Oligosaccharide Biosynthesis

Highly Efficient Biosynthesis of the Oligosaccharide Moiety of the GD3 Ganglioside by Using Metabolically Engineered *Escherichia coli***

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GD3 (Neu5Acα-8Neu5Acα-3Galβ-4GlcCer; Neu5Ac = *N*-acetylneuraminic acid, Gal = D-galactose, GlcCer = glycosylceramide) is a minor ganglioside found in most normal tissues in higher vertebrates including humans. The GD3 level has been shown to increase during some pathological situations, such as cancers (glioma, melanoma), and to have an important role in tumor angiogenesis.[1] Anti-GD3 monoclonal antibodies inhibit the growth of human melanoma cells both in vitro and in vivo.[2,3] In normal cells, GD3 is a cell-death effector that activates the mitochondrial-dependent apoptosome in response to apoptotic stimuli.[4] In addition, GD3 has a proapoptotic function by suppressing the nuclear factor-κb-dependent survival pathway.[5]

Chemical synthesis of the oligosaccharide moiety of gangliosides is difficult,[6] but efficient biotechnological techniques have recently been developed for the synthesis of GM3, GM2, and GM1 oligosaccharides.[7,8] The GM3 oligosaccharide (Neu5Acα-3Galβ-4Glc; Glc = D-glucose) was synthesized by a metabolically engineered *Escherichia coli* strain which overexpressed the *Neisseria meningitidis* genes for α-3 sialyltransferase and CMP-Neu5Ac synthase (CMP = cytidine monophosphate). Lactose and the neuraminic acid were supplied as exogenous precursors and were actively internalized by the β-galactosidase and Neu5Ac permease of the *E. coli*. To prevent catabolism of the precursors, a mutant strain devoid of both β-galactosidase and Neu5Ac aldolase activities was used.

In this work, we have extended this process to the synthesis of the oligosaccharide moiety of GD3 (Neu5Acα-8Neu5Acα-3Galβ-4Glc, referred to later as $II^3(Neu5Ac)_2$-lac) by using the alternative *Campylobacter jejuni* CstII sialyltransferase, which exhibited both α-3 and α-8 sialyltransferase activities.[9] The *cstII* gene occurs as either a monofunctional or a bifunctional sialyltransferase, depending on the *C. jejuni* strain. We chose to use the ATCC 43438 version, which has both high α3 and high α8 activity.[9]

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The metabolically engineered pathway for the biosynthesis of the GD3 oligosaccharide is described in Figure 1. The host strain, JM107*nanA*$^-$, was an *E. coli* K12 JM107 strain derivative in which the Neu5Ac aldolase activity was abolished by inactivating the *nanA* gene.[8] The *cstII* gene was amplified by PCR by using the genomic DNA of *C. jejuni* (ATCC 43438) as the template and was cloned into a pUC18 plasmid to yield pUC-*cstII*. The $II^3(Neu5Ac)_2$-lac production strain TA15 was constructed by transforming the host strain JM107*nanA*$^-$ with two compatible plasmids: pUC18-*cstII* and pBBnsy,[7] which was a pBBR1-*MCS1* derivative carrying the *N. meningitidis* gene for CMP-Neu5Ac synthase. The strain TA15 was cultured to high cell density with glycerol as the carbon source, as previously described.[7] Lactose (3 mM) and Neu5Ac (6 mM) were added at the beginning of the fed-batch phase, as well as isopropyl-β-D-thiogalactopyranoside (IPTG), which was the inducer of the Plac promoter of the two plasmids. The oligosaccharide content in the intracellular fraction of samples withdrawn at different cultivation times was analyzed by high-performance anion-exchange chromatography (HPAEC) analysis (see Supporting Information). Lactose and Neu5Ac transiently accumulated in the intracellular fraction and were entirely consumed after 7 and 24 h of incubation, respectively (Figure 2). Concurrently two compounds,**1** and **2**, putatively $II^3(Neu5Ac)$-lac and $II^3(Neu5Ac)_2$-lac, were produced, with their maximal concentrations reached 7 and 9 h after induction, respectively. Concentrations of compounds **1** and **2** then decreased during the final stage of culture and this decrease correlated with the appearance of compound **3**, which had a longer retention time in the HPAEC analysis (see Supporting Information) and which was believed to be $II^3(Neu5Ac)_3$-lac.

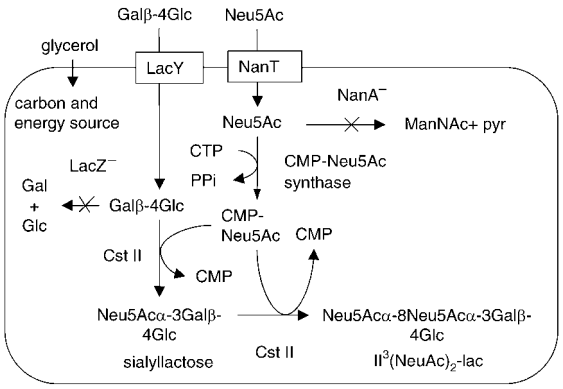

Figure 1. Metabolically engineered pathway of $II^3(Neu5Ac)_2$lac biosynthesis in *Escherichia coli* K12. Lactose and Neu5Ac, which were internalized by the specific β-galactoside and Neu5Ac permeases (LacY and NanT, respectively), could not be degraded because of β-galactosidase (LacZ) and aldolase (NanA) inactivation. Neu5Ac was converted into a nucleotide-activated form (CMP-Neu5Ac) by CMP-Neu5Ac synthase and then transferred onto lactose by the α-3 sialyltransferase activity of CstII to form sialyllactose. A second Neu5Ac was transferred onto the first sialic acid by the α-8 sialyltransferase activity of CstII to form $II^3(Neu5Ac)_2$-lac. CTP = cytidine triphosphate, PPi = inorganic pyrophosphate, pyr = pyruvate.

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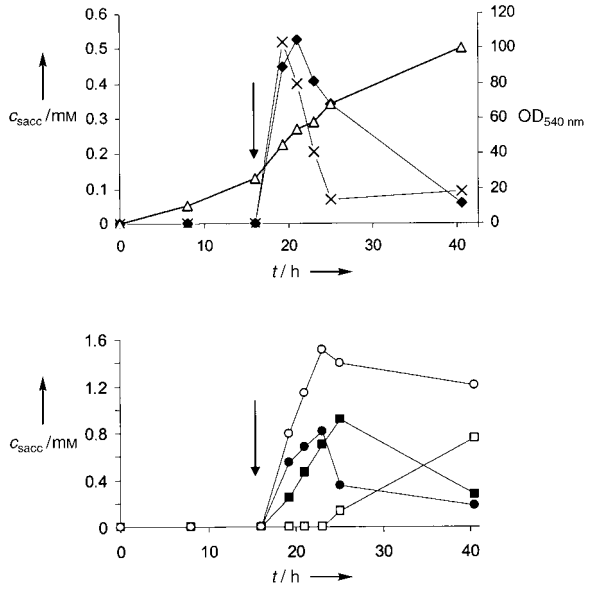
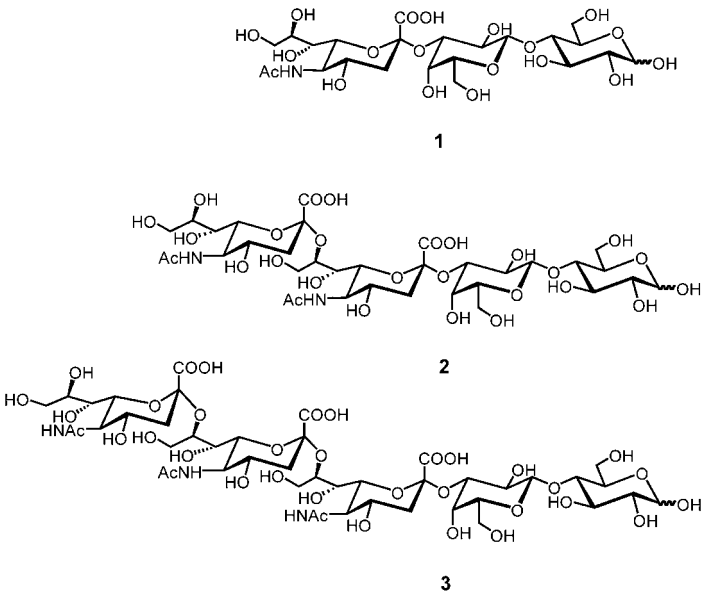


Figure 2. Production of oligosaccharides in a high-cell-density culture of strain TA15. The arrow indicates the start of induction and the addition of lactose (3 mM) and Neu5Ac (6 mM). △ cell growth (measured by optical density, OD); × lactose; ◆ Neu5Ac; ● II^3(Neu5Ac)-lac (**1**); ■ II^3(Neu5Ac)$_2$-lac (**2**); □ II^3(Neu5Ac)$_3$-lac (**3**); ○ sum of **1**–**3**. c_{sacc} = saccharide concentration.



Scheme 1. Structures of II^3(Neu5Ac)-lac (**1**), II^3(Neu5Ac)$_2$-lac (**2**), and II^3(Neu5Ac)$_3$-lac (**3**).

The structures of compounds **1**–**3** were confirmed by NMR spectroscopy and mass spectrometry of purified products. The ^{13}C NMR signal assignments of **1** and **2** (see Supporting Information) were in close agreement with previously described data.[10] The ^{13}C NMR signal assignment of **3** was made by cross-checking the ^{13}C NMR spectra of compound **2** and α-8-linked tri-Neu5Ac[11] (see Supporting Information). In the positive mode, mass spectrometry analysis showed the presence of quasi molecular ions $[M+Na]^+$ at *m/z* 656 and $[(M+Na-H)+Na]^+$ at *m/z* 678 in the spectrum of compound **1** and quasi molecular ions $[M+Na]^+$ at *m/z* 969 and $[(M+Na-H)+Na]^+$ at *m/z* 991 in the spectrum of compound **2.** The mass spectrum of compound **3** showed one peak in the positive mode at *m/z* 1304, corresponding to the quasi molecular ion $[(M+3Na-3H)+Na]^+$, and one peak in the negative mode at *m/z* 1258, corresponding to the quasi molecular ion $[(M+2Na-2H)-H]^-$. The structures of the carbohydrate moieties of the gangliosides GM3 (II^3(Neu5Ac)-lac; **1**), GD3 (II^3(Neu5Ac)$_2$-lac; **2**), and GT3 (II^3(Neu5Ac)$_3$-lac; **3**) are presented in Scheme 1.

The formation of compound **3** can be explained by a side activity of the CstII sialyltransferase, which would be able to add a third Neu5Ac onto the terminal Neu5Ac of II^3(Neu5Ac)$_2$-lac. The production of compound **3** was somewhat unexpected because no such trisialylated structure has been described in the lipooligosaccharides of *C. jejuni*, and there is no report of CstII being able to use a Neu5Acα-8Neu5Acα-3 motif as an acceptor. However, in other species, such as humans, polysialogangliosides are synthesized by a single enzyme, GD3/GT3 synthase.[12] GT3 is the precursor of C series gangliosides which are the major constituents in adult fish brain and are found abundantly in foetal brains of higher vertebrates.[13] They are also found in various neuroectodermal tumors and there is thus potentially great interest in having easy access to the GT3 oligosaccharide.

By varying the lactose and NeuAc initial concentrations on one hand and the culture time on the other, it is possible to favor either the production of GD3 or GT3 oligosaccharides. In our culture conditions the maximal production yields of the GD3 (0.83 g L^{-1}) and GT3 (0.91 g L^{-1}) oligosaccharides were observed 9 and 24 h after induction, respectively. The yields of purified products were much lower due to the multistep purification procedure which has thus to be considerably improved if these compounds are to be used in applications that require large quantities of material. Immunoadsorbents made with the trisaccharide Neu5Acα-8Neu5Acα-3Gal have recently been shown to deplete anti-GQ1b antibodies in autoimmune neuropathy sera from patients suffering from the Miller–Fisher syndrome.[14] The large-scale preparation of the GD3 oligosaccharide would thus make possible the development of effective immunoadsorption therapies for the treatment of this syndrome.

Experimental Section

The *cstII* gene was amplified by PCR from the *C. jejuni* (ATCC 43438) genome with the Pfu Turbo DNA polymerase from Stratagene. A ribosome-binding site (RBS) was inserted upstream of the original start codon in the forward primer. The PCR product was cloned into pCR4Blunt-TOPO (Invitrogen) and subcloned into the *EcoR1-Pst1* sites of pBluescript II KS (Stratagene).

Sugars were quantified by HPAEC with a decade detector equipped with a carbopac PA10 column (Dionex). Purified compounds **1**–**3** were used as standards. The elution program consisted of a linear gradient of sodium acetate of 0→0.5 M over 45 minutes with an isocratic background of sodium hydroxide (100 mM). The flow rate was 0.8 mL h^{-1}.

Compounds **1** and **2** were purified from one liter of a strain TA15 culture harvested 9 h after induction. The intracellular oligosaccharides were extracted from the cells and adsorbed on activated charcoal as previously described.[15] They were then separated by size-exclusion chromatography on a Biogel P6 column (4 × 100 cm) with $NaNO_3$ (50 mM) as the eluent and a flow rate of 45 mL h^{-1} (see Supporting Information). After being desalted on a TSK HW40F/50F column (50 × 2.1 cm) with water as the mobile phase and a flow rate of 4 mL min^{-1}, pure **1** and **2** were obtained with yields of 49 mg and 98 mg, respectively.

Compound **3** was purified from one liter of a strain TA15 culture harvested 24 h after induction. The procedure was the same as for **1** and **2** except that complete purification required an additional high-performance liquid cromatography step on an ion-exchange SP250/10 nucleosil column (10 × 1 cm). Aqueous $NaNO_3$ (150 mM) was used as the eluent and the flow rate was 4 mL min^{-1}. After a desalting step on a TSK HW40F/50F column, pure **3** was obtained with a final yield of 15 mg.

ESI mass spectra were recorded on a ZQ Waters micromass spectrometer (capillary 3.5 kV, cone voltage 80 V). The 1D NMR spectra of II^3(Neu5Ac)-lac and $II^3$$(Neu5Ac)_2$-lac were recorded on a Bruker Avance 300 spectrometer as previously described.[8]

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