Large-Scale In Vivo Synthesis of the Carbohydrate Moieties of Gangliosides GM1 and GM2 by Metabolically Engineered *Escherichia coli*

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Two metabolically engineered Escherichia coli *strains have been constructed to produce the carbohydrate moieties of gangliosides GM2 (GalNAcβ-4(NeuAcα-3)Galβ-4Glc; Gal = galactose, Glc = glucose, Ac = acetyl) and GM1 (Galβ-3GalNAcβ-4(NeuAcα-3)Galβ-4Glc. The GM2 oligosaccharide-producing strain TA02 was devoid of both β-galactosidase and sialic acid aldolase activities and overexpressed the genes for CMP-NeuAc synthase (CMP = cytidine monophosphate), α-2,3-sialyltransferase, UDP-GlcNAc (UDP = uridine diphosphate) C4 epimerase, and β-1,4-GalNAc transferase. When this strain was cultivated on glycerol, exogenously added lactose and sialic acid were shown to be actively internalized into the cytoplasm and converted into GM2 oligosaccharide. The in vivo synthesis of GM1 oligosaccharide was achieved by taking a similar approach but using strain TA05, which additionally overexpressed the gene for β-1,3-galactosyltransferase. In high-cell-density cultures, the production yields for the GM2 and GM1 oligosaccharides were 1.25 g L^{-1} and 0.89 g L^{-1}, respectively.*

KEYWORDS:

biosynthesis · gangliosides · glycolipids · glycosyltransferases · metabolic engineering

1. Introduction

Gangliosides are sialic acid containing glycosphingolipids that are present in all vertebrate cells. They are involved in a number of carbohydrate-dependent events in adhesion/recognition and signal transduction.[1–2] Gangliosides are notably abundant in brain tissues, where the majority of gangliosides are of the gangliotetraose (Galβ-3GalNAcβ-4Galβ-4Glc; Gal = galactose, Glc = glucose, Ac = acetyl) family with one (GM1) or more sialic acid residues (GD1a, GT1b). Neuronal gangliosides play an important role in the maintenance of myelin stability and in the control of nerve regeneration. The terminal epitope NeuAcα3-Galβ3GalNAc (Neu = neuraminic acid) of GD1a and GT1b is a specific ligand for the myelin-associated glycoprotein (MAG) that acts as an inhibitor of nerve regeneration.[3] The monosialic acid ganglioside GM1 fails to support any MAG-mediated adhesion but promotes neuronal growth and potentiates the action of Nerve Growth Factor and other neurotrophins.[4]

Several ganglioside carbohydrate antigens (GM2, GD2, GD3, fucosyl-GM1) are overexpressed in various types of cancer.[5] Neoconjugates of these tumor antigens to a protein carrier (keyhole limpet hemocyanin) have been shown to elicit antibody responses that might have a positive impact on the chance of survival.[6]

In spite of their biological significance and great potential for new drug development, ganglioside oligosaccharides are not readily available for fundamental or clinical research. The difficulty of chemical synthesis of complex carbohydrates means that ganglioside oligosaccharides are usually prepared from bovine brains.[7] There is thus huge interest in developing new reliable methods to synthesize appreciable amounts of various ganglioside carbohydrates.

Recently, new approaches for oligosaccharide synthesis that use genetically engineered bacteria have emerged.[8] Sialyllactose (NeuAcα-3Galβ-4Glc), the carbohydrate moiety of GM3, and the common trisaccharide core of all gangliosides have been produced in high yield either through bacterial coupling[9] or by living bacteria.[10] In the latter process, sialyllactose was directly produced by growing cells of metabolically engineered *Escher-*

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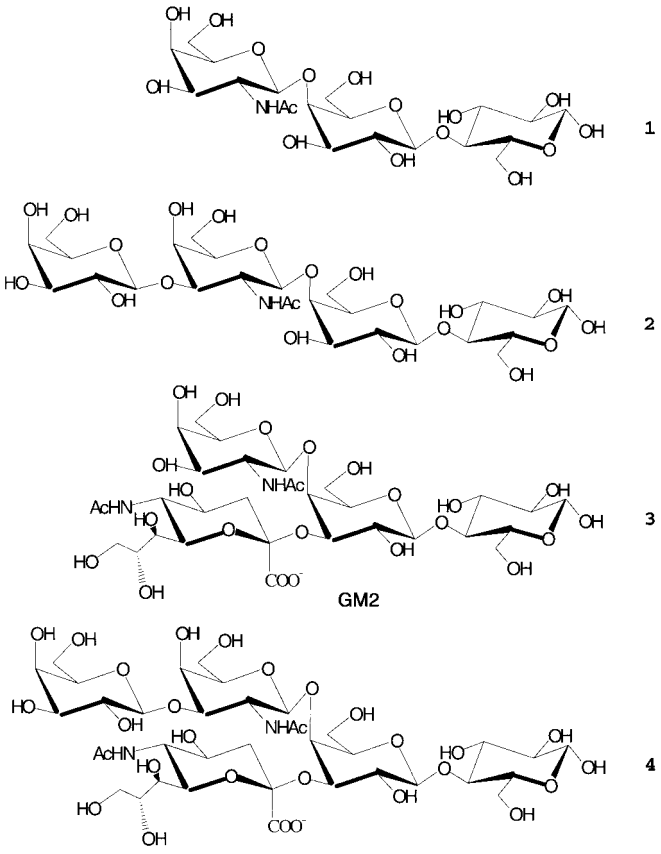
ichia coli strains that overexpress the *Neisseria meningitidis* genes for α-2,3-sialyltransferase and CMP-NeuAc synthase (CMP = cytidine monophosphate). The bacteria were grown to high cell density with glycerol as the carbon and energy source, while exogenous lactose and NeuAc were supplied as precursors for sialyllactose synthesis. Lactose and NeuAc were actively internalized by the *E. coli* β-galactoside and NeuAc permeases during growth of the bacteria. To prevent catabolism of lactose and NeuAc, a mutant strain devoid of β-galactosidase and NeuAc aldolase activity was used. Lactose and NeuAc accumulated in the cytoplasm, where NeuAc was converted into CMP-NeuAc to be transferred onto lactose and form sialyllactose.

The recent identification of the glycosyltranferase genes involved in the biosynthesis of ganglioside mimics in the lipooligosaccharide core of *Campylobacter jejuni*[11] has provided us with a unique opportunity to extend our process of sialyllactose microbial production to the synthesis of more complex ganglioside carbohydrates. However, these structures contain an *N*-acetylgalactosamine residue (GalNAc) and their in vivo synthesis thus requires an endogenous UDP-GalNAc (UDP = uridine diphosphate) supply from the *E. coli* bacterial cell. In mammalians, UDP-GalNAc comes from the epimerization of UDP-GlcNAc and the reaction is catalyzed by UDP-Gal C-4 epimerase, which is active against both UDP-Glc and UDP-GlcNAc. By contrast, the *E. coli* C-4 epimerase GalE has been shown to act only on UDP-Glc.[12] In fact, most bacteria do not need to synthesize UDP-GalNAc since it is not a basic (or housekeeping) constituent of the bacterial cell. GalNAc is only found in some specific carbohydrate antigens produced by a few bacteria that seem to have evolved specialized genes for UDP-GlcNAc C-4 epimerase. Two of these genes, *wbpP* and *gne*, have recently been identified in *Pseudomonas aeruginosa* O6[13] and *E. coli O55:H7*,[14] respectively.

In this work, we report that the carbohydrate moiety of gangliosides GM2 and GM1, along with their respective asialo-derivatives, aGM2 and aGM1 (Scheme 1, Table 1), can be efficiently produced by high-cell-density cultivation of an *E. coli* strain that overexpresses the genes for the appropriate glycosyltransferases and sugar-nucleotide synthases.



Scheme 1. *Structures of the ganglioside carbohydrates synthesized in this study: Gg3 (**1**); Gg4 (**2**); II³-α-Neu5Ac-Gg₃ (**3**); II³-α-Neu5Ac-Gg₄ (**4**).*

2. Results

Production of II³-α-Neu5Ac-Gg₃

The strategy for the in vivo production of II³-α-Neu5Ac-Gg_3 from exogenous lactose and NeuAc is described in Figure 1. The host strain TA01 is a derivative of *E. coli* K12 strain JM107, and was constructed by introducing a chromosomal deletion into the *nanA* gene, which resulted in the inactivation of NeuAc aldolase activity. The II³-α-Neu5Ac-Gg_3 producing strain TA02 was constructed by transforming the host strain TA01 with the three compatible plasmids: pBS-nsyt, which was a pBluescript derivative carrying the *Neisseria meningitidis* gene for CMP-NeuAc synthase and α2,3 sialyltransferase; pACT3cgtA, which is a pACT3[15] derivative carrying the *C. jejuni cgtA* gene for β1,4-GalNAc transferase;[9] and pBBRwbpP, which was a pBBR1 MCS-2[16] derivative carrying the *P. aeruginosa wbpP* gene for UDP-GlcNAc C4 epimerase.[17] The introduction of the *wbpP* gene was thought necessary because of the inability of *E. coli* K12 strains to produce UDP-GalNAc.[14]

Table 1. *Structures of the carbohydrate portion of gangliosides referred to in this study.*

Carbohydrate portion[a]		Ganglioside	
Structure	Abbreviation used in the text	IUPAC abbreviation	Svennerholm abbreviation
GalNAcβ-4Galβ-4Glc	Gg_3	Gg_3Cer	aGM2
Galβ-3GalNAcβ-4Galβ-4Glc	Gg_4	Gg_4Cer	aGM1
NeuAcα-3Galβ-4Glc	sialyllactose	II³-α-Neu5Ac-Gg_2Cer	GM3
GalNAcβ-4(NeuAcα-3)Galβ-4Glc	II³-α-Neu5Ac-Gg_3	II³-α-Neu5Ac-Gg_3Cer	GM2
Galβ-3GalNAcβ-4(NeuAcα-3)Galβ-4Glc	II³-α-Neu5Ac-Gg_4	II³-α-Neu5Ac-Gg_4Cer	GM1

[a] See Scheme 1 for a graphical representation of the structures.

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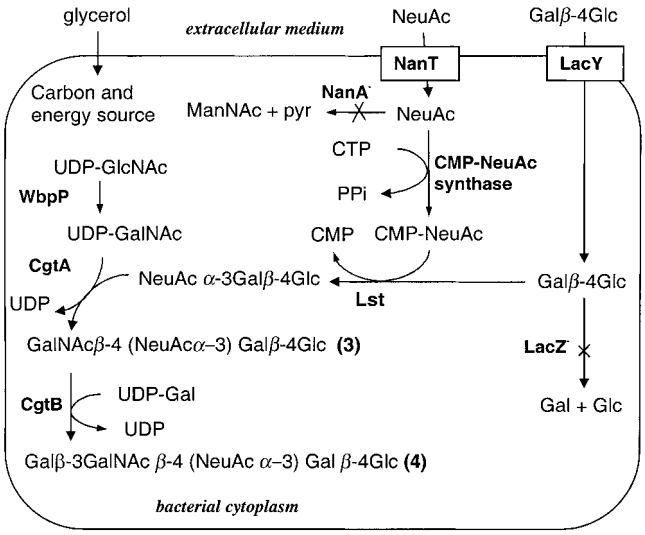

Figure 1. *Metabolically engineered pathway of II³-α-Neu5Ac-Gg₃ (**3**) and II³-α-Neu5Ac-Gg₄ (**4**) biosynthesis in* Escherichia coli *K12. Lactose and NeuAc, which are internalized by the specific permeases LacY and NanT, cannot be degraded because of β-galactosidase (LacZ) and aldolase (NanA) inactivation. NeuAc is converted into a nucleotide-activated form (CMP-NeuAc) by CMP-NeuAc synthase and then transferred onto lactose by α2,3-sialyltransferase (encoded by Lst), to form sialyllactose. Use of the endogenous pool of UDP-GalNAc produced by the recombinant UDP-GlcNAc C4 epimerase (WbpP) allows β1,4-GalNAc transferase (CgtA) to catalyze the glycosylation of sialyllactose to form II³-α-Neu5Ac-Gg₃. This compound serves as an acceptor for β1,3-galactosyltransferase (CgtB) and reacts to yield II³-α-Neu5Ac-Gg₄. CTP, cytidine triphosphate; Ppi, inorganic pyrophosphate.*

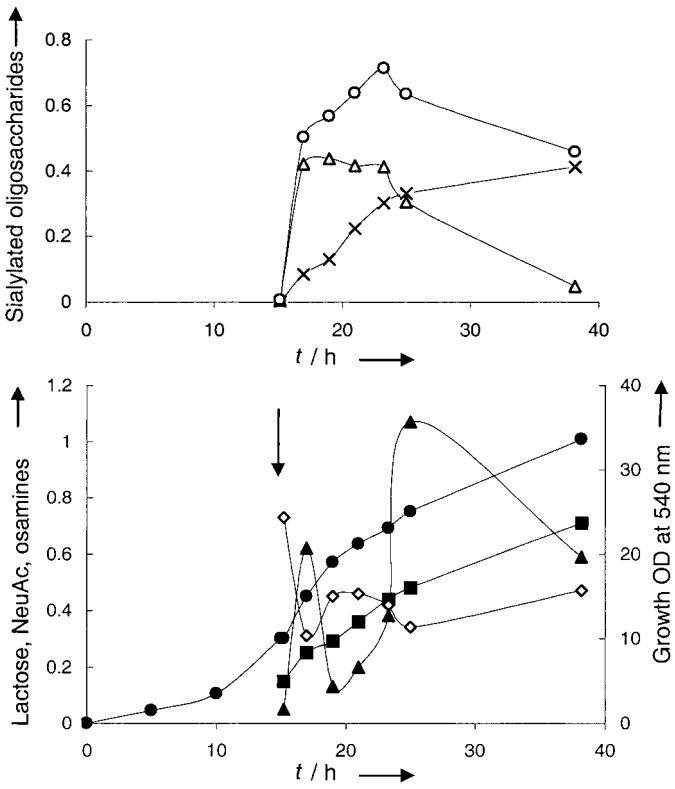

Figure 2. *Production of oligosaccharide in a medium-cell-density culture of strain TA02. ● cell growth; ▲, intracellular lactose; ■ acid-hydrolyzable hexosamine; ◇, extracellular NeuAc; △, sialyllactose; X, II³-α-Neu5Ac-Gg₃; ○, sum of the concentrations of sialyllactose and II³-α-Neu5Ac-Gg₃. The arrow indicates the start of the induction and the addition of lactose (2.9 mM) and NeuAc (0.7 mM). Concentrations are given in mM.*

The strain TA02 was cultivated to medium cell density (final absorbance at 540 nm, $A_{540} = 34$) with glycerol as the carbon and energy source (Figure 2). Lactose (2.9 mM) and NeuAc (0.70 mM) were added at the beginning of the fed-batch phase at the same time as the inducer (isopropyl-β-D-thiogalactopyranoside, IPTG) of *lacY* and of the four recombinant II³-α-Neu5Ac-Gg$_3$ biosynthetic genes, which were all under the control of the Lac repressor. A continuous increase in soluble acid-hydrolyzable hexosamine concentration was observed following induction and throughout the remaining time of culture growth. The level of acid-hydrolyzable hexosamine production was distinctly lower in the control cultures of strain TA03 (without pACT3cgtA) and strain TA04 (without pBBRwbpP), which suggests that strain TA02 had effectively produced II³-α-Neu5Ac-Gg$_3$. Analysis of the intracellular fraction of strain TA02 by TLC showed the appearance of a new compound that was not present in the extract of control strain TA03 and was later identified as II³-α-Neu5Ac-Gg$_3$. Unexpectedly, strain TA04 produced a small amount of a compound that comigrated with II³-α-Neu5Ac-Gg$_3$ on a TLC plate but was later identified as Galβ-4(NeuAcα-3)Galβ-4Glc.

At the end of the fermentation time course, the accumulation of approximately 0.4 mM II³-α-Neu5Ac-Gg$_3$ was detected by high performance anion exchange chromatography (HPAEC) in the intracellular fraction (Figure 2). A small amount of II³-α-Neu5Ac-Gg$_3$ (0.1 mM) was also detected in the culture supernatant. Transient accumulation of lactose and sialyllactose during II³-α-Neu5Ac-Gg$_3$ synthesis was detected. Lactose, which had been added in large excess, was still present at the end of the culture. Surprisingly, a sharp transient decrease in intracellular lactose concentration was observed after the first phase of rapid lactose internalization. The analysis of extracellular lactose concentration indicates this decline, which correlates with a slowdown of the lactose internalization rate. Whether or not this slowdown was due to an inhibition of lactose permease activity remains to be investigated.

An intracellular maximal accumulation of sialyllactose of around 0.4 mM was rapidly reached after 2 h of induction (Figure 2). The rate of sialyllactose synthesis then seemed to correlate with the rate of sialyllactose conversion into II³-α-Neu5Ac-Gg$_3$ for 6 h. Subsequent to that reaction, and likely a result of a shortage of NeuAc, the intracellular sialyllactose concentration decreased to almost complete exhaustion while II³-α-Neu5Ac-Gg$_3$ continued to be slowly produced. However, during this last phase, the amount of II³-α-Neu5Ac-Gg$_3$ being produced did not match the amount of sialyllactose consumed. This observation was partly explained by the presence of low concentrations of sialyllactose (0.23 mM) and II³-α-Neu5Ac-Gg$_3$ (0.1 mM) that had leaked out of the cells in the extracellular medium.

Production of II³-α-Neu5Ac-Gg$_4$

We previously reported that the capacity of *E. coli* to regenerate UDP-Gal was high enough to allow production of galactose-

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containing heterologous oligosaccharides without the need to overexpress or induce the gene for UDP-Gal biosynthesis.[8, 18] A β1,3-Gal transferase was thus the only additional enzyme that was necessary for the production of the carbohydrate moiety of GM1 (Figure 1). Therefore, we subcloned the *C. jejuni cgtB* gene for β1,3-Gal transferase in the pACT3cgtA plasmid to yield the plasmid pACT3cgtAB, in which the two genes *cgtA* and *cgtB* were both under the control of the strong tac promoter, with *cgtB* inserted downstream of *cgtA*. The II^3-α-Neu5Ac-Gg$_4$ producing strain TA05 was then constructed by transforming strain TA01 with the three plasmids pBS-nsty, pACT3cgtAB, and pBBRwbpP.

Strain TA05 was cultivated to medium cell density (final $A_{540} = 34$) under the same conditions as were used for the II^3-α-Neu5Ac-Gg$_3$ producing strain TA02. The kinetics of sialyllactose and acid-hydrolyzable hexosamine production were similar for the two cultures (Figure 3). In contrast, the kinetics of lactose

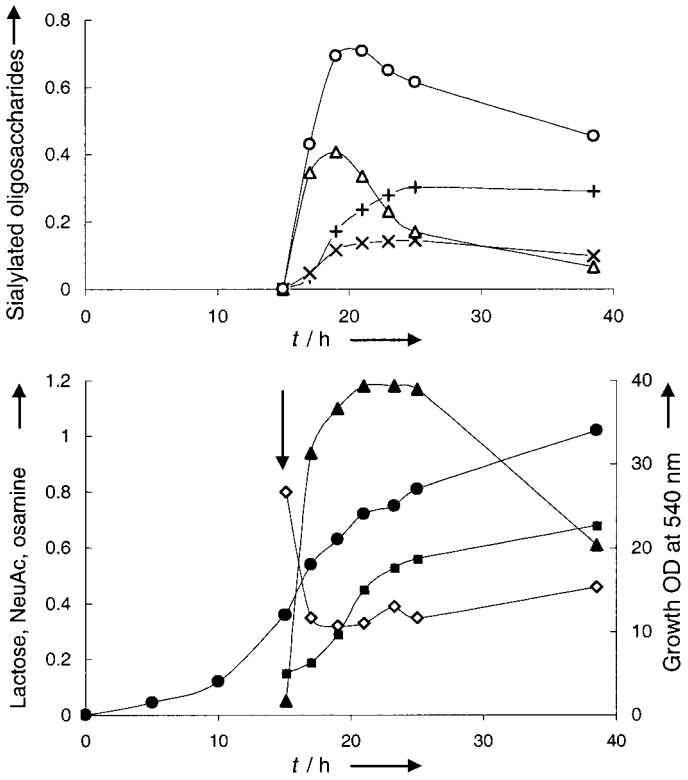

Figure 3. *Production of oligosaccharide in a medium-cell-density culture of strain TA05. ●, cell growth; ▲, intracellular lactose; ■, acid-hydrolyzable hexosamine; ◇, extracellular NeuAc; △, sialyllactose; X, II^3-α-Neu5Ac-Gg$_3$; +, II^3-α-Neu5Ac-Gg$_4$; ○, sum of sialyllactose, II^3-α-Neu5Ac-Gg$_3$, and II^3-α-Neu5Ac-Gg$_4$ concentrations. The arrow indicates the start of the induction and the addition of lactose (2.9 mM) and NeuAc (0.7 mM). Concentrations are given in mM*

internalization were quite different; strain TA05 did not exhibit the phenomenon of biphasic lactose accumulation. HPAE chromatography was used to search for the presence of II^3-α-Neu5Ac-Gg$_4$ in the intracellular fraction. A new product with a shorter retention time than II^3-α-Neu5Ac-Gg$_3$ was identified. This compound was not present in the culture of strain TA02 and its identification as II^3-α-Neu5Ac-Gg$_4$ was later confirmed by mass spectrometry and NMR spectroscopy. Maximal production of II^3-α-Neu5Ac-Gg$_4$ (0.28 mM) was achieved after 12 h of induction. At the end of the fermentation time course, significant amounts of both sialyllactose (0.08 mM) and II^3-α-Neu5Ac-Gg$_3$ (0.1 mM) intermediates were still present in the intracellular fraction. Sialyllactose (0.17 mM) and II^3-α-Neu5Ac-Gg$_3$ (0.07 mM) were detected in the extracellular medium, whereas II^3-α-Neu5Ac-Gg$_4$ could barely be detected.

Purification and characterization of oligosaccharides produced by *E. coli* strain TA02 and strain TA05

To obtain larger quantities of both II^3-α-Neu5Ac-Gg$_3$ and II^3-α-Neu5Ac-Gg$_4$, strains TA02 and TA05 were cultivated to high cell density and the concentration of added lactose and sialyllactose were proportionally increased to 8.7 mM and 2.1 mM, respectively. As a result, production of II^3-α-Neu5Ac-Gg$_3$ by strain TA02 reached 1.5 mmol per liter culture medium. Similarly, production of II^3-α-Neu5Ac-Gg$_3$ and II^3-α-Neu5Ac-Gg$_4$ by strain TA05 increased to 0.5 mM and 0.9 mM, respectively. After purification by charcoal adsorption and anion exchange chromatography on Dowex 1X4 – 400 (HCO_3^- form), the final yields of the acidic oligosaccharide fraction obtained from cellular extracts of one-liter cultures of strains TA02 and TA05 were 560 and 720 mg, respectively.

Results from HPAE chromatography (Figure 4) indicate that the acidic oligosaccharide fraction of strain TA02 consists almost exclusively of the compound that had been tentatively identified as II^3-α-Neu5Ac-Gg$_3$ in the cell extracts. This identification was confirmed by mass spectrometry analysis. The mass spectrum exhibited two peaks at $m/z = 837$ and 875 that correspond to the quasimolecular ions $[M+H]^+$ and $[M+K]^+$ derived from II^3-α-Neu5Ac-Gg$_3$. Size exclusion chromatography on BiogelP2 showed the neutral oligosaccharide fraction of strain TA02 to contain, in addition to lactose, a small amount of an oligosaccharide with the retention time of a trisaccharide. Mass spectrometry data for the purified compound revealed the presence of two quasimolecular ions, $[M+H]^+$ at $m/z = 546$ and $[M+Na]^+$ at $m/z = 568$, which suggests that the neutral oligosaccharide can be identified as Gg$_3$, the asialylated form of II^3-α-Neu5Ac-Gg$_3$.

HPAEC chromatography analysis showed that II^3-α-Neu5Ac-Gg$_4$ represented 67% (w/w) of the acidic fraction of strain TA05 oligosaccharide and that the remaining part consisted of II^3-α-Neu5Ac-Gg$_3$ and sialyllactose (18 and 15% (w/w), respectively). Complete purification of II^3-α-Neu5Ac-Gg$_4$ was achieved by paper chromatography with a 30% yield. The identity of II^3-α-Neu5Ac-Gg$_4$ was confirmed by its mass spectrum, which indicated the presence of three quasimolecular ions, $[M+H]^+$ at $m/z = 999$, $[M+Na]^+$ at $m/z = 1021$, and $[M+K]^+$ at $m/z = 1037$. A tetrasaccharide was purified from the neutral oligosaccharide fraction of strain TA05 by chromatography on Biogel P2. This compound was identified as Gg$_4$ by its mass spectral data, which show three peaks corresponding to the quasimolecular ions $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ at $m/z = 708$, 730, and 746, respectively.

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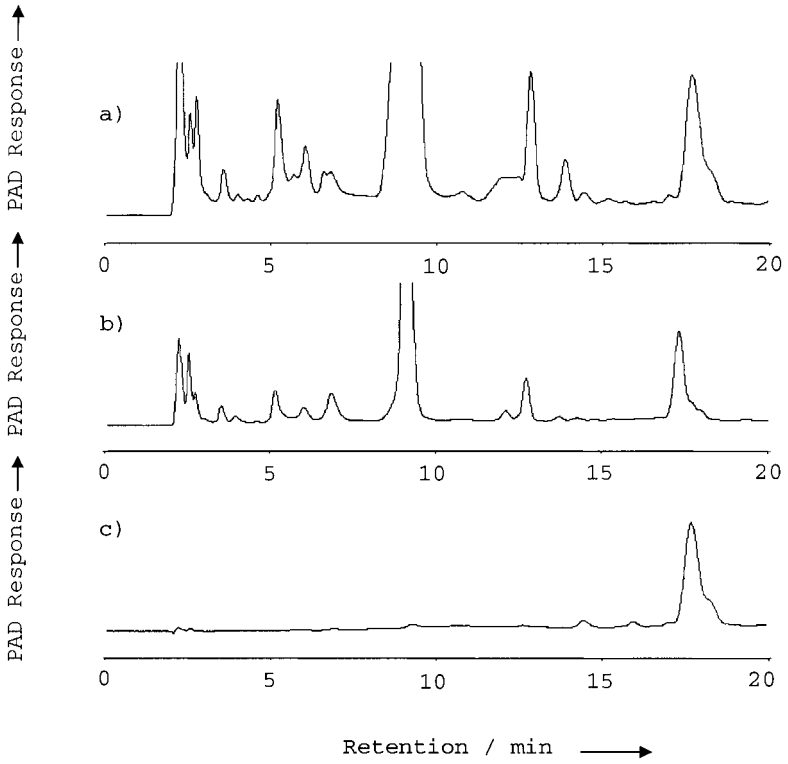

Figure 4. *Purification of II³-α-Neu5Ac-Gg₃ produced by strain TA02. HPAEC chromatograms at the different stages of the purification process: a) crude cellular fraction, b) after purification by charcoal adsorption, c) acidic oligosaccharide fraction separated on a Dowex 1x4 – 400 column.*

The structures of the oligosaccharides produced by strains TA02 and TA05 were also elucidated by ^{13}C and ^{1}H NMR spectroscopy. The data (see supplementary material) confirmed the identification of II³-α-Neu5Ac-Gg₃ and II³-α-Neu5Ac-Gg₄, Gg₃ and Gg₄. The ^{13}C spectrum of II³-α-Neu5Ac-Gg₄ was compared with the previously reported data and was in good agreement with the assignments given by Sabesan et al.[19] In addition, comparison of the ^{13}C spectrum of II³-α-Neu5Ac-Gg₄ with that of II³-α-Neu5Ac-Gg₃ showed that the chemical shift for C3 of the GalNAc residue was shifted downfield from $\delta = 71.57$ ppm (II³-α-Neu5Ac-Gg₃) to $\delta = 80.53$ ppm, which confirms that galactosylation occurs at this position. Comparison of the Gg4 and II³-α-Neu5Ac-Gg₄ spectra also confirmed that sialylation occurs at position 3 of the first Gal unit from the reducing end of the molecule (the C3 signal shows a downfield shift from $\delta = 72.83$ for Gg4 to $\delta = 77.0$ ppm for II³-α-Neu5Ac-Gg₄). The ^{1}H spectrum was also in accordance with previously published data (see the Supporting Information).

Purification and characterization of oligosaccharides produced by strain TA04

The acidic oligosaccharide fraction produced by the control strain TA04, which does not possess the *wbpP* gene for UDP-GlcNAc C4 epimerase, was purified by paper chromatography. The mass spectrometry data of the compound that migrated at the same rate as II³-α-Neu5Ac-Gg₃ on a TLC plate revealed the presence of three quasimolecular ions: $[M+H]^+$ at $m/z = 796$, $[M+Na]^+$ at $m/z = 818$, and $[M+K]^+$ at 834 $m/z = 818$. These results indicate that this compound is an analogue of II³-α-Neu5Ac-Gg₃ in which the terminal GalNAc residue has been replaced by a hexose residue. The compound was further analyzed by 300 MHz ^{1}H NMR spectroscopy. Signals were assigned by comparison with the ^{1}H chemical shifts of sialyllactose and II³-α-Neu5Ac-Gg₃ as references. The signals formed a pattern that is highly characteristic of a compound constituted of a galactose unit β-linked to C4 of the galactose residue of a sialyllactose. The structure Galβ-4(NeuAcα-3)Galβ-4Glc, which is compatible with the H-1 proton signal found at 4.65 ppm, could thus be proposed for the II³-α-Neu5Ac-Gg₃ analogue produced by strain TA04.

3. Discussion

These results clearly show that ganglioside oligosaccharides can be produced in high yields by metabolically engineered bacteria. The maximal yields of II³-α-Neu5Ac-Gg₃ and II³-α-Neu5Ac-Gg₄ are both around 1 g L^{-1}. We anticipate that this yield can be further increased by optimizing the expression of the recombinant genes and the culture conditions. However, the unexplained transient shortage of intracellular lactose observed during culturing of strain TA02 could limit a further improvement in II³-α-Neu5Ac-Gg₃ production.

High quantities of sialylated oligosaccharide were easily purified by using anion exchange chromatography to take advantage of their negative charge. However, the separation of individual sialylated oligosaccharides from one another is relatively difficult. In particular, the negative charge of the molecules limits the use of exclusion size chromatography such as Biogel P2 or P4, which has been proved very efficient for the separation of neutral oligosaccharides. An elegant way to facilitate the purification of sialylated oligosaccharide is to conduct the cultivation in such a way that only one single sialylated oligosaccharide is present when the cells are harvested. In the case of the II³-α-Neu5Ac-Gg₃ producing strain TA02, such cultivation was achieved by limiting the NeuAc supply to prevent excessive synthesis of sialyllactose. In contrast, it was not possible to produce II³-α-Neu5Ac-Gg₄ as the only end product of a strain TA05 culture. This problem probably resulted from insufficient activity of the β1,3-Gal transferase at the end of the culture period. Improvement of *cgtB* expression would thus be useful to both increase the II³-α-Neu5Ac-Gg₄ yield and facilitate its purification.

Synthesis of the asialylated trisaccharide Gg₃ confirmed previous results showing that the *cgtA* gene, which encodes β1,4-GalNAc transferase in *C. jejuni* strain OH4384, also encodes weak activity against lactose.[20] Although the in vitro activity of

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CgtA represented less than 1% of that measured with sialyllactose as the acceptor, this "side activity" of CgtA was high enough to allow the in vivo synthesis of a significant amount of Gg_3. It is noteworthy that CgtA proteins from other *C. jejuni* strains have different acceptor preferences: some are totally inactive against lactose and are able to use both mono- and disialylated acceptors, while others are only active against lactose. Utilization of the latter type of β1,4-GalNAc transferase would be interesting as a possible way to produce large quantities of Gg_3 and Gg_4, which have been shown to act as receptors for pathogens.[21–22]

The production of a small amount of Galβ-4(NeuAcα-3)Galβ-4Glc by strain TA04, which did not produce UDP-GlcNAc epimerase, showed that the β1,4-GalNAc transferase from *C. jejuni* strain OH4384 has low activity against UDP-Gal. The same behavior has been observed for other GalNAc transferases, such as the human blood group A glycosyltransferase.[23]

In conclusion, large-scale production of GM2 and GM1 oligosaccharides opens the way for the synthesis of other GalNAc-containing structures of biological interest, such as the blood group A epitope and the carbohydrate moiety of globoside.

4. Experimental section

Bacterial strains, plasmids, and cloning procedures: A summary of plasmids and strains used is presented in the Supporting Information. The chromosomal deletion in the *nanA* gene of strain TA01 was introduced by homologous recombination using an appropriate suicide vector derived from plasmid pMAK705.[24] Deletion mutants *nanA*$^-$ were screened for their inability to grow on M9 medium with *N*-acetylneuraminic acid as the sole carbon and energy source. To obtain the plasmid pBS-nsyt, the *Xba*I-*Xba*I fragment containing the CMP-NeuAc synthase gene was excised from the plasmid pNSY-01[25] and cloned into the *Xba*I site of plasmid pNST01[26] upstream from the α-2,3-sialyltransferase gene. The plasmid pBBRwbpP was constructed by inserting the *Xba*I-*Eco*RI fragment from pFV617 – 26a,[17] which contains the UDP-Glc*N*Ac-4-epimerase gene, into the *Xba*I-*Eco*RI sites of pBBR1MCS-2. To construct the pACT3cgtA plasmid, the *cgtA* sequence was first amplified by PCR from the plasmid pCJL-09, which carries the *cgtA* gene from *C. jejuni* strain OH4384 fused with *malE*. A ribosome binding site (RBS) sequence was inserted into the forward primer upstream of the original start codon of *cgtA*. The PCR product was cloned in pCR2.1-TOPO after amplification with the Taq polymerase from Promega. The 1.1-kb fragment containing the *cgtA* sequence was then cloned into *Kpn*I-*Sal*I sites in pACT3 to yield pACT3cgtA.

To construct pACT3cgtAB, the *cgtB* sequence was amplified by PCR from the plasmid pCJL-04, which carries the *cgtB* gene from *C. jejuni* strain NCTC 11168 fused with *malE*. A *Pst*I site and a RBS sequence were inserted into the forward primer upstream of the original start codon of *cgtB*. The PCR product was cloned in pCR4BLUNT-TOPO after amplification with the proof-start polymerase, purchased from QIAGEN. The 1-kb fragment containing the *cgtB* sequence was then excised from pCR4BLUNT-TOPO by *Pst*I digestion and cloned into the *Pst*I site of pACT3cgtA to yield pACT3cgtAB.

Medium- and high-cell-density cultivation: Cultivation was carried out in 2-litre reactors containing one litre of mineral culture medium as previously described.[27] Antibiotics were added in both preculture and culture at the following concentrations: ampicillin (50 mg L^{-1}), tetracycline (15 mg L^{-1}), chloramphenicol (20 mg L^{-1}). The temperature was maintained at 34 °C and the pH regulated to 6.8. The high-cell-density cultivation strategy included three phases: an initial exponential growth phase which started with the inoculation of the fermentor and lasted until exhaustion of the carbon substrate (glucose or glycerol) initially added to the medium at a concentration of 17.5 g L^{-1}; a 5-h fed-batch phase with a high substrate feeding rate of 4.5 g L^{-1} h^{-1}; a 10 – 20-h fed-batch phase with the lower feeding rate of 2.7 g L^{-1} h^{-1}. As a result of the relatively high cost of NeuAc, the first cultures were carried out at medium cell density with only one third of the normal glycerol supply. Lactose (2.9 mM) and sialic acid (0.7 mM) were added at the beginning of the first fed-batch phase (indicated by an arrow in Figures 2 and 3) at the same time as the inducer (IPTG, 50 mg L^{-1}) of the β-galactoside permease and of the recombinant genes that were under the control of the Lac repressor.

Quantification of oligosaccharides: Culture samples (3 mL) were centrifuged in microfuge tubes (5 min, 12 000 × *g*) immediately after collection. The supernatants were saved for quantification of extracellular oligosaccharides. The pellets were resuspended in distilled water (1 mL), boiled for 30 min, and centrifuged as described above. The second supernatant was kept for quantification of the intracellular oligosaccharides. TLC plate analysis was carried out on silica gel and the oligosaccharides were eluted with butanol/acetic acid/water (2:1:1, two runs). Sugars were detected by dipping the plate in orcinol sulfuric reagent and heating it. After acid hydrolysis, the hexosamine content was quantified colorometrically by using Ehrlich reagent. Lactose concentration was quantified with a Kit purchased from Roche Diagnostic for the enzymatic determination of lactose and galactose. Sialic acid was quantified colorometrically by using diphenylamine. Sialyllactose, II^3-α-Neu5Ac-Gg_3, and II^3-α-Neu5Ac-Gg_4 were quantified by high-performance anion-exchange chromatography with a DECADE detector equipped with a Carbopac PA10 column (Dionex). The temperature was kept at 30 °C. The elution program consisted of a linear gradient of sodium acetate from 0 to 20 mM in 20 min with an isocratic background of sodium hydroxide (167 mM). The flow rate was 0.6 mL h^{-1}.

Purification of oligosaccharides: At the end of the fermentation time course the bacterial cells were recovered by centrifugation (20 min at 7 000 × *g*). The pellets were suspended in a volume of distilled water equal to that of the original culture medium and the cells were permeabilized by autoclaving at 100 °C for 50 min. The solution was again centrifuged (20 min at 7 000 × *g*), the cell debris was discarded and the supernatant was mixed with activated charcoal. After filtration and thorough washing with distilled water, the oligosaccharides were eluted with aqueous ethanol 50% (v/v) as previously described.[28]

Negatively charged oligosaccharides were separated from neutral oligosaccharides by fixing on Dowex 1X4 – 400 (HCO_3^- form) resin. Sialylated oligosaccharides were then eluted with a linear $NaHCO_3$ gradient (10 – 50 mM). Sodium bicarbonate was then eliminated by treatment with Dowex 50X4 – 400 (H^+ form) resin. The neutral oligosaccharides were purified by size exclusion chromatography on a Biogel P2 column (1.5 × 200 cm) at 60 °C, with distilled water as the mobile phase. The flow rate was 3.5 mL h^{-1}. The acid oligosaccharides were separated by paper chromatography on Whatman No. 3 paper in ethyl acetate/pyridine/acetate/water (5:5:1:3 v/v).

Carbohydrate structural analysis: The 1D NMR spectra of II^3-α-Neu5Ac-Gg_3, Gg_3, and Gg_4 were recorded on a Bruker AVANCE 300 spectrometer by using the Bruker standard pulse sequences. ^{1}H NMR spectra were recorded at 303 K, with acetone ($\delta = 2.225$) as the standard. ^{13}C NMR experiments were carried out on the same

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spectrometer and on the same sample at 303 K (internal acetone ^{13}C (CH_3) $\delta = 31.5$ relative to tetramethylsilane (TMS)). The 1 D spectra of II^3-α-Neu5Ac-Gg_4 were recorded on a Bruker AVANCE 400 (^1H, frequency: 400.132 MHz; ^{13}C, frequency: 100.613 MHz) by using the Bruker standard pulse sequences. ^1H NMR spectra were recorded at 278 K, with HOD ($\delta = 4.99$) as the internal standard. ^{13}C NMR experiments were carried out on the same spectrometer and on the same sample at 303 K (internal acetone ^{13}C (CH_3) $\delta = 31.5$ relative to TMS). Low-resolution FAB mass spectra were recorded in the positive mode of an R1010C quadripolar mass spectrometer (model 2000, Nermag, Reuil-Malmaison, France) equipped with an M Scan Wallis-type gun (8 kV, 20 mA). The samples were dissolved in a glycerol matrix and submitted to Xe (9 kV) bombardment.

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