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Editor's Choice

Elucidation of the O-antigen structure of *Escherichia coli* O93 and characterization of its biosynthetic genes

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The structure of the O-antigen from the international reference strain *Escherichia coli* O93:−:H16 has been determined. A nonrandom modal chain-length distribution was observed for the lipopolysaccharide, a pattern which is typical when long O-specific polysaccharides are expressed. By a combination of (i) bioinformatics information on the gene cluster related to O-antigen synthesis including putative function on glycosyl transferases, (ii) the magnitude of NMR coupling constants of anomeric protons, and (iii) unassigned 2D ^{1}H, ^{13}C-HSQC, and ^{1}H,^{1}H-TOCSY NMR spectra it was possible to efficiently elucidate the structure of the carbohydrate polymer in an automated fashion using the computer program CASPER. The polysaccharide also carries *O*-acetyl groups and their locations were determined by 2D NMR experiments showing that ∼$\frac{1}{2}$ of the population was 2,6-di-*O*-acetylated, ∼$\frac{1}{4}$ was 2-*O*-acetylated, whereas ∼$\frac{1}{4}$ did not carry *O*-acetyl group(s) in the 3-*O*-substituted mannosyl residue of the repeating unit. The structure of the tetrasaccharide repeating unit of the O-antigen is given by: →2)-β-D-Man*p*-(1→3)-β-D-Man*p*2Ac6Ac-(1→4)-β-D-Glc*p*A-(1→3)-α-D-Glc*p*NAc-(1→, which should also be the biological repeating unit and it shares structural elements with capsular polysaccharides from *E. coli* K84 and K50. The structure of the acidic O-specific polysaccharide from *Cellulophaga baltica* strain NN015840^T differs to that of the O-antigen from *E. coli* O93 by lacking the *O*-acetyl group at O6 of the *O*-acetylated mannosyl residue.

Key words: bioinformatics; CarbBuilder; CASPER; lipopolysaccharide; NMR spectroscopy.

Introduction

Escherichia coli is a bacterium that belongs to the family *Enterobacteriaceae* and it is part of the colonic flora of humans, where the bacterium and the host mutually benefit from each other. However, clinical syndromes that result from pathogenic strains include enteric/diarrheal disease, urinary tract infection, and sepsis/meningitides (Stenutz et al. 2006; Vila et al. 2016; Liu et al. 2020).

Some *E. coli* make Shiga toxin(s) known as Stx1, one of the most potent bacterial toxins described to date (Melton-Celsa 2014), though a second type referred to as Stx2 is present in certain *E. coli* strains. Collectively these bacteria are referred to as Shiga toxin producing *E. coli* (STEC) and this pathogen is a potential health risk as it may cause, in particular, hemolytic uremic syndrome (HUS) resulting in kidney failure. The prevalence of STEC in healthy adults in Japan was investigated by collecting close to 3 million samples during a 2-year period and a large number of serogroups was identified carrying *stx* genes, among others, *E. coli* O93 (Morita-Ishihara et al. 2016). Monitoring of food-producing animals during handling and preparation of meat is therefore important in order to avoid STEC-based outbreaks and illness caused by these bacteria. The use of venison for human consumption called for a closer investigation of potential contamination by STEC,

e.g. from wild deer in Japan where 4 serogroups were isolated, one of them being *E. coli* O93 (Asakura et al. 1998). In a more recent study of 30 sika deer in Japan during 3 years the most dominant serogroup was O93 present in almost 40% of the isolated samples (Kabeya et al. 2017). In bovine ground meat from Brazil, *E. coli* O93 was not only identified as STEC but also as an enteroaggregative *E. coli* (EAEC) pathotype (Pelayo et al. 2019). Furthermore, bacterial *E. coli* isolates from cattle in Australia revealed that serogroup O93 was common and it was shown to have an antibiotic resistance phenotype, though the specific antibiotics to which the *E. coli* O93 bacteria were resistant did vary (Bettelheim et al. 2005). Avian pathogenic *E. coli* (APEC) were isolated from ducks in China during an 8-year period, where *E. coli* O93 was the predominant serogroup (Wang et al. 2010), whereas in ducks from Vietnam it was the least common of the isolated serogroups (Thu et al. 2019). A study of virulence factors in *E. coli* isolates from bovine mastitis (Fernandes et al. 2011) identified serogroups in a few cases, one of them being *E. coli* O93, where also the somatic antigen was possible to identify, i.e. the O-antigen. Furthermore, this *E. coli* O93 isolate was resistant to the antibiotics ampicillin and trimethoprim sulfa.

The potential severity as a human pathogen has prompted us to investigate the structure of the O-antigen polysaccharide

of *E. coli* O93 as knowledge thereof may give insight to how the bacterium can evade the immune system of the host, and it may also form the basis for future vaccine candidates. To this end we have used a combination of bioinformatics information, NMR spectroscopy and the structure prediction tool CASPER to unravel its primary structure. Subsequently, 3-dimensional sub-structure models of the polysaccharide were possible to construct revealing polymer extension and epitope accessibility of structural entities.

Results and discussion

Escherichia coli O93 was grown in a lysogeny broth medium and the LPS was extracted according to the hot phenol/water method using previously described procedures (Furevi et al. 2020). The LPS was analyzed by SDS-PAGE (Fig. 1, lane 3). This analysis revealed a typical ladder pattern for the LPS from *E. coli* O93 owing to the O-antigen having a number of repeating units (RU) expressed with a nonrandom modal chain-length distribution. For comparison, the rough LPS from *E. coli* CWG303 Δ*waaG* (lane 1; Yethon et al. 2000) and *E. coli* W3110 (lane 2; Klein et al. 2013) were included. *Escherichia coli* CWG303 ΔwaaG is truncated at the heptose region of the inner core whereas *E. coli* W3110 contains the full K12 core. An initial analysis by ^{1}H NMR spectroscopy of the LPS revealed the presence of signals in the spectral region 2.00–2.05 and 2.17–2.22 ppm where resonances from methyl groups of *N*-acetyl and *O*-acetyl groups of sugar residues reside, respectively.

The part of the genome of *E. coli* O93 related to O-antigen synthesis and expression, from *galF* to *HisI*, has been deposited at NCBI (GenBank AB812041.1), analyzed and putative functions of the gene products have been proposed and annotated (Fig. 2; Iguchi et al. 2015; DebRoy et al. 2016; Liu et al. 2020) The presence of the genes *wzx*, *wzy*, and *wzz* indicates that the O-antigen is synthesized via the Wzx/Wzy biosynthetic pathway (Reeves et al. 1996; Islam and Lam 2014; Liu et al. 2019; Ståhle and Widmalm 2019; Whitfield et al. 2020). The gene cluster also contained 2 NDP-sugar precursor-encoding genes, *manB* and *manC*, involved in the synthesis of the donor GDP-D-Man*p*, having an α-anomeric configuration. Furthermore, a putative *O*-acetyl transferase, *wexC*, was proposed and by identification of conserved regions it can be shown to be maltose O-acyltransferase (MAT)-like (Lo Leggio et al. 2003). A gene annotated as *ugd* was also present. This gene most likely encodes a UDP-glucose 6-dehydrogenase that converts UDP-glucose to UDP-glucuronic acid (Stevenson et al. 1996) (UDP-D-Glc*p*A with an α-anomeric configuration). Two genes coding for glycosyltransferases (GTs) were identified, viz., *wfdV* and *wfdW*, after *galF* and *wexC*, respectively. Analysis and identification of conserved regions revealed that *wfdV* belongs to GT family 2 that acts through an inverting mechanism whereas a corresponding analysis showed that *wfdW* belongs to GT family 1, which also acts through an inverting mechanism. The function of the product coded by the gene denoted by *orf5* has not yet been identified.

The Wzx/Wzy-dependent pathway employs WecA to make Und-PP-D-GlcNAc (Al-Dabbagh et al. 2016) as the initial sugar of the biological repeating unit (BRU). Since both *gnu*, coding an epimerase that interconverts *N*-acetyl-D-glucosamine-PP-Und to *N*-acetyl-D-galactosamine-PP-Und, and *gne*, coding for an epimerase that isomerases
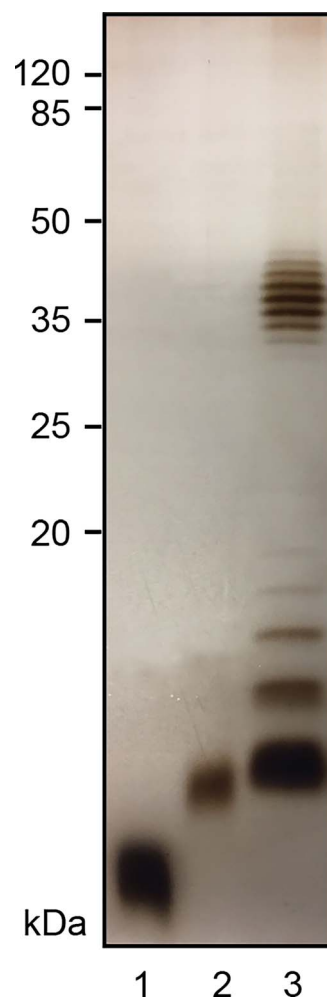

Fig. 1. SDS-PAGE analysis of LPS from different *E. coli* strains. Lane 1: LPS from *E. coli* CWG303 Δ*waaG*, Lane 2: LPS from *E. coli* W3110, and Lane 3: LPS from *E. coli* O93. A 14% Tricine SDS-PAGE gel was used to separate LPS samples, which subsequently were stained with AgNO$_3$. A pre-stained protein marker (Thermo Fisher Scientific) was utilized for calibration of molecular weights.
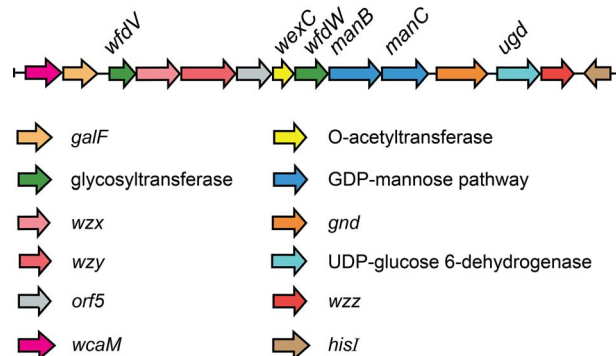

Fig. 2. The O-antigen gene cluster between *galF* and *hisI* in *E. coli* O93 (Iguchi et al. 2015; DebRoy et al. 2016; Liu et al. 2020).

UDP-D-Glc*p*NAc to UDP-D-Gal*p*NAc (Cunneen et al. 2013), are absent in the genome as judged from analysis hitherto performed, we conclude that the first sugar residue of the BRU is D-Glc*p*NAc and that D-Gal*p*NAc is not present in the O-antigen (unless *orf5* corresponds to one of the two genes). As there are not any other genes in the sequence between *galF* and *gnd* the sugar residues in the RU should contain
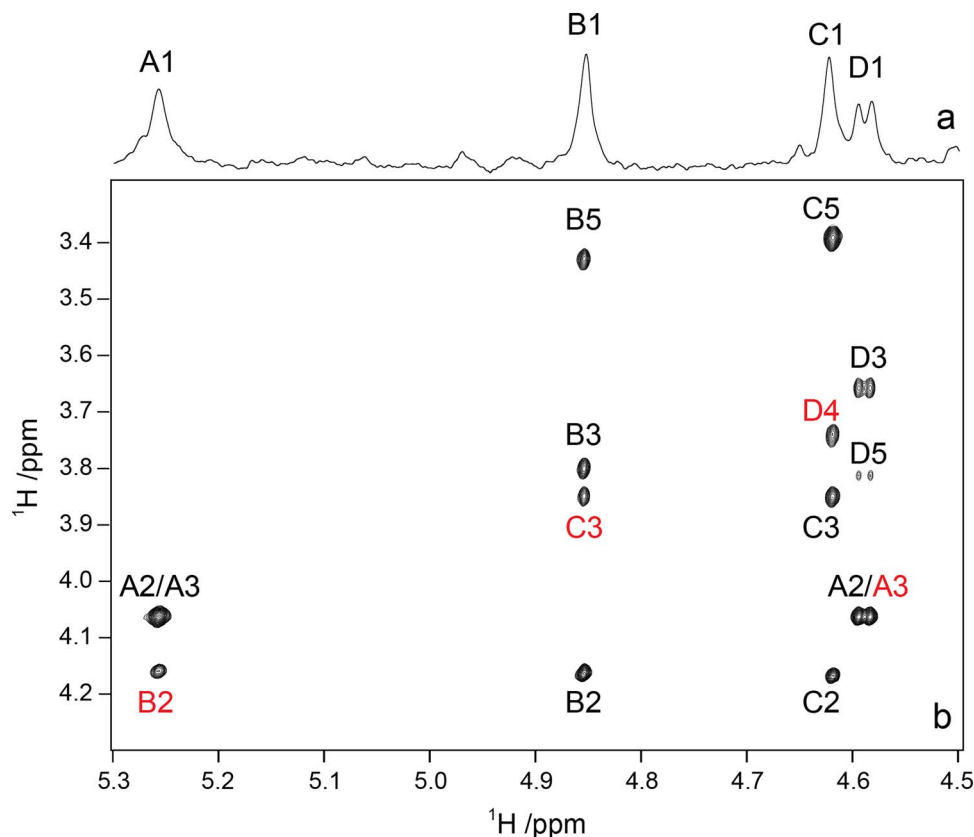
Fig. 3. The anomeric region of *O*-deacetylated *E. coli* O93 O-antigen polysaccharide (PS–OH) from a ^{1}H NMR spectrum (a) and a ^{1}H,[1]H-NOESY spectrum with a mixing time of 150 ms at 700 MHz (b). Transglycosidic cross-peaks are annotated by red color.

D-GlcNAc and D-Man as well as maybe D-GlcA; sugar residues coded outside the O-antigen cluster by housekeeping genes, e.g. D-Glc and D-Gal, may also be constituents of the O-polysaccharide.

Databases storing information on polysaccharide structures facilitate comparison among entries, which can be highly beneficial when unknown structures are to be elucidated. ECODAB stores (for each serogroup) available information from literature on *E. coli* O-antigen structures, their NMR chemical shifts and function of GTs used in the biosynthesis of the O-polysaccharides (Lundborg et al. 2010; Rojas-Macias 2015). Importantly, local BLAST+ searches have been performed within the database to compare amino acid sequences of putative GTs and the results from the sequence similarity searches are stored and ranked according to the e-value (the lower the value the more significant is the agreement) based on the similarity to other GTs within the database, for which function is known or has been predicted. In this way structural information can be gained resulting in a disaccharide structural element whose two constituent monosaccharides, the anomeric configuration at the glycosidic linkage and the substitution position of the acceptor sugar can be proposed. For *E. coli* O93 O-antigen biosynthesis, the first gene in the sequence coding for a putative GT, *wfdV*, is closely similar, with an e-value of 3×10^{-101}, to *weiG* from *E. coli* O161 (Li et al. 2010). *weiG* codes for a GT that makes the disaccharide structural element β-D-Glc*p*A-(1→3)-D-Glc*p*NAc, which is consistent with the fact that WfdV is proposed to operate via an inverting mechanism and that UDP-D-Glc*p*A with an α-anomeric configuration should then

be the donor molecule as the product has the β-anomeric configuration.

Polysaccharide material was prepared by treatment of the LPS with dilute acid, dialysis, and gel-permeation chromatography as well as by treatment under dilute basic conditions to yield material referred to as PS and PS–OH, respectively. The ^{1}H NMR spectrum of the PS–OH sample showed four resonances from anomeric protons and the sugar residues corresponding to these were denoted **A–D** by order of descending proton chemical shift (Fig. 3a). Notably, the $^3J_{H1,H2}$ of the anomeric protons of residues **B** and **C** were small and estimated to have $^3J_{H1,H2} < 2$ Hz, which is characteristic for *manno*-configured monosaccharides. The anomeric proton of **D** showed $^3J_{H1,H2}$ of ~8 Hz whereas H1 of **C** was significantly broader in comparison to those from **B** and **C** and was therefore estimated to have a medium-sized $^3J_{H1,H2} \approx 3$–4 Hz, which is characteristic for pyranose sugar residues having the *gluco/galacto*-configuration.

A ^{1}H,^{13}C-meHSQC (multiplicity-edited) NMR spectrum of the O93 PS–OH showed a total of 27 cross-peaks consisting of 4 cross-peaks in the spectral region for anomeric atoms (Fig. 4a), 15 cross-peaks with ^{13}C chemical shifts in the spectral range 65–82 ppm, 3 pairs of hydroxymethyl related resonances at δ_C 60–64, one cross-peak from a nitrogen-bearing carbon at δ_C 53.70 (Fig. 4b) and a resonance at δ_H/δ_C 2.04/22.87 in the *N*-acetyl spectral region. The bioinformatics analysis suggested 3 different sugar residues and together with the NMR data we conclude that the fourth sugar residue in the O-antigen should be a hexose. Furthermore, the ^{1}H NMR data of the resonances from anomeric protons are consistent
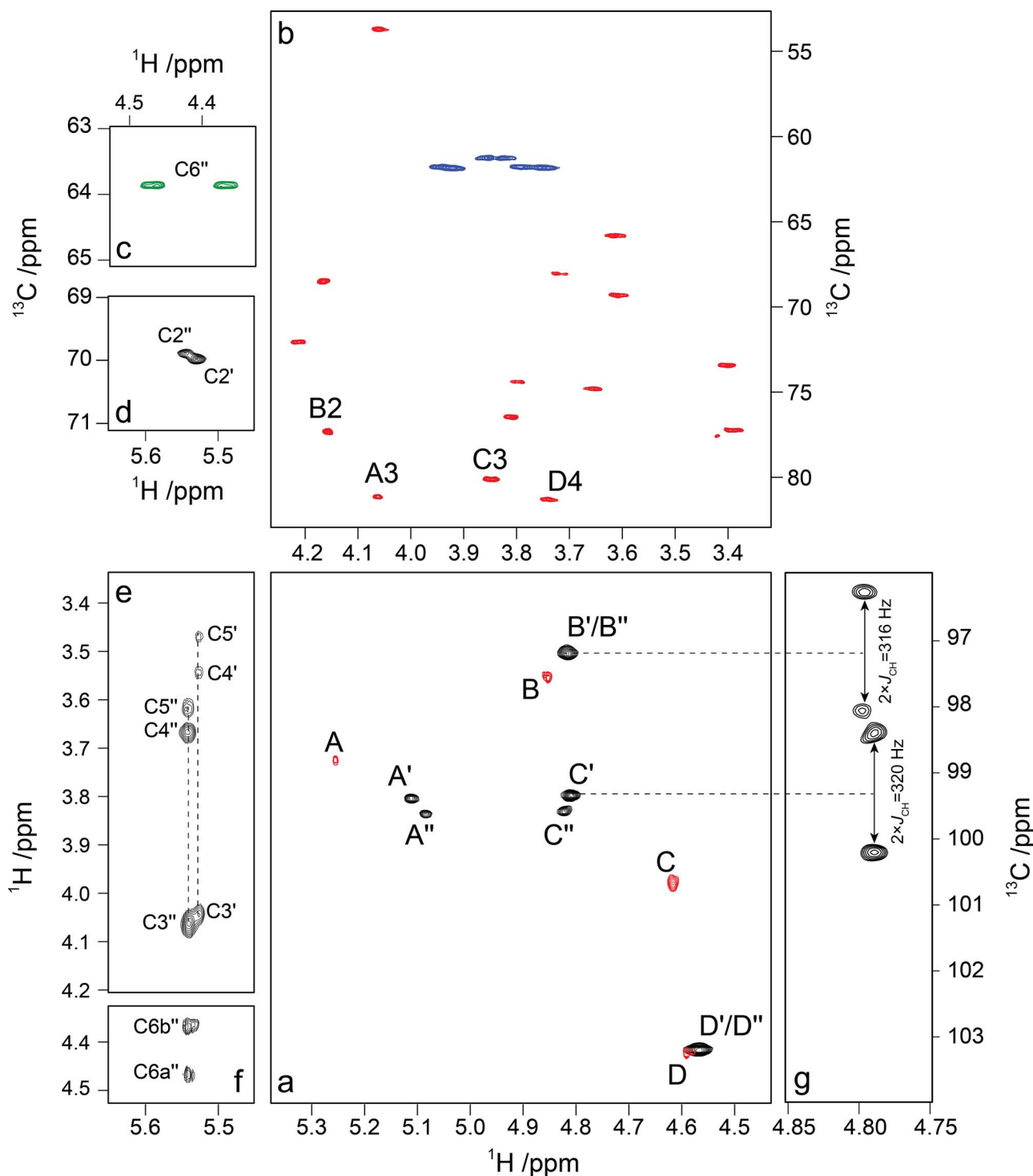
Fig. 4. NMR spectra of selected regions from the O-antigen polysaccharides (PS and PS–OH) of *E. coli* O93. (a) An overlay of ^{1}H,^{13}C-meHSQC NMR spectra of the anomeric region from PS (black) and PS–OH (red); (b) ^{1}H,^{13}C-meHSQC spectrum of PS–OH plotted in red color with linkage positions annotated and hydroxymethyl correlations plotted in blue color; (c, d) selected regions from the ^{1}H,^{13}C-meHSQC spectrum of the PS sample showing the *O*-acetylated positions in residues **C′/C″** with hydroxymethyl correlations plotted in green color; (e, f) ^{1}H,^{1}H-TOCSY spectrum with a mixing time of 120 ms displaying the cross-peaks from H2 resonances in residues **C2′** and **C2″**; (g) selected region from the coupling-enhanced (2×) ^{1}H,^{13}C-CT-CE-HSQC NMR spectrum resulting in $^1J_{C1,H1}$ for **B′/B″** (158 Hz) and **C′/C″** (160 Hz).

with the fact that two mannosyl residues should be present as part of the RU of the O-antigen polysaccharide. That the absolute configuration is D for the sugar residues in the O-antigen polysaccharide is deduced based on biosynthetic considerations. A sugar analysis of the polysaccharide showing that the major hexose component was mannose supports the

above conclusion based on the bioinformatics analysis. In addition, a minor hexose component was detected by the sugar analysis, viz., glucose, and it is assumed to derive from the core region of the LPS.

To determine the structure of the PS–OH, spectral data from a 1D ^{1}H NMR spectrum and the magnitude of
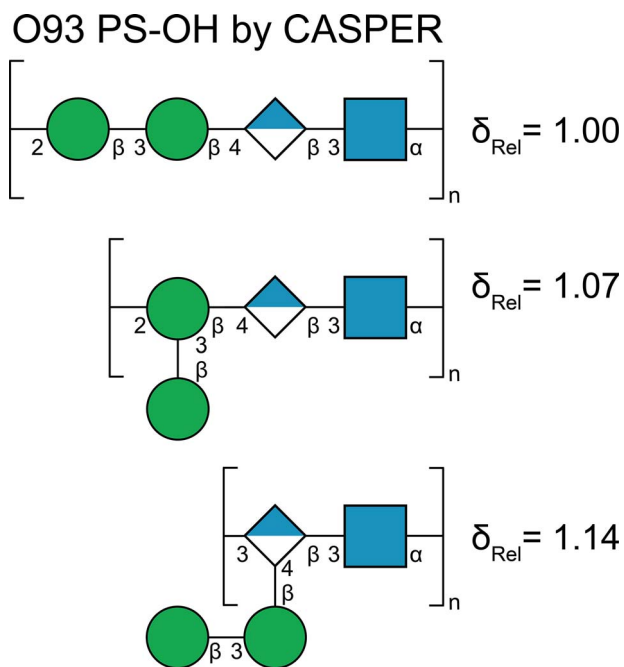
O93 PS-OH by CASPER

Fig. 5. Top 3 structures using the "Determine Glycan Structure" entry in CASPER for *E. coli* O93 PS–OH with NMR data obtained from ^{1}H,^{13}C-HSQC and ^{1}H,^{1}H-TOCSY experiments and magnitudes of coupling constants for input to CASPER, viz., those from $^1J_{C1,H1}$ and $^3J_{H1,H2}$ obtained from coupled ^{1}H,^{13}C-HSQC and ^{1}H NMR spectra, respectively.

$^3J_{H1,H2}$ coupling constants, 2D ^{1}H,^{13}C-meHSQC and F_2-coupled ^{1}H,^{13}C-meHSQC spectra where the latter reveals the magnitude of $^1J_{C1,H1}$ coupling constants (1 with $^1J_{C1,H1} > 170$ Hz and 3 with $^1J_{C1,H1} \approx 160$ Hz), and correlations from ^{1}H,^{1}H-TOCSY NMR spectra were used. The computer program CASPER can determine polysaccharide structure using NMR data in conjunction with some additional information such as sugar components (Jansson et al. 1987b; Lundborg and Widmalm 2011). The information obtained from the bioinformatics analysis and the sugar analysis, including the fact that the O-antigen is supposed to be synthesized via the Wzx/Wzy-dependent pathway (referred to as the biological WecA rule in CASPER), was given as input to CASPER, i.e. the sugar residues D-GlcNAc, D-GlcpA, and D-Man twice as well as the structural element β-D-GlcpA-(1→3)-D-GlcpNAc in conjunction with the above described *unassigned* NMR data (Supporting information).

The highest ranked O93 PS–OH structure proposed by CASPER has a linear tetrasaccharide RU in which both of the mannose residues have the β-anomeric configuration (Fig. 5), whereas the second and third ranked structures both have branched RUs. The proposed structure was confirmed by NMR resonance assignments of the PS–OH. The four sugar residues should have the pyranoid ring-form as indicated from ^{13}C NMR chemical shift data (Jansson et al. 1989, 1991) and that characteristic signals for furanosides (Ritchie et al. 1975; Beier et al. 1980) were absent in the ^{1}H,^{13}C-meHSQC spectrum. Residue **A** having $^1J_{C1,H1} > 170$ Hz shows that it has the α-anomeric configuration, whereas residues **B–D** have $^1J_{C1,H1} \approx 160$ Hz revealing that they all have the β-anomeric configuration (Table 1; Bundle and Lemieux 1976).

Residue **A** whose anomeric proton resonates at δ_H 5.25 showed at a short mixing a correlation to δ_H 4.06 in the

^{1}H,^{1}H-TOCSY spectrum and the latter correlated to the single ^{13}C resonance at δ_C 53.70 in the ^{1}H,^{13}C-meHSQC NMR spectrum; this sugar residue was therefore assigned to α-D-GlcpNAc. At longer mixing times the intensity of the cross-peak increased further and it was not until long mixing times were used that the full spin-system could be identified (Table 1). Thus, also H3 resonates at δ_H 4.06 and correlates to a glycosyloxylated carbon-13 nucleus resonating at 81.15 ppm, consistent with a large downfield chemical shift displacement, $\Delta\delta_{C3}$ 9.41, due to substitution at O3 in →3)-α-D-GlcNAc-(1→. The *manno*-configured sugars **B** and **C** had spin-systems that showed correlations between their anomeric protons and their H2 protons at δ_H 4.16 and δ_H 4.17, respectively, only when the longest mixing time of 120 ms was employed in the ^{1}H,^{1}H-TOCSY experiments. The remaining part of their spin-systems were assigned in a stepwise manner from the H2 autopeak using ^{1}H,^{1}H-TOCSY NMR spectra with incremented mixing times. In addition, strong intra-residue correlations were observed from H1 to H2, H3, and H5 in the ^{1}H,^{1}H-NOESY spectrum (Fig. 3b), which further confirms that both sugar residues have the β-anomeric configuration. The glycosylation shifts of residues **B** and **C**, $\Delta\delta_{C2}$ 5.20 and $\Delta\delta_{C3}$ 6.08, reveal that they correspond to →2)-β-D-Man-(1→ and →3)-β-D-Man-(1→, respectively. ^{1}H,^{1}H-TOCSY NMR spectra with increasing duration of the spin-lock times were used to assign ^{1}H NMR chemical shifts of residue **D**, the anomeric proton of which resides at δ_H 4.59, up to H5 having a chemical shift of 3.81 ppm. In the ^{1}H,^{13}C-meHSQC NMR spectrum its H4, δ_H 3.74, correlates to a signal at 81.26 ppm and the $\Delta\delta_{C4}$ 8.57 for the glucuronic acid then defines the position of substitution and the residue thus corresponds to →4)-β-D-GlcA-(1→. The ^{1}H and ^{13}C chemical shifts of the PS–OH were correlated using the ^{1}H,^{13}C-meHSQC NMR spectrum and the sequence between residues, [–**B**–**C**–**D**–**A**–], suggested by CASPER, was confirmed by the ^{1}H,^{1}H-NOESY spectrum (Fig. 3b, Table 2). The predicted ^{1}H and ^{13}C NMR chemical shifts by CASPER were in good agreement with those assigned by the 1D and 2D NMR experiments (Fig. 6). The structure of the PS–OH is also consistent with the fact that WfdW was proposed to act via an inverting mechanism and consequently the O-antigen should contain at least one β-D-Manp residue, since the donor molecule used would be GDP-D-Manp with an α-anomeric configuration.

The PS of *E. coli* O93 showed ^{1}H NMR resonances at 2.17 and 2.22 ppm and ^{13}C NMR resonances at 20.99 and 21.32 ppm for a minor (denoted by a prime) and a major population (denoted by a double-prime), respectively, which were not present in the material after treatment under dilute basic conditions, fully consistent with the presence of O-acetyl groups as substituents on the sugar residue(s) of the O-antigen polysaccharide. The ^{1}H,^{13}C-meHSQC NMR spectrum showed characteristic chemical shift displacements as a result of O-acetylation, inter alia, for a 6-O-acetylation resulting in cross-peaks between δ_C 63.88 and the two protons at δ_H 4.37 and 4.46 (Fig. 4c), i.e. $\Delta\delta_{C6} \sim 2$ and $\Delta\delta_{H6} \sim 0.5$ (Jansson et al. 1987a). Furthermore, cross-peaks at δ_H/δ_C 5.53/69.97 and δ_H/δ_C 5.55/69.87 (Fig. 4d) could be assigned to the respective protons and carbons of **C2′** and **C2″** resulting in a →3)-β-D-Man2Ac-(1→ residue. The two carbonyl carbons at δ_C 174.02 and 173.95 had correlations to δ_H 5.53 and δ_H 5.55, respectively, as deduced from a ^{1}H,^{13}C-BS-CT-HMBC experiment (Claridge and Pérez-Victoria 2003). Interestingly,

Table 1. ^{1}H and ^{13}C NMR chemical shifts of the O-antigen from *E. coli* O93 in which populations carrying *O*-acetyl groups are denoted by prime and double-prime residues and the corresponding *O*-deacetylated polysaccharide where sugar residues are devoid of the prime character.

Sugar residue		^{1}H and ^{13}C chemical shifts									
		1		2	3	4	5	6	2Ac	2CO	6Ac	6CO
A	→3)-α-D-GlcNAc-(1→	5.25	(M)[b]	4.06	4.06	3.61	4.21	3.83, 3.85	2.04			
		98.78	[179][c]	53.70	81.15	69.31	72.04	61.24	22.87	N.d.[a]		
A′	→3)-α-D-GlcNAc-(1→	5.11		4.03	4.03	3.66	4.04	3.73, 3.91	2.03			
		99.40	{174}[d]	53.77	81.21	68.91	71.90	60.81	22.94	175.16		
A″	→3)-α-D-GlcNAc-(1→	5.08		4.03	4.03	3.66	4.04	3.74, 3.93	2.03			
		99.66		53.77	81.30	68.80	71.86	60.70	22.94	175.16		
B	→2)-β-D-Man-(1→	4.85	(S)	4.16	3.80	3.72	3.42	3.79, 3.94				
		97.55	[159]	77.33	74.37	68.04	77.54	61.78				
B′	→2)-β-D-Man-(1→	4.82		3.83	3.77	3.71	3.44	3.79, 3.95				
		97.22	{158}	78.02	74.28	68.09	77.46	61.80				
B″	→2)-β-D-Man-(1→	4.82		3.81	3.77	3.71	3.44	3.79, 3.95				
		97.17		78.41	74.18	68.09	77.41	61.89				
C	→3)-β-D-Man-(1→	4.62	(S)	4.17	3.85	3.62	3.39	3.75, 3.92				
		100.66	[161]	68.50	80.11	65.80	77.21	61.82				
C′	→3)-β-D-Man2Ac-(1→	4.81		5.53	4.04	3.54	3.47	3.77, 3.94	2.22			
		99.35	{160}	69.97	77.63	66.04	77.41	61.65	21.32	174.04		
C″	→3)-β-D-Man2Ac6Ac-(1→	4.82		5.55	4.07	3.61	3.67	4.37, 4.46	2.22		2.17	
		99.61		69.87	77.33	65.73	74.73	63.88	21.32	173.95	20.99	174.67
D	→4)-β-D-GlcA-(1→	4.59	(L)	3.40	3.65	3.74	3.81					
		103.27	[162]	73.41	74.78	81.26	76.45	N.d.[a]				
D′	→4)-β-D-GlcA-(1→	4.57		3.40	3.65	3.78	3.79					
		103.22	{166}	73.51	74.73	81.58	76.24	174.65				
D″	→4)-β-D-GlcA-(1→	4.57		3.40	3.61	3.75	3.79					
		103.22		73.44	74.73	82.14	76.24	174.65				

[a]N.d. = not determined. [b]$^3J_{H1,H2}$ value used as input for "Determine glycan structure" in CASPER: S < 2 Hz, M = 2–7 Hz and L > 7 Hz. [c]$^1J_{C1,H1}$ values given in hertz in square brackets and obtained from a coupled ^{1}H,^{13}C-HSQC NMR spectrum. [d]$^1J_{C1,H1}$ values given in hertz in curly brackets and calculated from a ^{1}H,^{13}C-CT-CE-HSQC NMR spectrum.

Table 2. Inter-residue correlations from ^{1}H,^{1}H-NOESY, 1D ^{1}H,^{1}H-NOESY, and ^{1}H,^{13}C-HMBC spectra for *E. coli* O93 O-antigen polysaccharide (PS) and de-*O*-acetylated polysaccharide (PS–OH).

Sugar residue of PS–OH		Atom	NOE correlations to	HMBC via $^3J_{CH}$
A	→3)-α-D-GlcNAc-(1→	H1, **A**	H2, **B**	
B	→2)-β-D-Man-(1→	H1, **B**	H3, **C**	
C	→3)-β-D-Man-(1→	H1, **C**	H4, **D**	
D	→4)-β-D-GlcA-(1→	H1, **D**	H3, **A**	
	PS			
A′	→3)-α-D-GlcNAc-(1→	H1, **A′**	H2, **B′**	C2, **B′**
		C1, **A′**		H2, **B′**
B′	→2)-β-D-Man-(1→	H1, **B′**	H3, **C′**	C3, **C′**
		C1, **B′**		H3, **C′**
C′	→3)-β-D-Man2Ac-(1→	H1, **C′**	H4, **D′**	C4, **D′**
		C1, **C′**		H4, **D′**
		Me^{2Ac}, **C′**	H1, H5, H6, **A′**	
		Me^{2Ac}, **C′**	H1, H2, **B′**	
D′	→4)-β-D-GlcA-(1→	H1, **D′**	H3, **A′**	C3, **A′**
		C1, **D′**		H3, **A′**

cross-peaks from δ_H 5.55 (H2 of **C″**) to δ_H 4.37 and δ_H 4.46 (H6 of **C″**) were observed in the ^{1}H,^{1}H-TOCSY acquired with a mixing time of 120 ms (Fig. 4e and f) showing that two *O*-acetyl groups substitute positions 2 and 6 in **C″**, i.e. a →3)-β-D-Man2Ac6Ac-(1→ residue. The ^{1}H chemical shift displacements due to the *O*-acetylation at position 2 follow the pattern observed for α-L-Rha2Ac-OMe where downfield shifting by >1 ppm takes place for H2 and smaller effects in the same direction occur for H1 and H3; for the ^{13}C chemical shifts the magnitude of the displacements is in the range of ~1.5–2.5 ppm, downfield for C2 and upfield for C1

and C3 (Table 1; Rönnols et al. 2013). For the PS material the anomeric configuration of, in particular, the 2 mannosyl residues was further confirmed by a ^{1}H,^{13}C-CT-CE-HSQC NMR experiment (Tian et al. 2001) in which 2 × $^1J_{C1,H1}$ is determined along the F_1 dimension of the spectrum (Fig. 4g, Table 1) as a way to resolve spectral overlap that may occur in an F_2-coupled HSQC NMR spectrum. The degree of *O*-acetylation of residue **C** was determined by integration of the anomeric signals in the ^{1}H,^{13}C-HSQC NMR spectrum and showed that ~$^1/_2$ of the population was 2,6-di-*O*-acetylated, ~$^1/_4$ was 2-*O*-acetylated, whereas ~$^1/_4$ of the population
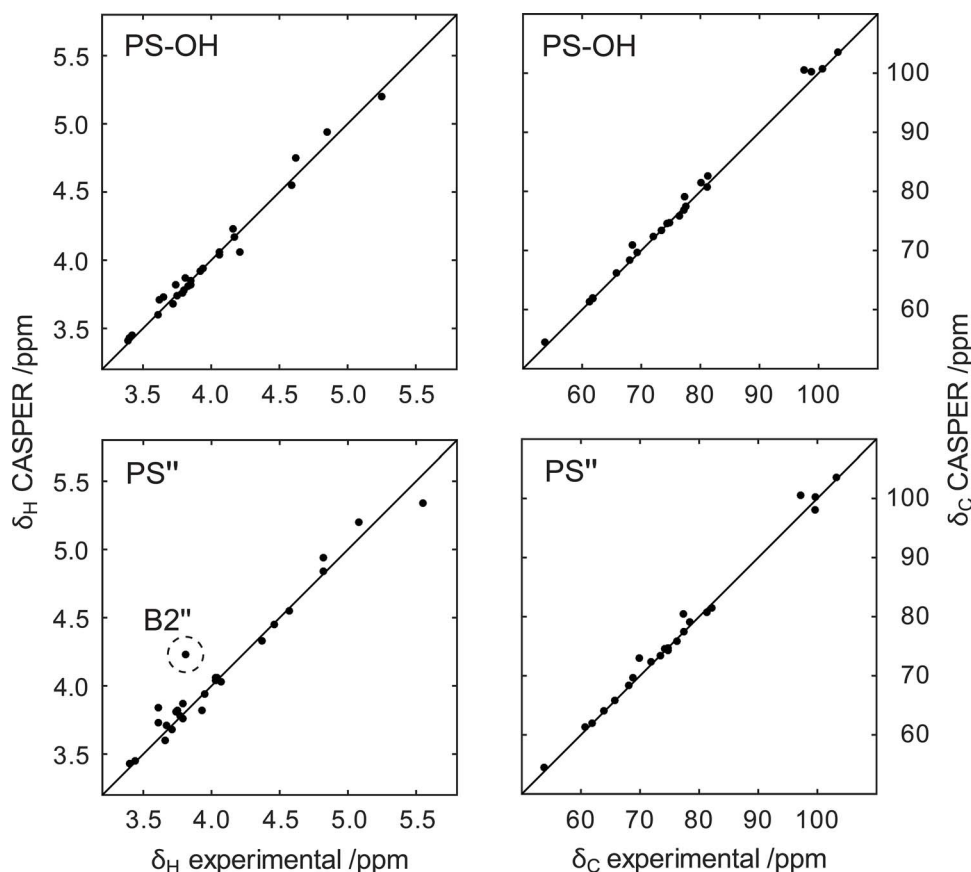
Fig. 6. Comparison of ^{1}H (left) and ^{13}C (right) NMR chemical shifts predicted by CASPER versus assigned from NMR experiments for the *O*-deacetylated (PS–OH) and the 2,6-di-*O*-acetylated population of the polysaccharide (PS) from the *E. coli* O93 O-antigen.

did not carry *O*-acetyl groups. Whether the observed di-*O*-acetylation of a specific mannosyl residue in the RU results from the action of two different *O*-acetyl transferases, on migration (Lassfolk et al. 2019, 2022; Oh et al. 2022), or on the conditions of growth and purification of polysaccharide material (Ilg et al. 2013; Knirel et al. 2015), remains an open question.

The sequential arrangement of the sugar residues in the O93 PS was determined from ^{1}H,^{13}C-HMBC and ^{1}H,^{1}H-NOESY NMR spectra (Table 2) and is summarized in the "sequence plot" (Fig. 7), in which the correlations "from" the anomeric atoms in residues **A–D** "to" adjacent sugar residues are shown. To further validate the NMR resonance assignments of the PS, the suggested structure was submitted, along with the assigned ^{1}H and ^{13}C NMR chemicals shifts, using the "Predict NMR Chemical Shifts" entry in CASPER. The experimentally assigned and predicted NMR chemical shifts of the PS having a →3)-*β*-D-Man2Ac6Ac-(1→ residue as part of its RU were compared (Fig. 6) and found to be in good agreement, but for a conspicuous, but significant, ^{1}H outlier for **B2″**. Significant upfield ^{1}H NMR chemical shifts displacements of 0.33 and 0.35 ppm were observed for **B2′** and **B2″**, respectively, compared to **B2** in the PS–OH. Intriguingly, whereas the ^{1}H NMR chemical shift of **B2** was predicted well for the PS–OH by CASPER it deviated when 2-*O*-acetyl substitution was present in residue **C**. Moreover, in residue **A**, which is not directly linked to residue **C**, the anomeric proton also showed a significant upfield chemical shift displacement of 0.14 and 0.17 ppm for **A1'** and **A1″**, respectively.

We hypothesize that these upfield ^{1}H NMR chemical shift displacements may be due to shielding by the carbonyl group, which has a strong magnetic anisotropy (Field et al. 2020) of the *O*-acetyl group in residue **C**. To further analyze this, we set out to investigate spatial proximity of protons between sugar residues and acquired 1D ^{1}H,^{1}H-NOESY experiments with mixing times of 300 and 600 ms where the selective irradiation was set on the methyl group of the 2-*O*-acetyl substituent (**C2″Ac/C2′Ac**; Fig. 8). Interestingly, strong inter-residue correlations to **A1″**, **A5″**, **A6″**, and **B2″** (and the corresponding mono-*O*-acetylated sugar residue which has degenerate or closely similar NMR chemical shifts) were observed (Fig. 8). This allowed us to construct a 3D model where **C2″Ac** was oriented in such a way that it was proximate to the protons that it had NOEs to. In this model torsion angles of $\phi_H,\psi_H \approx -35°, -45°$ for the glycosidic linkage of *α*-D-Glc*p*NAc-(1→2)-*β*-D-Man*p* and $\phi_H,\psi_H \approx +25°, +60°$ for that of *β*-D-Man*p*-(1→3)-*β*-D-Man*p*2Ac6Ac were adopted. The orientation of the carbonyl group then is such that the anisotropic shielding can affect **B2″** and the **A1″** protons, consistent with the significant upfield chemical shift displacement of these (Fig. 8).

The capsular polysaccharides (CPS) from *E. coli* K84 and *E. coli* K50 containing hexa- and pentasaccharide RUs have structural the elements →4)-*β*-D-Glc*p*-(1→3)-*α*-D-Glc*p*NAc-(1→2)-*β*-D-Man*p*-(1→3)-*β*-D-Man*p*NAc-(1→ and *α*-D-Glc*p*NAc-(1→2)-*β*-D-Man*p*4,6(*S*)Pyr-(1→3)-D-Man*p*NAc, respectively (Whittaker et al. 1994; Grue et al. 1994), which are similar to part of the O-antigen from *E. coli* O93. The ^{1}H NMR chemical shifts of H1 in the *α*-D-Glc*p*NAc residue
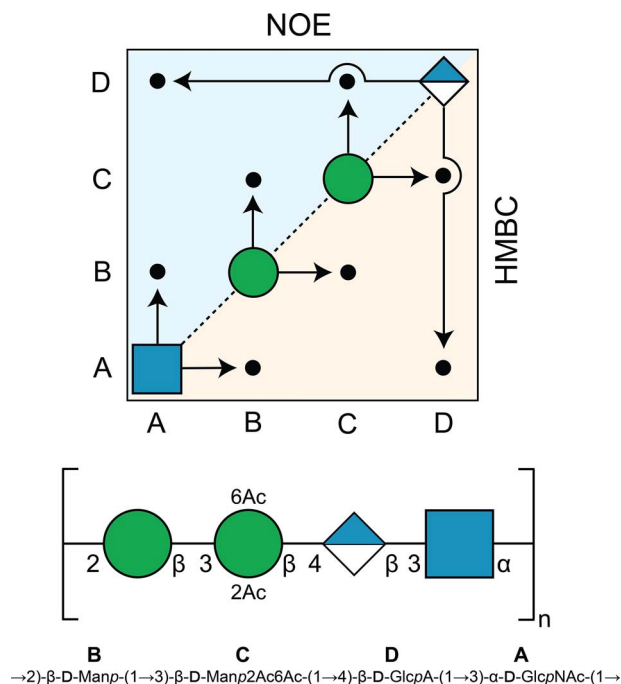
Fig. 7. (top) A "Sequence plot" summarizing the connectivities observed in a ^{1}H,^{1}H-NOESY spectrum of *E. coli* O93 PS–OH (upper triangle) and a ^{1}H,^{13}C-HMBC spectrum of *E. coli* O93 PS (lower triangle) where the connectivities emanate "from" the anomeric position "to" the linkage position, (middle) *E. coli* O93 PS repeating unit in SNFG-representation (Neelamegham et al. 2019) and (bottom) standard nomenclature with residues annotated by **A–D**.

were 5.17 and 5.12 ppm and those for H2 in the β-D-Man$p$ were 3.91 and 3.93 ppm, respectively, reminiscent of the corresponding ones in the 2-*O*-acetylated populations of the *E. coli* O93 O-antigen. Based on structural and ^{1}H NMR chemical shift similarities we deduce that the presence of the *N*-acetyl group in D-Man$p$NAc of the two CPS has a similar effect on protons in adjacent sugar residues as the 2-*O*-acetyl group in D-Man$p$ has on protons in the corresponding residues in the O-antigen from *E. coli* O93.

From the established tetrasaccharide BRU of the *E. coli* O93 O-antigen (Fig. 7) 3D structures of the polysaccharides without and with *O*-acetyl substituents were generated by CarbBuilder (Kuttel et al. 2016; Fig. 9) revealing the *O*-acetyl groups as additional epitopes to the main chain polymer. Notably, the *E. coli* O93 O-antigen structure only differs to that of the O-polysaccharide of the gram-negative marine bacterium *Cellulophaga baltica* strain NN015840^T (Tomshich et al. 2007) by the additional 6-*O*-acetylation. Whereas *E. coli* O93 was grown at 37 °C, the optimal growth conditions for *C. baltica* occur at a lower temperature in the range of 26–30 °C and importantly it does not grow above 32 °C (Johansen et al. 1999). If one of the bacteria obtained the gene cluster coding for the O-antigen from the other via "horizontal gene transfer" (Liu et al. 2020; Arnold et al. 2022) remains to be elucidated.

The analysis of function of gene products, from *galF* to *HisI* (vide supra), in relation to the determined structure of the BRU of the O-antigen from *E. coli* O93 calls for the presence of a third GT (or that WfdW has a dual activity). The function of the gene product from *orf5* was still uncharacterized and the information deposited by Iguchi et al. (2015) translated into a putative protein sequence containing 334 amino acids. A protein BLAST resulted in the identifier WP_000697476.1,

which was entered to UniProt to give A0A0A8J5U3_ECOLX, followed by searching the AlphaFold Protein Structure Database (Jumper et al. 2021; Varadi et al. 2022) resulting in a hit of an uncharacterized protein, the structure of which is shown in Fig. 10. The protein has two Rossmann-like domains with $\beta\alpha\beta$-segments (Hanukoglu 2015) and displays a GT-B fold (Chang et al. 2011). Interestingly, Orf5 has a recurrent C-terminal α-helix that extends back onto the N-terminal domain, like in the GT WaaG that also has the GT-B fold (Martinez-Fleites et al. 2006; Riu et al. 2022). Moreover, whether Orf5 has a membrane-interacting region (MIR), which has been shown to present in WaaG (Liebau et al. 2015), requires further investigations. Based on the information acquired we propose that Orf5 is a GT responsible for the third sugar transfer starting from the initial GlcNAc-*PP*-undecaprenyl acceptor to result in a tetrasaccharide-*PP*-undecaprenyl entity, which subsequently is translocated from the cytoplasmic side of the inner membrane to the periplasmic side by Wzx where the oligosaccharide will be polymerized by the Wzy–Wzz complex to give the O-antigen polysaccharide.

Conclusions

The initial analysis of the gene cluster related to the biosynthesis of the O-antigen polysaccharide from *E. coli* O93 revealed putative functions of proteins that were encoded in this part of the genome (Iguchi et al. 2015; DebRoy et al. 2016; Liu et al. 2020). This information gave clues about the biosynthesis of the O-polysaccharide, such as the presence the Wzx/Wzy biosynthetic pathway, possible constituent sugar components as well as probable *O*-acetyl decoration on the polysaccharide. Further information was obtained from ECODAB, in which the function of one of the GTs had been proposed with high fidelity, viz., the structural element β-D-Glc$p$A-(1→3)-D-Glc$p$NAc, and was consistent with the presence of UDP-glucuronic acid as the donor molecule and Und-*PP*-D-GlcNAc as the acceptor molecule in conjunction with the proposed GT family acting by an inverting mechanism.

To gain deeper insight into the structure of the O-antigen polysaccharide the bioinformatics information was coupled with an NMR analysis. Using the computer program CASPER, structure proposals were generated in a highly efficient way without the need to carry out NMR chemical shift assignments; the top-ranked proposal was subsequently confirmed to be correct by means of additional NMR experiments. Detailed NMR analysis was employed to identify the *O*-acetylation pattern of the O-antigen polysaccharide, which has tetrasaccharide RUs, →2)-β-D-Man$p$-(1→3)-β-D-Man$p$2Ac6Ac-(1→4)-β-D-Glc$p$A-(1→3)-α-D-Glc$p$NAc-(1→, consisting of three populations, viz., ∼$\frac{1}{2}$ of them are 2,6-di-*O*-acetylated, ∼$\frac{1}{4}$ are 2-*O*-acetylated, and ∼$\frac{1}{4}$ do not carry *O*-acetyl groups on the penultimate sugar (a mannosyl residue) of the BRU. From 3D models of the different O-antigen populations it is evident that the *O*-acetyl groups can form exposed epitopes on the polysaccharide backbone, but whether the dual substitution pattern on a single sugar residue in the RU renders the bacterium any advantages as a pathogen during infection remains to be established.

Materials and methods
Bacterial strains and polysaccharide preparations
Escherichia coli reference strains of O93 (*E. coli* O93:−:H16) was obtained from SSI Diagnostica A/S (Hillerød, Denmark).
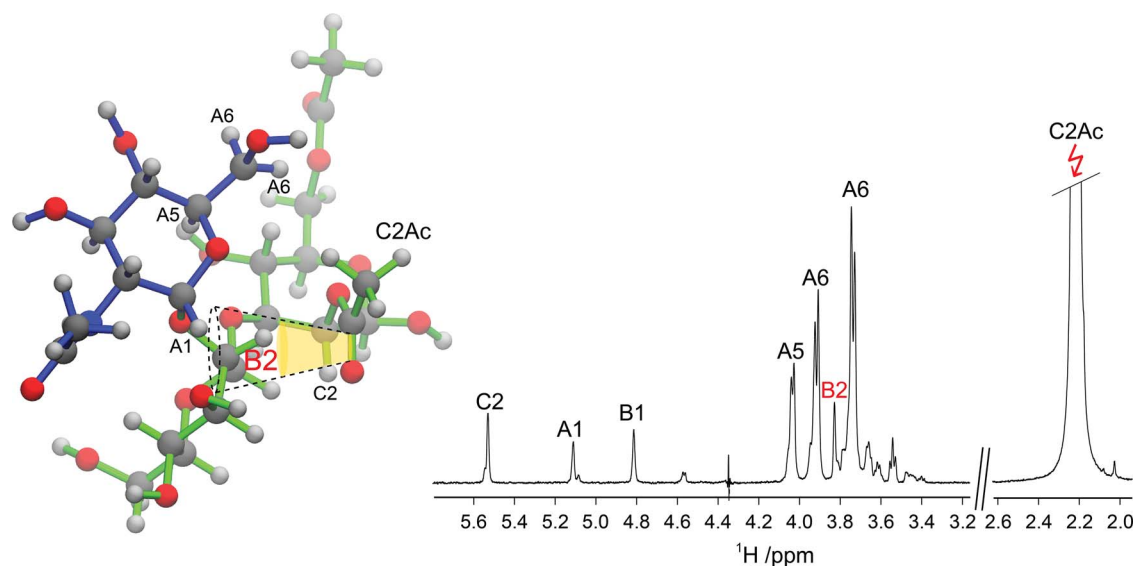
Fig. 8. (left) 3D representation of the trisaccharide α-D-GlcNAc-(1→2)-β-D-Man-(1→3)-β-D-Man2Ac6Ac from the *E. coli* O93 PS in a conformation consistent with the 1D ^{1}H,^{1}H-NOESY spectrum (right) with an irradiation at 2.22 ppm of the methyl group of the *O*-acetyl group at **C2** recorded with a 600 ms mixing time at 700 MHz showing inter-residue correlations. The yellow conical shape represents the proposed anisotropic shielding effect by the carbonyl group of the *O*-acetyl group at position **C2** affecting H2 in residue **B** by an upfield chemical shift displacement, in comparison to the *O*-deacetylated polysaccharide. Primes used in the text to differentiate *O*-acetylated populations in the polysaccharide are not included in the figure for clarity.

Bacterial culture, LPS extraction and preparation of lipid-free polysaccharides (PS) were carried out as previously described (Furevi et al. 2020). *O*-deacetylated polysaccharide (PS–OH) was prepared by treatment of the PS with aq. NaOH (0.5 M) at 20 °C for 18 h followed by extensive dialysis against deionized water using Spectra/Por 4 Membrane Tubing with a MWCO of 12–14 kDa (fisher scientific).

Sugar analysis by GLC

Hydrolysis of the PS was carried out using 2 M TFA at 120 °C for 30 min followed by reduction with $NaBH_4$ in aq. ammonia (1 M) at ambient temp for 30 min and acetylation at 100 °C for 30 min using Ac_2O and pyridine (1:1). The resulting mixture of alditol acetates was analyzed by gas–liquid chromatography (GLC) whereby components were separated on a PerkinElmer Elite-5 column using a temperature program of 150 °C for 2 min, 1 °C·min^{-1} up to 173 °C, followed by 8 °C·min^{-1} to 220 °C with hydrogen as the carrier gas (25 psi). The column was fitted to a PerkinElmer Clarus 400 gas chromatograph equipped with flame ionization detectors. The retention times of the derivatives were compared with those of authentic standards as references.

NMR spectroscopy

The PS sample was deuterium-exchanged by freeze-drying from 99.9% D_2O and examined by NMR spectroscopy at 70 °C as solutions in 99.96% D_2O (10 mg in 0.55 mL) with a trace amount of NaN_3 and was used for 1D ^{13}C NMR, 2D ^{1}H,^{13}C-HMBC, ^{1}H,^{13}C-H2BC and ^{1}H,^{13}C-BS-CT-HMBC, ^{1}H,^{13}C-HSQC, and ^{1}H,^{13}C-CT-CE-HSQC experiments. A PS–OH sample purified by gel-permeation chromatography was used for further analysis employing 1D ^{1}H and diffusion-filtered ^{1}H NMR experiments, F_2 coupled ^{1}H,^{13}C-HSQC, ^{1}H,^{13}C-HSQC, ^{1}H,^{1}H-TOCSY, and ^{1}H,^{1}H-NOESY NMR experiments (<1 mg in 0.55 mL)

acquired at 60 °C. NMR spectra were recorded using a Bruker Avance III 700-MHz spectrometer equipped with 5-mm TCI (^{1}H/^{13}C/^{15}N) Z-Gradient (53.0 G cm^{-1}) CryoProbe or a Bruker Avance III 600-MHz NMR spectrometer equipped with a 5-mm TXI (^{1}H/^{13}C/^{15}N) Z-gradient (55.7 G cm^{-1}) inverse probe. Chemical shifts are reported in ppm using internal sodium 3-trimethylsilylpropanoate-2,2,3,3-d_4 (TSP, δ_H 0.00) for ^{1}H NMR and external 1,4-dioxane 10% in D_2O (δ_C 67.40) for ^{13}C NMR as references. Chemical shift differences were obtained by comparison with NMR data of the corresponding monosaccharides (Jansson et al. 1989). NMR experiments suitable for resonance assignments of carbohydrates (Widmalm 2021) were recorded essentially as previously described (Furevi et al. 2020); specific additional experimental conditions are given below.

^{1}H,^{1}H-TOCSY experiments were acquired with mixing times of 20, 30, 60, 90, and 120 for the PS–OH and 30, 60, 90, 120, and 200 ms for the PS using DIPSI-2 spin-locks. For the PS–OH, a phase-sensitive ^{1}H,^{1}H-NOESY experiments at 700 MHz was obtained with a mixing time of 150 ms employing homospoil gradient pulses (1 ms) during mixing time with a strength of 40% of the maximum; both types of experiments used 2,048 × 256 data points in the F_2 and F_1 dimensions, respectively. For the PS material, a ^{1}H,^{1}H-NOESY experiment, with suppression of effects from zero-quantum coherence (Thrippleton and Keeler 2003), was carried out at 600 MHz with mixing times of 60 and 150-ms employing 14 k × 256 data points in F_2 and F_1, respectively, an acquisition time of 1.7 s and a relaxation delay of 5 s.

Multiplicity-edited ^{1}H,^{13}C-HSQC experiments were acquired for PS and PS–OH at a ^{1}H frequency of 700 MHz with 1,176 × 512 data points in F_2 and F_1, respectively, covering 100 ppm in the indirect dimension for PS and covering 105 ppm in F_1 for the PS–OH. Both experiments were acquired with non-uniform sampling of 50% sparsity and an exponential weighting set to 100 ms for the T_2

PS-OH

PS'

PS''

Core

Lipid A

Core

Lipid A

Core
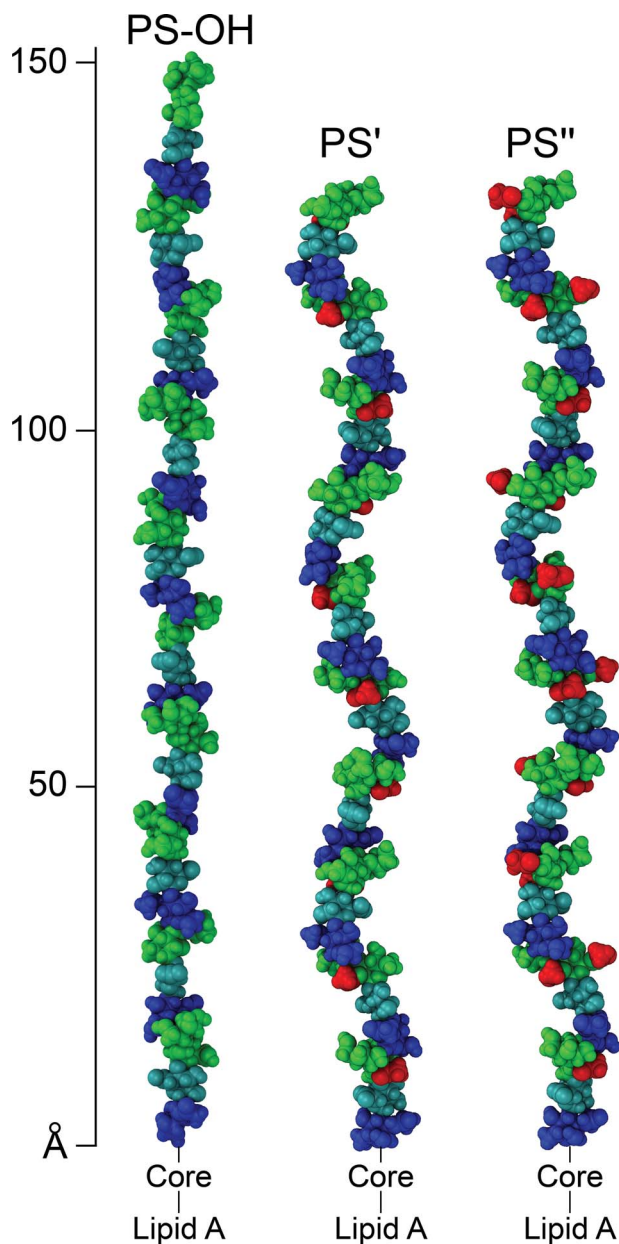
Lipid A

150

100

50

Å

Fig. 9. Molecular models of 10 repeating units of the *O*-deacetylated (PS–OH), 2-*O*-acetylated (PS'), and 2,6-di-*O*-acetylated (PS'') O-antigens built using CarbBuilder (Kuttel et al. 2016) and visualized with VMD (Humphrey et al. 1996). Sugar-residues are colored with D-GlcNAc in blue, D-Man in green, and D-GlcA in cyan. The *O*-acetyl groups present in (PS') and (PS'') are depicted in red color.
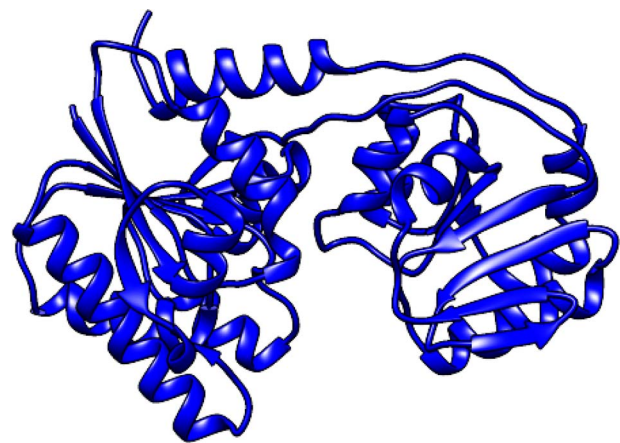

Fig. 10. Protein structure obtained from the AlphaFold Protein Structure Database of a putative glycosyltransferase (*orf5* in the O-antigen gene cluster) from *E. coli* O93 carrying out the last glycosylation reaction in formation of the oligosaccharyl-*PP*-undecaprenyl entity. The molecular model was made using UCSF Chimera (Pettersen et al. 2004).

for long-range couplings corresponding to 10 Hz, spectral widths of 8×6 ppm in the F_2 and F_1 dimensions, respectively, with the transmitter frequency at 175 ppm in the F_1 dimension and a Q3 Gaussian cascade pulse for selective ^{13}C excitation in the spectral region for carbonyl groups. A ^{1}H,^{13}C-H2BC experiment (Nyberg et al. 2005) for the PS material was recorded with a 3-fold low-pass *J*-filter, $^1J_{\text{CH-min}}$ and $^1J_{\text{CH-max}}$ set to 145 and 170 Hz, respectively, and a constant-time delay of 22 ms.

Bioinformatics analysis

The bioinformatics analysis using the genome of *E. coli* O93 related to its O-antigen synthesis and expression (Iguchi et al. 2015; DebRoy et al. 2016; Liu et al. 2020) and previously deposited at NCBI (GenBank AB812041.1) by Iguchi et al. (2015) relied also on ECODAB (Lundborg et al. 2010; Rojas-Macias et al. 2015), BLAST (Camacho et al. 2009), and CAZy (Drula et al. 2022).

Supplementary material

Supplementary material is available at *GLYCOB Journal* online.

Funding

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relaxation time. The F_2 coupled ^{1}H,^{13}C-HSQC experiment applied to the PS–OH was recorded with $1{,}886 \times 128$ data points in the F_2 and F_1 dimensions, respectively, using an evolution time corresponding to $^1J_{\text{CH}}$ of 145 Hz. A ^{1}H,^{13}C-CT-CE-HSQC experiment (Tian et al. 2001) was acquired for the PS with $1{,}176 \times 512$ data points in F_2 and F_1, respectively, and an evolution time corresponding to $^1J_{\text{CH}}$ of 165 Hz.

A ^{1}H,^{13}C-HMBC experiment for the PS was acquired using a 3-fold low-pass *J*-filter, $^1J_{\text{CH-min}}$ and $^1J_{\text{CH-max}}$ set to 120 and 170 Hz, respectively, and a delay for the evolution of the long-range couplings corresponding to 8 Hz. The band-selective constant-time ^{1}H,^{13}C-HMBC experiment (Claridge and Pérez-Victoria 2003) for the PS was acquired with a delay

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