Carbohydrate Research, 197 (1990) 171–180
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

THE STRUCTURE OF *Escherichia coli* K31 ANTIGEN

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(Received February 16th, 1989; accepted for publication, June 5th, 1989)

ABSTRACT

The capsular polysaccharide of *Escherichia coli* K31 has been found by methylation analysis and n.m.r. spectroscopy to be based on the hexasaccharide shown. The sequence of the repeating unit was deduced from the combined results of β-elimination, lithium–ethylenediamine degradation, and hydrogen-fluoride and selective hydrolyses. The nature of the anomeric linkages, established by chromic acid oxidation, was confirmed by ^{1}H-coupled ^{13}C-n.m.r. spectroscopy. Two dimensional n.m.r. studies on a low molecular weight polymer obtained by bacteriophage depolymerization are also reported.

→2)-α-D-Glc*p*-(1→3)-β-D-Gal*p*-(1→3)-α-D-Glc*p*A-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→
4
↑
1
α-L-Rha*p*

INTRODUCTION

The capsular (K) antigen of *Escherichia coli* K31 has been designated[1] as heat stable (type A) and may, therefore, in the absence of amino sugars, be expected to resemble the *Klebsiella* antigens. A partial structure of *E. coli* K31 was proposed earlier by other workers[2], and we now report the complete structural elucidation of this antigen.

RESULTS AND DISCUSSION

Composition. — Analysis of the native polysaccharide before and after reduction[3] of the uronic-acid component gave galactose, glucose, and rhamnose in the ratios of 1.00:0.98:2.95, and 1.00:1.89:2.98, respectively. A composition of galactose, glucose, glucuronic acid, and rhamnose in the ratios of 1:1:1:3 was consistent with the n.m.r. data (see later), suggesting a hexasaccharide repeating unit.

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0008-6215/90/$ 03.50

TABLE I

METHYLATION ANALYSES OF *E. coli* K31 POLYSACCHARIDE AND DERIVED PRODUCTS

Methylated sugars[a] *(as alditol acetates)*	*Mole %*[b]						
	I[c]	*II*	*III*	*IV*	*V*	*VI*	*VII*
3,4-Rha	39.7	33.9	43.6	26.8		41.54	27.6
2,3,4-Rha	14.0	16.9	5.1	31.1		22.72	29.3
2,4,6-Gal	20.8	17.3	16.2	20.4	50.9		21.4
2-Glc		13.8	5.2			17.54	
2,4-Glc			9.0				
3,4,6-Glc	18.2	18.1	21.0	21.8		19.54	21.7
2,3,4,6-Glc					49.1		

[a]2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, etc. [b]Values are corrected using the effective carbon response factors given by Sweet *et al.*[6]; determined on a DB 17 column programmed for 1 min at 180° then to 250° at 2°/min. [c]I, K31 polysaccharide; II, K31 polysaccharide, uronic ester reduced after methylation; III, product **PH** from selective partial hydrolysis; IV, product from β-elimination and remethylation; V, product **A2** from HF hydrolysis; VI, product from chromium-trioxide oxidation; VII, product **F1** from Li-ethylenediamine degradation.

Methylation analysis. — Methylation[4] analyses without and with reduction of the uronic acid gave the results shown in Table I, columns I and II, from which it may be deduced that a rhamnose residue occupies a terminal position and the glucuronic acid unit constitutes the branch point. Methylation analysis of the product (**PH**) obtained by selective partial hydrolysis indicates (Table I, column III) that the lateral rhamnose unit is linked to the glucuronic acid at C-4. The glucuronic acid was shown by a β-elimination experiment[5] on the methylated polysaccharide to be linked to C-2 of a rhamnose unit (Table I, column IV).

In order to confirm the identity of the sugar to which the glucuronic acid is linked, a portion of the hydrolyzate used to determine the sugar composition of the native polysaccharide was methylated and analyzed directly by g.l.c. Subsequent to the elution of a mixture of permethylated monosaccharide methyl glycosides a single peak appeared which was shown by c.i.-m.s. to give a molecular ion $(M + NH_4)^+$ at *m/z* 456 consistent with the formulation of the aldobiouronic acid as GlcA–Rha. The potential of capillary columns to separate neutral and acidic materials, as has been noted previously[7], enables one to avoid tedious separations on ion-exchange resins.

On the basis of these methylation experiments the partial structure may be written as below.

$$\begin{array}{l} \rightarrow 3)\text{-Glc}p\text{A-}(1\rightarrow 2)\text{-Rha}p\text{-}(1\rightarrow \\ \qquad\quad 4 \\ \qquad\quad \uparrow \\ \qquad\quad 1 \\ \quad \alpha\text{-L-Rha}p \end{array}$$

The configuration of the rhamnose was established as L and of the other sugars as D by comparison of the circular dichroism spectra of their methylated derivatives with those of standards[8].

Lithium-ethylenediamine degradation[9]. — The polysaccharide was treated with lithium in ethylenediamine and the reducing oligosaccharide (**F1**) present in the mixture was isolated by gel-permeation and paper chromatography. N.m.r. spectroscopy (^1H and ^{13}C) and methylation data (Table I, column VII) showed the product **F1** to be a tetrasaccharide containing two deoxy sugar residues. The reducing end was determined by the method of Morrison[10] to be galactose, which therefore in the polymer is linked to glucuronic acid at C-3.

The tetrasaccharide **F1** is therefore (Glc,Rha,Rha)-Gal, and since two of the three rhamnose residues have already been located it remains to be established whether the sequence in **F1**, and hence in the native polysaccharide, is Rha-Rha-Glc-Gal or Rha-Glc-Rha-Gal. Partial hydrolysis of the native polysaccharide with hydrofluoric acid distinguished between these possibilities.

Hydrofluoric-acid hydrolysis[11]. — From this hydrolysis two pure disaccharides (**A2** and **A3**) were isolated, the latter in only small amount. Methylation analysis of **A2** (Table I, column V) showed it to be Glc-(1→3)-Gal, and c.i.-m.s. gave $(M + NH_4)^+$ at *m/z* 472, consistent with **A2** being a hexose–hexose disaccharide. Methylated **A3** similarly gave $(M + NH_4)^+$ at *m/z* 412, indicative of a disaccharide composed of two deoxyhexose units, which from the methylation results of the original polysaccharide must be Rha-(1→2)-Rha.

It therefore follows that the sequence in the main chain is

Glc—Gal—GlcA—Rha—Rha.

N.m.r. spectroscopy. — The proton spectrum of the native polysaccharide (at 363 K) showed six signals in the anomeric region between δ 5.24 and δ 4.82. Each signal integrated to approximately 1 H, but only the signal at δ 4.82 showed a measurable coupling constant of 8 Hz, indicative of a β linkage. The others were broad, presumably because of the viscosity of the solution. In addition two signals integrating to approximately 9 H were observed at δ 1.31 and δ 1.28.

The proton-decoupled ^{13}C spectrum likewise showed six signals in the anomeric range between 104.73 and 95.76 p.p.m., together with signals at 176.94, 17.57, and 17.45 p.p.m. indicative of uronic acid and 6-deoxysugar, respectively. In a proton-coupled spectrum the signal at 104.73 p.p.m. exhibited a coupling constant (J_{CH}) of 160.35 Hz, whereas the others gave values between 168.52 and 172.88 Hz, confirming the presence of a single β linkage[12]. Both the proton and the ^{13}C spectra suggest that the K31 polysaccharide is composed of a hexasaccharide repeating unit having a single β glycosidic linkage. This was confirmed by a chromium-trioxide oxidation[13], when galactose was the only sugar completely degraded.

The sequence of the sugar residues in the repeating unit of the polysaccharide and the anomeric configurations were confirmed by 2D n.m.r. spectroscopy. A

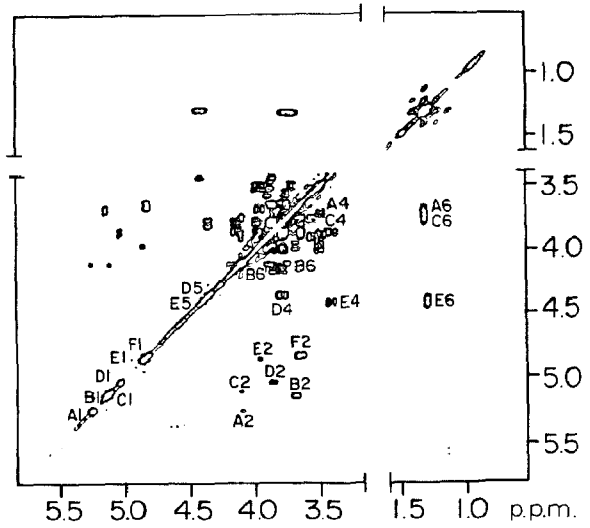


Fig. 1. ^1H-^1H Spin-correlated (COSYHG) spectrum of *E. coli* K31 polysaccharide **Pn** (see text for details).

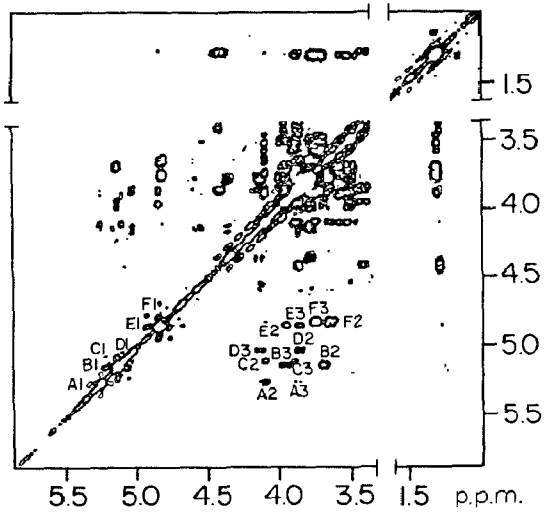


Fig. 2. One-step relay ^1H-spin coherence transfer (COSYR1HG) spectrum of *E. coli* K31 polysaccharide **Pn**.

polysaccharide (**Pn**) of significantly lower molecular weight and viscosity than the native polysaccharide was prepared by depolymerization with a viral endoglycanase and used in all 2D experiments.

The results of COSY[14] (Fig. 1) and one- and two-step relay-COSY[15] spectra (Figs. 2 and 3) allowed the assignment of H-1, H-2, H-3, and H-4 of most of the sugar residues in the repeating unit (Table II). The H-1 resonances of the sugar

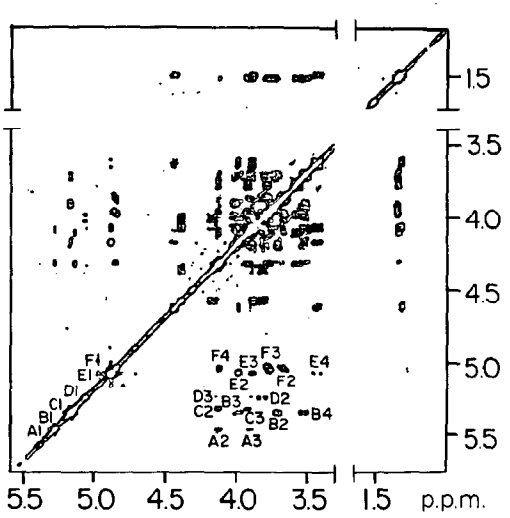


Fig. 3. Two-step relay ^{1}H-spin coherence transfer (COSYR2HG) spectrum of *E. coli* K31 polysaccharide **Pn**.

residues were arbitrarily labelled A to F in order of decreasing chemical shift. The assignment of the H-1 signals for the α-linked 6-deoxy residues A, C, and E was then greatly facilitated by the additional window provided by the methyl resonances. Thus, by following the cross-peaks from H-1 of A, C, and E the respective H-2, H-3, and H-4 resonances were established, while from the H-6 resonances the resonances for H-5 and their respective connectivities to H-4 were established. The assignment of the resonances for the glucuronic acid residues was likewise facili-

TABLE II

^{1}H-N.M.R. DATA FOR *E. coli* K31 POLYSACCHARIDE

Symbol	*Residue*	*Chemical shifts in p.p.m.*[a]						
		H-1 ($J_{1,2}$ *in Hz*)	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*
A	2-Rhaα-	5.251 (<1)	4.113	3.860	3.500	3.710	1.320	
B	2-Glcα-	5.126 (3)	3.689	3.958	3.501	3.950	3.781	3.860
C	2-Rhaα-	5.096 (<1)	4.102	3.891	3.556	3.750	1.320	
D	3,4-GlcAα-	5.033 (3)	3.865	4.130	3.770	4.354		
E	Rhaα-	4.837 (<1)	3.958	3.853	3.421	4.420	1.288	
F	3-Galβ-	4.816 (8)	3.658	3.740	4.091			

[a]Determined at 400 MHz, measured from internal acetone at δ 2.23. Spectra were recorded at 363 K. The sample used was the low M.W. polymer **Pn**. See text for details.

TABLE III

N.O.E. DATA FOR *E. coli* K31 POLYSACCHARIDE

Symbol	*Sugar residue (H-1)*	*Inter-residue contact (δ, assignment)*[a]	*Intra-residue contact (δ, H-2)*
A	2-Rhaα-	3.689, H-2B	4.113
B	2-Glcα-	3.740, H-3F	3.689
C	2-Rhaα-	no	4.103
D	3,4-GlcAα-	4.103, H-2C	3.865
E	Rhaα-	3.788, H-4D	3.958
F	3-Galβ-	4.131, H-3D	3.658[b]

[a]See footnote *a*, Table II. [b]May be due to scalar correlation effect.

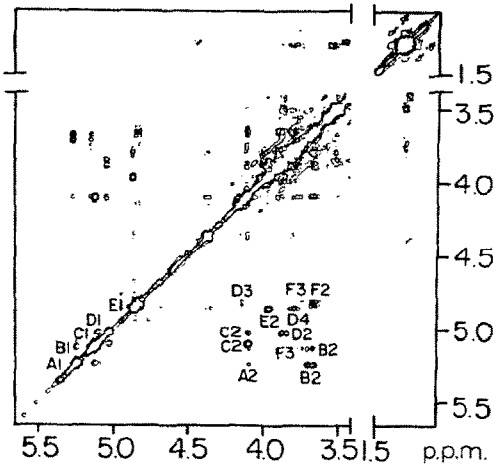


Fig. 4. Homonuclear dipolar correlated 2D-n.m.r. (NOESY) spectrum of *E. coli* K31 polysaccharide **Pn**.

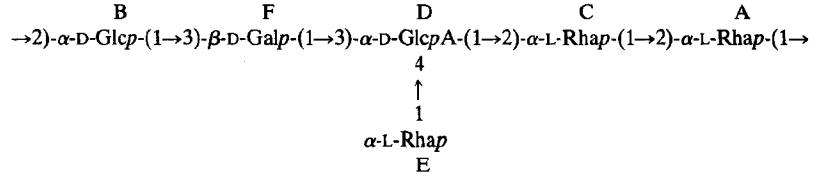
tated by the easily recognized H-5 doublet (^3J 11.0 Hz) at δ 4.354. Only the resonances of residue F could not be completely assigned.

Based on the composition and methylation results for the native polysaccharide, and the comparison of the ^{1}H-n.m.r. data for **Pn** with those for monosaccharide methyl glycosides, residue E was identified as the terminal α-rhamnose, residues A and C as 2-linked α-rhamnoses, B as a 2-linked α-glucose residue, D as a 3,4-linked α-glucuronic acid unit, and F as the 3-linked β-galactose residue.

The sequence of the sugar residues in the repeating unit was confirmed by data from a NOESY experiment[16] (Table III and Fig. 4). Interresidue n.O.e. contacts[17] established that residue A was linked to B, D to C, and E and F to D, giving the sequence shown. Interestingly, n.O.e.'s were observed between H-1A and H-1B, and H-1C and H-1D. In both cases, H-1 of the glycosyl residue is located between the proton at the bridge (H-2) and the equatorial neighbouring proton

(H-1) of the glycosyl-substituted sugar residue. This observation is common for α-mannose-type residues carrying a glycosyl group at C-2[18–23].

The proposed structure differs in several respects from the partial one published previously in a review. The main difference is the absence in the latter of a lateral rhamnose unit, although a cross reaction with *Pneumococcus* XXIII suggested its presence[2]. It is possible, however, that in the previous investigation the lateral rhamnose residue was fortuitously eliminated during the purification of the polysaccharide.



EXPERIMENTAL

General methods and the instrumentation used have been described previously[24,25].

Analytical paper chromatography was carried out on Whatman No. 1 paper using either solvent 1, 18:3:1:4 ethyl acetate–acetic acid–formic acid–water, or solvent 2, 4:1:5 1-butanol–ethanol–water (upper phase). Preparative gas-liquid chromatography was carried out in a fused-silica capillary column (DB 17, 15 m), using flame ionization detectors. G.l.c.-m.s. analysis was performed on a Varian Vista 6000 series gas chromatograph coupled directly to a Delsi Nermag R10-10C quadrupole mass spectrometer. This mass spectrometer was employed for desorption-chemical-ionization mass spectrometry, with ammonia as the reagent gas, source pressure 0.1 Torr, ion-source temperature 175°, and ionizing voltage 72 eV. ^{1}H-N.m.r. spectra were recorded on a Bruker WH-400 FT spectrometer. ^{13}C-N.m.r. spectra were obtained on a Varian 300 FT spectrometer. Samples in D_2O were deuterium-exchanged by lyophilizing twice from 99.96% D_2O. Acetone (δ 2.23 for ^{1}H and 31.07 p.p.m. for ^{13}C, measured against aqueous sodium 4,4-dimethyl-4-silapentane-1-sulfonate) was used as internal standard.

Isolation of K31 polysaccharide. — An actively growing culture was used to inoculate 100 mL of Mueller–Hinton broth, which was incubated for 4 h at 37° to give a turbid suspension, then poured into a metal tray (60 × 40 cm) containing Mueller–Hinton agar medium. After 4 d at 37° the bacterial slime was scraped off and treated with 1% phenol, and the polysaccharide was purified with Cetavlon[26] following centrifugation and precipitation in ethanol. Further purification was achieved by use of a Bio-Gel P-2 column (92 cm × 2.6 cm).

Sugar analysis and composition. — Hydrolysis of a sample (20 mg) of K31 polysaccharide with 2M trifluoroacetic acid (TFA) for 20 h at 95° and removal of excess acid by coevaporation with water, followed by paper chromatography

(solvent 1), showed glucose, galactose, rhamnose, and an aldobiouronic acid. The neutral sugars released were analyzed as alditol acetates by g.l.c. on column DB 17, programmed from 180° to 220° at 5°/min.

A portion of the hydrolyzate was methylated[4] and subjected to g.l.c. using the program 145°, 1 min, then 2°/min to 155°, 1 min, 3°/min to 220°, 1 min, and then to 250° at 3°/min. Under these conditions the permethylated monsaccharides were eluted between 1.9 and 4.9 min and the methylated aldobiouronic ester methyl glycoside at 16.0 min. When this latter component was analyzed by c.i.-m.s. a value of $(M + NH_4)^+ = 456$ was obtained.

A portion (49.5 mg) of K31 polysaccharide (H^+ form) was dissolved in water (30 mL), 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CMC, 423 mg) was added, and reduction was achieved by the addition of aqueous sodium borohydride (3M, *ca.* 100 mL) with continuous adjustment of the pH to 7. The product (61 mg), isolated by dialysis and lyophilization, was analyzed as alditol acetates following hydrolysis with 2M TFA.

Methylation analysis. — The capsular polysaccharide (30 mg, H^+ form) was methylated by the method of Hakomori[4], dialyzed, partitioned between dichloromethane and water, and purified on Sephadex LH 20 to give a product that showed no absorption at 3625 cm^{-1}. Analytical results on this material are given in Table I, column I, and those for a portion refluxed overnight with lithium aluminum hydride in oxolane in Table I, column II.

Uronic acid degradation. — A sample (25 mg) of methylated K31 polysaccharide was dried and then, with a trace of *p*-toluenesulfonic acid, dissolved in 19:1 dimethyl sulfoxide–2,2-dimethoxypropane (12 mL), and the flask was sealed under nitrogen. Methylsulfinylmethyl anion (5 mL) was added and allowed to react for 18 h at room temperature. Methyl iodide (3 mL) was added to the cooled, stirred reaction mixture and stirring was continued for an hour. The methylated, degraded product was then isolated by partition between chloroform and water. The isolated product was purified by gel-permeation chromatography on Sephadex LH-20, hydrolyzed with 2M TFA for 8 h at 95°, and analyzed by g.l.c. (Table I, column IV).

Selective partial hydrolysis. — Polysaccharide (60 mg) was hydrolyzed with 0.1M TFA for 25 min at 95°. Following dialysis the retentate (**PH**) was examined by ^1H- and ^{13}C-n.m.r. and subjected to methylation[4] followed by reduction of the carboxyl function with lithium aluminum hydride. The analytical results are presented in Table I, column IV.

Reaction with lithium in ethylenediamine. — Dry K31 polysaccharide (150 mg) was suspended in dry ethylenediamine (21 mL) and six pieces of lithium wire (3 mm × 3 mm, hexane washed) were added. The resulting intense blue color was maintained for 1 h by the addition of smaller pieces of lithium. The reaction was terminated by the addition of dry methanol (4 mL) to the flask cooled in ice water. The excess ethylenediamine and methanol were removed *in vacuo* over sulfuric acid and sodium hydroxide. To decompose the residual lithium methoxide glacial

acetic acid (3 mL) was added, with external cooling, followed by an equal volume of water. The resultant product was purified by ion-exchange chromatography (Bio-Rad AG 50-X8 resin) and gel-permeation chromatography (Bio-Gel P-2). A pure oligosaccharide (**F1**) was isolated by preparative paper chromatography. Methylation data on **F1** are reported in Table I, column VII. The proton n.m.r. spectrum showed signals at δ 5.25 (br., 1 H), 5.13 (d, J 3 Hz, 1.2 H), 4.99 (br., 1.3 H), 4.95 (br., 0.2 H), and 4.65 (d, J 8 Hz, 0.8 H).

Hydrofluoric-acid hydrolysis. — The native polysaccharide (50 mg) was hydrolyzed in anhydrous HF at −40° for 15 min and the products **A2** (~8 mg) and **A3** (~2 mg) were separated by paper chromatography. Methylation data for **A2** are given in Table I, column V. G.l.c.–c.i.-m.s. of methylated **A2** gave $(M + NH_4)^+$ at *m/z* 472 and methylated **A3** gave $(M + NH_4)^+$ at *m/z* 412.

Chromium-trioxide oxidation. — Acetylated polysaccharide (25 mg) was dissolved in acetic acid and treated with chromium trioxide (100 mg) for 2 h at 50°. The results of methylation analysis of the product are shown in Table I, column VI.

Bacteriophage depolymerization. — Bacteriophage was isolated from Vancouver sewage and propagated by tube and flask to a concentration of 1.2×10^{11} p.f.u. mL^{-1}. Phage suspension was then added to an aqueous solution of 150 mg of K31 polysaccharide. Depolymerization was carried out for 5 h at 37°, after which the solution was heated to 85° for 5 min and lyophilized. The crude product was deionized by passage through a column of Amberlite IR 120 (H^+) and the eluate was concentrated and added to a column of Bio-Gel P-2 (400 mesh), which was eluted with water at 20 $mL.h^{-1}$. The low molecular weight polymer (**Pn**) was collected between 133 and 150 mL, and the fractions were evaporated to give 80 mg of product.

2D-N.m.r. spectroscopy. — Phage degraded polymer (**Pn**) was lyophilized twice from deuterium oxide and dissolved at a concentration of 25 $mg.mL^{-1}$. Spectra were recorded at 400 MHz for samples in 5 mm diameter tubes, using a Bruker WH 400 spectrometer equipped with an Aspect 3000 computer. For homonuclear two-dimensional spectroscopy Bruker D1SB871 software was used. COSY[14] and NOESY[16] experiments were performed with suppression of the HOD resonance. A mixing time of 250 ms was employed in NOESY experiments. Relayed COSY[15] experiments were done with fixed delays of 35 ms. All homonuclear experiments were performed with quadrature detection in the F1 dimension and a total of 256 t_1 increments of 96 scans each were recorded with a minimum delay between pulses of 1.3 s, a sweep width of 3118 Hz, and 1024 real data points in t_2. All the 2D n.m.r. experiments were conducted at 343 K. All 2D spectra were recorded in the non phase-sensitive mode with zero filling and symmetrization.

ACKNOWLEDGMENTS

The authors thank Dr. I. Ørskov, State Serum Institute, Copenhagen for a culture of *E. coli* 09:K31:H4 (Su 3973/41) and Dr. E. Altman for some preliminary

experiments. The continued financial support of N.S.E.R.C. (Ottawa) is gratefully acknowledged.

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