gb5 by an *E. coli* strain coexpressing *lgtD* and one of these above mentioned β1,3-galactosyltransferase genes. However, in the course of these investigations, we surprisingly found that *E. coli* cells expressing *lgtD* alone converted Gb4, which was produced from Gb3, into Gb5. We now present evidence that the LgtD protein from *H. influenzae*, which normally acts as a β1,3-*N*-acetylgalactosaminyltransferase in presence of Gb3, has an additional β1,3-galactosyltransferase activity in presence of Gb4 as the acceptor.

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Abbreviations: SSEA-3, stage-specific embryonic antigen-3; Gb5, globopentaose; Gb4, globotetraose; Gb3, globotriaose; GT, glycosyltransferase; GalT, galactosyltransferase; GalNAcT, *N*-acetylgalactosaminyltransferase; PCR, polymerase chain reaction; IPTG, isopropyl 1-thio-β-galactopyranoside; TLC, thin layer chromatography; UDP-Gal, uridine diphosphate-galactose; UDP-GlcNAc, uridine diphosphate-*N*-acetylglucosamine; UDP-GalNAc, uridine diphosphate-*N*-acetylgalactosamine; TFA, trifluroacetic acid; HPAEC, high pH anion exchange chromatography; GC–MS, gas chromatography–mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; NMR, nuclear magnetic resonance

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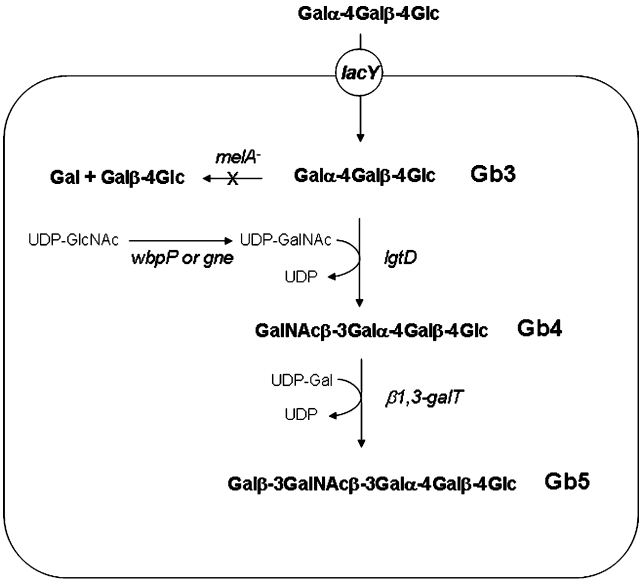

Fig. 1. Metabolically engineered pathway for globopentaose (Gb5) production from exogenous globotriaose (Gb3) in *Escherichia coli K 12*. Gb3 is internalized by the β-galactoside permease LacY and cannot be degraded because the DM strain is a *lacZ melA* mutant devoid of α- and β-galactosidase activities. The expression of *Pseudomonas aeruginosa wbpP* or *Campylobacter jejuni gne* enables the epimerization of UDP-GlcNAc into UDP-GalNAc. The Gb3 acceptor is converted into Gb4 by β1,3-GalNAcT encoded by *Haemophilus influenzae* LgtD. The subsequent conversion of Gb4 into Gb5 requires the expression of β1,3-GalT.

2. Materials and methods

2.1. Bacterial strains, plasmids and cloning procedures

To construct the pBS-*lgtD* plasmid, the *H. influenzae lgtD* gene was subcloned from pBluescript-*lgtDC* [1] into the SalI–PstI sites of pBluescript II KS. The *C. jejuni gne* gene was amplified by polymerase chain reaction (PCR) from the genomic DNA of the NCTC 11168 strain using the following primers 5′-AGGAGGATTATTGATGAAAATTCTTATTAGCGGTG and 5′-AGAGCTCGCCAAAGTGATAAAAGGCATTA. The PCR product was cloned into pCR4Blunt-TOPO and subcloned into the XhoI–EcoRI sites of the pBBR1-MCS3 vector [16] to yield pBBR-*gne* and into the PstI–SacI sites of pBS-*lgtD* to yield pBS-*lgtD-gne*.

The DM strain was constructed from the DC strain [17] by inactivating the *melA* gene encoding α-galactosidase activity as previously described in [18]. MR13, MR14 and MR15 strains were obtained by transforming the DC strain with the plasmids pBS-*lgtD*, pBBR-*gne* and pBS-*lgtD-gne*, respectively.

2.2. Production of oligosaccharides in high cell density culture

High cell density cultures were carried out at 34 °C and pH 6.8 in 2-liter reactors containing mineral culture medium (1 l) as previously described [19]. They consisted of three phases: an exponential-growth phase, which started with inoculation of the fermenter and lasted until exhaustion of the initially added glycerol (17.5 g/l), a 5 h fed-batch phase with a high substrate-feeding rate (4.5 g/l/h) and a 19 h fed-batch phase with a lower feeding rate (2.4 g/l/h). The acceptor (Gb3, 3 g/l) and the inducer (IPTG, 50 mg) were added at the end of the exponential phase. Culture samples were prepared and analyzed by thin layer chromatography (TLC) as previously described [1].

2.3. Assay for β1,3-GalNAcT and β1,3-GalT activities

For protein expression, cell extracts of DM, MR13 and MR14 strains were prepared as previously described [20]. The enzymatic assays were performed at 30 °C for 2 h in a final volume (100 µl) containing 50 mM Tris–HCl (pH 7.0), 10 mM $MnCl_2$, 1% BSA, 3 mM acceptor (Gb3 or Gb4) and 10 µl of protein extract from DM and MR13 strains. For the β1,3-GalT assay, 0.3 mM uridine diphosphate-galactose (UDP-Gal) and 1.68 µM ^{14}C-UDP-Gal (Amersham, 2.5 µCi) were used as donors while 0.3 mM uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) and 2.12 µM ^{14}C-UDP-GlcNAc (Amersham, 10 µCi) were used as donors for the β1,3-GalNAcT assay. In the latter assay, the mixture was incubated at 30 °C for 1 h with protein extract from the MR14 strain to provide uridine diphosphate-*N*-acetylgalactosamine (UDP-GalNAc) before adding the MR13 strain. Reactions were stopped by adding Dowex 1 × 8-400 chloride anion-exchange resin (500 µl, 0.25 g/ml in water). After centrifugation, the supernatant was added to liquid scintillation cocktail (4 ml, Amersham) to quantify the incorporated radioactivity.

2.4. Purification of oligosaccharides

At the end of the fermentation period, bacterial cells were recovered by centrifugation (7000×*g*, 30 min), and the supernatant was designated the extracellular fraction. The cells contained in the pellet were resuspended in water, and permeabilized by autoclaving at 100 °C for 50 min. After an additional identical centrifugation, the intracellular oligosaccharides were found in the supernatant. They were adsorbed on activated charcoal/celite (1:1) and eluted with 50% aqueous ethanol. Finally, they were separated by size-exclusion chromatography on a Biogel P2 column (200 × 1.5 cm) at 60 °C, with water as the mobile phase and at a flow rate of 35 ml/h.

2.5. Carbohydrate structural analysis

Glycosyl composition analysis. Three milligrams of pentasaccharide were hydrolyzed in 500 µl, 2 M trifluoroacetic acid (TFA) containing *m*-inositol (0.964 mg/ml) at 100°C for 4 h. TFA was removed by repeated evaporations with distilled water to obtain a neutral pH. The resulting sample was analyzed by high pH anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) on a Dionex CarboPac PA-10 column (250 × 4 mm) [21].

Alditol acetates of the monosaccharides were obtained after reduction ($NaBH_4$ at 37 ° for 4 h) and acetylation (acetic anhydride/pyridine (1/1, v:v) at room temperature for 20 h). They were analyzed on a HP 5890 gas chromatograph (Hewlett–Packard, France) fitted with a flame-ionization detector with a SP-2380 column (30 m × 0.53 mm), using He as the carrier gas.

GC–MS. Reduced pentasaccharide (3 mg) was dissolved in dimethylsulfoxide (200 µl), methylsulfinyl carbanion (200 µl) was added and the mixture was flushed with N_2 overnight. Methyl iodide (200 µl) was added gradually. The methylated sample was extracted three times with chloroform, washed with distilled water and dried with Na_2SO_4. After evaporation, 500 µl of 90% formic acid was added and heated under reflux for 1 h. A second hydrolysis was performed with 2 M TFA at 100 °C for 2 h. After evaporation, the resulting monosaccharides were reduced with $NaBD_4$ and acetylated. The resulting partially methylated alditol acetates were analyzed by GC–MS as previously described [22].

Electrospray ionization mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR). ESI mass spectra were recorded on a ZQ Waters micromass spectrometer (capillary 3.5 kV, cone voltage 80 V). ^{1}H and ^{13}C NMR spectra were performed with a 300 MHz Bruker AVANCE spectrometer.

Gb5: ESI-MS: *m/z* 892 [M+Na$^+$]; ^{1}H NMR (D_2O, 303 and 323 K): δ = 5.23 (H-1α, d, J = 3.4 Hz, 1H), 4.93 (H-1″, d, J = 2.7 Hz, 1H), 4.72 (H-1‴, d, J = 7.15 Hz, 1H), 4.66 (H-1β, d, J = 10 Hz, 1H), 4.52 (H-1′, d, J = 7.5 Hz, 1H), 4.46 (H-1⁗, d, J = 7.6 Hz, 1H), 4.38(H-5″, t, J = 6.4 Hz, 1H), 4.26 (H-4″, d, J = 4.2 Hz, 1H), 4.18 (H-4‴, s, 1H), 3.64 (H-2β, t, J = 8 Hz, 0.6H), 2.04 (Ac, s, 3H); ^{13}C NMR (D_2O) : δ = 175.78 (CO), 105.6 (C-1⁗), 104.12 (C-1′), 103.76 (C-1‴), 101.29 (C-1″), 96.61 (C-1β), 92.68 (C-1α), 80.44 (C-3‴), 79.74 (C-3″), 61.89 - 60.9 (all C-6), 52.41 (C-2‴), 23.22 (CH_3).

3. Results

3.1. Globopentaose production by metabolically engineered *E. coli* cells

In our previously published system for Gb4 production with the TA21 strain [1], the *lgtD* and *wbpP* genes were placed on two separate plasmids, pACT3-*lgtD* and pBBR-*wbpP*. In order

to reduce the number of plasmids and to optimize Gb4 production, the *wbpP* gene was replaced by the *C. jejuni gne* gene, which has been shown to encode a more active UDP-GlcNAc C4-epimerase, and both *gne* and *lgtD* genes were cloned together on the high copy number pBluescript plasmid to yield pBS-*lgtD*-*gne*. Prior to investigating the production of Gb5 by expressing an additional gene for β1,3-galactosyltransferase, the control MR15 strain carrying the pBS-*lgtD*-*gne* plasmid was examined for Gb4 production. TLC analysis of the intracellular oligosaccharide content of the MR15 strain indicated that Gb4 production rates were significantly improved when compared with the results of the TA21 strain. Gb3 (3 g/l) was entirely converted into Gb4 within 4 h of incubation with the MR15 strain, whereas it took 6 h for the TA21 strain to convert only 1 g/l. TLC analysis of the intracellular fraction of the MR15 strain (Fig. 2) also clearly revealed the presence of an unidentified compound which started to be formed after the complete exhaustion of Gb3 (4 h). After 20 h of incubation, Gb4 had almost entirely disappeared and the unidentified compound had become the major detectable product. Its full characterization showed that it was Gb5 (see Section 3.2). A mixture of oligosaccharides (2.7 g) was isolated from the cell medium and purification on a Biogel P2 column showed that it was composed of a mixture of Gb5, Gb4 and Gb3 in a ratio of 85/5/10.

3.2. Characterization

At the end of the MR15 strain fermentation, the major product was first characterized by ESI mass spectrometry. Its ESI^+ mass spectrum showed the presence of a quasi molecular ion $[M+Na]^+$ at *m/z* 892, which could have originated from a pentasaccharide having 4 hexoses and one *N*-acetylhexosaminyl residue. The determination of the monomeric sugar composition by HPAEC-PAD analysis after acid hydrolysis indicated that this pentasaccharide was composed of Glc, Gal and GalNAc monomers in a ratio of 1/3/1. This strongly suggested that the pentasaccharide was formed by the transfer of one galactosyl unit onto a molecule of Gb4. The linkage position of the additional galactose was determined by gas chromatography–mass spectrometry (GC–MS) analysis after methylation, acid-hydrolysis, reduction and acetylation of the pentasaccharide. The presence of fragment ions at *m/z* 261 and 161 derived from the *N*-acetylgalactosaminyl residue unambiguously demonstrated that the linkage was at the third position. Compared with the ^{13}C NMR spectrum of Gb4, the spectrum of the pentasaccharide showed an additional signal at 105.6 ppm indicating that the third galactosyl residue was attached to the GalNAc unit with a β linkage. These results showed that the pentasaccharide produced by the MR15 strain from Gb3 has the structure of Gb5. This identification was confirmed by the overall ^1H and ^{13}C NMR data that agreed closely with values previously published for 3-aminopropyl galactosylgloboside [23].

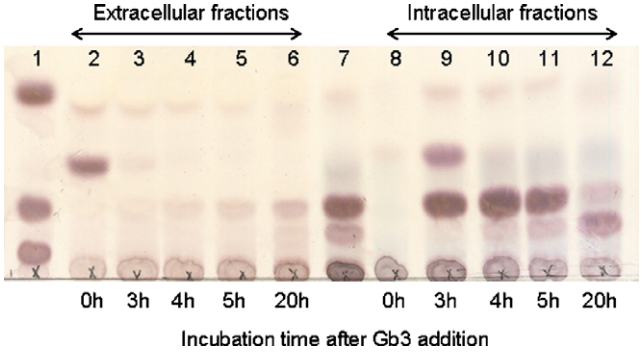

Fig. 2. TLC plate analysis of the oligosaccharide content in extracellular (lanes 2–6) and intracellular (lanes 8–12) fractions of samples withdrawn from the culture of the globopentaose-producing MR15 strain. Standards are in lane 1 (lactose, lacto-*N*-neotetraose, lacto-*N*-neohexaose) and lane 7 (Gb4 and Gb5).

3.3. Specificity of LgtD

Enzymatic assays showed that crude extracts from the MR13 strain expressing the LgtD protein had both β1,3-GalNAcT and β1,3-GalT activities in the presence of Gb3 or Gb4 as acceptors (Table 1). Control experiments with extracts from the DM strain showed no detectable activity indicating that both glycosyltransferase activities resulted from the expression of LgtD. The maximum velocities of the two glycosyltransferases (GT) were in the same range regardless of the acceptor and sugar donor used. However large differences in the affinity of the enzyme for UDP-Gal or UDP-GalNAc were observed as a function of the acceptor used. When Gb3 was the acceptor, the K_m was 6 times lower for UDP-GalNAc than for UDP-Gal, indicating that LgtD acted primarily as a GalNAc-transferase, converting Gb3 into Gb4. When Gb3 was replaced by Gb4 as the acceptor, there was a 20-fold increase in the K_m for UDP-GalNAc but, on the other hand, in a 3.5-fold decrease in the K_m for UDP-Gal. In the presence of Gb4 as the acceptor, the enzyme, which had an 11-fold greater affinity for UDP-Gal than for UDP-GalNAc, can thus be regarded as a galactosyltransferase which specifically directs the synthesis of Gb5.

3.4. Sequence analysis

The *lgtD* gene encodes a polypeptide of 323 aminoacids. Protein sequence analysis revealed the presence of the N-terminal domain which comprises residues [1–240] corresponding to the catalytic domain. It shows a strong level of sequence similarity (60–70% identity) to a number of bacterial GTs involved in lipopolysaccharide biosynthesis, which belong to the large CAZY GT2 family (http://afmb.cnrs-mrs.fr/CAZY/). This domain is assumed to adopt the mixed α/β GT-A fold first observed in the inverting GT SpsA [24]. An Â″Asp-X-AspÂ″ motif (where X is any amino acid) implicated in divalent metal ion binding is formed by Asp92 and Asp94. The size of the catalytic domain is indicative of a classical monofunctional enzyme belonging to the GT-A family and only one catalytic center is clearly identified using fold recognition methods [25]. Therefore the dual enzyme activity (β1,3-GalT/β1,3-

Table 1
Kinetic parameters for the β1,3-GalNAcT and β1,3-GalT activities of LgtD

Donor\acceptor	Globotriaose (Gb3)		Globotetraose (Gb4)	
	K_m[a]	V_{max} (pmol/min)	K_m[a]	V_{max} (pmol/min)
UDP-GalNAc	0.81	21.27	16.66	10.00
UDP-Gal	5.00	15.15	1.42	16.60

[a] K_m (mM) is the Michaelis–Menten constant for the Gb3 and Gb4 acceptors with concentrations covering the range 0.5–20 mM.

GalNAcT) can be attributed to a marked plasticity in donor sugar specificity that appears to be dictated by the acceptor molecule. In contrast, the C-terminal region which comprises residues [21–323] does not match any known GT catalytic domain. It is only observed in a small subset of closely related LgtD protein sequences and is predicted to adopt an α-helical fold. It is currently not known if this region is required for the catalytic activity of LgtD but it can be suggested that this C-terminal region may be involved in protein oligomerization as was recently proposed for a mannosylglycerate synthase that also displayed an extra C-terminal α-helical domain [26].

4. Discussion

There is no endogenous β1,3-galactosyltransferase in *E. coli* and the formation of Gb5 using the MR15 strain expressing the *lgtD* gene clearly results from a galactosyltransferase activity of the LgtD protein. It is not surprising that LgtD could act as a galactosyltransferase because it has already been shown that this enzyme can use UDP-Gal with a low efficiency with Gb3 as the acceptor [27]. In addition, there are several reports of GalNAc-transferases that have a side galactosyltransferase activity [28] and the blood groups A and B glycosyltransferases are known to differ only by a few amino acids [29]. What is quite remarkable in Gb5 production by the MR15 strain is the fact that LgtD specifically adds a galactosyl residue onto Gb4 but does not galactosylate Gb3 to produce the globotetraose analog Galβ-3Galα-4Galβ-4Glc. It is also notable that LgtD does not produce the globopentaose analog GalNAcβ-3GalNAcβ-3Galα-4Galβ-4Glc from Gb4 in vivo. This very high specificity could be explained by the presence of two separate active sites, as was demonstrated in other GTs such as hyaluronan synthase [30] and chondroitin synthase [31] which catalyze the successive transfer of two distinct sugars. However, LgtD is a relatively small protein of only 323 aminoacids and its sequence analysis reveals only one putative catalytic site. The presence of only one active site is also consistent with the in vitro assay results which showed that the two activities were observed with both Gb3 and Gb4 as acceptors. The explanation for the unusual specificity of LgtD probably lies in the difference in affinity for UDP-GalNAc and UDP-Gal that was observed as a function of the acceptor. In the presence of Gb3, LgtD acts as a *N*-acetylgalactosaminyltransferase because it has a high affinity for UDP-GalNAc and a low affinity for UDP-Gal. Conversely in the presence of Gb4, LgtD acts as a galactosyltransferase because it has a higher affinity for UDP-Gal than for UDP-GalNAc. In this hypothesis, the mechanism by which the acceptor can modulate the affinity for the sugar donor remains to be established.

An important question raised by these results is whether the double specificity of LgtD is fortuitous or has a real physiological significance. LgtD is involved in the synthesis of the Gb4 epitope in the lipopolysaccharide of *H. influenzae* [32]. The expression of the globoside mimics is believed to contribute to pathogenicity by allowing the bacteria to evade the host immune response. In this respect it could be interesting to determine if Gb5 is expressed on the *H. influenzae* cell surface as a result of the galactosyltransferase activity of LgtD.

Regardless of its biological meaning, the β1,3-galactosyltransferase activity of LgtD is of great biotechnological importance and LgtD is the first reported enzyme that can be used for the practical enzymatic synthesis of Gb5. Furthermore the expression of LgtD in the metabolically engineered *E. coli* strain is a very efficient method to produce large quantities of Gb5. Since we have shown that it is possible to synthesize fucosyl α1,2-linked [20] and sialyl α2,3-linked oligosaccharides [33] in *E. coli*, it should certainly be possible in the future to extend the method to the production of Globo-H and SSEA-4 antigens.

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