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# Chemo-bacterial synthesis of conjugatable glycosaminoglycans

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Conjugatable glycosaminoglycans hold promise for medical applications involving the vectorization of specific molecules. Here, we set out to produce bacterial chondroitin and heparosan from a conjugatable precursor using metabolically engineered *Escherichia coli* strains. The major barrier to this procedure was the glucuronylation of a lactosyl acceptor required for polymerization. To overcome this barrier, we designed *E. coli* strains expressing mouse  $\beta$ -1,3-glucuronyl transferase and *E. coli* K4 chondroitin and K5 heparosan synthases. These engineered strains were cultivated at high density in presence of a lactose-furyl precursor. Enzymatic polymerization occurred on the lactosyl precursor resulting in small chains ranging from 15 to 30 kDa that accumulated in the cytoplasm. Furyl-terminated polysaccharides were produced at a gram-per-liter scale, a yield similar to that reported for conventional strains. Their efficient conjugation using a Diels-Alder cycloaddition reaction in aqueous and catalyst-free conditions was also confirmed using *N*-methylmaleimide as model dienophile.

## 1. Introduction

Bacterial glycosaminoglycans (GAGs) are capsular components found in some pathogenic and probiotic bacteria (DeAngelis, 2002). GAGs offer exciting perspectives for medical applications because they are natural, non-immunogenic, biocompatible polymers with promising potential as nanocarriers for drug delivery, medical device coatings or conjugating vehicles to extend the plasma half-life of therapeutic proteins (DeAngelis, 2015). The latter application in particular requires the production of conjugatable GAGs to enable their efficient coupling with the molecules to be conveyed. There are at least two ways of preparing conjugatable GAGs. The first way is to utilize the reactivity of the reducing end to modify the polymer using conventional chemical means such as reductive amination, hydrazone formation and oxime conjugation. For example, reductive amination of hyaluronic acid (HA) with propargyl amine has been used for the production self-assembled nanocapsules that can detect pathogenic bacteria (Haas et al., 2015). Although alkynylation occurred with a satisfactory yield of 80%, it required a long reaction time (5 days) and the use of toxic sodium cyanoborohydride as the reducing agent. In an alternative enzymatic approach to introduce an alkyne group at the reducing end of chondroitin sulfate (Yamaguchi et al., 2006), an *endo*- $\beta$ -xylosidase was used to catalyze a transglycosylation reaction between a native proteoglycan donor and propargyl alcohol producing the expected functionalized polysaccharide. However, no information on the

coupling efficiency was provided. More recently, oxime ligation has been advocated as a simple and broadly applicable method for the reducing-end conjugation of glycosaminoglycans (Thakar et al., 2014). This method, assessed using a quartz crystal microbalance-based biosensor approach, overcomes the limited stability and yield of the popular hydrazone ligation method. The second way of preparing conjugatable GAGs is to prepare a (small) glycosylation precursor already chemically activated at its reducing end, and then activate enzymatic polymerization. This approach is very promising because adding a conjugatable group to mono- or disaccharides is very efficient due to the availability, solubility in organic solvents and homogeneity of these small precursors. Synthetic HAS have been efficiently produced *in vitro* from UDP-sugar donors and the *Pasteurella* HA synthase enzyme (pmHAS) in the presence of natural or modified acceptors (Jing & DeAngelis, 2003). The elongation process was synchronized in the presence of the acceptor and produced polysaccharides with limited polydispersity. In addition, the use of modified acceptors also allows the production of HA polymers containing tags (e.g. fluorescent, radioactive).

Our laboratory has extensive experience in the bacterial conversion of beta-galactosides into oligosaccharides (e.g. human milk oligosaccharides, blood group oligosaccharides and tumour carbohydrate antigens) (Priem, Gilbert, Wakarchuk, Heyraud, & Samain, 2002). Bacterial conversion relies on the bacterial internalization of an exogenously added precursor, which accumulates and serves as the substrate of glycosylation catalyzed by recombinant glycosyltransferases. Various chemical groups such as propargyl and allyl can be added to the precursor, and they remain linked to the final oligosaccharide product ready for conjugation (Fort et al., 2005). We recently described the efficient anomeric modification of blood

group oligosaccharides with a feryl group and their efficient grafting onto maleimide-activated polymer particles via a Diels-Alder reaction (Petrelli, Samain, Pradeau, Halila, & Fort, 2017). The feryl group has the advantage of reacting with maleimides in water without the addition of any catalyst. For application to the synthesis of bacterial GAGs, the key issue is to determine how recombinant GAG synthases act on an exogenous precursor and make it serve as an acceptor of glycosylation. GAG synthases can act on small oligosaccharides *in vitro*, but only on GAG hydrolysates carrying at least two disaccharide units [UA-GN]. We previously carried out the glucuronylation of lactose and allyl-lactoside by overexpressing a mammal  $\beta$ -1,3-glucuronyltransferase in engineered *Escherichia coli* (Bastide, Priem, & Fort, 2011; Yavuz, Drouillard, Samain, Roberts, & Priem, 2008). Glucuronyl- $\beta$ -1,3-lactose (GlcA-Lac) is not believed to be a substrate for bacterial GAG synthases. Here, we nonetheless based our strategy on its glycosylation by GAG synthases *in vivo*, assuming that a high GlcA-Lac concentration would compensate for the low affinity of the enzymes for this glucuronide. To do so, GAG synthases were overexpressed in a recombinant *E. coli* strain synthesizing GlcA-Lac. The selected strain was a K-12 derivative, lacking *kps* genes and thus deficient in the initiation and exportation mechanisms (Studier, Daegelen, Lenski, Maslov, & Kim, 2009; Willis & Whitfield, 2013).

The present work describes the engineering of bacterial strains to produce conjugatable GAGs *in vivo*. Lactose or lactose-feryl was added to the culture medium with the goal of incorporating it at the reducing end of nascent GAG polysaccharides. Several recombinant strains were prepared for the synthesis of conjugatable chondroitin and heparosan. This is the first study to prepare conjugatable GAGs in bacteria, offering new perspectives for the chemistry of GAGs and biomaterials.

## 2. Materials and methods

### 2.1. Chemicals and molecular biology supplies and services

Chemicals, unless otherwise indicated, were purchased from Sigma-Aldrich Chemie (Saint-Quentin Fallavier, France). DNA polymerases, DNA modification and restriction enzymes were procured from Thermo-Fisher Scientific Inc (city, country). DNA sequencing was performed by MWG Eurofins Genomics (Planegg, Germany). Recombinant DNA was isolated using miniprep columns (Qiagen Inc, Valencia, CA, USA). Gene synthesis was performed by Genscript (Piscataway, NJ, USA). The *E. coli* strain TopTen was used for plasmid constructions.

### 2.2. Synthesis of lactosyl-N-acetamido-N-furfurylamine (i.e. lactose-feryl)

A solution of lactose (12 g, 35 mmol) in furfurylamine (24 mL, 247 mmol) was stirred at 50 °C for 48 h. The reaction mixture was then precipitated by addition of ethyl acetate (200 mL). The solid was isolated by filtration, washed with ethyl acetate (100 mL) and dried under vacuum. N-Acetylation was conducted in a mixture of methanol and acetic anhydride (10:1 v/v, 330 mL) for 16 h at room temperature. Toluene (200 mL) was added to the reaction mixture and the solution was evaporated to dryness. The solid was dissolved in water (200 mL), washed with ethyl acetate (3 × 200 mL) and the aqueous solution was concentrated to half its initial volume. After freeze-drying, pure lactosyl-N-acetamido-N-furfurylamine was isolated at a yield of 96% (15.6 g). Proton nuclear magnetic resonance was performed to confirm the structure of the produced molecule:  $^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$  7.49–7.43 (m, 1H,  $-\text{O}-\text{CH}=\text{CH}$  rotamers), 6.44–6.34 (m, 2H,  $-\text{O}-\text{CH}=\text{CH}-$  rotamers), 5.51 (d, 0.3H,  $J=9.0$  Hz,  $\text{H}_1^{\text{Glc}}$  rotamers), 5.10 (d, 0.7H,  $J=9.0$  Hz,

$\text{H}_1^{\text{Glc}}$  rotamers), 4.69–4.44 (m, 3H,  $\text{CH}_2\text{NAC}$ ,  $\text{H}_1^{\text{Gal}}$ ), 3.94–3.45 (m, 12H,  $\text{H}_2^{\text{Glc-Gal}}$ ,  $\text{H}_5^{\text{Glc-Gal}}$ ), 2.29–2.25 (2s, 3H,  $\text{NCOCH}_3$  rotamers);  $^{13}\text{C}$  NMR (100 MHz,  $\text{D}_2\text{O}$ )  $\delta$  176.56, 175.35, 151.16, 150.59, 142.85, 142.16, 110.69, 108.57, 108.23, 102.91, 86.67, 82.57, 77.63, 76.91, 76.75, 75.41, 75.07, 72.53, 70.97, 70.11, 69.55, 68.60, 61.11, 60.06, 60.00, 41.00, 37.45, 21.87, 21.28; ESI-HRMS:  $m/z$  calculated for  $\text{C}_{19}\text{H}_{29}\text{NO}_{12}$   $[\text{M}+\text{H}]^+$  464.1768, gave 464.1765.

### 2.3. Bacterial strains and recombinant vectors

A summary of bacterial strains used in this study is shown in Table 1.

#### 2.3.1. Plasmids pBBR-glcAT-kfiD and pBBR-glcAT-kfiDB

The gene duplex *glcAT-kfiD* was released from the pBS-*glcAT-kfiD* plasmid with restriction enzymes *KpnI* and *SpeI*, and cloned in the same sites of the pBBR-MCS3 plasmid to produce the pBBR-*glcAT-kfiD* plasmid.

The sequence of *kfiB* was amplified by PCR from pBBR-*kfiAB* (Barreateau, Richard, Drouillard, Samain, & Priem, 2012) using primers 3'-AACTAGTAAAGAGGAAATATGATGAATAAATTAGTGCTAG-5' and 3'-AGAGCTCAGCCCTTGATTTTGTCTCTCTATATTTA-5' containing the *SpeI* and *SacI* sites, digested and inserted in the same sites of the pBBR-*glcAT-kfiD* plasmid to produce the plasmid pBBR-*glcAT-kfiDB*.

#### 2.3.2. Plasmid pSU-glcAT-kfiD

The gene duplex *glcAT-kfiD* was released from plasmid pBS-*glcAT-kfiD* using *KpnI* and *SpeI*, and cloned in the *KpnI* and *XbaI* sites of plasmid pSU2718.

#### 2.3.3. Plasmid pBS-kfiCA

The sequence of *kfiA* was amplified by PCR from plasmid pBBR-*kfiAB* (Barreateau et al., 2012) with the 3'-AAGAATTCCGAAGGAGATATACATATGATTGTTGCAAATATGTCATCATACCCACCTCGA-5' and 3'-ACTGCAACTAGTACCCTTCCACATTATACACTAATTCG-5' primers containing *EcoRI* and *SpeI* sites and cloned in the same sites of pBluescript II KS to produce pBS-*kfiA*. Then, the *kfiC* sequence was amplified by PCR from plasmid pSU-*kfiCD* (Barreateau et al., 2012) with the 3'-AGGTACCCTCGAGTGAAGAGGTATACATGGATCCTATGAACGCAGAATATATAAATTTA-5' and 3'-TTTCTCGAGCTATTGTTCAATTATTCCTGATACATCTTTAAAC-5' primers containing *KpnI* and *XhoI* sites, then digested and inserted into the same sites of pBS-*kfiA* to produce the pBS-*kfiCA* plasmid.

#### 2.3.4. Plasmid pBS-kfoCG

Plasmid pBS-*kfoC* carrying the *kfoC* gene encoding the bi-functional chondroitin synthase of *E. coli* K4 was kindly provided by I.S. Roberts (Univ. Manchester, UK). However, the ribosome binding site (rbs) sequence was weak because it is composed of the *BamHI* site of the polylinker. A stronger rbs sequence was introduced by inserting an adapter in the restriction sites *SacI* and *BamHI*, thus providing the following sequence AGAGGATCCGATG having a strong rbs sequence upstream from the start codon. The adapter was prepared by pairing the primers 3'-CCCTGCAGTAAATAAGAG-5' and 3'-GATCCTCTTATTTACTGCAGGGAGCT-5' with *SacI* and *BamHI* cohesive ends as well as an internal *PstI* site for easy screening to detect good clones for producing a pBS-*kfoC* plasmid. Then, a synthetic sequence of *kfoG* identical to the native sequence GI 21326779 (Genscript, USA Inc) and flanked with two *KpnI* sites was cloned into the *KpnI* site of pBS-*kfoC* in the sense orientation with respect to transcription, giving pBS-*kfoCG*.

**Table 1**Genes, plasmids and *E.coli* strains used in this study.

Genes	Description	Ref. or source
glcAT	Mouse glucuronyltransferase	GI 568960207
kfiA	GlcNAc-transferase from <i>E. coli</i> K5	GI 496605
kfiB	Initiating protein from <i>E. coli</i> K5	GI 47600702
kfiC	GlcAU-transferase from <i>E. coli</i> K5	GI 735964
kfiD	UDP-glucose-6-dehydrogenase from <i>E. coli</i> K5	GI 735963
kfoC	Chondroitin synthase from <i>E. coli</i> K4	GI 21326784
kfoG	Chondroitin synthase protein helper from <i>E. coli</i> K4	GI 21326779
wbpP	UDP-GlcNAc C4 epimerase from <i>P. aeruginosa</i>	GI 6690131
Plasmids		
pBBR1MCS3	Cloning vector, Tet, Plac promoter, bhr replicon	Kovach et al. (1995)
pSU2718	Cloning vector, Cm, Plac promoter, P15A replicon	Martinez et al. (1988)
pWKS130	Cloning vector, Km, Plac promoter, pSC101 replicon	Wang and Kushner (1991)
pBluescript II KS	Cloning vector, Amp, Plac promoter, ColE1 replicon	Stratagene
pBluescript II SK	Cloning vector, Amp, Plac promoter, ColE1 replicon	Stratagene
pBBR-glcAT-kfiD	pBBR1MCS3 derivative carrying glcAT, kfiD	This study
pBBR-glcAT-kfiDB	pBBR1MCS3 derivative carrying glcAT, kfiD, and kfiB	This study
pSU-glcAT-kfiD	pSU2718 derivative carrying glcAT and kfiD	This study
pBSkfiCA	pBluescript II KS derivative carrying kfiC and kfiA	This study
pBSkfoCG	pBluescript II SK derivative carrying kfoC and kfoG	This study
pWKS-wbpP	pWKS derivative carrying wbpP	This study
Strains		
DH 1	F <sup>-</sup> , endA1, gyrA96, thi-1, hsdR17(rK <sup>-</sup> ,mK <sup>+</sup> ), supE44, relA1	DSMZ <sup>a</sup>
DJ	DH1 lacZ, lacA, wcaJ	Yavuz et al. (2008)
K-cho	DJ, pBSkfoCG, pBBR-glcAT-kfiD, pWKS-wbpP (chondroitin synthesis)	This work
K-hep	DJ, pBSkfiCA, pBBR-glcAT-kfiD-kfiB (heparosan synthesis)	This work

<sup>a</sup> Leibniz-Institute DSMZ-German Collection of Microorganisms.

### 2.3.5. *pWKS-wbpP*

The sequence of wbpP was released from pBBR-wbpP (Antoine et al., 2003) with *EcoRI* and *XbaI*, and cloned in the same sites of pWKS130.

### 2.4. Culture media and culture conditions for production of glycosaminoglycans

Ampicillin, kanamycin, and tetracycline were used at 100, 25, and 15 µg/mL, respectively.

Cultures were carried out in 0.5L reactors (Infors HT Multifors, Bottmingen, Switzerland) containing 0.2L of mineral culture medium, as previously described (Barreteau et al., 2012). The high-cell density culture consisted of two phases: (1) an exponential-growth phase at 33 °C, which started with inoculation of the bioreactor and lasted until exhaustion of the initially added glucose (17.5 g/L), (2) a 48 h fed-batch phase at 28 °C, with a 50% feeding solution glycerol, provided at a flow rate of 1.25 mL/h. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.2 mM) and lactosides (0.22 mmol, i.e. 0.75 g of lactose or 1 g of lactose-furyl) were added to the culture medium 4 h after the beginning of the glycerol feeding.

### 2.5. Extraction and purification of glycosaminoglycans

At the end of the fed-batch culture, the cells were harvested by centrifugation (8400 × g, 25 min, RT) and the supernatant was designated as the extracellular fraction. The cell pellet was then suspended in distilled water and cells were disrupted by autoclaving at 105 °C for 20 min. The mixture was centrifuged (8400 × g, 25 min, RT), the cell debris discarded and the supernatant designated as the pellet fraction. The polysaccharide purification began with the precipitation of the proteins using IR120H<sup>+</sup> Amberlite resin to reach pH 3. After a centrifugation step, the supernatant was treated with 3 vol of ethanol overnight at 4 °C. The pellet was recovered by centrifugation, dissolved in 100 mL of a 10 mM sodium acetate buffer pH 4.0, 25 mM NaCl buffer and loaded on a QAE-Sephadex A25 column (10 × 4 cm) equilibrated in the same buffer. The column was

washed with 200 mL of buffer, and polysaccharides were eluted with an NaCl gradient (1 L from 25 mM to 1.5 M). Uronic acids were detected by colorimetric assay; all positive fractions were pooled and recovered by ethanol precipitation. The pellet was recovered by centrifugation, and dissolved in water. The pH was adjusted to 7 with NaOH; extracts were dialyzed against water in 14,000 kDa cut-off tubes, and then freeze-dried.

### 2.6. On-line SEC-MALLS

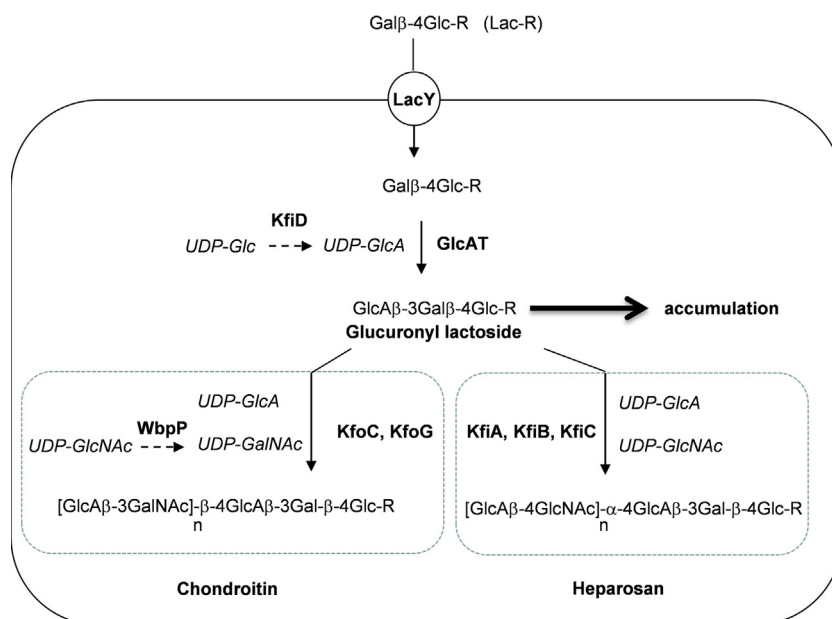
Average molecular weight and molecular weight distributions were determined by high-performance size-exclusion chromatography (HPSEC) [LC-20AD, Shimadzu] with on-line multi-angle light scattering (MALS) [MiniDAWN TREOS], Wyatt Technology Corp. (Santa Barbara, CA, USA) fitted with a K5 cell and a laser wavelength of 660 nm], a refractive index detector and a viscometer. Columns [OHPAK SB-G guard column and OHPAK SB 802-803 and 803-804 HQ columns (Shodex)] were eluted with 0.1 M NaNO<sub>3</sub> containing 0.03% NaN<sub>3</sub> at 0.5 mL/min. Solvent and samples were filtered through 0.1 µm and 0.2 µm filter units (Millipore), respectively.

### 2.7. Conjugation of GAG-furyl with maleimide derivatives

Polysaccharide-furyl (5 mg) was dissolved in D<sub>2</sub>O (500 µL). *N*-Methylmaleimide (10 equiv.) was then added and the solution was kept at 37 °C for 24 h. The reaction was monitored *in situ* by <sup>1</sup>H NMR without any further purification.

### 2.8. NMR spectroscopy

<sup>13</sup>C and <sup>1</sup>H NMR spectra were recorded with a BRUKER Avance 400 spectrometer operating at a frequency of 100.618 MHz for <sup>13</sup>C and 400.13 MHz for <sup>1</sup>H. Samples were solubilized in D<sub>2</sub>O and analyzed at 293 K. Residual signal of the solvent was used as an internal standard: HOD at 4.75 ppm.



**Fig. 1.** *In vivo* biosynthetic pathway for the production of conjugatable glycosaminoglycans in engineered *Escherichia coli*. Lactoside (Lac-R) is internalized by the permease LacY and cannot be degraded due to LacZ inactivation. It is glycosylated by recombinant mouse  $\beta$ -1,3-glucuronyltransferase GlcAT, with UDP-GlcA being furnished by the action of recombinant UDP-Glc dehydrogenase KfiD from the *E. coli* strain K5. For the production of chondroitin (left-hand pathway (K-cho strain)): a small proportion of accumulated glucuronyl-lactoside is used by the recombinant chondroitin synthase complex KfoCG to form chondroitin. Recombinant epimerase WbpP furnishes the UDP-GalNAc necessary for chondroitin synthesis, and UDP-GlcNAc is constitutively produced in the bacterium. For the production of heparosan (right-hand pathway) (K-hep strain): a small proportion of accumulated glucuronyl-lactoside is used by the recombinant heparosan synthase complex KfiABC to form heparosan.

### 3. Results and discussion

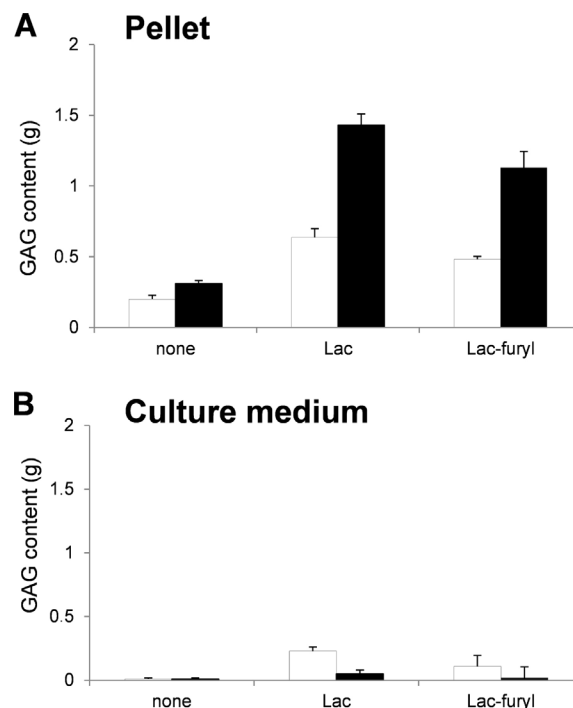
#### 3.1. Engineering of *E. coli* to produce GAG-lactose-(furyl)

A schematic representation of the engineering pathway is shown in Fig. 1. A recombinant strain overexpressing the mammal  $\beta$ -1,3-glucuronyltransferase (GlcAT) for the bacterial conversion of exogenous lactose into glucuronyl-lactose was common to the synthesis of chondroitin and of heparosan. Then, depending on the type of GAG to be produced, specific genes were overexpressed in several compatible plasmids. Strain K-cho overexpressed the *kfoC* and *kfoG* genes involved in chondroitin synthesis in *E. coli* K4, together with the *kfiD* and *wbpP* genes involved in the synthesis of nucleotide sugars UDP-GlcA and UDP-GalNAc, respectively. Strain K-hep overexpressed the genes *kfiABCD* involved in heparosan synthesis in *E. coli* K5. Gene expression for both pathways was verified with the addition of isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG).

#### 3.2. Production of GAG-lactose-(furyl) in fed-batch cultures

The two recombinant strains K-cho and K-hep were cultivated under fed-batch conditions to produce the GAGs chondroitin and heparosan, respectively. Lactose or lactose-furyl was added upon IPTG induction for 48 h of glycerol feeding. GAG concentration was estimated in the extracellular medium and in the bacterial pellet (Fig. 2).

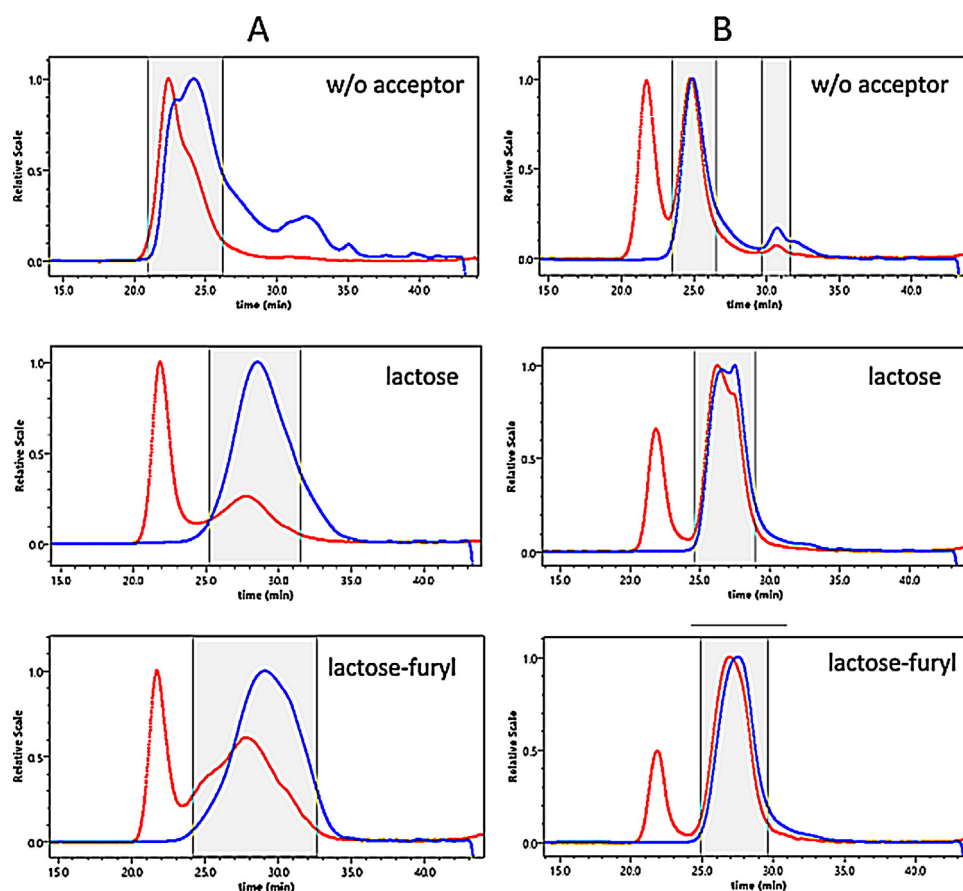
The production of GAGs without any acceptor was low and intracellular, because strain K-12 lacks the *kps* genes involved in the synthesis of the endogenous membrane acceptor and in the export system (Studier et al., 2009). When lactose or lactose-furyl were added, the production of GAGs increased by a factor of 3 or 4, suggesting that the lactoside was used as an acceptor in polymerization and GAG synthesis. A large proportion of GlcA-Lac or GlcA-Lac-furyl was not consumed and was found in the pellet and in the extracellular fraction (data not shown). The size of polysaccharides formed in the absence of lactose was similar to values



**Fig. 2.** Production of glycosaminoglycans from lactose (Lac) or lactose-furyl (Lac-furyl) after 48 h of feeding following IPTG induction. (A) Bacterial pellet content and (B) extracellular content in the K-cho strain (white bars) producing chondroitin and the K-hep strain (black bars) producing heparosan. Amounts were calculated from the uronic acid content of the ethanol-precipitate fractions from 200 mL cultures. Error bars represent the standard deviation of at least three independent cultures.

reported for natural chondroitin and heparosan produced in *E. coli* (Barreteau et al., 2012; He et al., 2015). In the presence of lactose or lactose-furyl, however, the size of recombinant polysaccharides was much smaller, probably due to the high acceptor concentra-





**Fig. 3.** SEC-MALS analysis of recombinant GAGs produced with or without (w/o) exogenous precursor (acceptor). A: The K-cho strain (chondroitin). B: The K-hep strain (heparosan). The blue chromatogram corresponds to differential refraction detection, and the red chromatogram to light scattering detection. The gray window corresponds to the area of the chromatogram used for the calculation of molecular mass. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 2**  
GPC/SEC analysis of recombinant glycosaminoglycans.

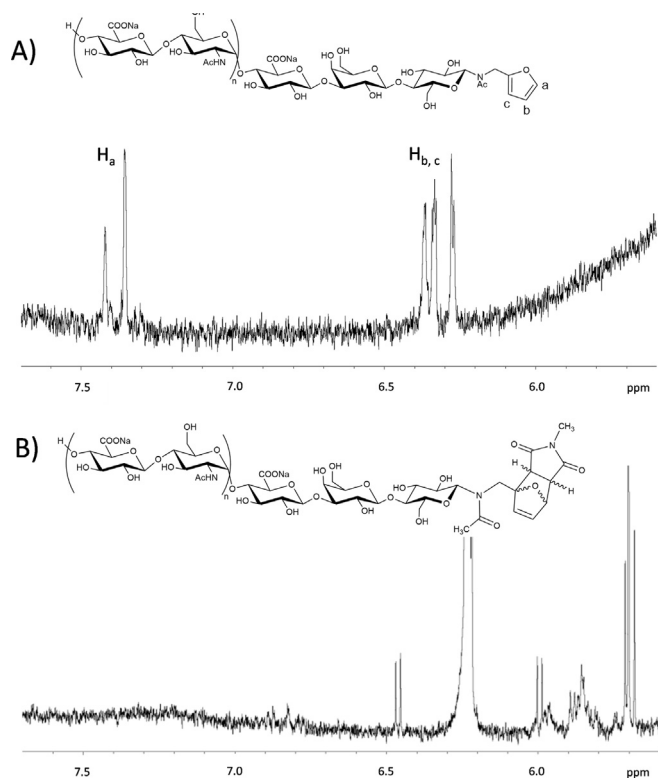
Strain	Acceptor	Peak # (%)	Mn	Mw	Mw/Mn
KCB4 (chondroitin)	none	1	114,000	177,000	1.56
	lactose	1	18,400	22,400	1.22
	lactose-furyl	1	14,300	20,100	1.40
KHB21 (heparosan)	none	1 (90)	74,000	79,000	1.06
		2 (10)	34,100	34,800	1.02
	lactose	1	34,600	36,400	1.05
	lactose-furyl	1	29,400	31,300	1.07

Mn, number average molecular weight; Mw, weight average molecular weight.

tion: chain initiation was higher, resulting in less chain elongation (Table 2, Fig. 3). GAG-lactose-(furyl) dispersity was quite low, particularly for heparosans for which the Mw-to-Mn ration narrowed 1. The NMR analysis of the products purified by anion-exchange chromatography confirmed the presence of lactose or lactose-furyl in the structure.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of synthetic polysaccharides obtained from bacterial culture without the lactose precursor (see Supporting information) were similar to those reported earlier for the K4 (Rodríguez, Jann, & Jann, 1988) and K5 (Wang et al., 2010) strain polysaccharides. On the  $^1\text{H}$  NMR spectra of the polysaccharides that were biosynthesized from a lactose-furyl acceptor, there was clear evidence of the furyl group as demonstrated by the presence of characteristic ethylenic protons at 7.40–7.50 ppm and 6.35–6.45 ppm as well as the presence of the protons of the *N*-acetyl group of the precursor at 2.25–2.29 ppm.

Although a precise determination of the average molecular weight of the polysaccharides could not be determined using this technique, it is obvious from signal integration that the degree of polymerization of the heparosan was roughly one and a half times higher than that of chondroitin. Moreover, the fact that the protons of the chain ends can be observed by NMR is indicative of relatively low-molecular-weight polysaccharides.

The ability of the biosynthesized polysaccharides to be conjugated via a furan-maleimide Diels-Alder reaction was further investigated by  $^1\text{H}$  NMR using *N*-methylmaleimide as a dienophile. Chondroitin- and heparosan-furyl were incubated in water at 37 °C with a 10-fold excess of the maleimide reagent and the reaction was monitored for 48 h. Complete conversion was observed for the heparosan polysaccharide within 24 h, whereas some remaining furyl derivative was still present in the chondroitin reaction even after 48 h (see Supporting Information). The NMR spectra of the Diels-Alder adducts are rather complex due to the formation of multiple isomers in the reaction and to the presence rotamers arising from the *N*-acyl group at the anomeric position of lactose. Nevertheless, the efficiency of the conjugation was unambiguously demonstrated by comparing the  $^1\text{H}$  NMR spectra of polysaccharide glycoconjugates with those of the adduct between lactose-furyl and *N*-methylmaleimide. Upon coupling between the furyl group and maleimide, ethylenic protons at 7.40–7.50 ppm disappeared completely (Fig. 4) and new signals appeared between 6.0 ppm and 7.1 ppm and at 2.75 ppm. This glycoconjugation approach needs to be applied to more appropriate molecules than *N*-methylmaleimide, but the method already



**Fig. 4.** Comparison of the <sup>1</sup>H NMR spectra (zoom of the ethylenic region) of (a) heparosan-furyl and (b) its Diels-Alder adduct with *N*-methylmaleimide.

appears extremely promising given the positive results obtained with heparosan and chondroitin polysaccharides.

#### 4. Conclusion

We successfully developed a new approach to produce conjugatable GAGs at scale of several grams per liter, a level comparable to that obtained with normal, non-modified bacterial GAGs in non-pathogenic recombinant strains. A furyl group was incorporated in the GAG and other types of conjugatable groups such as allyl or propargyl could be incorporated as well (Fort et al., 2005). The size of the GAG-lactosides was quite small, which is an advantage for drug vectorization and bio-material coating. In addition, the β-1,3-GlcA-β-galactoside motif at the end of the GAG structure is also present in vertebrates GAGs, making these structures more similar to those produced in vertebrates. These conjugates will be very useful for the design and production of brush-like structures found in proteoglycans.

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