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Determination of the structure of the capsular antigen of *Escherichia coli* O8:K46:H30, using FABMS and 2D-NMR spectroscopy

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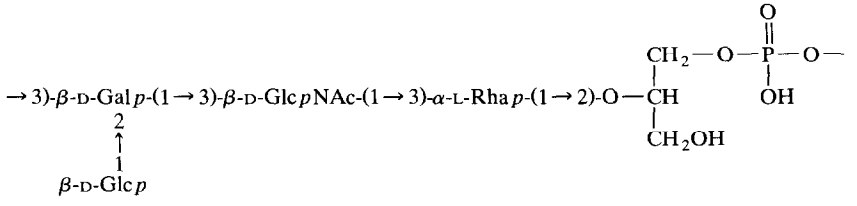
ABSTRACT

The structure of the capsular antigen from *Escherichia coli* O8:K46:H30 was elucidated by methylation analysis and 1D and 2D ^{1}H- and ^{13}C-NMR spectroscopy, and by methylation analysis, 1D- and 2D-NMR spectroscopy, and FABMS of the oligosaccharide-alditol obtained after dephosphorylation of the polymer with aqueous hydrofluoric acid. The capsular polymer is of the teichoic acid type and has the following repeating unit.



INTRODUCTION

The structures of 53 *Escherichia coli* capsular antigens have been reported[1–6], of which ten are of the teichoic acid type and belong to Group II (thermolabile antigens)[7]. Serotypes K2a, K2ab, K24, and K62 incorporate glycerol phosphate, K18, K22, and K100 contain ribitol phosphate, and K11, K51, and K52 have

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glycosyl phosphates in their repeating units. The capsular antigen of *E. coli* O8:K46:H30, the structure of which is now described, is the first antigen of the teichoic acid type in the *E. coli* series which belongs to Group I (thermostable antigens)[7].

RESULTS AND DISCUSSION

E. coli O8:K46:H30 bacteria were grown on Mueller–Hinton agar, and the polymeric material was isolated by precipitation into ethanol of the supernatant aqueous 1% phenol solution obtained after ultracentrifugation. The crude polymer was fractionated with cetyltrimethylammonium bromide and purified by gel-permeation chromatography on Sephacryl S400.

GLC of the alditol acetates derived from a hydrolysate of the purified polymer (PS) revealed Gal, GlcN, Glc, Rha, and glycerol in the molar ratios 1.0:0.9:1.0:0.7:0.5. That the Rha was L and the other residues were D was established by GLC of the acetylated (−)-2-octyl glycosides[8]. ^{31}P-NMR spectroscopy confirmed the presence of phosphate in PS. The ^{1}H-NMR spectrum of PS contained signals at δ 4.97 (unresolved doublet), 4.79 ($J_{1,2}$ 8 Hz), 4.75 ($J_{1,2}$ 8 Hz), and 4.66 ($J_{1,2}$ 8 Hz) for H-1, at δ 2.05 for NAc, and at δ 1.30 ($J_{5,6}$ 7.5 Hz) for Me of Rha. The ^{13}C-NMR spectrum of PS contained signals at 103.73, 102.86, 101.55, and 99.71 ppm for C-1, at 23.41 and 175.02 ppm for $NCOCH_3$, and at 17.56 ppm for CH_3 of Rha. The ^{13}C-NMR spectrum (Fig. 1) also revealed at least four phosphorus-coupled carbon atoms, indicating a structure of the teichoic acid type for PS.

Treatment of PS with cold aqueous 48% hydrofluoric acid afforded a dephosphorylated product (DP). The ^{1}H- and ^{13}C-NMR spectra showed that DP contained one major (~85%) and at least two minor components. The ^{1}H-NMR spectrum of the major component contained H-1 signals at δ 4.97, 4.81, 4.80, and 4.60. The ^{13}C-NMR spectrum contained 29 resonances, which included signals for

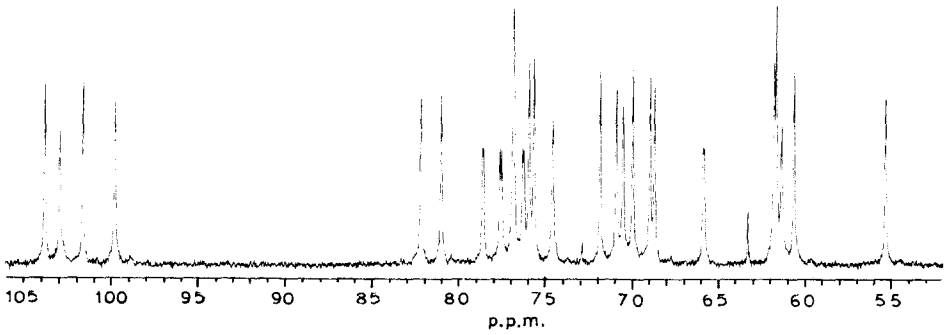


Fig. 1. Partial proton-decoupled ^{13}C-NMR spectrum of the *E. coli* K46 capsular polymer showing carbon–phosphorus couplings.

TABLE I

Methylation analyses of *E. coli* K46 capsular polymer (PS) and its dephosphorylated degradation product (DP)

Methylated sugars [a] (as alditol acetates)	Mole %	
	PS	DP
2,4-Rha	27.28	23.03
4,6-Gal	30.10	
2,4,6-GlcNAc	21.77	24.71
3,4,6-Gal		26.03
2,3,4,6-Glc	20.10	28.23

[a] 2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, *etc.*

$NHCOCH_3$ at 23.41 and 175.14 ppm, for C-2 of GlcNAc at 55.61 ppm, for CH_3 of Rha at 17.40 ppm, for CH_2OH at 60.90, 61.40, 61.68, 61.69 and 62.17 ppm, and for C-1 at 103.51, 103.37, 101.82, and 99.77 ppm. These data suggest that the major component of DP was an oligosaccharide composed of Glc, Gal, GlcNAc, Rha, and glycerol.

Methylation analyses (Table I) of PS and DP indicated the former to contain terminal Glc, 3-linked Rha, 3-linked GlcNAc, and 2,3-linked Gal, and established the phosphate to be 3-linked to the branching Gal.

The sequence of the sugar residues in the repeating unit was established by 2D-NMR experiments on PS and by FABMS of DP. The location of the phosphate diester bridges was confirmed by comparison of the 2D-NMR data for PS and DP.

2D-NMR studies of PS.—Complete assignment of the ^1H and ^{13}C resonances of the sugar residues and glycerol was made from COSY[9], one- and two-step RELAY COSY[10], and ^1H–^{13}C shift-correlated (HETCOR)[11] experiments.

The H-1 resonances of the four sugar residues in the repeating unit were labelled **A–D** in order of decreasing chemical shift. Partial COSY and two-step RELAY COSY contour plots of PS are shown in Figs. 2 and 3, and the ^1H assignments are presented in Table II. Commencing from the resonance for H-1, the chemical shifts of the other ^1H resonances of each residue were established by tracing the connectivities via the cross-peaks.

Residue **A.**—The Rha Me resonance provided a second window into the spin system. Connectivities from H-1 to H-3 were established from the COSY spectrum, whereas those from H-1 to H-4 were noted from the H-1 track and those for H-6 to H-4 from the H-6 track in the two-step RELAY COSY spectrum.

Residue **B.**—The chemical shifts for H-1 to H-3 were traced easily on the COSY contour map, whereas, in the two-step RELAY COSY, the H-1 and H-2 tracks unexpectedly showed connectivities for the entire spin system as though a total correlation experiment had been performed.

Residue **C.**—Only the connectivity to H-2 could be distinguished from the COSY spectrum, whereas connectivities up to H-5 were observed from the H-1

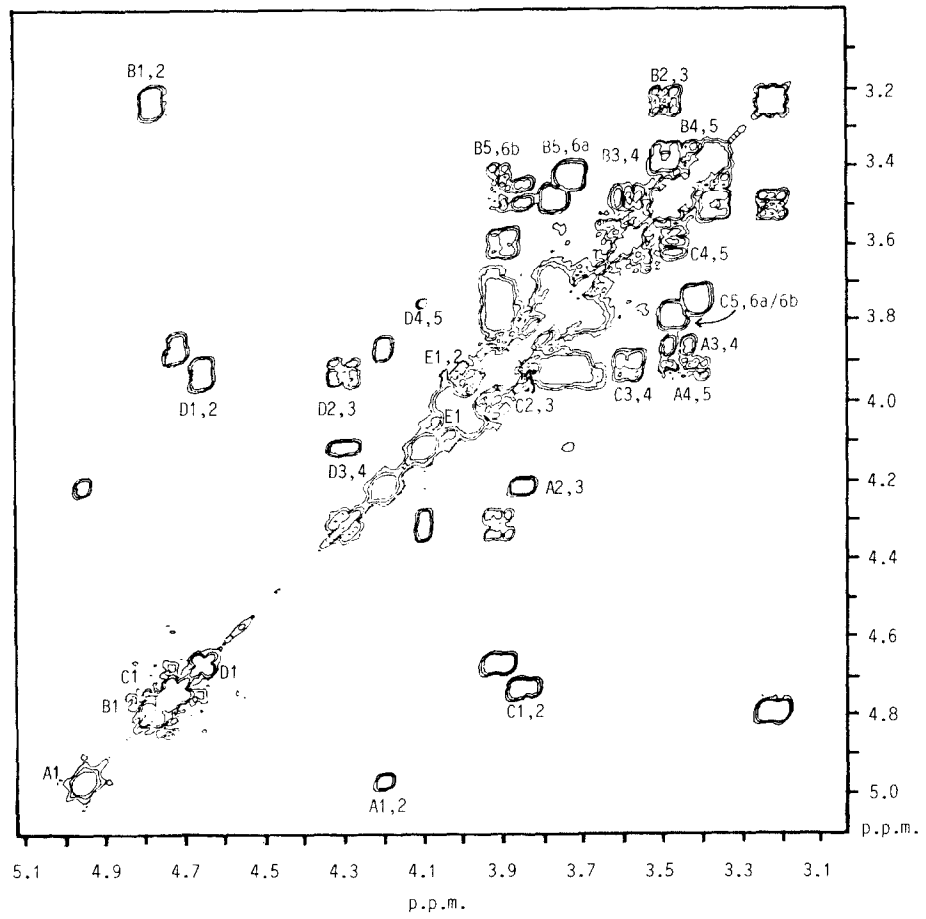


Fig. 2. COSY contour plot of the region 5.1–3.1 ppm of the *E. coli* K46 capsular polymer. The ^{1}H resonances of the *J*-coupled spin systems are labelled A–E.

track in the two-step RELAY COSY contour map. Returning to the COSY spectrum, the chemical shifts for the H-6a/H-6b resonances were then traced from that for H-5.

Residue **D**.—The chemical shift for the H-2 resonance was established from the COSY spectrum, and those for the H-3 and H-4 resonances from the H-1 track in the two-step RELAY COSY spectrum. In addition, a weak H-4/H-5 cross-peak was noted; the chemical shift of the latter resonance was confirmed by the expected intramolecular NOE between H-4 and H-5 in a homonuclear dipolar-correlated (NOESY) experiment[12].

The ^{1}H resonances assigned for residues **A–D** were then compared with the data obtained from the HETCOR experiment (Fig. 4 and Table II). In this way, all the ^{13}C resonances for residues **A–C** and C-1/5 for residue **D** were assigned. Of the four unassigned sets of $^{13}C/^{1}H$ chemical shifts, one must have arisen from

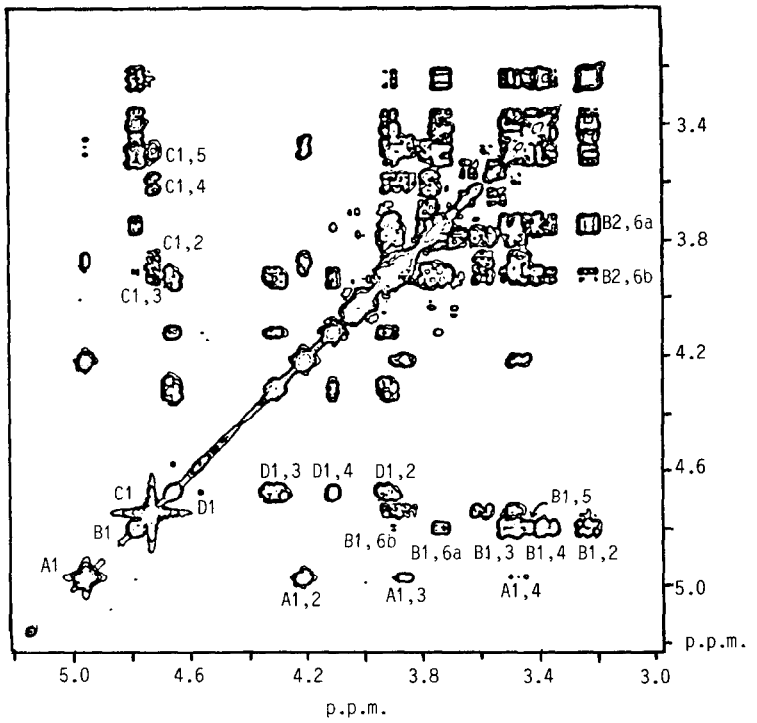


Fig. 3. Two-step RELAY COSY spectrum of the region 5.2–3.0 ppm of the *E. coli* K46 capsular polymer.

C-6/H-6a and H-6b of residue **D**, whereas the other three must have arisen from the glycerol moiety **E**.

Further inspection of the COSY spectrum now identified the H-1 resonance of residue **E** and its cross-peak to H-2. The chemical shift for the H-3 resonance of **E** was obtained from the H-1/H-3 connectivity noted in the two-step RELAY COSY spectrum. Finally, the remaining set of $^{13}C/^{1}H$ chemical shifts were assigned to C-6 and H-6a and H-6b of residue **D**.

Comparison of the ^{1}H and ^{13}C chemical shift data with those in the literature[13–17] permitted the residues in the repeating unit to be identified as indicated in Table II. The considerable deshielding of C-1,3 of **A**, C-1 of **B**, C-1,3 of **C**, C-1,2,3 of **D**, and C-1,2 of **E** established these as the positions of the linkages. The results of the methylation analysis for the Hex*p* residues in the repeating unit supported these findings. The NMR chemical shift data, the $J_{1,2}$ values for H-1, the $J_{C\text{-}1,H\text{-}1}$ values for C-1, and the intramolecular NOE contacts observed in the NOESY experiment (Table III) indicated **A** to be α and residues **B**–**D** to be β. The $^{2}J_{C,P}$ values for C-3 of **D** and C-1 of **E** (Table II) and the large $^{3}J_{C,P}$ values for C-2 of **D** and **E** indicated the monophosphate diester group to be located at O-3 of **D** and O-1 of **E**.

TABLE II

^{1}H- and ^{13}C-NMR data for *E. coli* K46 polymer [a]

Residue	**A** → 3-α-Rha	**B** β-Glc	**C** → 3-β-GlcNAc	**D** → 2,3-β-Gal	**E** → 2-Glycerol-1-PO_4
H-1a	4.970	4.790	4.750	4.660	4.050
H-1b					4.050
C-1	99.71 (170) [b]	102.86 (164) [b]	103.73 (164) [b]	101.55 (163) [b]	65.83 (3.6) [d]
H-2	4.220	3.247	3.886	3.928	3.927
C-2	70.81	74.54	55.34	77.52 (7.9) [c]	76.23 (5.9) [c]
H-3	3.875	3.500	3.923	4.312	3.741
H-3a					3.741
C-3	80.97	76.92	82.14	78.55 (4.3) [d]	60.62
H-4	3.487	3.380	3.590	4.123	
C-4	71.80	70.47	68.92	68.68	
H-5	3.911	3.430	3.490	3.738	
C-5	69.91	76.81	75.90	75.63	
H-6a	1.298	3.737	3.775	3.830	
H-6b		3.907	3.775	3.920	
C-6	17.59	61.78	61.65	61.33	

[a] Chemical shifts with acetone as internal reference, δ 2.23 and 31.07 ppm, respectively, for ^{1}H and ^{13}C. [b] $^1J_{C,H}$ (Hz). [c] $^3J_{P,C}$ (Hz). [d] $^2J_{P,C}$ (Hz).

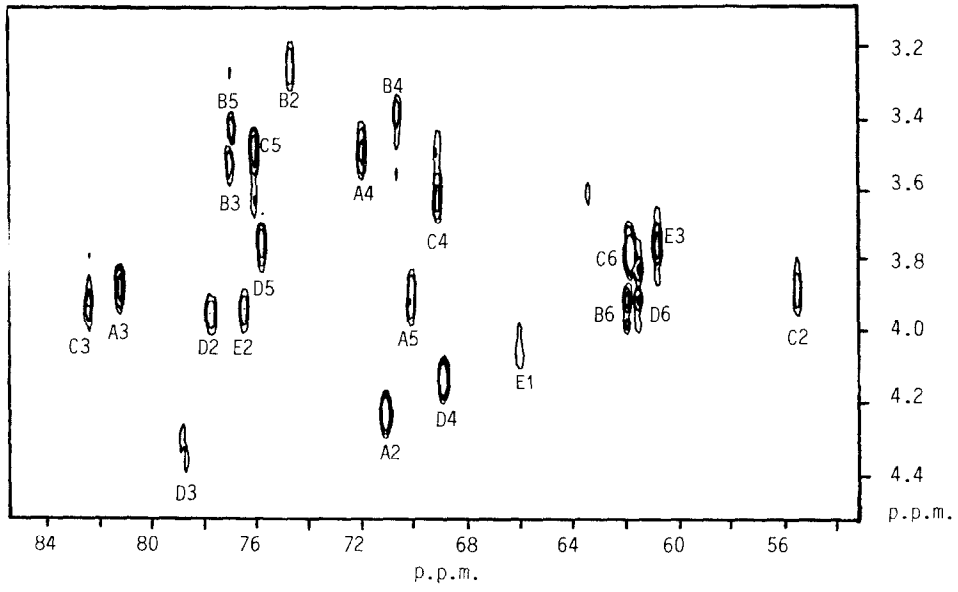


Fig. 4. ^{1}H–^{13}C shift correlation map of the spectral region F2 (84–54 ppm) and F1 (4.4–3.2 ppm) for the *E. coli* K46 capsular polymer. The correlated resonances are labelled A–E.

TABLE III

Observed NOE contacts for the K46 polymer

Proton	NOE contact
A, H-1	4.220 (**A**, H-2), 3.927 (**E**, H-2)
A, H-2	3.870 (**A**, H-3)
B, H-1	3.500 (**B**, H-3), 3.430 (**B**, H-5)
	3.928 (**D**, H-2)
C, H-1	3.875 (**A**, H-3)
C, H-3	3.490 (**C**, H-5)
D, H-1	4.312 (**D**, H-3), 3.738 (**D**, H-5)
	3.923 (**C**, H-3)
D, H-3	4.123 (**D**, H-4)
D, H-4	3.738 (**D**, H-5)

The sequence of the Hex*p* residues in the repeating unit of the polymer was investigated by a NOESY experiment. The inter- and intra-residue NOE contacts observed are presented in Table III. Strong inter-residue NOEs between H-1 and the proton across the glycosidic linkage were observed for each of the residues and established the sequence: **B**-(1 → 2)-**D**-(1 → 3)-**C**-(1 → 3)-**A**-(1 → 2)-**E**.

FABMS.—Further proof for the sequence of the residues in the repeating unit of PS was provided by FABMS of DP. The positive-ion spectrum of DP contained ions at *m/z* 766 and 783 for $[M + H]^+$ and $[M + NH_4]^+$, respectively, corresponding to Hex_2RhaGlcNAcGlycerol. The fragment ion at *m/z* 528 is most likely an A-type ion produced by cleavage of the GlcNAc linkage. The mass of this fragment is consistent with the composition Hex_2GlcNAc. Additional information on the structure was obtained from methylated DP, the spectrum of which (Fig. 5) contained peaks for one major (*m/z* 962) and several minor molecular ions (*m/z* 554, 700, 758, and 874). The major signal had an *m/z* value expected for the

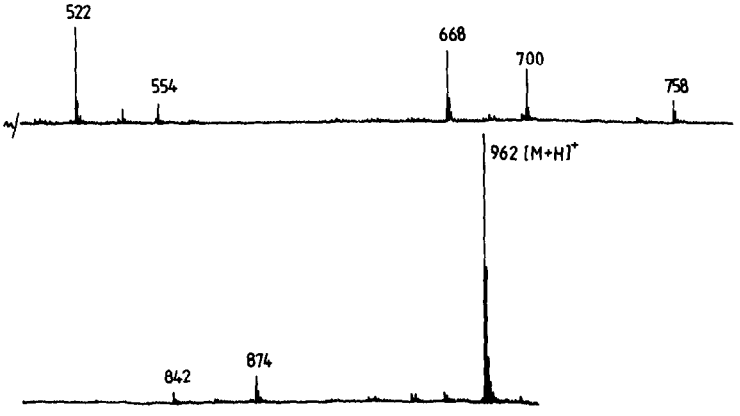


Fig. 5. Positive-ion FAB-mass spectrum of DP (the dephosphorylated and methylated *E. coli* K46 capsular polymer).

TABLE IV

^{1}H-NMR data [a] for *E. coli* K46 dephosphorylated product DP

Proton	Residue			
	A 3-α-Rha	**B** β-Glc	**C** 3-β-GlcNAc	**D** 2-β-Gal
H-1	4.973	4.814	4.797	4.596
H-2	4.218	3.304	3.816	3.791
H-3	3.876	3.514	3.933	3.858
H-4	3.514	3.421	3.599	3.925
H-5	3.864	3.422	3.488	
H-6a	1.290	3.744	3.778	
H-6b		3.939	3.778	

[a] As for Table II.

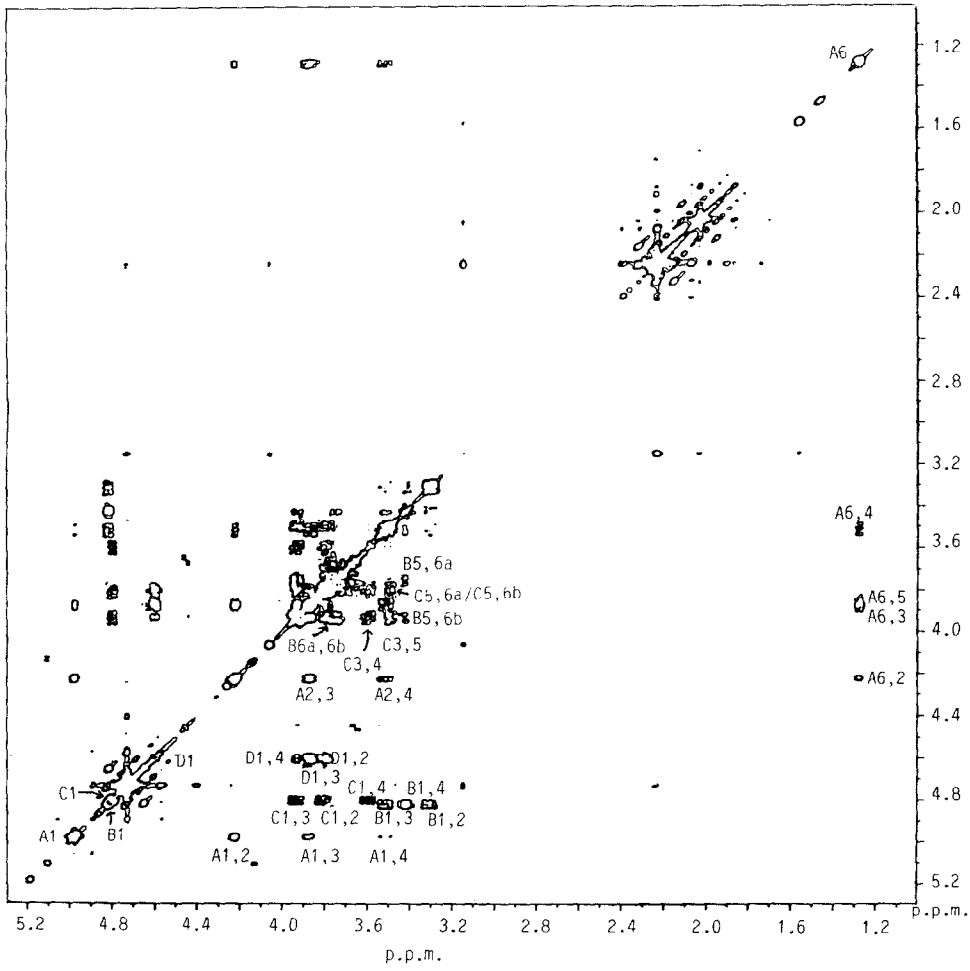
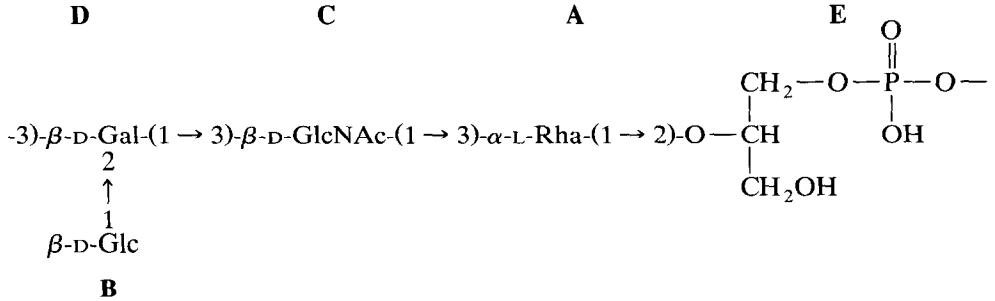


Fig. 6. Two-step RELAY COSY contour map of the region 5.2–1.1 ppm of DP (the dephosphorylated *E. coli* K46 capsular polymer).

tetrasaccharide-glycerol; the minor molecular ions corresponded to GlcNAcRhaGlycerol, Hex_2GlcNAc-methyl glycoside, HexGlcNAcRhaGlycerol, and Hex_2GlcNAcRha-methyl glycoside, respectively. Prominent fragment ions were present at m/z 522 and 668. The latter is the methylated analogue of the fragment ($Hex_2GlcNAc^+$) observed in the spectrum of DP; the former is most likely formed by elimination of Hex_2 from m/z 962. In order to confirm that these ions were derived from the molecular ion at m/z 962 and not from other components, the mixture was subjected to reverse-phase HPLC. The FAB-mass spectrum of the most strongly retarded component contained major signals at m/z 522, 668, and 962. Monitoring of the time course of methanolysis (at 60°) of the purified tetrasaccharide-glycerol by FABMS showed a rapid loss of glycerol and Rha and, after 20 min, the major signals were at m/z 668 and 700 (A-type and protonated molecular ions, respectively, for Hex_2GlcNAc-methyl glycoside). The above data are consistent with the sequence (Glc,Gal)GlcNAcRhaGlycerol for the major component of DP. The absence of a fragment ion corresponding to loss of methanol from the GlcNAc-cleavage ion at m/z 668 is consistent[18] with 3-*O*-glycosylation of the GlcNAc.

2D-NMR studies of DP.—The chemical shifts of all the ^{1}H resonances of residues **A–C** and the resonances for H-1/4 of residue **D** were determined (Table IV) for the major component of DP by COSY and one- and two-step RELAY COSY (Fig. 6). Comparison of the ^{1}H-NMR data for DP (Table IV) and the polymer (Table II) show that the residue most affected by the removal of phosphate is **D**. In the spectrum of DP, the resonances of H-2,3,4 of residue **D** occur 0.137, 0.454, and 0.198 ppm, respectively, upfield of the corresponding resonances in PS. These data confirm the location of the phosphate ester at O-3 of residue **D**.

The combined NMR, FABMS, and methylation analysis data permit the structure of the repeating unit of PS to be written as



The capsular antigens of *E. coli* have been divided into two groups on the basis of their chemical, physical, and microbiological characteristics[7]. Thermolability at pH 5–6 is one of the characteristics of the Group II antigens, many of which contain phosphate in their repeating units. Group I antigens, on the other hand, are thermostable. The capsular antigen of *E. coli* K46, which is the first antigen of

Group I to be described that incorporates phosphate in its repeating unit, probably owes its stability to the absence of a labile sugar such as Kdo, Neu5Ac, Gal*f*, or Fru*f*, which are constituents of all but one of the repeating units of Group II antigens of the teichoic acid type.

EXPERIMENTAL

General methods.—These and the instrumentation used have been described[19]. In addition, samples were hydrolysed at 25° with anhydrous HF for 6 h and PC was carried out on Whatman No. 1 paper, using either EtOAc–acetic acid–formic acid–water (18:3:1:4) or 1-butanol–EtOH–water (4:1:5, upper phase). GLC was carried out with a J and W Scientific fused-silica DB-17-bonded-phase capillary column (15 m × 0.25 mm) having a film thickness of 0.15 μm. GLC–MS was performed with a Varian Vista 6000 series chromatograph coupled directly to a Delsi Nermag R10-10C quadrupole mass spectrometer. Reverse-phase HPLC was carried out on a Waters model 510 system equipped with a Spherisorb S5 ODS2 column (4.6 × 250 mm) by elution with mixtures of *A*, nanopure water and *B*, acetonitrile at 1 mL/min. The column was equilibrated in 90% *A*:10% *B*, the samples were eluted using a gradient from 90% *A* to 40% *A* over 100 min, and 1-mL fractions were collected. The components giving quasimolecular ions at *m/z* 700 and 758 were present in fraction 47, whereas that giving *m/z* 875 was present in fraction 50 together with the trailing edge of that which gave an ion at *m/z* 700. The third component (*m/z* 962) was present in fraction 61. FAB-mass spectra were recorded with a VG Analytical High Field ZAB-HF mass spectrometer equipped with an M-Scan FAB gun operated at 10 kV. Samples were dissolved in aq 5% acetic acid (native) or MeOH (methylated derivatives) prior to loading into the thioglycerol matrix. Spectra were recorded on oscillographic chart paper and manually counted.

^{1}H-NMR spectra were recorded for solutions in D_2O with a Bruker WH-400 spectrometer, and the ^{13}C- and ^{31}P-NMR spectra were obtained with a Varian XL 300 spectrometer. Acetone (δ 2.23 for ^{1}H, and 31.07 ppm for ^{13}C) was the internal reference.

Isolation and purification of E. coli *K46 capsular polymer (PS).*—An authentic culture of *E. coli* O8:K46:H30 was obtained from Dr. I. Ørskov (Copenhagen) and the bacteria were grown on Mueller–Hinton agar. The harvested bacteria were suspended in aq 1% phenol and stirred at 4° for 6 h, and the suspension was then ultracentrifuged. The supernatant solution was poured into EtOH, the precipitate was collected, a solution in water was treated with a few drops of aq 5% cetyltrimethylammonium bromide, and the precipitate produced was removed by centrifugation. The supernatant solution was treated with more cetyltrimethylammonium bromide solution, and the precipitate was isolated and treated with 4 M NaCl in the usual way to afford the capsular polymer. The polymer was purified

further by gel-permeation chromatography on a column of Sephacryl S400 (100 × 2.5 cm) to give PS.

Dephosphorylation of PS.—A solution of the polymer (100 mg) in aq 48% HF was kept at 4° for 72 h, then neutralised, concentrated, and desalted on Bio-Gel P2 to afford a dephosphorylated product (DP, 60 mg).

Methylation analysis.—Samples of PS and DP were methylated[20], and the products were isolated by extraction with CH_2Cl_2 and then purified by passage down a column of Sephadex LH20.

2D-NMR spectroscopy.—All 2D experiments were recorded at 27° on a Bruker WH-400 or AM-400 Spectrometer, equipped with an Aspect 3000 computer and an array processor, using standard Bruker software.

^{1}H-Homonuclear shift-correlated experiments (COSY[9] and one- and two-step RELAY COSY[10]) and a homonuclear dipolar-correlated experiment (NOESY)[12] were performed on PS, using a spectral width of 2118 Hz. Data matrices of 256 × 1024 data points were collected for 96 transients for each t_1 delay. The matrices were zero-filled in the t_1 dimension and, after resolution enhancement in both dimensions by a non-shifted sine-bell window function, the data were transformed and symmetrised. Digital resolution in the resulting 512 × 1024 matrices was 4.1 Hz per point. Relaxation delays of 1.2 or 1.3 s were used. For the RELAY COSY experiments, fixed delays of 0.035 s were used. The mixing delay in the NOESY experiment was 0.255 s.

A ^{13}C–^{1}H shift-correlated (HETCOR)[11] experiment was recorded on the capsular polymer, using a spectral width in f_2 of 11 000 Hz (109.3 ppm) and 1 720 Hz (4.3 ppm) in f_1. The initial matrix of 128 × 2048 data points was zero-filled to 256 × 2048 points and processed with a sine-bell window function prior to transformation. Digital resolution in f_2 was 10.7 Hz per point and in f_1 13.4 Hz per point. A recycle delay of 1.5 s was employed and 1072 transients per fid were collected.

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