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The living factory: *In vivo* production of N-acetyllactosamine containing carbohydrates in *E. coli*

Emmanuel Bettler[1], Eric Samain[1], Valerie Chazalet[1], Claude Bosso[1], Alain Heyraud[1], David H. Joziasse[2], Warren W. Wakarchuk[3], Anne Imberty[1], and Roberto A. Geremia[1]*

[1]*Centre de Recherches sur les Macromolécules Végétales CNRS (Affiliated with the Joseph Fourier University) B.P. 53, 38041 Grenoble cedex 9, France*
[2]*Department of Medical Chemistry, Vrije Universiteit, van der Boechstraat 7, 1081 BT Amsterdam, The Netherlands*
[3]*Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, K1A 0R6, Canada*

Scientific and commercial interest in oligosaccharides is increasing, but their availability is limited as production relies on chemical or chemo-enzymatic synthesis. In search for a more economical, alternative procedure, we have investigated the possibility of producing specific oligosaccharides in *E. coli* that express the appropriate glycosyltransferases. The *Azorhizobium* chitin pentaose synthase NodC (a β(1,4)GlcNAc-oligosaccharide synthase), and the *Neisseria* β(1,4)galactosyltransferase LgtB, were co-expressed in *E. coli.* The major oligosaccharide isolated from the recombinant strain, was subjected to LC-MS, FAB-MS and NMR analysis, and identified as βGal(1,4)[βGlcNAc(1,4)]$_4$GlcNAc. High cell density culture yielded more than 1.0 gr of the hexasaccharide per liter of culture. The compound was found to be an acceptor *in vitro* for βGal(1,4)GlcNAc α(1,3)galactosyltransferase, which suggests that the expression of additional glycosyltransferases in *E. coli* will allow the production of more complex oligosaccharides.

Keywords: **oligosaccharides, glycosyltransferases, glycobiology, N-acetyllactosamine**

Introduction

Oligosaccharides, often as glycoconjugates, are involved in biological recognition processes such as occur during fertilization, embryogenesis, metastatis, inflammation, and host pathogen adhesion. Therefore there is a large pharmaceutical interest in developing novel carbohydrate-based therapeutic agents, and considerable effort has gone into developing chemical or chemo-enzymatic syntheses [1]. Unfortunately chemical methods are laborious and time-consuming, and because of the large number of steps involved they are difficult to scale-up. On the other hand, chemo-enzymatic approaches suffer from the relatively poor availability of glycosyltransferases and the need to regenerate *in situ* nucleotide sugars.

An alternative strategy is to produce the desired oligosaccharides in recombinant organisms expressing the appropriate glycosyltransferases. This method presents several advantages when compared to chemo-enzymatic methods. First, overproduction and purification of glycosyltransferase is not needed. A basal level of expression, that can be reached using a weak promoter, could be enough to produce catalytic amounts of enzyme. Second, the metabolic pathway for the synthesis of most sugar nucleotides donors is already present. Additionally, the culture medium is inexpensive, and fermenter technology is well proven. Therefore, only simple cloning methods, inexpensive culture media and fermenters are needed. Finally, as *E. coli* is one of the best known living organisms, the technology for modifying its metabolic pathways, by physiological or by genetic means, is readily available.

The NodC protein is a chitin oligosaccharide synthase from bacterial *Rhizobium* species [2] that was shown to be functional *in vitro* and *in vivo* when expressed in *E. coli* [2,3,4]. NodC has four transmembrane helices [5], and was found to localize in a total membrane fraction [2]. The globular domain was located in the cytoplasmic site of the inner membrane. In high cell density culture of a recombinant *E. coli* strain expressing both NodC and the chitin oligosaccharide deacetylase NodB, the resulting NodBC

*To whom correspondence should be addressed: *CERMAV/CNRS, BP 53, Grenoble Cedex 9; France Tel* + 33 (0)4 76 03 76 47, Fax + 33 (0)4 76 54 72 03. E-mail: geremia@cermav.cnrs.fr

metabolite (tetra N-acetyl chitopentaose) was produced with a yield of one gram per liter of culture [6]. If only NodC is expressed, the non-reducing GlcNAc residue can be used as an acceptor for the addition of new carbohydrate residues, leading to the synthesis of complex oligosaccharides. For example, the co-expression of NodC and a lactosamine synthase (UDP-galactose:N-acetyllactosamine β(1,4)galactosyltransferase), is anticipated to lead to the synthesis of a N-acetyllactosamine (LacNAc) moiety at the non-reducing end. LacNAc is part of several oligosaccharides of biological interest such as blood group substances and "complex-type" N-glycans (see Fig. 1A). This disaccharide is also the core of several carbohydrate drugs that are designed to prevent the recognition of specific carbohydrate epitopes such as sialyl Lewis^x (in inflammation processes) and αGal(1,3)βGal(1,4)βGlcNAc (in hyperacute rejection of xenografts).

The co-expression of NodC and β(1,4)galactosyltransferase may lead to the synthesis of a terminal N-acetyllactosamine moiety. To test this hypothesis, we have introduced the *Neisseria meningitidis lgtB* gene in an *E. coli* strain expressing NodC. LgtB is a lactosamine synthase [7] involved in the biosynthesis of lacto-N-neotetraose terminal lipopolysaccharide structure. When produced in *E. coli,* LgtB was found to be located mainly in the membrane, but a significant amount is located in the cytoplasm [8]. In the present work we report the production of a N-acetyllactosamine-containing oligosaccharide Gal-$(GlcNAc)_5$, by an

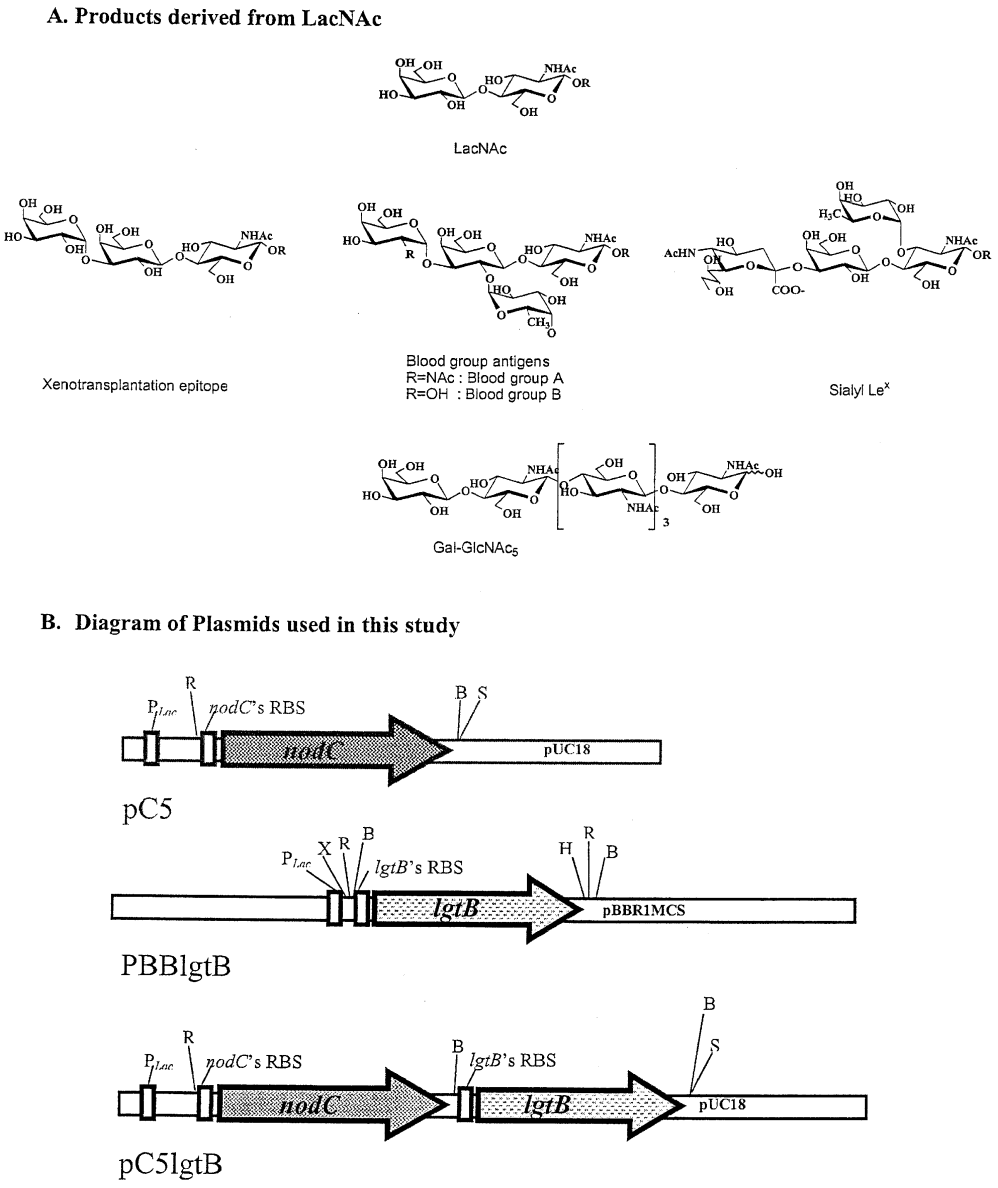

Figure 1. A) Oligosaccharides derived from LacNAc. B) Diagram of plasmids used with relevant restriction sites. R: *Eco*RI, B: *Bam*HI, X: *Xho*I; S: *Sph*I, RBS: Ribosomal Binding Site.

E. coli strain expressing both NodC and the β(1,4)galactosyltransferase LgtB, with yields close to 1 g/liter of culture.

Experimental Protocols

Bacterial strains, plasmids and growth conditions

E. coli XL-1Blue (Stratagene) was used for cloning procedures, and *E. coli* JM109, DH1 or DH5α for production of oligosaccharides. *E. coli* strains harbouring *nodC* were grown at 34°C. Low cell density cultures were performed in 10–100 ml of M9 medium supplemented with 2% glycerol. High cell density cultures were performed as previously described [6], but antibiotics were added at the following concentrations: ampicillin (50 mg/l), chloramphenicol (34 mg/l). After consumption of the initial glycerol (Fig. 2, phase 1), the feeding of a concentrated glycerol solution was started. After 6 hours of cultivation (phase 2), the feeding rate was lowered and kept constant until the end of the culture (phase 3).

Cloning procedures

The 853 bp *Bam*HI-*Hind*III fragment derived from pCWlgtB, containing the *lgtB*'s ribosomal binding site and open reading frame, was subcloned into *Bam*HI/*Hind*III-digested pLitmus28 (NEN Biolabs), to give pLitlgtB. The 0.9 kb *Xho*I-*Hind*III fragment containing *lgtB* was excised from pLitlgtB, and cloned into pBBR1MCS [9], to give pBBLgtB. PC51gtB was constructed by cloning the 0.95 kb *Eco*RV fragment containing *lgtB* from pBBLgtB, into the *Sph*I site of pC5, downstream *nodC*.

Quantification of Gal-chitin oligosaccharide.

The Gal-$(GlcNAc)_5$ was quantified using a differential enzymatic degradation method, based on the mechanism of action of the β(1,4)galactosidase-free *Serratia marcescens* chitobiase [10]. This enzyme starts releasing GlcNAc units located at the non reducing end [11], thus it does not degrade chitin oligosaccharides substituted at the non reducing end. Total recombinant oligosaccharides was quantified using an *Aspergillus oryzae* β(1,4)galactosidase contaminated with a chitobiase [12]. Between 75 µl and 3 ml of culture sample was centrifuged (5 min. 12,000 g) in microfuge tubes. The pellet was resuspended in 150 µl of distilled water, boiled for 30 min to release oligosaccharides, and centrifuged as above. 80 µl of the supernatant were digested with *S. marcescens* chitobiase and/or *A. oryzae* enzymes for 3 h at 37 °C. The GlcNAc content was determined [13] using chitin pentaose as standard.

Chromatographic methods

Pure βGal(1,4)[βGlcNAc(1,4)]$_4$GlcNAc was obtained after degradation of non-galactosylated oligosaccharide by chitobiase and purified on a column (1.5 x 200 cm) of Bio-Gel

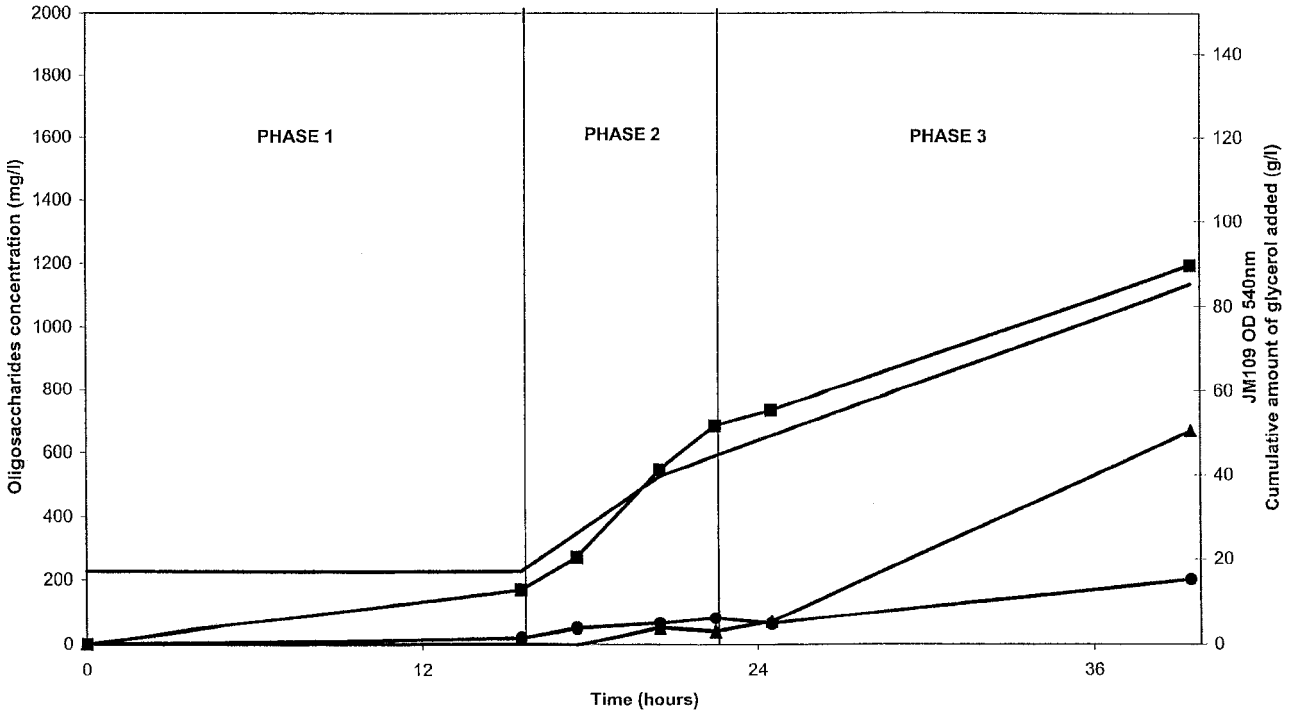

Figure 2. High-cell density culture of *E. coli* JM109 harbouring both pC5 and pBBLgtB. —▲—: Total recombinant oligosaccharides, —●—: chitin oligosaccharides, —■—: bacterial growth, ——: total glycerol added. Total oligosaccharides were quantified using *A. oryzae* β(1,4)galactosidase and chitobiase, and chitin oligosaccharides using *S. marcescens* chitobiase.

P2, equilibrated and run in deionized water at a flow rate of 0.5 ml/min at 60°C.

Spectroscopic analysis

The mass spectra of various compounds were obtained in two ways: first, by Fast Atom Bombardment (FAB) on quadripolar 1010C (NERMAG) version 2000 uma spectrometer, with glycerol matrix. Second, by coupling liquid chromatography–mass spectrometry (LC/MS) on Platform II (Micromass Instruments) spectrometer equiped with electrospray source. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AC300 spectrometer at 300 MHz.

α(1,3)galactosyltransferase assay

Production, purification and enzymatic assay of soluble bovine α(1,3)galactosyltransferase were performed as described previously [14,15]. Briefly, a reaction mixture (50µl) containing acceptor substrate, 2.5 µmol Na-cacodylate buffer pH6.5, 1.0 µmol $MnCl_2$, 200 nmol ATP, 25ng BSA, 25 nmol UDP-[^{14}C]Gal, and recombinant bovine α(1,3) galactosyltransferase, was incubated for 60 min at 37°C. The acceptor was lactose (48 mM), LacNAc (7 mM), pure Gal-$(GlcNAc)_5$ (7 mM), or crude Gal-$(GlcNAc)_5$ (130 µg/50 µl). Reactions were stopped by the addition of 400 µl cold water, unreacted UDP-Gal was removed by chromatography on a column of DEAE-Sephacell (Pharmacia), and the radioactivity in eluate and washes determined by liquid-scintillation counting.

Results

Sub-cloning of *lgtB* and oligosaccharide production in low cell density batch culture

Since the acceptor of LgtB is similar to the product of NodC, and since *E. coli* has an internal pool of UDP-Gal, we have hypothesized that co-expression of both genes in *E. coli* will lead to the following reactions:

$$5\,\text{UDP-GlcNAC} \xrightarrow{\text{NodC}} [\beta\text{GlcNac}(1,4)]_3\text{GlcNAc} + 5\text{UDP}$$

$$\beta\text{GlcNAc}(1,4)[\beta\text{GlcNAc}(1,4)]_3\text{GlcNAc} + \text{UDP-Gal} \xrightarrow{\text{LgtB}} \beta\text{Gal}(1,4)[\beta\text{GlcNAc}(1,4)]_4\text{GlcNAc} + \text{UDP}$$

Since pC5 plasmid (expressing *nodC*) was already available [6], the *lgtB* gene was cloned either in a compatible plasmid, or into pC5 downstream of *nodC* (Fig. 1B), to give pBBLgtB and pC5LgtB respectively. In both cases these genes were under the control of P_{Lac} a leaky promoter allowing a low-level expression of the downstream genes. Both the pC5 and pBBLgtB plasmids, or pC5LgtB alone were introduced into *E. coli* JM109. This *lacZ* strain was used to prevent degradation of the Gal-(GlcNAc)5 product. Since the production of oligosaccharides was optimized for the pC5 plasmid, *lgtB* was first provided in *trans* to *nodC*. *E. coli* JM109 harbouring both pC5 and pBBLgtB was grown in small scale culture and the oligosaccharides were isolated and analyzed by FAB-MS. Two quasimolecular ions at m/z: 1196 ($[M+H]^+$) and 1218 ($[M+Na]^+$), corresponding to the expected hexosyl-$(GlcNAc)_5$ were detected.

Production of more complex or modified oligosaccharides in *E. coli* will require expression of several additional genes, making it necessary to clone more than one gene in a single plasmid. The influence of *lgtB* localization on the production of recombinant oligosaccharide was tested by comparing the production of chitobiase resistant oligosaccharides (see Experimental Protocols) either in *E. coli* JM109 pC5 and pBBLgtB or *E. coli* JM109 pC5LgtB in low cell density cultures. The strain harboring pC5 and pBBLgtB produced 12 µg/ml, while strain harboring pC5LgtB produced 15.8 µg/ml, indicating that the position of *lgtB* has no major effect in the oligosaccharide production.

Interestingly, *E. coli* DH1 (a $LacZ^+$ strain) harbouring pC5 and pBBLgtB, produced only *S. marcesens* chitobiase sensitive oligosaccharides, indicating that in the presence of endogenous β-galactosidase, the non-reducing end of chitooligosaccharide is not substituted.

To establish the feasibility of large-scale production and to obtain sufficient amounts of the hexasaccharide for structural analysis, we proceeded to high cell density cultures (HCDC).

Production of Gal-$(GlcNAc)_5$ in high cell density cultures

E. coli strain JM109 harboring pC5LgtB was grown in HCDC conditions, as described in Experimental Protocols. After 40 hours of culture (Fig. 2), the total amount of oligosaccharides produced was 1.35 g/l of culture. *S. marcescens* chitobiase-sensitive oligosaccharides represented only 20% of the total, suggesting that ~80% of the chitin oligosaccharides are substituted at the non-reducing end. The strain *E. coli* JM109 pC5 and pBBLgtB produced comparable amounts of oligosaccharides.

Characterization of recombinant oligosaccharides

The oligosaccharides were released from bacteria by boiling the cells, adsorbed on charcoal, and eluted with 50% ethanol as previously described [6]. To assess the complexity of the oligosaccharide mixture, this fraction was submitted to LC-MS analysis. Several peaks were resolved by HPLC (data not shown). Peaks eluting at 18.89 min and

13.56 min were attributed respectively to the β and α anomers of a hexosyl-$(GlcNAc)_5$ (quasimolecular ion at *m/z* 1196 $[M + H]^+$, rupture ions of intersugar linkages at *m/z* 366, 569, 772 and 975). $(GlcNAc)_5$ anomers elute at 11.03 (β and 15.74 (α) min (quasimolecular ion at *m/z* 1034 $[M + H]^+$, *m/z* 1056 $[M + Na]^+$, rupture ions at *m/z* 204, 407, 610 and 813). Interestingly, a product eluting at 34.19 min corresponding to hexosyl-$(GlcNAc)_5$-glycerol (*m/z* 1270 $[M + H]^+$, *m/z* 635.7 $[M+2H/2]^+$, rupture ions at *m/z* 366, 569, 772, 975 and 1178) was detected. Products with lower retention time were difficult to identify, nevertheless, peaks at 5.7 and 7.27 min represent hexosyl-$(GlcNAc)_2$ and hexosyl-$(GlcNAc)_3$ respectively. The fragmentation pattern of recombinant hexosyl-chitinoligosaccharides is consistent with the presence of the hexose residue at the non-reducing end. ^{1}H-NMR analysis of the crude sample showed an H-1(Gal):H(CH_3 Acetate) ratio of 1:14, indicating a degree of galactosylation higher than 90 %.

To estimate the purity of Gal-$(GlcNAc)_5$, and for further purification, the mixture was resolved by gel filtration chromatography on Bio-Gel P2 (Fig. 3, A). Only one major peak, eluting at 140 min (Fig. 3, A, peak I) was observed. This product was analyzed by NMR and FAB-MS (see below) and was concluded to be βGal(1,4)[βGlcNAc(1,4)]$_4$GlcNAc (Fig. 1A) The minor shoulder (Fig. 3, A, peak II) was assigned to $(GlcNAc)_5$ by comparison with available standards, and its sensitivity to chitobiase treatment. The low amounts of material in peak IV and another small peaks precluded structural analysis.

Structural analysis of Gal-$(GlcNAc)_5$

To obtain pure material for structural analysis, the crude extract was digested with *S. marcescens* chitobiase, and submitted to Bio-Gel P2 chromatography (Fig. 3, B). The H-1(Gal):H(CH_3 Acetate) ratio of peak I was of 1:15 as expected for the pure product. The structure of peak I (Fig. 3, A) was deduced from ^{1}H-NMR, ^{13}C-NMR, and MS spectra. The 1D ^{1}H NMR spectrum (data not shown) contains four anomeric signals. By comparison with data obtained on chitin oligosaccharides [16], the signals at δ 5.07 ($J_{1,2}$ 1.8 Hz) and 4.58 ($J_{1,2}$ 7.5 Hz) ppm were assigned to the H-1 of the α and β forms of the reducing GlcNAc residue. The large peak at δ 4.45–4.51 ppm (4 H) was attributed to the four protons of the (1,4) linked βGlcNAc residues and the resonance at δ 4.34 ppm (1 H, $J_{1,2}$ 7.6 Hz) corresponds to the H-1 of the βgalactose residue at the non-reducing end. The high-field signal at δ 1.45 ppm (15 H), is consistent with the presence of 5 N-acetyl groups. The assignment of the ^{13}C-NMR spectrum (Fig. 4) was done on the basis of data from related oligosaccharides [6,16,17]. These results are consistent with a structure in which a βgalactosyl residue is 1–4 linked to the non-reducing end of chitin pentaose (Fig. 1A).

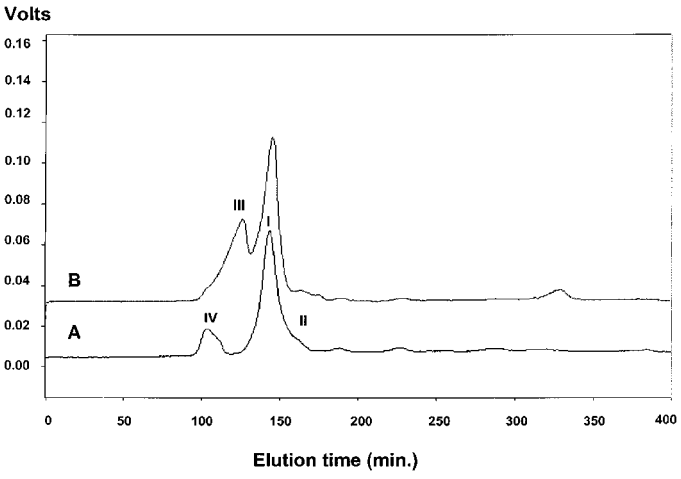

Figure 3. Bio-Gel P2 Chromatography of recombinant oligosaccharides. A: Crude Gal-$(GlcNAc)_5$, **B:** Gal-$(GlcNAc)_5$ after *S. marcescens* chitobiase treatment. Eluting material was detected with a refractometer. Peak I corresponds to Gal-$(GlcNAc)_5$ compound, peak II to $(GlcNAc)_5$ and peak III to the chitobiase.

Biochemical activity of βGal(1,4)[β(GlcNAc)]$_4$GlcNAc

Since the chitin tetrasaccharide located at the reducing end of the recombinant LacNAc may interfere in recognition of LacNAc by proteins, we have tested the Gal-$(GlcNAc)_5$ compound as acceptor for bovine UDP-Gal:β(1,4)GlcNAc α(1,3)galactosyltransferase, which uses either free or conjugated LacNAc as acceptor [14]. Lactose, LacNAc, crude charcoal desorbed Gal-$(GlcNAc)_5$ and pure Gal-$(GlcNAc)_5$ were compared in the assay. After incubation of the oligosaccharides in the presence of α(1,3)galactosyltransferase and UDP[^{14}C]Gal (see Experimental Protocols) radioactivity was incorporated into the neutral fraction as a function of time and dependent on the presence of oligosaccharide (Table 1). These results indicate that Gal-$(GlcNAc)_5$ is recognized as an acceptor. The presence of chitin oligosaccharides either free, or linked to the LacNAc, does not interfere with galactosyl transfer.

Discussion

A novel system for the production of complex oligosaccharides in *E. coli* was developed. NMR and FAB-MS spectral data firmly established that galactosyl-chitin pentaose was produced. The oligosaccharide is sensitive to β-galactosidase, consistent with its absence in a $LacZ^+$ strain, and is an acceptor for α(1,3)galactosyltransferase. Based on colorimetric data, Bio-Gel P2 chromatography, and ^{1}H NMR, the Gal-$(GlcNAc)_5$ represents at least 90% of the total "recombinant" oligosaccharides. Other minor products were also detected: chitin pentaose, derivatives with a lower degree of polymerization, and derivatives substituted with glycerol. These compounds were also detected when growing *E. coli* pC5 cells in batch culture (Geremia *et al.*,

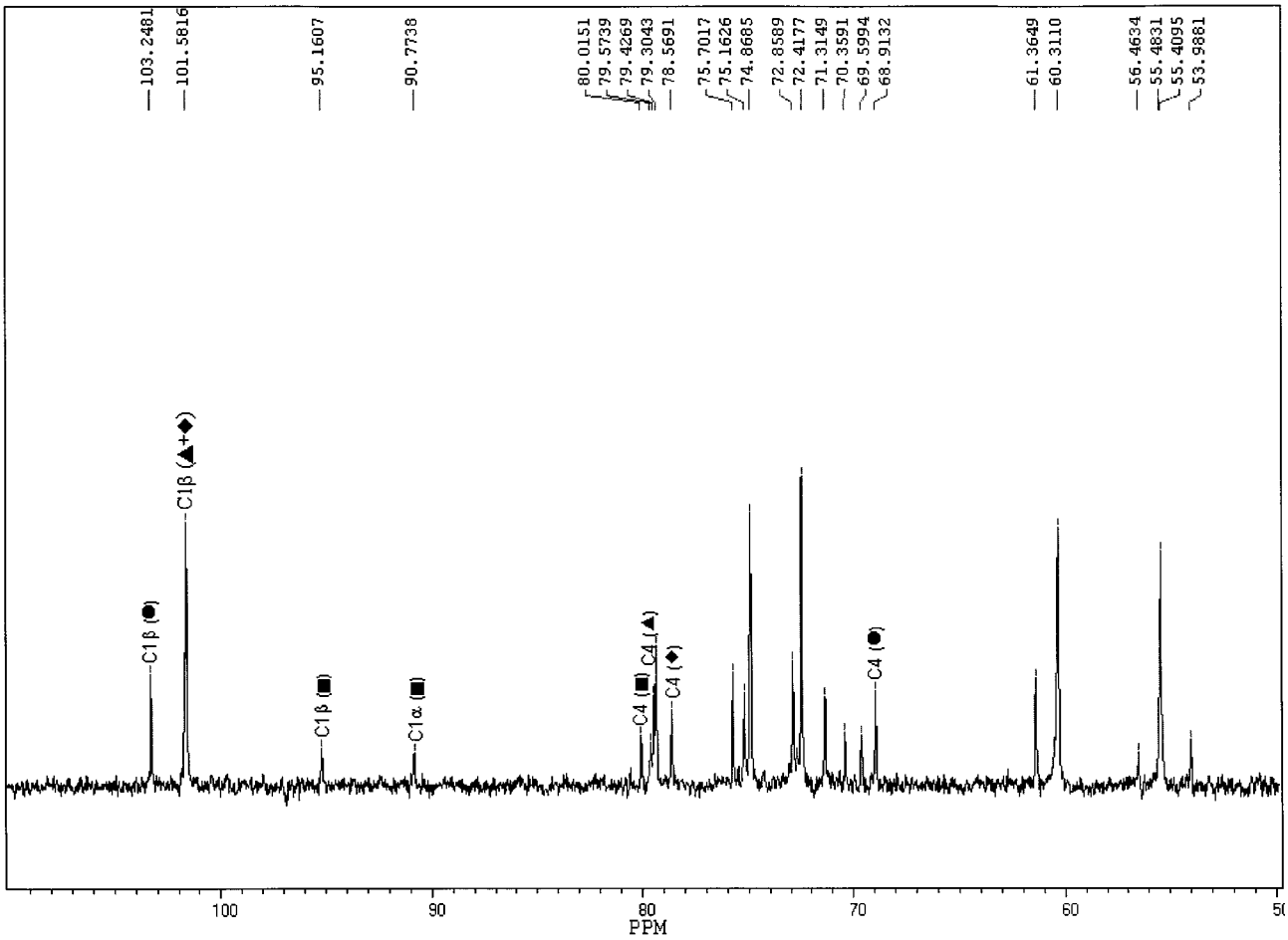

Figure 4. ^{13}C-NMR spectra of Peak I showing relevant structural features. The spectrum was recorded at 20°C using D_2O as solvent, and H_2O as reference. ■: reducing-end GlcNAc, ●: Gal, ◆: GlcNAc linked to Gal, ▲: internal GlcNAc

unpublished results). Our results show that the metabolism of *E. coli* can support the production of an heterologous oligosaccharide in relevant amounts.

The production of sufficient amounts of biologically active oligosaccharides is today a major challenge for the progress of glycobiology. Unfortunately, chemical and chemo-enzymatic methods do not yet meet the demand. Production of the oligosaccharides by the native organism renders tiny amounts of product. Here we have shown that oligosaccharide production by recombinant *E. coli* is feasible. This method produces high yields (grams/liter of culture media), and in addition is time-efficient (one week including culture of the bacteria and purification), inexpensive, reproducible and technically simple. It is conceivable that more complex oligosaccharides can be produced in this system using the appropriate glycosyltransferases. Furthermore, the presence of the chitin tail does not interfere with further extension, as shown by the action of α1,3-galactosyltransferase. In fact, the chitin tail may be used as a reactive linker that will allow coupling to affinity matrices, or to other moieties providing interesting pharmacological properties.

Table 1. Enzymatic Activity of α(1-3) galactosyltransferase with various acceptors.

Acceptor	*Activity (mU/ml)*
Lactose	8.4
LacNAc	8.9
Crude Gal-$(GlcNAc)_5$	5.8
Pure Gal-$(GlcNAc)_5$	8.3

The use of oligosaccharides as therapeutics has been proposed in the field of immunology. Hyperacute rejection occurring in pig-to-primate organ transplantation has been successfully suppressed in baboon by infusion of the oligosaccharide αGal(1,3)βGal(1,4)GlcNAc , which neutralises the pre-existing antibodies [18]. These antibodies could also be immunoabsorbed on a column containing the same carbohydrate epitope [19]. The sialyl Lewis$^{\times}$ structure is

considered as a putative anti-inflammatory agent because this epitope, present on the leukocytes surface, interacts with endothelial selectins in the early step of recruitment to sites of inflammation [20]. Furthermore, other sugars can be added to the terminal galactose to produce epitopes of pharmaceutical interest. Oligosaccharides are also involved in adhesion of pathogenic bacteria, thus oligosaccharides may be used as blocking agents to reduce the dose or time of exposure to standard antibiotics [21]. For example, the terminal αNeuAc(2,3)βGal-R structure inhibits the adherence of *Helicobacter pylori* to gastric cells and is developed as a potential cure for peptic ulcer and gastritis [22]. The synthesis of these and similar drugs can possibly be accomplished in *E. coli,* when the proper glycosyltransferases are provided.

The production of such interesting oligosaccharides can possibly be achieved using *E. coli* as a living factory. For instance, for the production of the xenotransplantation antigen, a supplementary α1-3 galactosyltransferase should be introduced in *E. coli.* It is worth to mention that very recently, the production of a cytosolic active form of the bovine α1-3 galactosyltransferase in *E. coli* was reported [23]. The stem region of this protein was removed by genetic engineering, which led to a fully active enzyme. In the case of Sialyl Le^x, much more work is necessary. The first problem is the production of GDP-fucose and CMP-sialic acid. The GDP-fucose pathway is present in *E. coli,* for the synthesis of colanic acid, but is only activated during particular growth conditions [24,25], thus the adaptation of the culture conditions may be sufficient to express the corresponding genes. Regarding to the production of CMP-sialic acid, recently the expression of a fusion protein comprising the activity for the production of this molecule has been reported [26]. Also certain pathogenic *E. coli* strains produce CMP-sialic acid for the synthesis of colominic acid. In both cases, the corresponding genes can be introduced into the *E. coli* chromosome by genetic engineering to achieve constitutive expression. In a second instance, it will be necessary to add to the system both a fucosyltransferase and a sialytransferase. The source of these genes may be both eukaryotic or prokaryotic organisms. If eukaryotic genes are used, removal of the stem region will be recommended. Otherwise homologous bacterial sialyl- and fucosyltransferase genes (like 1*st from N. meningitidis* of *fucT gene* from *Helycobacter pylori*) have been described [27,28,29]. Obviously the prokaryotic genes are preferable over the eukaryotic ones, and more effort should be devoted for the search and study of novel bacterial glycosyltransferases.

In this report we have demonstrated the feasibility of the production of galactosyl β(1,4)chitin pentaose by recombinant *E. coli.* The production of more complex oligosaccharides (like the drugs described above) will require the expression of multiple glycosyltransferases. At least two genes can be carried in the same plasmid and other compatible plasmids can be used to provide additional glycosyltransferases. Also the synthesis of other sugar nucleotides such as GDP-fucose, or even the overproduction of the already present sugar nucleotides [30] can be engineered in *E. coli.* The production of more complex oligosaccharides by expression of the appropriate glycosyltransferases in *E. coli* is now under study.

Acknowledgments

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