Articles

Structural Determination and Biosynthetic Studies of the O-Antigenic Polysaccharide from the Enterohemorrhagic *Escherichia coli* O91 Using ^{13}C-Enrichment and NMR Spectroscopy†

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Received May 10, 1999; Revised Manuscript Received July 12, 1999

ABSTRACT: The structure of the O-antigenic polysaccharide from the enterohemorrhagic *Escherichia coli* O91 has been determined using primarily NMR spectroscopy on the ^{13}C-enriched polysaccharide. The O-antigen is composed of pentasaccharide repeating units with the following structure: →4)-*β*-D-Gal*p*-(1→4)-*β*-D-Glc*p*NAc-(1→4)-*β*-D-Glc*p*A-6-*N*-Gly-(1→3)-*β*-D-Glc*p*NAc-(1→4)-α-D-Qui*p*-3-*N*-[(*R*)-3-hydroxybutyramido]-(1→. The bacterium was grown with D-[UL-^{13}C]glucose in the medium which resulted in an overall degree of labeling of ∼65% in the sugar residues and ∼50% in the *N*-acyl substituents, indicating some metabolic dilution in the latter. The ^{13}C-enrichment of the polysaccharide proved valuable since NMR assignments could be made on the basis of ^{13}C,^{13}C-connectivity in uniformly labeled residues. The biosynthesis of the (*R*)-3-hydroxybutyramido substituent via C_2 fragments was identified by NMR spectroscopy. The (*R*)-configuration at C3 is in accord with fatty acid biosynthesis. Additional cultures with specifically labeled D-[1-^{13}C]glucose or D-[6-^{13}C]glucose corroborated the direct incorporation of glucose as the building block for the hexose skeletons in the polysaccharide and the biosynthesis of acyl substituents occurring via the triose pool followed by decarboxylation to give acetyl building blocks labeled with ^{13}C at the methyl group.

Enterohemorrhagic *Escherichia coli* (EHEC)[1] cells produce a bacteriophage-mediated Shiga-like toxin (SLT). This toxin is also called verotoxin (VT) or Vero cytotoxin because of its effect on cultured Vero cells. EHEC strains may produce two different Shiga-like toxins (SLT-1 and/or SLT-2). Both toxins are closely related to Shiga toxin produced by *Shigella dysenteriae* type 1. They consist of an A subunit and five B subunits. The B subunit binds to a globotriasylceramide (Gb_3) receptor in the intestine, and the A subunit possesses the toxic activity. Both SLT-1 and -2 are very similar in terms of the mode of action, structure, and general biochemical characteristics. However, there is no cross neutralization between the two toxins, and the amino acid sequences differ. In addition, the EHEC strains produce an enterohemolysin. Its production is strongly correlated with the production of SLT. It is a cell-associated, pore-forming cytolysin. The SLT-producing EHEC strains are the causative agents of hemorrhagic colitis often followed by a severe and life-threatening complication, hemolytic uremic syndrome (HUS). *E. coli* O157 is the serotype most often isolated from these patients. However, during the past few years, several other *E. coli* serotypes have been isolated from patients with hemorrhagic colitis and HUS, and identified as SLT-producing EHEC strains (*1*−*7*). Next to *E. coli* O157, the *E. coli* O26:H11 is the most common EHEC serotype. The structure of the O-antigenic polysaccharides of *E. coli* O157 and O26 have been elucidated (*8*, *9*). Recently, several reports described the presence of *E. coli* O91 in patients with HUS (*2*, *10*). In this study, we report the structure of the O-antigenic polysaccharide of a non-O157 EHEC strain, viz., that of *E. coli* O91.

Specific isotope labeling has been used in studies of biosynthetic pathways for a long time. These include radioactive isotopes, e.g., ^{3}H or ^{14}C, or stable isotopes, e.g., ^{13}C for NMR measurements, also combined with ^{2}H or ^{18}O for the identification of isotope shifts on a ^{13}C NMR resonance (*11*). A decade ago, ^{13}C isotope enrichment was reported for the capsular polysaccharide from *Klebsiella* K3 (*12*). A detailed investigation of the polysaccharide by NMR spectroscopy employing, inter alia, two-dimensional ^{13}C,^{13}C-COSY experiments with cross-peak analysis revealed that ∼30% of the glucose given to the bacterium for growth was catabolized prior to incorporation (*13*). The phosphogluconate pathway was confirmed by biosynthetic enrichment with

† Research supported by the Swedish Natural Science Research Council and the Magn. Bergvalls Foundation.

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[1] Abbreviations: EHEC, enterohemorrhagic *Escherichia coli*; UL, uniformly labeled.

10.1021/bi9910629 CCC: $18.00
Published on Web 09/03/1999

[2-^{13}C]glucose. More recently, alginate biosynthesis in *Azotobacter vinelandii* NCIB 8789 was studied with a variety of ^{13}C-labeled glucose precursors and NMR spectroscopy (*14*). It was shown that the entire catabolism of glucose occurs through the Entner−Doudoroff pathway and that the triose pools are in equilibrium. The use of ^{13}C-enrichment combined with NMR spectroscopical studies has been reported for oligosaccharides, e.g., from cyanobacteria (*15*) and yeast (*16*), and for polysaccharides, e.g., from *E. coli* K1 (*17*), *Streptococcus mitis* J22 (*18*), and *Cryptococcus neoformans* serotype A (*19*). The above-mentioned structural determination of the O-antigen polysaccharide from *E. coli* O91 was facilitated by supplying the bacterium with uniformly labeled [^{13}C]glucose. The resulting ^{13}C-enriched sugars in the polysaccharide revealed information about preferable biosynthetic pathways in the bacterium. Additional cultures with specifically labeled [1-^{13}C]Glc or [6-^{13}C]Glc further supported direct incorporation of the sugar backbone and catabolism via the triose pool to give two carbon building blocks for the different acyl substituents of the sugar residues in the lipopolysaccharide.

MATERIALS AND METHODS

Growth of the Organism. E. coli O91:K$^-$:H$^-$ (CCUG 11393) was grown in flasks, either in TY medium (Difco Laboratories) composed of tryptone (10 g/L) and yeast extract (5 g/L) supplemented with NH_4Cl (47 mM), Na_2HPO_4 (84 mM), KH_2PO_4 (44 mM), and Na_2SO_4 (1.6 mM) (Merck, Darmstadt, Germany), in Martek 9 Microbial Growth Medium (Martek Biosciences Co.), or in Celtone medium (Martek) supplemented with K_2HPO_4 (10 mM), KH_2PO_4 (10 mM), $MgSO_4$ (4 mM), and $CaCl_2$ (0.1 μM). The above media were further supplemented with different concentrations (0.05−0.4%) of D-glucose or ^{13}C-labeled D-glucose, viz., uniformly ^{13}C-enriched D-glucose (98−99 at. % ^{13}C, >99% pure, Cambridge Isotope Laboratories, Woburn, MA) or specifically labeled D-[1-^{13}C]glucose or D-[6-^{13}C]glucose (99 at. % ^{13}C, Omicron Biochemicals, Inc.). The growths were carried out in 250−1000 mL cultures. The bacteria were grown for 6 h at 37 °C under a constant agitation of 120 rpm using a New Brunswick incubator shaker (model G25, New Brunswick Scientific Co. Inc., Edison, NJ). A preculture (2 h) in the corresponding medium was used to inoculate the flasks.

After incubation, the bacteria were killed by adding formalin (1% w/v final concentration), and the cultures were stored at 4 °C overnight. The cultures were then centrifuged (8000*g* at 4 °C for 20 min), washed with phosphate-buffered saline [0.01 M potassium phosphate and 0.14 M NaCl (pH 7.2)], suspended in distilled water, and finally subjected to hot phenol/water extraction (*20*). The aqueous phase was dialyzed at 4 °C for 3−5 days against tap water, and then overnight against distilled water, concentrated under reduced pressure, and lyophilized. The yields of LPS under the different culture conditions were ∼80 mg/L of TY culture, ∼10 mg/L of Martek 9 culture, and ∼15 mg/L of Celtone culture.

Preparation of Lipid-Free Polysaccharides. The lipopolysaccharides were purified from any contaminating nucleic acids by treatment with nucleases (RNase and DNase, Sigma) in 0.1 M sodium acetate (pH 5) and subsequent dialysis. The nucleic acid content was determined spectrophotometrically to be <5% as described previously (*21*), and the protein content was estimated to be <0.5% according to Lowry et al. (*22*), with BSA as the standard.

Lipid-free polysaccharide was prepared by treatment of the lipopolysaccharide with 1% HOAc at 100 °C for 1 h. Lipid A was removed by centrifugation (10000*g* at 4 °C for 20 min). The polysaccharide was purified by gel-permeation chromatography on a column of Sephacryl S100 (Pharmacia, Uppsala, Sweden). Effluents were monitored using a differential refractometer (Waters, Milford, MA). The final yield of polysaccharide was ∼20 mg from the culture with the TY medium supplemented with uniformly labeled glucose. The preparations in which specifically labeled glucose in a Celtone medium was employed gave a final yield of ∼5 mg of polysaccharide.

Gas−Liquid Chromatography. GLC of alditol acetates, acetylated (*S*)-2-butyl glycosides, and (*S*)-2-butyl glycoside (*S*)-2-butyl esters was performed on a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) fitted with a flame-ionization detector using a temperature program of 180 °C for 1 min followed by an increase at a rate of 3 °C/min to 210 °C. For the trifluoroacetylated (*R*)-2-octyl ester of the β-hydroxybutyric acid, a temperature program of 110 °C for 1 min followed by an increase at a rate of 3 °C/min to 200 °C was used, and for the (*S*)-2-butyl ester of acetylated glycine, a temperature program of 130 °C for 5 min followed by an increase at a rate of 10 °C/min to 210 °C was used. Hydrogen was used as carrier gas. GLC−MS of the partially methylated alditol acetates was performed on a Hewlett-Packard 5970 MSD mass spectrometer with a temperature program of 190 °C for 3 min followed by an increase at a rate of 3 °C/min to 250 °C. Helium was used as the carrier gas. Both gas chromatographs were equipped with HP-5 capillary columns.

Component Analysis. The samples were hydrolyzed with 4 M hydrochloric acid at 100 °C for 20 min. After reduction with sodium borodeuteride and acetylation as described previously (*23*), the samples were analyzed by GLC. Absolute configurations of the sugar residues were determined essentially as described previously (*24*), but with (*S*)-2-butanol (*25*). The configuration of the β-hydroxybutyric acid was determined essentially as described previously (*26*, *27*). The polysaccharide was hydrolyzed with 0.5 M trifluoroacetic acid (0.5 mL) for 16 h at 100 °C to cleave the amide linkage of the β-hydroxybutyramido group. After evaporation to dryness, the hydrolysate was treated with (*R*)-2-octanol (0.2 mL) in the presence of concentrated trifluoroacetic acid (0.05 mL) at 120 °C for 16 h and evaporated to dryness. The formed 2-octyl esters and 2-ocyl glycosides were dissolved in CH_2Cl_2 (0.4 mL) and trifluoroacetylated with trifluoroacetic anhydride (0.05 mL) at room temperature for 30 min. The trifluoroacetylated (*R*)-2-octyl ester of the β-hydroxybutyric acid was analyzed by GLC using the trifluoroacetylated (*R*)- and (*S*)-2-octyl esters of authentic (*R*)-β-hydroxybutyric acid.

Methylation Analysis. The methylation was performed according to the Hakomori method (*28*) using sodium methylsulfinylmethanide as the base. The methylated compound was recovered from Sep-Pak C_{18} cartridges (Waters-Millipore) (*29*). The purified methylated sample was hydrolyzed (4 M hydrochloric acid at 100 °C for 20 min), and the

sugars were converted to partially methylated alditol acetates and analyzed by GLC−MS.

^{1}H and ^{13}C NMR Spectroscopy. For NMR experiments, about 15 mg of either ^{13}C-enriched or ^{13}C natural abundance polysaccharide was dissolved in D_2O, with a pD corrected to 9.0 with NaOH or HCl, lyophilized, and redissolved in D_2O. The pD was checked, and if necessary the procedure was repeated. Spectra were recorded for solutions in D_2O at pD 9.0 and 65 °C. Chemical shifts are referenced to external TSP [sodium 3-(trimethylsilyl)propanoate-d_4, δ_H 0.0] or acetone (δ_C 31.0 ppm). NMR experiments were performed on a Varian INOVA 600 MHz spectrometer equipped with a Varian 5 mm triple-resonance PFG probe (proton-detected experiments) or a Varian 5 mm broad-band probe (^{13}C-detected experiments).

The ^{13}C,^{13}C-DQFCOSY experiment was performed using a sweep width of 14 kHz in both dimensions and 250 t_1 increments of 64 scans, each with 4096 data points in t_2. A ^{13}C,^{13}C-DQFCOSY experiment with a spectral width of 25 kHz and 128 increments in t_1 of 32 scans with 8192 data points in t_2 was also performed to ascertain the assignments of the carbonyl signals, which had been aliased in the first ^{13}C,^{13}C-DQFCOSY experiment. The ^{13}C,^{13}C-relay-COSY experiment was performed with a sweep width of 14 kHz in both dimensions, and 96 transients of 4096 data points were accumulated for 256 increments. WALTZ-16 ^{1}H broad-band decoupling with a $\gamma B_1/2\pi$ of ~4.4 kHz was used throughout all the ^{13}C-detected experiments, and in all ^{13}C-detected two-dimensional experiments, data were zero-filled to 1024 × 8192 points and treated with a Gaussian apodization window function in both dimensions prior to Fourier transformation.

The gHSQC (*30*) and the HCCH-TOCSY (*31*) experiments were performed with spectral widths of 2.7 kHz for ^{1}H and 10.5 kHz for ^{13}C, and four transients of 1024 complex data points were accumulated for 128 increments. In the HCCH-TOCSY experiments, a DIPSI-3 (*32*) spin lock of 7.7 kHz was applied for 7.7, 15.5, and 23.3 ms. In total, six different HCCH-TOCSY experiments (three experiments each in the f_1f_3 and f_2f_3 planes) were performed. GARP broad-band decoupling with a $\gamma B_1/2\pi$ of 8.9 kHz was used for ^{13}C decoupling in the gHSQC and HCCH-TOCSY experiments. The gHMBC experiment (*30*) was performed with spectral widths of 2.7 kHz for ^{1}H and 25 kHz for ^{13}C, and eight transients of 1024 complex data points were accumulated for 256 increments. Prior to Fourier transformation, the data from the ^{1}H-detected two-dimensional experiments were zero-filled to 512 × 2048 data points and treated with a Gaussian apodization window function in both dimensions. The gHSQC experiment performed on the unlabeled material was performed as described above, but with 28 scans.

RESULTS

The *E. coli* O91 was grown in different media, namely TY medium, which is commonly used for growth of these bacteria, and Martek 9 and Celtone, both of which can be supplied with stable isotopes for NMR studies. The bacteria grown in the TY medium (supplemented with D-glucose) gave a sufficient amount of material for chemical characterization and preliminary NMR studies. Subsequent growth with a supplement of uniformly ^{13}C-labeled D-glucose gave ^{13}C-labeled material for NMR studies. ^{1}H NMR spectroscopy of lipopolysaccharides (LPS) grown in the three different media and extracted by the hot phenol/water method showed no structural changes in the LPS, as expected. The LPS grown in the TY medium were delipidated under acidic conditions and purified by gel-permeation chromatography to give (i) a natural abundance polysaccharide (PS) used for chemical analysis and some NMR spectroscopy and (ii) a ^{13}C-enriched polysaccharide (PS-^{13}C).

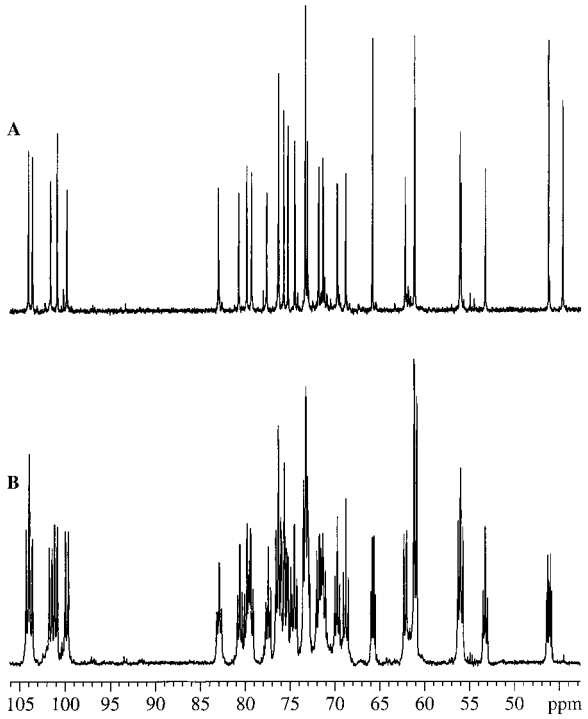

FIGURE 1: Part of the ^{13}C NMR spectrum of the O-antigenic polysaccharide from *E. coli* O91 at natural abundance (A) and ^{13}C-enriched (B).

Analysis of neutral sugar components after hydrolysis of the PS, reduction, and acetylation revealed Qui3N, Gal, GlcN, and an N-substituted Qui3N residue in a 0.3:1.0:1.2:0.7 relative proportion. Treatment of the PS with acidic methanol followed by acetylation showed further components to be present, namely, Gly, 3-hydroxybutyric acid, and GlcA. The absolute configurations could be determined by treatment of the PS with optically active (*S*)-2-butanol or (*R*)-2-octanol under acidic conditions which resulted in glycosides as well as esters where applicable. The acetylated products were compared to authentic standards, and D-Gal, D-GlcNAc, and D-GlcA were found. By comparison to the D-Qui3NAc residue present in the LPS of *Hafnia alvei* 1216 (*33*), it was concluded that the *E. coli* O91 LPS contains a D-Qui3N residue. The absolute configuration of this residue was confirmed by NMR (vide infra). The asymmetric center of the 3-hydroxybutyric acid was determined to be (*R*). Methylation analysis of the PS showed 2,3,6-tri-*O*-methyl-D-galactose, 3,6-dideoxy-2-*O*-methyl-3-(*N*-methylacetamido)-D-glucose, 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucose, 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucose, and 3,6-dideoxy-2-*O*-methyl-3-*N*-methyl[(*R*)-3-*O*-methylbutyramido]-D-glucose in a 1:0.1:0.7:0.3:0.5 relative proportion.

The ^{13}C NMR spectrum of the PS at natural abundance (Figure 1A) contains, inter alia, in the anomeric region five signals of equal intensity and in the carbonyl region three

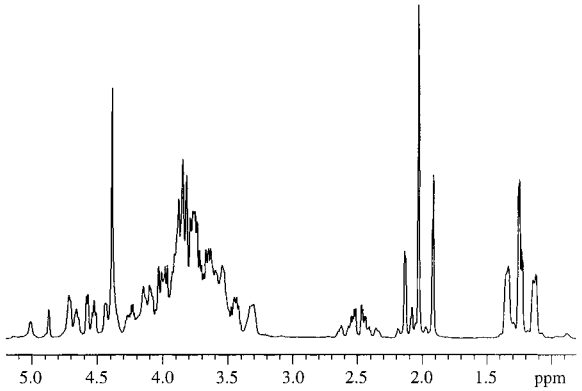

FIGURE 2: Part of the ^{1}H NMR spectrum of the ^{13}C-enriched O-antigenic polysaccharide from *E. coli* O91.

signals with a 1:3:1 relative intensity, the most upfield one being at δ_C 169.9 ppm. ^{1}H and ^{13}C NMR spectra further show that sugar residues have pyranoid ring forms and that the PS contains two *N*-acetyl groups and the (*R*)-3-hydroxybutyric acid in stoichiometric amounts. The presence of 3-deoxy-3-*N*-acetyl-D-Qui in the sugar analysis can thus be explained by cleavage of the acylamido linkage during acidic hydrolysis followed by N-acetylation during the workup. The above data indicate that the *E. coli* O91 O-polysaccharide is composed of pentasaccharide repeating units containing a 4-substituted D-Gal residue, a 3-substituted D-GlcN residue, a 4-substituted D-GlcN residue, a 4-substituted D-Qui-3-*N*-[(*R*)-3-hydroxybutyramido] residue, a D-GlcA residue, two *N*-acetyl groups, and a glycine residue.

At this point, the ^{13}C-enriched material grown in TY medium was used (Figure 1B). The overall degree of labeling in PS-^{13}C could be determined from the ^{1}H NMR spectrum (Figure 2) on the basis of integration of the satellites and was found to be ~65% in the sugar residues and ~50% in the *N*-acyl substituents, indicating some metabolic dilution in the latter. In a ^{13}C,^{13}C-DQFCOSY spectrum, the analysis of cross-peaks (*13*) revealed that where labeling had occurred within a sugar residue, all positions (C1−C6) were ^{13}C-enriched. The labeling of substituents will be discussed in detail below. All signal assignments were determined with the ^{13}C-enriched material, starting with assignment of the ^{13}C spin systems using ^{13}C,^{13}C-DQFCOSY and ^{13}C,^{13}C-relay-COSY experiments. ^{13}C,^{1}H correlations were then assigned using ^{1}H,^{13}C-gHSQC and ^{1}H,^{13}C-gHMBC experiments. Tentative assignments were clarified with the proton-detected HCCH-TOCSY experiment, and the sequence of the sugar residues was elucidated using the gHMBC experiment. ^{13}C and ^{1}H chemical shifts are given in Table 1. The sugar residues are denoted **A**−**E** according to decreasing chemical shift of the signal for their respective anomeric carbon.

Residues **A**−**D** all have large $J_{H1,H2}$ values (8.0−8.1 Hz), showing that they are β-linked, while residue **E** has a smaller $J_{H1,H2}$ value (3.4 Hz), which shows that this residue is α-linked. Residue **A** is assigned as the 4-substituted galactopyranosyl residue because of the small proton−proton coupling constants of position 4. The downfield chemical shift displacement of C4 is in agreement with the substitution pattern of the galactopyranosyl residue found in the methylation analysis. Residue **B** is the glucuronic acid as its C6 signal is found at δ_C 169.9 ppm. The upfield chemical shift of C6 at this slightly basic pD indicates that the uronic acid has a substituent linked to the carboxyl group via an amide linkage. Glycine was found in the chemical analyses and a cross-peak was found, in the gHMBC spectrum (Figure 3), between the chemical shifts of the carbonyl carbon of the uronic acid and the α-protons of the glycine residue. It is concluded that the uronic acid is substituted at C6, via an amide linkage, with a glycine residue. The downfield chemical shift of C4 of the uronic acid shows that the residue is substituted at O4. Residues **C** and **D** are the two glucosamine residues because of the upfield chemical shift of their C2 signals, and they are subsequently N-acetylated. As both the ^{13}C and ^{1}H chemical shifts of position 2 of these residues as well as their anomeric proton signals completely overlapped, it was necessary to use a ^{13}C,^{13}C-relay-COSY experiment to assign the two spin systems. Residue **C** exhibits a downfield chemical shift for C3 and residue **D** for C4. This shows that residue **C** is the 3-substituted and residue **D** is the 4-substituted 2-acetamido-2-deoxyglucopyranosyl residue identified in the methylation analysis. Residue **E**, finally, is the Qui3NAcyl residue found in the chemical analyses because of the upfield chemical shift of C3 and the downfield chemical shift displacement of C4 corroborating its 4-substitution. The fact that there is only one set of signals rules out the possibility of heterogeneity, and thus, the acyl group of this residue is derived from the (*R*)-3-hydroxybutyric acid. This amide linkage is further corroborated by the long-range connectivity found between the chemical shift of the carbonyl group of the substituent and H3 of residue **E**. A one-dimensional DPFGSE NOESY experiment, using three different mixing times and selective excitation of the anomeric proton of residue **E** (which substitutes position 4 of residue **A**, vide infra), showed interglycosidic NOEs to H4, and both H6 protons of residue **A** (Figure 4). These NOEs should be found if an α-D-sugar substitutes the D-galactose residue, but not if an α-L-sugar does (*34*). Thus, it is concluded that the absolute configuration is D, which corroborates the finding in the chemical analysis.

The sequence of the sugar residues was determined using the gHMBC experiment with a delay time of 50 ms, and long-range connectivities between the different sugar residues and between the sugar residues and their respective substituents are given in Table 2. Correlations were found from all anomeric protons and anomeric carbons to the linkage carbons and protons at the glycosyloxylated carbons, respectively, in all sugar residues. Therefore, the sequence of sugars in the repeating unit could be unambiguously determined as **A**-**D**-**B**-**C**-**E**, and the structure of the repeating unit of the *E. coli* O91 PS is →4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*A-6-*N*-Gly-(1→3)-β-D-Glc*p*NAc-(1→4)-α-D-Qui*p*-3-*N*-[(*R*)-3-hydroxybutyramido]-(1→.

The β-hydroxybutyric acid is synthesized partly from the ^{13}C-enriched D-glucose as labeling has occurred at this residue. However, the multiplicities of the signals in the ^{13}C NMR spectrum of the PS-^{13}C were for the signals from α- and β-carbons of the (*R*)-3-hydroxybutyramido substituent more complex than anticipated from a coupling pattern of a fully labeled spin system (Figure 5). A ^{13}C,^{13}C-DQFCOSY cross-peak analysis of the spin system of this residue (Figure 6) demonstrates that when the methyl carbon was ^{13}C-enriched, so was the β-carbon and when the carbonyl carbon was ^{13}C-labeled, so was the α-carbon. The trace of the cross-peak between the β-carbon and the methyl carbon at the f_2

Table 1: Chemical Shifts (parts per million) of the Signals in the ^{13}C and ^{1}H NMR Spectra of the *E. coli* O91 Polysaccharide[a]

sugar residue		^{13}C and ^{1}H chemical shifts									
		1	2	3	4	5	6	CO	C/H-α	C/H-β	Me
→4)-β-D-Gal*p*-(1→	**A**	104.1	71.7	73.1	77.4	76.3	61.1				
		4.52 (~8.0)	~3.67	3.75[b]	4.04	3.78	3.88, 3.81				
→4)-β-D-Glc*p*A-6-*N*-Gly-1-(→	**B**	103.7	73.3	74.6	79.4	75.3	169.9	176.8	44.5		
		4.54 (~8.0)	3.42[b]	3.66	3.90	3.97			3.84		
→3)-β-D-Glc*p*NAc-(1→	**C**	101.6	56.1	83.0	69.8	76.4	62.2	175.3			23.1[b]
		4.58 (8.1)	3.74	3.81	3.47	3.46	3.77, 3.98				2.03
→4)-β-D-Glc*p*NAc-(1→	**D**	101.1	56.1	73.2	79.9	75.7	61.1	175.3			23.2[b]
		4.58 (8.1)	3.74	3.72[b]	3.72	3.63	3.82, 4.02				2.03
→4)-α-D-Qui*p*-3-*N*-[(*R*)-3-hydroxybutyramido]-(1→	**E**	99.8	71.3	53.3	80.5	68.8	17.1	175.6	46.3	65.8	22.6
		4.87 (~3.4)	~3.67	4.08	3.44	4.27	1.23		2.46, 2.53	4.24	1.26

[a] $J_{H1,H2}$ values are given in hertz in parentheses. [b] Could be interchanged.

Table 2: Interglycosidic Correlations from the Anomeric Atoms and Intraglycosidic Correlations from the Carbonyl Atoms Observed in the gHMBC Spectrum of the *E. coli* O91 Polysaccharide

sugar residue		anomeric atom (δ_C or δ_H)	carbonyl	HMBC	connectivity
→4)-β-D-Gal*p*-(1→	**A**	104.1		3.72	**A** C1 to **D** H4
		4.52		79.9	**A** H1 to **D** C4
→4)-β-D-Glc*p*A-6-*N*-Gly-1-(→	**B**	103.8		3.81	**B** C1 to **C** H3
		4.54		83.0	**B** H1 to **C** C3
			169.9	3.84	**B** C6 to Gly Hα
			176.8	3.84	Gly CO to Gly Hα
→3)-β-D-Glc*p*NAc-(1→	**C**	101.6		3.44	**C** C1 to **E** H4
		4.58		80.5	**C** H1 to **E** C4
			~176.0	3.74	*N*-acetyl CO to **C** H2
→4)-β-D-Glc*p*NAc-(1→	**D**	101.1		3.90	**D** C1 to **B** H4
		4.58		79.4	**D** H1 to **B** C4
			~176.0	3.74	*N*-acetyl CO to **D** H2
→4)-α-D-Qui*p*-3-*N*-[(*R*)-3-hydroxybutyramido]-(1→	**E**	99.8		4.04	**E** C1 to **A** H4
		4.87		77.4	**E** C1 to **A** H4
			~176.0	4.24	*N*-acyl CO to *N*-acyl H3
			~176.0	4.08	*N*-acyl CO to **E** H3

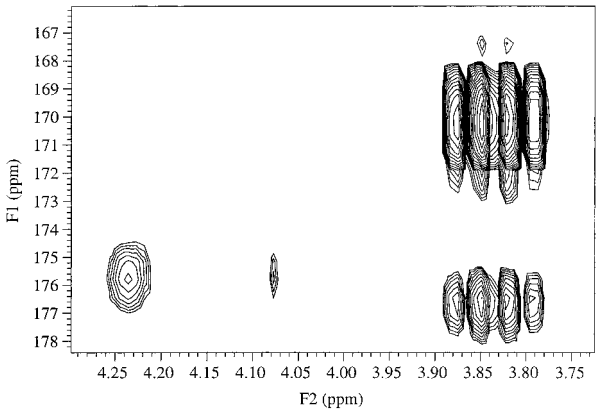

FIGURE 3: HMBC spectrum of the ^{13}C-enriched O91 PS showing correlations from the carbonyl region.

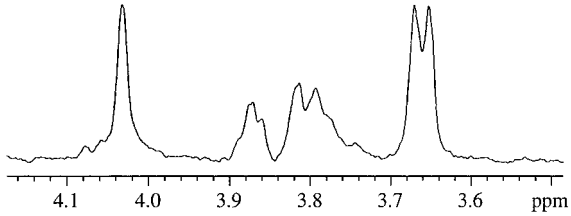

FIGURE 4: One-dimensional DPFGSE NOESY spectrum of the O91 PS showing NOEs from H1 of residue **E** to protons in residue **A**.

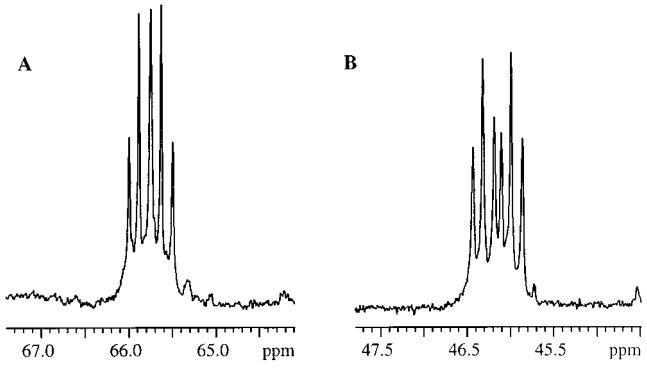

FIGURE 5: Signals for the β-carbon (A) and the α-carbon (B) of the β-hydroxybutyramido group in the ^{13}C NMR spectrum of the ^{13}C-enriched O91 PS.

chemical shift of the β-carbon reveals a cross-peak that is split antiphase by $^{1}J_{C(\beta)C(Me)}$ and split approximately 50% further by a passive coupling between the β-carbon and a ^{13}C atom at the α-position. The cross-peaks between the α-carbon and the carbonyl carbon exhibit the same pattern.

This demonstrates that ^{13}C labels are incorporated in the β-hydroxybutyric acid residue as C_2 units, in which either both carbons or neither carbon is ^{13}C-labeled. Of the labeled C_2 fragments, approximately 50% have been joined to form a $^{13}C_2$+$^{13}C_2$ chain, while the rest have been joined as $^{12}C_2$+$^{13}C_2$ or $^{13}C_2$+$^{12}C_2$ carbon chains.

The glycine residue was identified via chemical analysis and NMR spectroscopy of the natural abundance material. In the PS-^{13}C, however, enrichment did not take place to any significant degree in the glycine residue, as determined from the ^{13}C NMR spectrum. The amino acid is therefore obtained by the bacterium from the growth medium, either

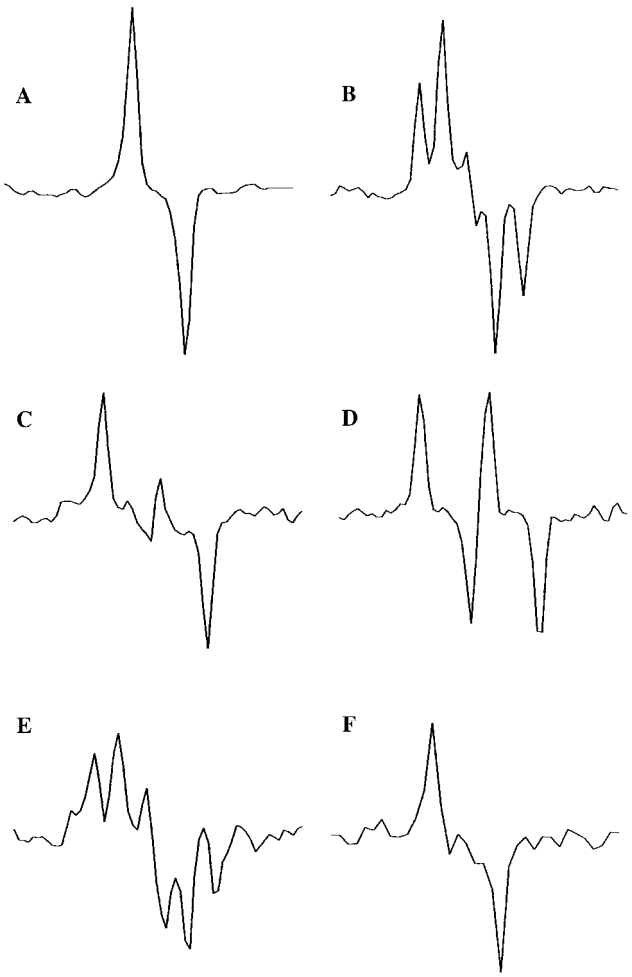

FIGURE 6: $^{13}C,^{13}C$-DQFCOSY cross-peaks of the ^{13}C spin system of the β-hydroxybutyramido group from the ^{13}C-enriched O91 PS.

directly or originating from another amino acid, but not from glucose as the carbon source.

The *E. coli* O91 was also grown in Celtone by addition of specifically labeled D-[1-^{13}C]glucose or D-[6-^{13}C]glucose, delipidated, and purified as described above. This resulted in polysaccharide preparations in which the carbon label originated from C1 or C6 in glucose. The degree of labeling was similar to that observed for the PS-^{13}C. The resulting ^{13}C NMR spectra are shown in Figure 7. Three results can readily be identified: (i) specific ^{13}C labels occur at C1 or C6 signals, (ii) the degree of scrambling is small in the residues, and (iii) ^{13}C labels are found at some positions in the acyl groups. The incorporation at C1 when the bacterium was given [1-^{13}C]Glc and at C6 when supplied with [6-^{13}C]-Glc is consistent with direct incorporation of glucose when it is present. However, in the acyl substituents, the labels are found at the methyl group of the *N*-acetyl groups and at C_α and or the methyl group of the (*R*)-3-hydroxybutyramido group, i.e., at C2 of the former and at C2 and/or C4 of the latter, as a result of catabolism (vide infra).

DISCUSSION

The $^{13}C,^{13}C$-DQFCOSY cross-peak analysis shows that the *E. coli* O91 incorporates the ^{13}C-enriched D-glucose in polysaccharide biosynthesis without prior degradation, since ^{13}C-enrichment is complete within each labeled sugar residue. Such direct incorporation was the major pathway in the

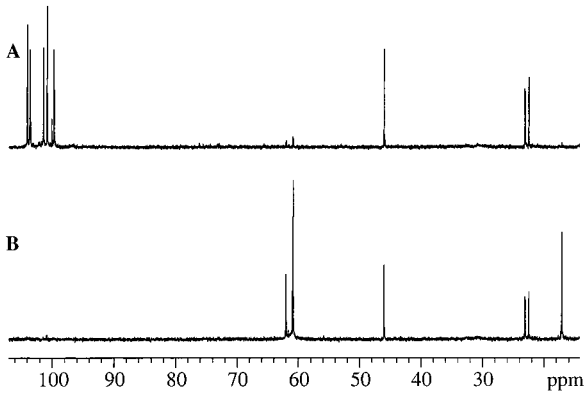

FIGURE 7: Part of the ^{13}C NMR spectrum of the O-antigenic polysaccharide from *E. coli* O91 grown with C1 (A) and C6 (B) ^{13}C-enriched D-glucose.

synthesis of the capsular polysaccharide of *Klebsiella* K3 (*13*). However, in the latter strain, approximately 30% of the glucose substrate was catabolized via the phosphogluconate pathway prior to incorporation into the polysaccharide. The *A. vinelandii* NCBI 8789, on the other hand, uses the Entner−Doudoroff pathway as a primary pathway to catabolize glucose prior to incorporation in the alginate chain (*14*).

The direct incorporation of the D-glucose can be explained by the bacteria converting D-glucose to UDP-glucose (*35*), which can then be modified into any carbohydrate residue needed in the polysaccharide synthesis without disturbing the ^{13}C isotope distribution. Also, the use of specifically labeled glucose rules out other pathways as major ones for the biosynthesis of the sugar units, namely, (i) glycolysis (Embden−Meyerhof pathway) followed by glyconeogenesis, since C1 or C6 is not scrambled via the triose pool, (ii) the Entner−Doudoroff pathway since C1 is not lost to a large extent or when C6 is the origin of label, it does not end up at both C1 and C6, and (iii) the phosphogluconate pathway (pentose phosphate shunt) since C1 is not lost as CO_2 early in the pathway.

The glycine residue substituting C6 of the glucuronic acid has not been ^{13}C-enriched at all. Note that the signals at δ_C 176.8 and 44.5 ppm (the chemical shifts for the carbonyl and the α-carbon, respectively, of the glycine residue) are missing in the ^{13}C-enriched material. The cross-peaks in the gHMBC spectrum of the PS-^{13}C, vide supra, demonstrated that the glycine residue was present also in the ^{13}C-enriched material. This can be explained by the presence of amino acids, including glycine, in the growth medium. Instead of the glycine residue being synthesized from the ^{13}C-enriched D-glucose, it can simply be incorporated directly, or obtained from conversion of another amino acid to glycine.

The finding of ^{13}C atoms in noncarbohydrate entities such as the *N*-acyl groups shows that part of the ^{13}C-labeled D-glucose is catabolized by the bacteria and incorporated in various parts. In all ^{13}C-enriched growths, a smaller amount of labeling was present in the *N*-acyl groups compared to that in the ring skeleton of the carbohydrate moieties, suggesting a slight degree of metabolic dilution. It was evident that C_2 building blocks were obtained via the triose pool followed by decarboxylation. When uniformly labeled D-glucose was used as a precursor, the various combinations of ^{13}C incorporation gave a complex appearance of the ^{13}C signals from the β-hydroxybutyramido group. The use of

either [1-^{13}C]Glc or [6-^{13}C]Glc gave the same labeling pattern of the β-hydroxybutyramido group, i.e., at C2 and/or at C4, supporting equilibrium at the triose pool. Also, the C_2 block synthesis of this *N*-acyl group is in accordance with a product from the fatty acid biosynthesis (*36*). Furthermore, the (*R*)-configuration suggests that the residue is derived from fatty acid synthesis rather than from fatty acid degradation.

The structure of the repeating unit of the *E. coli* O91 polysaccharide is almost identical to that of the repeating unit from the *H. alvei* 1216 O-polysaccharide (*33*). The differences between the repeating units are found in the substituents. The *H. alvei* 1216 PS repeating unit contains an *O*-acetyl group linked to position 6 of the galactose residue. A corresponding *O*-acetyl group is not present in the O91 PS. The repeating unit of the O91 PS, however, contains the glycine residue amidically linked to position 6 of the glucuronic acid residue, and such a substituent was not found in the *H. alvei* 1216 PS. To our knowledge, possible serological cross reactivity has not been studied.

ACKNOWLEDGMENT

We thank Dr. Y. Knirel for a sample of the O-antigenic polysaccharide from *H. alvei* 1216 and Omicron Biochemicals, Inc., for samples of specifically labeled [^{13}C]glucoses.

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BI9910629