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Note

Identification of a novel glycosyltransferase involved in LOS biosynthesis of *Moraxella catarrhalis*

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Abstract—*Moraxella catarrhalis* is an important human mucosal pathogen that contributes to otitis media in infants and exacerbates conditions such as chronic obstructive pulmonary disease in the elderly. This study describes the identification of a novel gene, *lgt5* that encodes a glycosyltransferase involved in the LOS biosynthesis of *M. catarrhalis*. Analysis of NMR data of LOS-derived oligosaccharide from a Serotype A *lgt5* mutant strain of *M. catarrhalis* indicate that *lgt5* encodes an α-(1→4)-galactosyltransferase.

Keywords: Glycosyltransferases; Lipooligosaccharide; Insertional inactivation; *Moraxella catarrhalis*; NMR spectroscopy

Moraxella catarrhalis, a Gram-negative bacterium, is the causative agent of approximately 20% of cases of acute otitis media in infants. The organism's only known host is the human and its importance as a host-adapted pathogen is now widely recognised. In addition to causing childhood ear infections, *M. catarrhalis* also plays a significant role in respiratory tract disease in adult patients, causing or exacerbating ailments such as sinusitis, pneumonia, and chronic obstructive pulmonary disease (COPD), which are particularly serious for the elderly.[1,2]

As a Gram-negative organism, the outer membrane of *M. catarrhalis* contains lipopolysaccharide as its major glycolipid. *M. catarrhalis* does not express the repeating O-antigen found in enteric bacteria and therefore the glycolipid is termed lipooligosaccharide (LOS). There are three major serotypes, A, B and C, of *M. catarrhalis* LOS, representing 61%, 28.8% and 5.3%, respectively.[3] The expression of each serotype within a strain is not phase-variable, and no phase-variable LOS biosynthesis genes have been identified to date. The structure of LOS from strains of each serotype has been determined[4–8] and the structure of wild type OS serotype A (strain 2951) is shown in Figure 1.

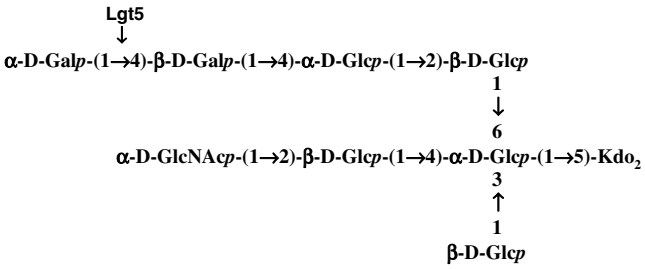

Figure 1. Structure of wild type serotype A OS (strain 2951). The glycosidic linkage catalysed by *lgt5* is indicated.

The role of LOS in colonisation and pathogenesis of *M. catarrhalis* has not been addressed in any depth,[1] in part, presumably because of a lack of an appropriate model, and also because stepwise defined truncations of LOS have not been available. Recent reports implicate a role for LOS in serum resistance,[9,10] and in adherence.[11]

A recent study identified three genes (*lgt1*, *lgt2* and *lgt3*) involved in LOS biosynthesis in a serotype B strain of *M. catarrhalis*[12] and ascribed functions to each of these putative glycosyltransferase genes for that serotype. Further to that study, a multiplex PCR assay was developed to identify glycosyltransferase genes for each of the seroptypes A, B and C,[13] in which a gene encoding an additional putative glycosyltransferase,

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lgt4, was noted in serotypes A and C strains, and allelic variations between serotypes for *lgt2* were described. In the present study, we have identified an additional gene, *lgt5*, in the same genomic locus as the previously identified LOS glycosyltransferase-encoding genes. We generated a strain of *M. catarrhalis* in which *lgt5* is mutated resulting in LOS of this strain being truncated relative to the wild-type LOS. This truncated LOS has been structurally characterised by NMR and mass spectrometry. From these data, the function of the *lgt5* gene product is assigned as an α-galactosyltransferase responsible for addition of the terminal galactose residue of *M. catarrhalis* LOS (Fig. 1).

In this study, the DNA sequence from *M. catarrhalis* strain ATCC43617 containing genes with similarity to glycosyltransferases of other organisms was examined (Accession number AX067456). Some of the genes within this region, namely *lgt1*, *lgt2*, *lgt3* have previously been described.[12] PCR amplification and sequencing of the corresponding locus from a serotype A strain, 2951, revealed the presence of an additional gene, *lgt4*, within that locus. The presence of *lgt4* has previously been noted in serotypes A and C strains, although its function requires elucidation.[13] Further in silico analysis of the ATCC43617 LOS biosynthesis locus revealed the presence of a previously undescribed gene with similarity to glycosyltransferases. This gene, which we have named *lgt5*, is located immediately adjacent to, and in the opposite orientation to *lgt1* (Fig. 2) and is 795 base pairs in length, encoding a predicted protein of 264 amino acids.

Lgt5 was amplified by PCR from strains 2951 (serotype A) and CCUG3292 (serotype B) (chosen because the structure of their LOS is known[5,7,8]). Comparison of *lgt5* revealed a highly conserved gene encoding an amino acid sequence 100% identical between 2951 and ATCC43617.

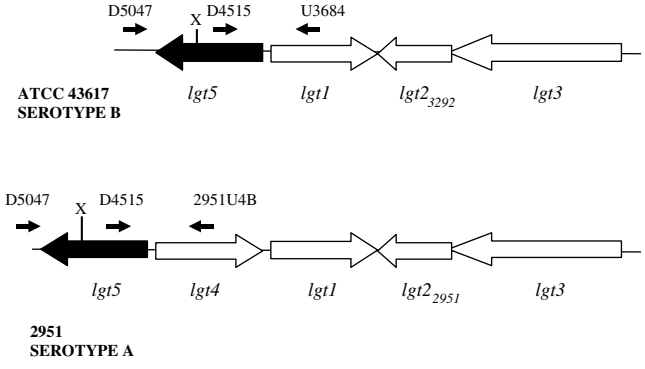
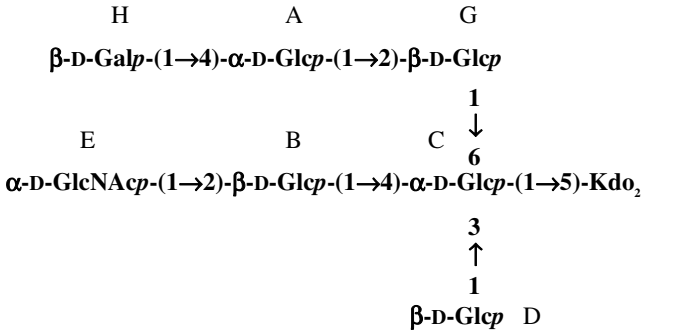


Figure 2. Schematic representation of the LOS biosynthesis locus of strain ATCC43617 and strain 2951. Large filled arrows represent *lgt5*. Open arrows indicate previously reported or characterised genes. Small arrows represent site of oligonucleotides used for cloning and sequencing. X indicates *Xba*I restriction site used for insertional mutagenesis.

Functional analysis of glycosyltransferase *lgt5* by mutation. To determine whether *lgt5* is involved in LOS biosynthesis, and to assign a function to this gene, mutant strains 2591*lgt5*Δ and 3292*lgt5*Δ were made. The *lgt5* gene was cloned from strain CCUG3292 and from 2951, and disrupted by insertion of Kan^r into a convenient *Xba*I restriction site within the reading frame (see Fig. 2). Linearised plasmid containing the mutant allele was transferred into strains 2951, and CCUG33292 by natural transformation. Analysis by PCR revealed that the mutant allele had integrated within *lgt5* of the chromosome. As *lgt5* is in the opposite orientation to the next characterised glycosyltransferase-encoding gene in the locus (*lgt1*), it is unlikely that this mutation had any effect on other genes in the locus. LOS was separated by T-SDS-PAGE and visualised using a silver stain (data not shown). LOS from all mutants is truncated relative to parental strains, indicating that *lgt5* encodes a functional glycosyltransferase in both 2951 and CCUG3292.

Structural analysis of mutant LOS. LOS was isolated and purified from the 2951*lgt5*Δ mutant strain, and structurally characterised by NMR and MS. The phenol–chloroform–petroleum ether extraction method generally yielded between 9 and 15 mg of LOS per gram of dry bacteria. After acid hydrolysis and purification, a single oligosaccharide (OS) component was isolated from the *lgt5*Δ mutant strain. In some preparations, two forms of Kdo monosaccharide (α-pyranose and β-furanose) were also recovered after size exclusion chromatography. As *M. catarrhalis* LOS contains two Kdo residues, the source of these Kdo monosaccharides is likely to be from the acid hydrolysis reaction where the acid-labile Kdo bonds are cleaved to yield the OS containing one Kdo, lipid A, and one Kdo monosaccharide.

Using a combination of 1D and 2D NMR experiments, it was possible to assign ^{1}H and ^{13}C chemical shifts for the OS isolated from the 2951*lgt5*Δ mutant strain (Table 3). Moreover, negative-ion ESI-MS spectra for the OS showed a parent ion, with a *m*/*z* ratio of 1413.4, within one atomic mass unit of the calculated molecular weight ($C_{52}H_{87}NO_{43}$ 1414.23 amu). The anomeric region of the ^{1}H–^{13}C HSQC spectrum (Fig. 3) reveals that OS from strain 2951*lgt5*Δ is truncated by one terminal galactose residue, when compared to the wild type ^{1}H NMR spectra for serotype A *M. catarrhalis*.[5] This is evident by a missing peak at 4.96 ppm corresponding to the anomeric proton of the terminal α-D-galactose residue in 2951*lgt5*Δ OS. In addition to the chemical shift assignments determined from ^{1}H, selective 1D TOCSY, ^{13}C APT, COSY, ^{1}H,^{13}C HSQC and ^{1}H,^{13}C HSQC-TOCSY experiments, the sequence of the sugar residues was confirmed by examination of 250 and 400 ms NOESY experiments. Table 4 shows the inter-residue NOE correlations that were identified from these spectra to confirm the sugar linkage



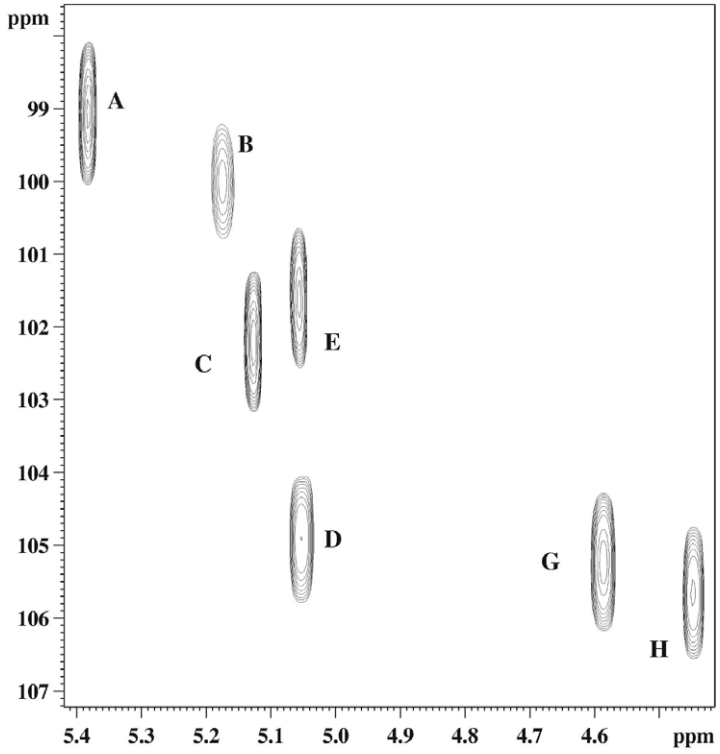


Figure 3. Anomeric region of the $^{1}H,^{13}C$ HSQC spectrum (600 MHz, 298 K, D_2O) 2951*lgt5*Δ mutant OS.

sequence. The terminal sugar H has inter-residue NOEs from its anomeric proton at 4.48 ppm to H-4 (3.65 ppm) of A. No other inter-residue NOEs were observed from any other residue to residue H indicating that H is terminal.

For all sugar residues of 2951*lgt5*Δ OS, with the exception of H, (the terminal β-D-Gal*p*-(1→4)) the ^{1}H and ^{13}C chemical shifts were in accordance to those published.[5] For this reason, the assignment of residue H only, is described in detail as follows. The location of the anomeric signal H-1 (4.48 ppm) in the COSY spectrum of residue H immediately gave the position of the H-2 (3.56 ppm). The ^{13}C shift of these signals were then located in the $^{1}H,^{13}C$ HSQC-TOCSY spectrum (C-1 105.7 and C-2 73.8 ppm), and were confirmed in the $^{1}H,^{13}C$ HSQC spectrum. In the $^{1}H,^{13}C$ HSQC spectrum, the H-3 proton is clearly resolved at 3.69 ppm revealing that C-3 resonates at 75.4 ppm, with TOCSY correlations clearly observed to H-2 and H-4. Most noticeably, along the C-3 ^{13}C-line (75.4 ppm) of residue H, offset by −0.6 ppm there are $^{1}H,^{13}C$-correlations that have strong TOCSY peaks back into the H-6/C-6 (3.70–374/63.8 ppm) region of the spectrum. This line of correlations strongly suggested this was the C-5 ^{13}C chemical shift (74.8 ppm) just resolved from the C-3 line (75.4 ppm). Accordingly, the $^{1}H,^{13}C$-correlation for H-5/C-5 was located in the $^{1}H,^{13}C$ HSQC spectrum at 4.01/74.8 ppm, with TOCSY correlations confirmed to H-3, H-4 and H-6 protons. The H-4 resonance was located from the anomeric ^{13}C TOCSY line (105.7 ppm) at 3.94 ppm, and therefore, its corresponding ^{13}C shift located from the $^{1}H,^{13}C$ HSQC-TOCSY spectrum at 71.3 ppm and confirmed in the $^{1}H,^{13}C$ HSQC spectrum. TOCSY correlations at the C-4 ^{13}C chemical shift

(71.3 ppm) were clearly observed to H-1 and H-3 of residue H. In general, the ^{1}H chemical shifts of residue H 2951*lgt5*Δ OS differed by <0.12 ppm from the equivalent residue H in wild type OS (serotype A), except for H-5 which differed by 0.2 ppm.[5] The ^{13}C shifts differed most notably from the published values for wild type serotype A OS residue H for C-4 (2951*lgt5*Δ OS 71.3 ppm vs wild type 78.1 ppm) and C-5 (2951*lgt5*Δ OS 74.8 ppm vs wild type 76.2 ppm), which is not unexpected since residue H has a terminal α-D-galactose (1→4) linked at C-4 in the wild type serotype A OS. The terminal location of H in 2951*lgt5*Δ OS is further supported by comparison of the chemical shift of C-4 in monomeric β-D-galactose (69.7 ppm) with the chemical shift of C-4 of H (71.3 ppm).

The novel gene, *lgt5*, was identified as a homologue of glycosyltransferase encoding genes in genomic sequence of *M. catarrhalis* ATCC43617. *Lgt5* is orientated divergently from *lgt1*. NMR and MS analysis of LOS from a serotype A derived *lgt5* mutant strain indicate that *lgt5* encodes an α-(1→4)-galactosyltransferase. An α-(1→4)-linked galactose is present as the terminal residue of major or minor glycoforms of all serotypes.[5–7] PCR and sequence analysis confirms that at least two serotype B strains (CCUG3292 and ATCC 43617) contain *lgt5*, and electrophoretic examination of a serotype B strain CCUG3292 *lgt5*Δ mutant confirms that *lgt5* is involved in LOS biosynthesis in this strain. Full characterisation of the LOS biosynthesis pathway of known serotypes of *M. catarrahalis* will assist in the determination of the role of LOS in pathogenesis.

1. Experimental

1.1. Bacterial growth and transformation

Bacterial strains are described in Table 1. *Escherichia coli* was grown at 37 °C in Luria Bertani (LB) shaking at 200 rpm or on LB agar, with ampicillin at 50 μg/mL, kanamycin at 100 μg/mL and X-Gal at 40 μg/mL as appropriate. *M. catarrhalis* was grown at 37 °C on Brain Heart Infusion (BHI) agar, with kanamycin added at 15 μg/mL where appropriate. For structural analysis, *M. catarrhalis* was grown at 37 °C in BHI broth supplemented with 0.5% yeast extract, shaken at 220 rpm.

1.1.1. Transformation of *M. catarrhalis*. After overnight growth, bacteria were suspended into 500 μL of sterile phosphate buffered saline (PBS). A 20 μL sample of bacterial suspension was placed on a BHI plate and left to dry. Approximately 1 μg of restriction digested plasmid dissolved in 30 μL PBS was added to the bacteria and incubated at 37 °C for 3–4 h, before transfer of bacteria to BHI/kanamycin plates and overnight incubation at 37 °C. Sterile PBS (30 μL) was added instead of the DNA as a control in each transformation.

1.2. Electrophoretic analysis of LOS

T-SDS-PAGE: Bacteria from overnight growth were resuspended in PBS, treated with proteinase K and SDS (0.05% w/v final) before electrophoretic separation on tricine-SDS-PAGE gels using Tris–tricine buffer.[14] LOS was visualised using an ammoniacal silver stain method that was previously described.[15]

1.3. In silico analysis

Nucleotide sequence of Accession number AX067456 was obtained and analysed using the suite of nucleotide and protein analysis tools hosted by the Australian National Genomic Information Service (www.angis.org.au), or locally using MacVector 7.0 (Oxford Molecular). Open reading frames flanking *lgt1* and *lgt3* were identified, and translated into amino acid sequence. These amino acid sequences were used as search terms for interrogating nucleotide and protein sequence databases using the BLAST and PSI-BLAST algorithms[16,17]

Table 1. Strains and plasmids

Strain	Genotype, relevant phenotype, or comment	Source/reference
Moraxella catarrhalis		
2951	Wild type, serotype A, LOS structure determined	Zaleski et al.[10]
CCUG3292	Wild type, serotype B, LOS structure determined	CCUG
2951*lgt5*Δ	*lgt5*::Kan^{r}	This study
3292*lgt5*Δ	*lgt5*::Kan^{r}	This study
Escherichia coli		
DH5α	ϕ80 dlacZΔM15 recA1 endA1 gyrA96 thi-1 hsdR17 (r_k-, m_k+) supE44 relA1 deoR Δ(lacZYA-argF)U169	Invitrogen
Plasmid/vector		
pGemT-Easy	F1 ori lacZ Amp^{r}	Promega
pUC4kan	pBR322 ori lacZ Amp^{r}Kan^{r}	Pharmacia
p4:3292	UORF4:3684 & DORF4:5047 in pGemT-Easy	This study
p4:3292K	As p4:3292, with Kan^{r} in *Xba*I site	This study

hosted by the National Library of Medicine (www.ncbi.nlm.nih.gov).

1.4. Recombinant DNA techniques

Standard DNA manipulation techniques were employed, essentially as previously described.[18,19] Chromosomal DNA was purified as previously described, and plasmid DNA was purified from *E. coli* by alkali-lysis method, or using purification kits obtained from Qiagen. Enzymes were obtained from New England Biolabs, and used as recommended. DNA was purified from agarose gel using kits obtained from Eppendorf.

The *lgt5* gene and flanking region were amplified from strain 3292 using primers UORF4:3684 & DORF4:5047 and cloned into pGEMTeasy to generate p4:3292 and p4:2951. Kan^r, excised from pUC4Kan with *Hin*cII, was ligated into the unique *Xba*I site (blunted with Klenow). The resulting plasmid, p4:3292K, has Kan^r in the opposite orientation relative to *lgt5* (Table 2).

1.5. Isolation of OS

Bacteria harvested from 6 to 12 L of culture were dried by successive washes with ethanol, acetone and petroleum ether (bp 40–60 °C). LOS was extracted from the

Table 2. Oligonucleotide primers

Oligonucleotide primers used to amplify and/or sequence *lgt5*	Sequence 5′–3′	Location (see Fig. 2)
UORF4:3684	TCA ATT TGC TCA TGT AAT GGC	Within *lgt1*
DORF4:4515	TTT CTA GAT TTA TAC CAT GGT G	Within *lgt5*
DORF4:5047	TTA TCG GTA CAT ATT GAT TGG	3′ to *lgt5*
2951U4B	AAA AGG TGT CGT AAT CTC ACC	Within *lgt4*

Table 3. ^{1}H and ^{13}C Chemical shifts (ppm) for OS isolated from the 2951*lgt5*Δ mutant *M. catarrhalis* in D_2O referenced to DSS (0 ppm), at 298 K, on a Bruker Avance spectrometer operating at 600 and 150 MHz, respectively

Sugar residue	^{1}H Chemical shift (δ, ppm), and $^3J_{1,2}$ (Hz) in parentheses							
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	NH(C=O)CH_3
(A)→4)-α-D-Glc*p*-(1→	5.41 [3.1]	3.45	3.89	3.65	4.13	3.77	4.00	
(B)→2)-β-D-Glc*p*-(1→	5.20 [6.6]	3.39	3.61	3.45	3.51	3.75	3.85	
(C)→3,4,6)-α-D-Glc*p*-(1→	5.16 [3.1]	3.90	4.53	3.95	4.62	4.03	4.14	
(D)β-D-Glc*p*-(1→	5.08 nd[a]	3.35	3.54	3.36	3.55	3.99	4.01	
(E)α-D-GlcNAc*p*-(1→	5.09 nd[a]	4.03	3.75	3.58	3.83			2.19
(G)→2)-β-D-Glc*p*-(1→	4.62 nd[b]	3.49	3.59	3.41	3.45	3.78		
(H)β-D-Gal*p*-(1→	4.48 [7.7]	3.56	3.69	3.94	4.01	3.70–3.74		
	H-3ax	H-3eq	H-4	H-5	H-6	H-7	H-8a	H-8b
→5)-α-Kdo*p*	2.04	1.89	4.16	4.08	3.85	4.06	3.81	3.64
	^{13}C Chemical shift (δ, ppm)							
	C-1	C-2	C-3	C-4	C-5	C-6	NH(*C*=O)*C*H_3	
(A)→4)-α-D-Glc*p*-(1→	99.0	74.2	73.7	81.2	73.2	62.9		
(B)→2)-β-D-Glc*p*-(1→	100.0	83.8	77.9	72.3	78.9	63.2		
(C)→3,4,6)-α-D-Glc*p*-(1→	102.3	76.4	77.9	76.4	74.2	70.5		
(D)β-D-Glc*p*-(1→	105.0	76.6	78.9	72.7	78.9	64.0		
(E)α-D-GlcNAc*p*-(1→	101.6	56.4	74.8	72.5	74.1	62.9[c]	177.0/25.4	
(G)→2)-β-D-Glc*p*-(1→	105.3	78.1	77.5	72.3	78.3	63.6		
(H)β-D-Gal*p*-(1→	105.7	73.8	75.4	71.3	74.8	63.8		
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
→5)-α-Kdo*p*	179.3	99.1	37.2	68.5	77.9	74.2	71.1	66.0

[a] The anomeric protons of residue D and E overlap and so $^3J_{1,2}$ coupling constants were not determined for these residues.
[b] The anomeric protons of residue G and H5 of residue C overlap and so a $^3J_{1,2}$ coupling constant was not determined for residue G.
[c] Tentative assignment, could not be unambiguously determined due to overlap.

Table 4. 2951*lgt5*Δ mutant OS NOE correlations identified from a 400 ms NOESY spectrum (600 MHz, 298 K, D_2O) that confirm the sequence of the sugar residues

Sugar residue	Inter-residue	NOEs identified
(A)→4)-α-D-Glc*p*-(1→	A1–G4	A1–G2
(B)→2)-β-D-Glc*p*-(1→	B1–C4	B1–C3
(C)→3,4,6)-α-D-Glc*p*-(1→	C1–Kdo5	
(D)β-D-Glc*p*-(1→	D1–C3	
(E)α-D-GlcNAc*p*-(1→	E1–B2	
(G)→2)-β-D-Glc*p*-(1→		
(H)β-D-Gal*p*-(1→	H1–A4	

dry cell mass using the phenol–chloroform–petroleum ether extraction method[20] with modifications.[21] The water-soluble oligosaccharide component was isolated from the LOS by acid hydrolysis as outlined by Phillips et al.[22]

The oligosaccharides were passed through conditioned Alltech Maxi-Clean™ C18, 300 mg cartridge to remove lipophilic components before being centrifuge-filtered with Rainin Microfilterfuge™ tubes (0.45 μm positive charged Nylon-66) at 2000*g*. The filtrate was lyophilised before size exclusion chromatography using a Bio-Rad Bio-Gel™ P2 extra fine column (15 × 500 mm) and eluted with 0.05 M pyridinium acetate buffer, pH 5.4, at a flow rate of 16 mL/h. Fractions (1 mL) were assessed for carbohydrate content by charring on thin layer chromatography plates (H_2SO_4/ethanol). Fractions containing carbohydrate were analysed by NMR spectroscopy.

1.6. Structural analysis of the OS

1.6.1. NMR spectroscopy. Purified oligosaccharides were dissolved in D_2O (CIL 99.998%) and cycled through three steps of lyophilisation/dissolution to remove exchangeable protons. ^1H and ^{13}C NMR experiments were performed at 600 and 150 MHz, respectively, at 298 or 278 K in D_2O using a Bruker Avance spectrometer. Chemical shifts are reported in ppm referenced to DSS (0 ppm). Spectral assignment was aided by the recording of 1D ^1H, COSY, TOCSY (60 and 120 ms mixing time), NOESY (250 and 400 ms), ^{13}C attached proton test (APT), ^1H–^{13}C HSQC and ^1H–^{13}C HSQC-TOCSY (60 and 120 ms mixing time) spectra. Additionally, 1D selective TOCSY (120 ms) spectra using a selective Gaussian pulse (100 ms) at the frequency of each of the anomeric resonances were acquired. All spectra were acquired using unmodified pulse sequences from the Bruker pulse sequence library.

1.6.2. Structural analysis by electrospray ionisation mass spectrometry. Lyophilised oligosaccharide samples were resuspended in acetonitrile–water, 70:30 v/v to a concentration of 1 mg/mL and injected directly into a Bruker Esquire 3000 ion-trap mass spectrometer in negative ion mode at a flow rate of 550 μL/h.

1.7. Sequence data

Sequences have been deposited under the accession numbers DQ071425 (2951 locus), and DQ071426 (3292 locus).

Acknowledgements

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