NMR analysis of succinoglycans from different microbial sources: partial assignment of their ^{1}H and ^{13}C NMR spectra and location of the succinate and the acetate groups ☆

Mária Matulová [a,1], Renato Toffanin [a,*], Luciano Navarini [a], Roberto Gilli [a], Sergio Paoletti [a,b], Attilio Cesàro [a,b]

[a] *Centro Ricerche POLY-biós, LBT-Area di Ricerca, Padriciano 99, I-34012 Trieste, Italy*
[b] *Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università degli Studi di Trieste, Via L. Giorgieri 1, I-34127 Trieste, Italy*
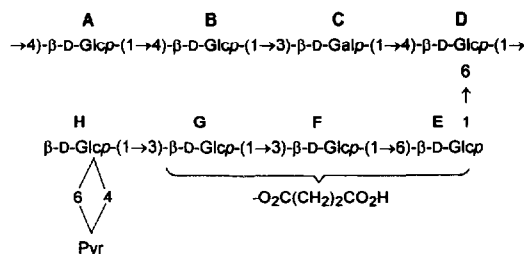
Abstract

In order to obtain information on the location of succinate and acetate groups, comparative NMR analyses were carried out on succinoglycans from different microbial sources by using conventional and advanced NMR techniques. In particular, one-dimensional ^{1}H and ^{13}C NMR spectra were recorded for qualitative and quantitative analysis on native high-molecular-weight succinoglycans (both in the Na$^+$ salt and free-acid forms) from *Pseudomonas* sp. NCIB 11592, *Agrobacterium radiobacter* A201-25, *Rhizobium meliloti* YE-2, and *Rhizobium* sp. isolated from *Vicia faba* and compared with those of the deacylated and deacylated–depyruvated, partially depolymerised exopolysaccharides from *Rhizobium meliloti* YE-2. Moreover, a series of two-dimensional experiments was performed on all the exopolysaccharides aiming at the partial assignment of the NMR spectra. The NMR data showed that succinate is located on O-6 of either one or both of the two side chain 3-linked β-D-Glc residues, whereas the acetate (when it is present) is located on one of the O-6 of backbone 4-linked β-D-Glc units, but the specific site could not be determined. In addition, the spectral features of the succinate substituent were found to be sensitive to pH changes.

Keywords: NMR analysis; Succinoglycans, an NMR analysis of

☆ Presented in part at the VIIth European Carbohydrate Symposium, 22–27 August 1993, Cracow, Poland.

* Corresponding author.

[1] Permanent address: Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, 84238 Bratislava, Slovakia.

| A | B | C | D |

→4)-β-D-Glcp-(1→4)-β-D-Glcp-(1→3)-β-D-Galp-(1→4)-β-D-Glcp-(1→

```
                                                          6
                                                          ↑
        H            G            F            E  1
   β-D-Glcp-(1→3)-β-D-Glcp-(1→3)-β-D-Glcp-(1→6)-β-D-Glcp
          / \        └──────────────┬──────────────┘
         6   4                -O2C(CH2)2CO2H
          \ /
          Pyr
```

Scheme 1. Chemical structure of the octasaccharide repeating unit [5] of succinoglycans.

1. Introduction

Succinoglycans are ionic exopolysaccharides (EPSs) produced by many species of microorganisms belonging to the family Rhizobiaceae [1], in particular *Rhizobium meliloti* and *Agrobacterium* sp. They are also produced by *Alcaligenes faecalis* var. *myxogenes* [2] (taxonomically close to *Agrobacterium*) and *Pseudomonas* sp. [3]. The polymer chains have a neutral backbone with regularly positioned tetrasaccharide side-chains composed of β-D-Glcp residues. The side-chain, which contains pyruvate acetal linked at positions O-4 and O-6 on the terminal glucose residue, is connected to the main chain via a β-(1→6) glycosidic linkage. Each octasaccharide repeating unit [4] (Scheme 1) is further substituted by succinate and acetate groups at undetermined sites [2,5]. Unpublished data, obtained by neutral permethylation and collision mass spectrometry, are quoted for the location of succinate and acetate groups in the succinoglycan from *Rhizobium meliloti* Rm 1021 [6]. The discriminating feature among the succinoglycans from different microbial sources seems to reside in the acylation pattern. In fact, a variable content of acetyl and succinyl substituents can be present depending on microbial source and fermentation conditions [7]. To the best of our knowledge, no detailed NMR analysis of succinoglycans has hitherto been performed. In fact, previous ^{1}H NMR data have mainly concerned minor substituents [5–8] and only one ^{13}C NMR spectrum has been published for the succinoglycan octasaccharide of *Rhizobium meliloti* YE-2 with partial description of the signals [9]. In this work we report the comparative NMR analysis of native succinoglycans from different microbial sources in order to identify the location of the succinate and the acetate groups.

2. Experimental

Materials.—Several samples of exopolysaccharides, identified as having similar carbohydrate composition to the commercially available succinoglycan, were used in the present work (see also Table 1).

The succinoglycan exopolysaccharide from cultures of *Rhizobium meliloti* YE-2 (hereafter designated YE2) was obtained from CO.E.PO (Catania, Italy) as a yellow-brown cell-free supernatant (pH 5.9–6.0). The supernatant (4 L) was concentrated (400 mL) by rotary evaporation at 50°C and the exopolysaccharide was recovered by dropwise addition of 3 vol of EtOH under constant stirring. The precipitate was washed several times with EtOH and then dissolved in 400 mL of Milli-Q water. The polymer was again precipitated

Table 1
Molar ratio of substituents in the repeating unit of the exopolysaccharides [a]

EPS	Succinate	Acetate	Pyruvate
YE2*	0.2	0.8	1.0
YE2	1.0	0.8	1.0
YE2a	0.0	0.0	1.0
YE2b	0.0	0.0	≈ 0.2
Vf	0.5	0.0	1.0
Ar	0.8	0.0	1.0
Ps	1.2	0.0	1.0

[a] Values obtained from ^{1}H NMR spectra, as content relative to pyruvate. Abbreviations for polysaccharides: YE2 and YE2* are the EPS produced by *Rhizobium meliloti* strain YE-2 cultivated under different conditions; YE2a is a deacylated YE2 sample; YE2b is an acyl-pyruvate free and partially depolymerised YE2; Vf is the EPS produced by *Rhizobium* sp. isolated from *Vicia faba*; Ar is the EPS from *Agrobacterium radiobacter* A201-21; and Ps is the EPS isolated from *Pseudomonas* sp. NCIB 11592.

from the aqueous solution by dropwise addition of 3 vol of EtOH and the mixture was maintained overnight at 5°C. The EPS was recovered by centrifugation (4500 rpm at room temperature), washed several times with EtOH, and redissolved in 200 mL of Milli-Q water. The aqueous solution was dialysed against 0.025 M sodium citrate in 1 M NaCl, and then exhaustively against Milli-Q water, filtered (10 μm), and freeze-dried to give ca. 6 g of a white fluffy purified product containing 15% of water (Karl–Fischer titration).

Another sample of succinoglycan from *Rhizobium meliloti* YE-2 cultivated under different conditions (here designated YE2*) was kindly supplied as a purified freeze-dried sample by Dr. S. Leonhartsberger (unpublished data).

The EPS from the *Rhizobium* sp. isolated from *Vicia faba* (designated Vf) was obtained from CO.E.PO (Catania, Italy) as a crude precipitate. A solution of the crude polysaccharide in Milli-Q water (2 g/L) was dialysed against 0.025 M sodium citrate in 1 M NaCl and then exhaustively against Milli-Q water, filtered (10 μm), and freeze-dried to give a white fluffy purified product (yield 80%, w/w) containing 15% of water (Karl–Fischer titration).

A crude sample of succinoglycan from *Agrobacterium radiobacter* A201-25 (here designated Ar), kindly supplied by Dr. C. Bertocchi, was purified following the aforementioned procedure.

The purified sample of succinoglycan from *Pseudomonas* sp. NCIB 11592 (designated Ps), kindly supplied by Dr. M. Dentini, is from the same batch of that previously used by the author in the physico-chemical characterisation in dilute aqueous solution [10].

In addition to these native samples, two aliquots of the purified succinoglycan from *Rhizobium meliloti* YE-2 were chemically treated in order to remove acyl substituents and pyruvate as follows. In one case, aq 1 M NaOH was added to an aq 0.33% (w/v) polymer solution to give a final pH of 12.5. After 1 h under stirring at room temperature the solution was neutralised, exhaustively dialysed against Milli-Q water, and then the deacylated sample (YE2a) was recovered by freeze-drying. In a second batch 1 M NaOH was added to an aq 0.43% (w/v) polymer solution to give a final pH of 12.5 as above and, after 1 h under stirring at room temperature, the solution was neutralised. In order to remove the pyruvate groups and to decrease the molecular weight, after neutralisation dil HCl was added to a

final pH of 3 and the solution was heated for 6 h at 100°C with stirring under N_2. The mixture was then cooled to room temperature, neutralised, and dialysed exhaustively against Milli-Q water. The acyl-free and pyruvate-free, partially depolymerised sample (designated YE2b) was recovered by freeze-drying. The samples were dissolved at room temperature in D_2O at a concentration of 5–10 mg/mL for ^1H NMR and 20–30 mg/mL for ^{13}C NMR spectroscopy.

Methods.—All NMR experiments were performed at 70°C, unless stated otherwise, on a Bruker AM 300 WB spectrometer (^1H, 300.13 MHz and ^{13}C, 75.47 MHz) equipped with a process controller, a selective excitation unit (SEU), and an Aspect 3000 computer. The ^1H and ^{13}C NMR chemical shifts are with reference to internal sodium 4,4-dimethyl-4-silapentanoate-d_4 (δ 0.00) and external acetone (δ 31.07), respectively. Two-dimensional (2D) COSY, multistep relayed COSY, phase-sensitive (PS) COSY spectra using the method of time-proportional phase incrementation (TPPI) [11] with double quantum filtering [12,13], and 2D TOCSY experiments with the MLEV-17 pulse sequence for spin lock [14] (mixing time 21–95 ms) were acquired. For all 2D ^1H NMR experiments, typical parameters were 1400 Hz spectral width, 1K × 256 time-domain data matrix zero-filled to 1K × 512, 32–64 scans for each FID with a relaxation time of 1.3 s. A Lorentz–Gauss multiplication of the FID signals was used prior to Fourier transformation (FT), except for COSY and relayed COSY experiments, where a nonshifted sine-bell function was applied and the magnitude spectra obtained were symmetrised about the diagonal. Quantitative 1D ^{13}C NMR experiments were performed with inverse-gated decoupling, a relaxation delay of 4 s, and an acquisition time of 0.6 s. ^1H–^{13}C correlation data were obtained with ^{13}C detection [15] and ^1H decoupling in F_1 or in the inverse mode (^1H detection) using the pulse sequence HMQC [16] with GARP1 for decoupling during acquisition and employing the transmitter in the low-power mode. For ^{13}C-detected experiments the following parameters were used: spectral width of 6700 and 620 Hz, in F_2 and F_1, respectively, with 2K × 256 time-domain data matrix zero-filled to 2K × 512, 1500–1728 scans, and a relaxation delay of 1 s. In F_2, a sine-bell squared function shifted by $\pi/3$ and in F_1 a Lorentz–Gauss multiplication before FT were used. For ^1H-detected experiments, spectral widths of 1200 and 2050 Hz in F_2 and F_1, respectively, were applied with the time-domain data matrix 2K × 128 zero-filled to 2K × 512 and 128 scans for each FID were accumulated with a relaxation delay of 1 s. A Lorentz–Gauss function was applied in both dimensions before FT. 1D TOCSY experiments [17] were performed by selective excitation with a half-Gaussian pulse of 150 ms [18] followed by a pulsed MLEV-17 sequence for spin lock (mixing time 21–95 ms), using the transmitter in the low-power mode.

3. Results and discussion

General structural features.—The ^1H and ^{13}C NMR spectra of EPSs produced by *Rhizobium meliloti* YE-2 (both the YE2 and the YE2*), *Rhizobium* sp. isolated from *Vicia faba* (Vf), and *Agrobacterium radiobacter* A201-25 (Ar) had a pattern similar to that of the EPS from *Pseudomonas* sp. NCIB 11592 (Ps) already characterised as succinoglycan. However, for all of the ^1H and ^{13}C NMR spectra, some differences were observed, caused by different contents of nonsaccharidic substituents (Table 1).

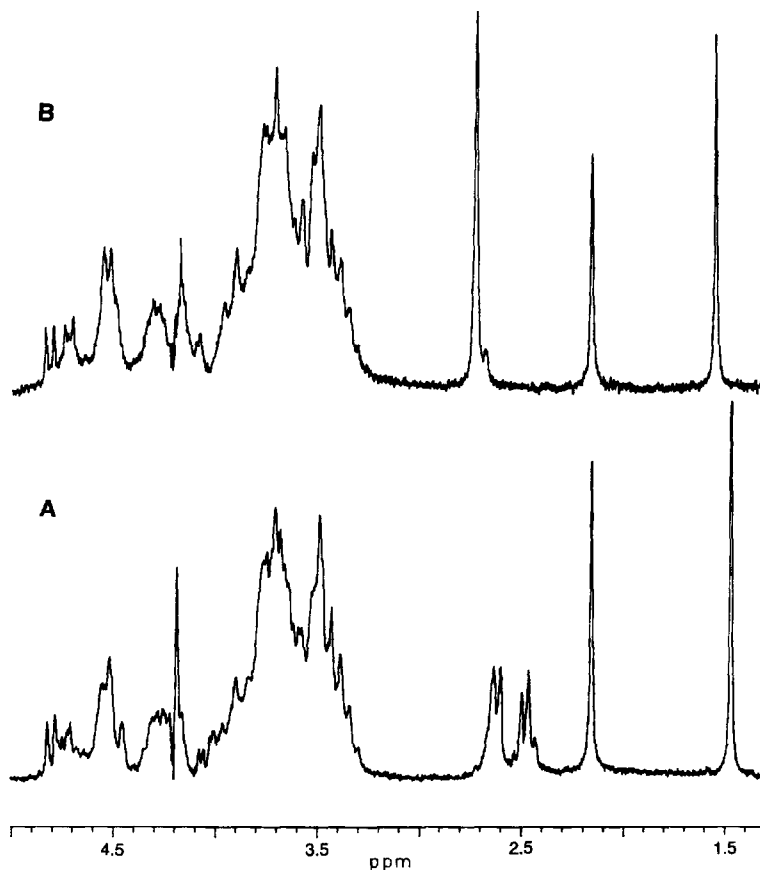Fig. 1. ^{1}H NMR spectra of the native YE2, indicating the dependence of the shape and δ of succinate CH$_2$ signals upon pH change: neutral conditions (pH 6.98) (A) and acidic conditions (pH 3.43) (B).

For all samples investigated the chemical shifts of the CH$_3$ signal of pyruvate were δ 1.48 and 25.7 in the ^{1}H and ^{13}C NMR spectra, respectively. These values, together with the ^{13}C δ value $\approx$ 102.2 for its acetal carbon atom, gave evidence of the S absolute configuration of the pyruvic acid acetal [19]. This configuration corresponds to the equatorial position of the methyl group and the axial position of the carboxyl group, a configuration found to be the most stable both by experimental methods and theoretical calculations on model compounds [20].

The chemical shift and the multiplicity of the succinate signal were found to be sensitive to thermal treatment, in agreement with the literature data [8,21]. Moreover, by comparison of the ^{1}H NMR spectra obtained for neutral and acidic solutions of YE2 and Vf, a peculiarity of the pH dependence of the spectral features of succinate was revealed (Fig. 1). Under neutral conditions, in the ^{1}H NMR spectra, two broad multiplets of the succinate signal occurred at δ 2.60 for nonequivalent protons of CH$_2$ groups. Also, in the ^{13}C NMR spectra, two CH$_2$ signals of succinate at $\delta \approx$ 32.4 and 31.2 were present, whereas free succinate after long-time accumulation appeared at $\delta \approx$ 33.5. At pH $\approx$ 3.5 the shape of the succinate signal was reversibly changed to a broad singlet at δ 2.74 in the ^{1}H NMR spectra, indicating the

equivalency of both CH_2 groups. The same spectral feature was observed in the ^{13}C NMR spectrum of the native YE2 EPS in acidic conditions, in which one broad singlet at δ 29.8 was present for both carbon atoms of the CH_2 groups of the succinate. Further studies, including model molecules, will be necessary to clarify this interesting behaviour.

Analysis of ^1H NMR spectra.—In order to achieve further evidence for the identity of the succinoglycan structure of all the native samples, and moreover to find the location of minor substituents, a partial assignment of the NMR spectra on the basis of a series of two-dimensional experiments was performed on each exopolysaccharide. The deacylated YE2a and the acyl-free and pyruvate-free YE2b were also used in this comparative analysis, taking advantage of the absence of substituents and to the lower molecular weight of YE2b. In their ^1H NMR spectra, only four anomeric signals were resolved at 300 MHz for the octasaccharide repeating-unit of the succinoglycans (Scheme 1). For the native polysaccharides YE2, Vf, and Ps the spectral features of the anomeric region were slightly changed because of the substitution; however, four types of anomeric signals could still be distinguished.

For all of the samples, similar two-dimensional patterns were observed in PS COSY, TOCSY, relayed COSY, and also in the 2D heteronuclear correlated spectra. From all of the spectra, two sets of ^1H NMR data were extracted. The assignment starting from the anomeric protons is presented in set A, whereas the very well resolved H-6a protons (Fig. 2) were used as a second starting point for the assignment presented in set B (Table 2). For all EPSs examined, the assignment of the signals for the whole spin system of the terminal pyruvated Glc unit was obtained and verified in a series of 1D TOCSY experiments by selective irradiation of H-6a at δ 4.04 (Table 2, set A, and Fig. 3). The 1D TOCSY experiment with irradiation of H-6 signals at δ 4.20 of the two different 6-linked Glc units indicated a corresponding anomeric signal at δ 4.54, attributed to 6-linked β-D-Glc of the side chain. The anomeric signal at δ 4.56, with a peak area corresponding to three protons, showed peculiar cross-peak patterns in the 2D TOCSY and relayed COSY spectra (Fig. 4). Regardless of the mixing time in the TOCSY or of the steps used in the relayed COSY experiments, only the cross-peaks between H-1 protons of this region and their H-2 and H-3 neighbours were observed in these spectra of all the EPSs, thus indicating the degeneracy of the spin system (Fig. 4). From the ^1H–^{13}C correlation plot of YE2b the chemical-shift values of the glycosidic proton signals were as follows: H-3 of both 3-linked β-D-Glc at δ 3.78, H-4 of 4-linked β-D-Glc at δ 3.76 and 3.68, and H-3 of 3-linked β-D-Gal at δ 3.84; similar values were obtained from heterocorrelated spectra of YE2a and native YE2 (Table 2). To differentiate between 4-linked and 3-linked β-D-Glc units, our data have been usefully compared with those in the literature [22], which resulted in the assignments presented in Table 2. As the H-3 signal of β-D-Gal at $\delta \approx$ 3.84, obtained from the heterocorrelated plot, was not observed in any ^1H NMR experiment performed, the spectral data for the β-D-Gal unit could not be assigned.

In the 2D PS COSY spectrum of Ps (Fig. 2) and Vf the cross-peak between the signals at δ 4.50 and 4.32 was observed and attributed to H-6a and H-6b, respectively, of a sugar residue having succinate linked at C-6. This assignment was supported by the fact that these signals were observed as the cross-peaks for C-6 with linked succinate in the heterocorrelated plot of the native YE2. The high content of succinate and acetate for YE2 was reflected in the complexity of the cross peak patterns in 2D spectra. However, the cross-peak between
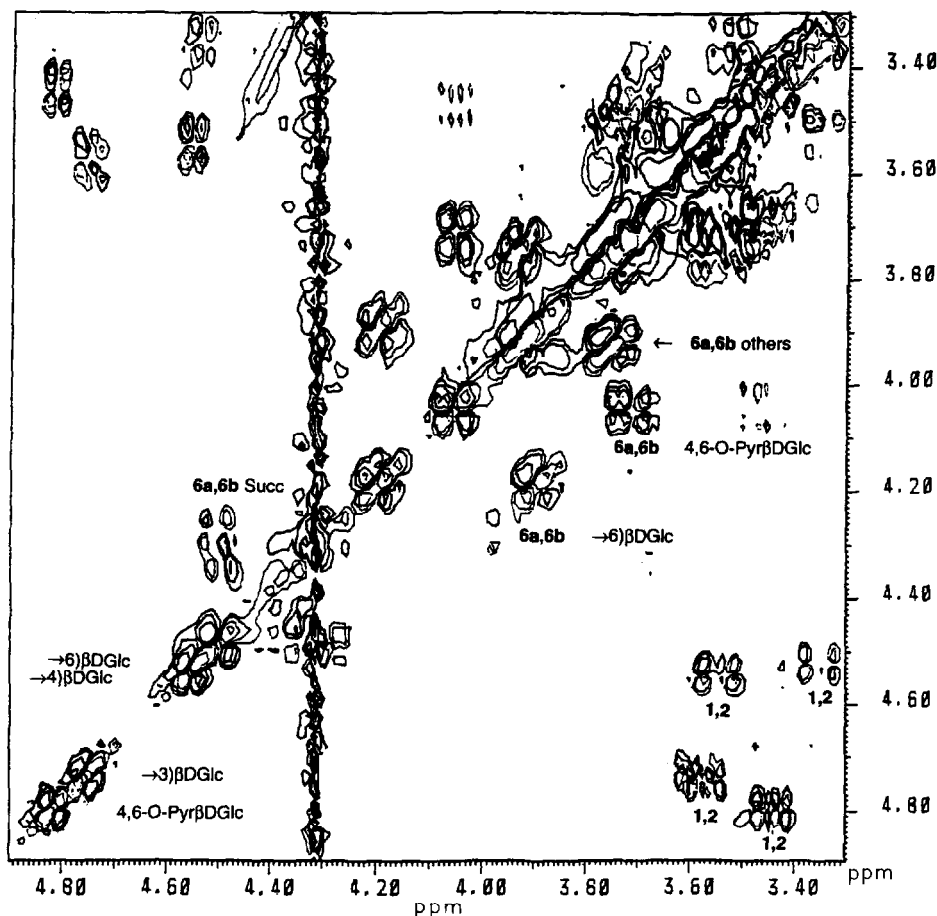
Fig. 2. The 2D phase-sensitive COSY spectrum of the native succinoglycan exopolysaccharide produced by *Rhizobium* sp. isolated from *Vicia faba* (Vf).

the broad signals of H-6a and H-6b at δ 4.50 and 4.32, respectively, could nevertheless be observed. This cross-peak was also supposed to contain the cross-peak between the signals of some sugar residue shifted downfield due to linked acetate. YE2* was the only EPS investigated containing a lower content of succinate and higher amount of acetate. However, because of the broad signals, it was impossible to find the correlation peak between the downfield-shifted signals caused by acetate substituents in the 2D PS COSY spectrum.

Analysis of the ^{13}C NMR spectra.—The ^{13}C NMR spectra of all the samples investigated were similar to the ^{13}C NMR spectrum previously published [9] for the octasaccharide isolated from supernatants of cultures of *Rhizobium meliloti* YE-2. In the quantitative ^{13}C NMR spectrum of YE2b, the values of the integrals for glycosylated carbon atoms were in agreement with the proposed structure for the repeating unit of succinoglycans [4]. Comparison of ^{13}C NMR spectra of the exopolysaccharides investigated with the spectrum of YE2b, free of any substituents, showed other signals with different intensities as a consequence of the different contents of succinate (Fig. 5). Two of these signals, at $\delta \approx 86.5$ and

Table 2
^{1}H NMR chemical shifts for β-D-Glc of the investigated exopolysaccharides

EPS	Set of data [a]	Residue	Chemical shift/δ						
			H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
YE2	A	4,6-O-Pyr-β-D-Glc	4.82	3.44	3.69	3.48	3.73	4.05	3.73
		→3)-β-D-Glc	4.75	3.58	[b]	3.50			
		→4)-β-D-Glc	4.55	3.53	3.75	[c]			
		→6)-β-D-Glc	4.54	3.35	3.53				
	B	→6)-β-D-Glc						4.21	3.71
		β-D-Glc-Succ, Ac [d]						4.50	4.32
		Others						3.93	3.78
Vf	A	4,6-O-Pyr-β-D-Glc	4.83	3.45	3.71	3.50			
		→3)-β-D-Glc	4.76	3.58	3.77	3.53			
		→4) + →6)-β-D-Glc	4.54	3.55	3.77				
	B	4,6-O-Pyr-β-D-Glc				3.50	3.73	4.07	3.73
		→6)-β-D-Glc						4.20	3.91
		β-D-Glc-Succ [d]						4.50	4.32
		Others					3.52	3.94	3.76
Ps	A	4,6-O-Pyr-β-D-Glc	4.81	3.45	3.70	3.50	3.73	4.05	3.72
		→3)-β-D-Glc	4.76	3.57	3.76	3.51			
		→4)-β-D-Glc	4.56	3.54	3.78				
		→6)-β-D-Glc	4.54	3.35	3.57				
	B	4,6-O-Pyr-β-D-Glc				3.49	3.73	4.06	3.72
		→6)-β-D-Glc						4.20	3.91
		Glc-Succ [d]						4.50	4.32
		Others					3.51	3.94	3.76
YE2a	A	4,6-O-Pyr-β-D-Glc	4.82	3.43	3.68	3.47	3.73	4.05	3.71
		→3)-β-D-Glc	4.75	3.56	3.72	3.53			
		→4)-β-D-Glc	4.54	3.54	3.74	3.68			
		→6)-β-D-Glc	4.54	3.36	3.56				
	B	4,6-O-Pyr-β-D-Glc				3.48	3.73	4.05	3.71
		→6)-β-D-Glc						4.19	3.90
		Others					3.50	3.93	3.75
YE2b	A	β-D-Glc-terminal	4.74	3.40	3.56	3.44	3.51	3.94	3.75
		→3)-β-D-Glc	4.78	3.59	3.78	3.54			
		→4)-β-D-Glc	4.55	3.55	3.78	[e]			
		→6)-β-D-Glc	4.54	3.36	3.50				
	B	→6)-β-D-Glc					3.63	4.19	3.91
		Others					3.51	3.49	3.75

[a] A, Set of data obtained from all types of experiments with starting point in the anomeric region; B, set of data obtained from all types of experiments with starting point at H-6 signals.
[b] Four H-3 cross-peaks in heterocorrelated plot were resolved; H-3 of nonsuccinylated units at δ 3.76 and 3.74 and H-3 of succinylated units at δ 3.75 and 3.73.
[c] Two cross-peaks observed at δ 3.72 and 3.67.
[d] The succinate and/or the acetate are linked to C-6.
[e] One H-4 cross-peak at δ 3.76 and others at δ 3.68.

≈ 85.4 in the spectra of native polysaccharides, could be recognised in the region of the C-3 atoms of the 3-linked β-D-Glc, together with other signals at $\delta \approx 64.5$, which were identified as C-6 signals by a DEPT experiment. Relatively large downfield shifts of these
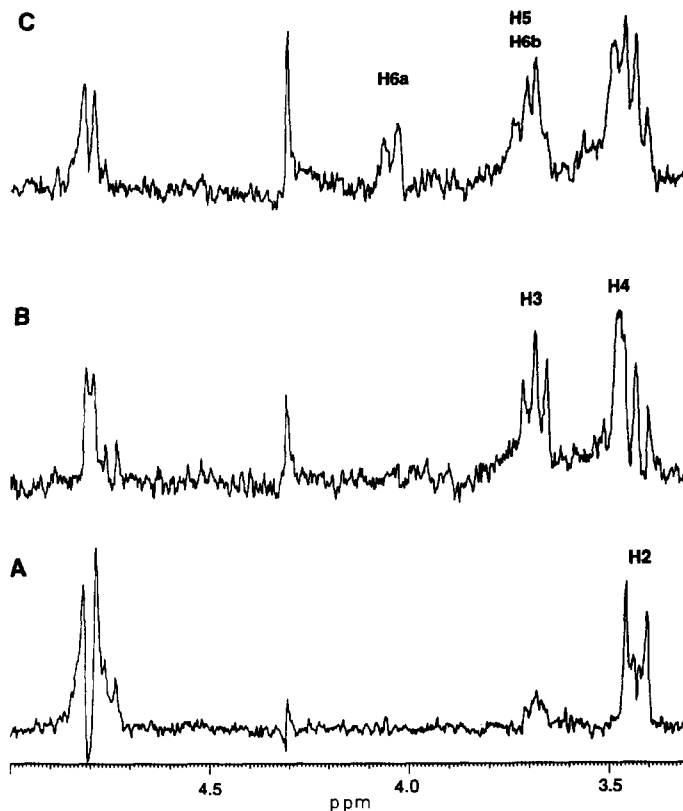
Fig. 3. 1D TOCSY spectra of the deacylated EPS YE2a, obtained after chemical treatment of EPS isolated from *Rhizobium meliloti* strain YE-2. The spectra were obtained after selective irradiation of the anomeric signal at δ 4.80 for different mixing times: 21.3 ms (A), 53.9 ms (B), and 94.6 ms (C).

C-6 signals ($\Delta \approx 3.2$ ppm) were found for all the samples (with intensities related with the different content of succinate), indicating their location at C-6. Because the intensities of the two C-3 signals were unequal, it was deduced that the succinate is located on C-6 of either one or both of the two 3-linked β-D-Glc units. (residues F and G in Scheme 1). In the case of succinoglycan from *Rhizobium meliloti* Rm 1021 Reuber and Walker [6] noted the presence of succinate on residue G only, but without mentioning the actual degree of molar substitution. The variability of the location of succinate on the two 3-linked glucose units (residues F and G) seems to be an additional feature characterising the acylation pattern of succinoglycans.

Among the samples examined, only succinoglycans from the *Rhizobium meliloti* strain YE-2 contained the acetate substituent. The relatively high content of acetate (Table 1) made it quite easy to identify these differences between the ^{13}C NMR spectra of the exopolysaccharides examined. In the spectra of YE2 and YE2* in the glycosidic C-4 signal region of the 4-linked β-D-Glc units, a downfield shift of one of the C-4 signals (Δ -0.5 ppm), together with another C-6 signal at δ 63.8 could be observed in comparison with the spectra of the samples lacking the acetate group (Fig. 5). Because the chemical shift of the C-4 signal was found to be dependent on the substitution at C-6 [23], and the C-6 chemical
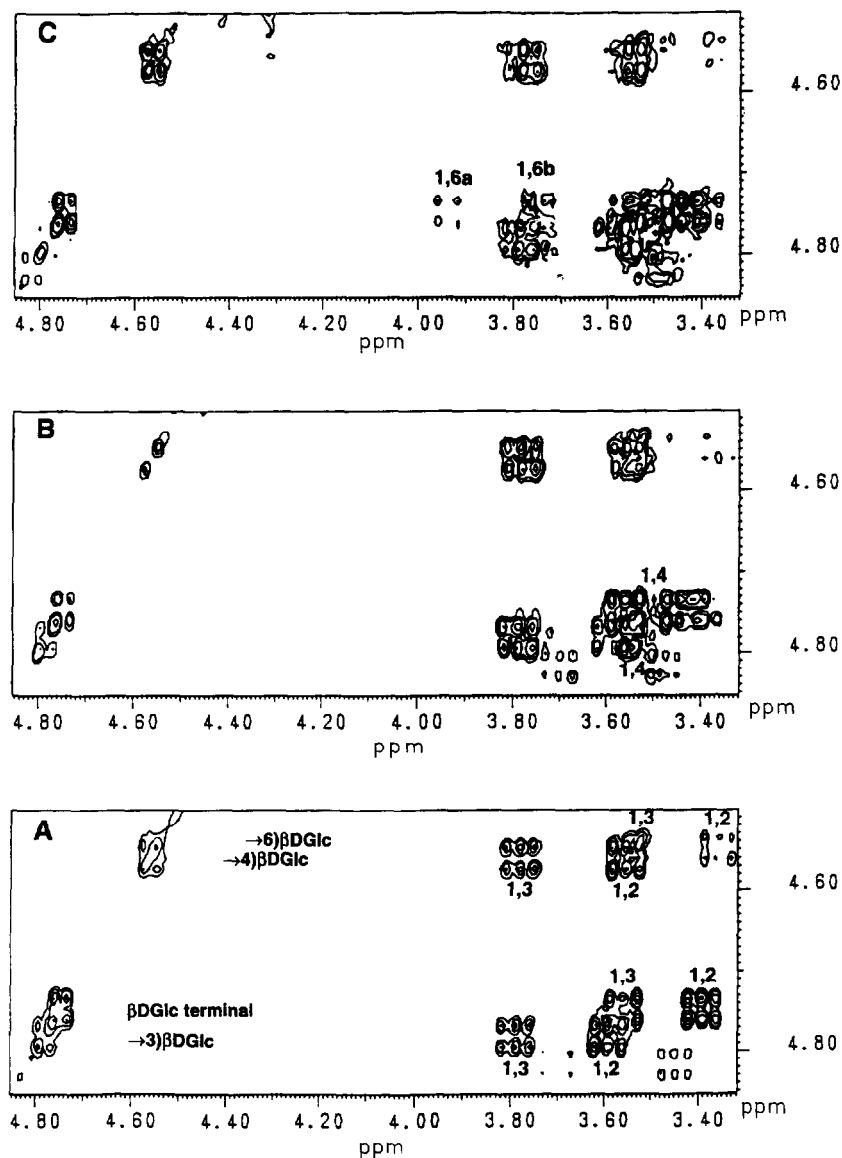
Fig. 4. A series of 2D relayed COSY spectra of the acyl-pyruvate free partially depolymerised EPS YE2b, obtained after chemical treatment of EPS isolated from *Rhizobium meliloti* strain YE-2: one step with D2 = 29.4 ms (A), two steps D2 = 29.4 ms and D3 = 30 ms (B), three steps with D2 = 29.4 ms, D3 = 30 ms, and D4 = 30 ms (C).

shift of the 4-linked α-D-Glc unit due to acetylation was found at δ 63.4 [24], the location of the acetate in the succinoglycans was deduced to be at C-6 of one of the 4-linked β-D-Glc units (A or B in Scheme 1) of the backbone.

Some other signals could be assigned by comparison of all quantitative ^{13}C NMR spectra. The C-5 and C-6 signals of pyruvated β-D-Glc of the side chain were found at $\delta \approx 66.9$ and 64.9, respectively. From heterocorrelated plots the assignment of C-2 and C-5 atoms of β-
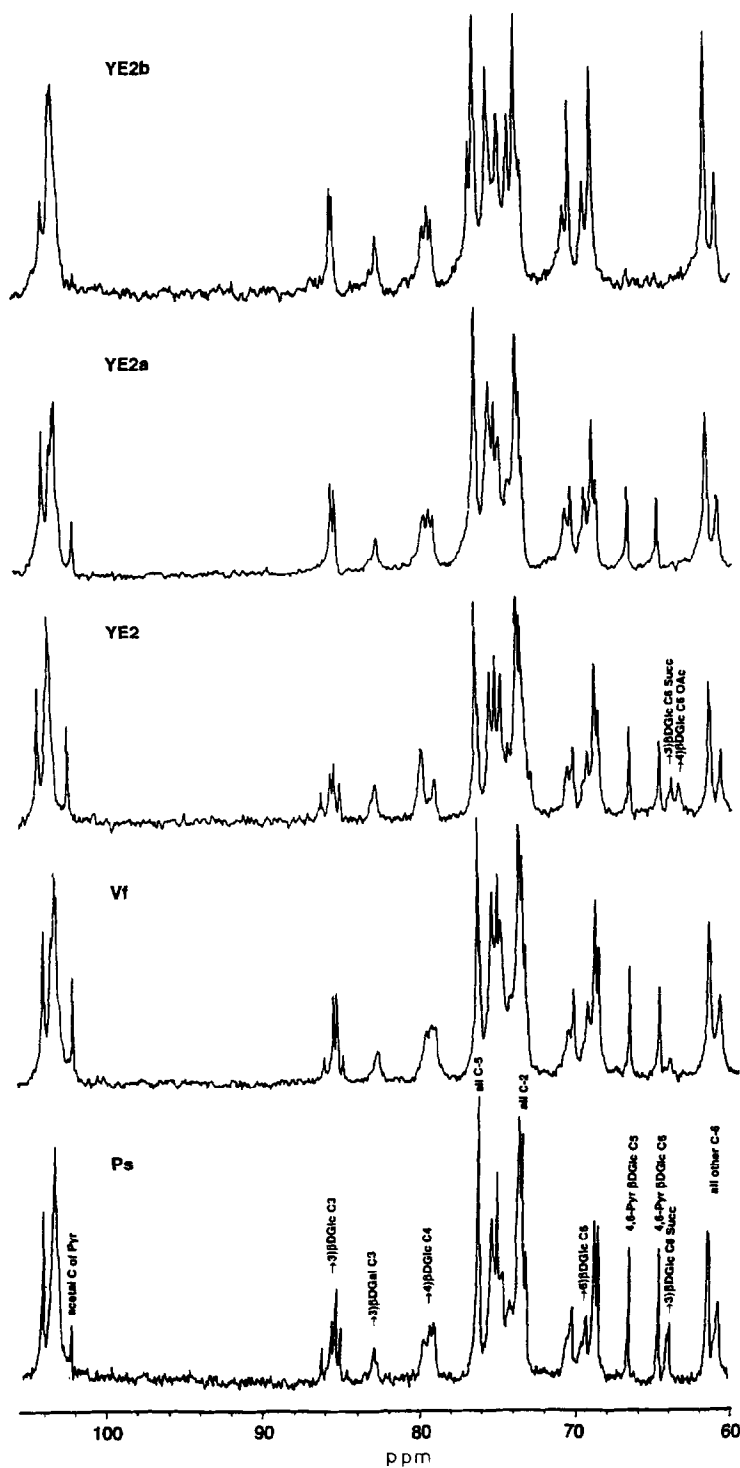
Fig. 5. ^{13}C NMR spectra of the native succinoglycan exopolysaccharides produced by *Pseudomonas* sp. strain NCIB 11592 (Ps), *Rhizobium* sp. isolated from *Vicia faba* (Vf), *Rhizobium meliloti* strain YE-2 (YE2), and chemically treated EPS isolated from *Rhizobium meliloti* strain YE-2: deacylated (YE2a) and acyl-pyruvate free, partially depolymerised (YE2b). Only the region of anomeric and skeletal carbons showed.

D-Glc residues were established (Fig. 5). The carbonyl signals could also be assigned as follows: acetate δ 174.5, pyruvate δ 175.7, and succinate δ 176.1.

4. Conclusions

Strong similarities of the EPSs examined from *Agrobacterium radiobacter* A201-25, *Rhizobium meliloti* YE-2, and *Rhizobium* sp. isolated from *Vicia faba* with that from *Pseudomonas* sp. NCIB 11592, already characterised as a succinoglycan, were evident from ^{13}C NMR and from two-dimensional NMR spectra. Advanced and conventional NMR techniques permitted location of the succinate and the acetate groups and to perform a partial assignment of the ^1H and ^{13}C NMR signals. The NMR data showed that the succinate is located on the 3-linked β-D-Glc units of the side chain, whereas acetate, in the samples in which it is present, is located on the 4-linked β-D-Glc units of the backbone. The substituents are always linked to the O-6 position. In addition to the expected effects of the pH changes on the carboxylate groups, the spectral features of the succinate substituent show some peculiarities. From two multiplets of nonequivalent CH_2 protons under neutral conditions, the shape of the succinate signal was reversibly changed to a broad singlet at δ 2.74 at pH $\approx$ 3.5, thus indicating their equivalence. The EPS produced by *Rhizobium* sp. isolated from *Vicia faba* has been also identified as a succinoglycan, thus eliciting further interest in this new finding, as *Rhizobium* strains producing succinoglycans have not been hitherto isolated from *Vicia faba*. The strain of *Rhizobium meliloti* YE-2 cultivated under two different conditions (but with the same culture medium) produced succinoglycans having different contents of the succinate substituents.

Acknowledgements

References

[1] D.C. Jordan, in N.R. Krieg and J.G. Holt (Eds.), *Berglyis Manual of Systematic Bacteriology*, Vol. 1, Williams and Wilkins, Baltimore, 1984, p 234.
[2] T. Harada, P.-E. Jansson, L. Kenne, B. Lindberg, H. Ljunggren, J. Lönngren, U. Rudén, and S. Svensson, *J. Am. Chem. Soc.*, 99 (1977) 3812–3815.
[3] G. Gravanis, Ph.D. Thesis, University of Grenoble, 1985.
[4] T. Harada, *Biochem. Soc. Symp.*, 48 (1985) 97–116.
[5] P. Åman, M. McNeil, L.E. Frazén, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 95 (1981) 263–282.

[6] T.L. Reuber and G.C. Walker, *J. Bacteriol.*, 175 (1993) 3653–3655.

[7] T. Harada, A. Amemura, P.E. Jansson, and B. Lindberg, *Carbohydr. Res.*, 77 (1979) 285–288.

[8] A. Heyraud, M. Rinaudo, and B. Courtois, *Int. J. Biol. Macromol.*, 8 (1986) 85–88.

[9] L.P.T.M. Zevenhuizen, in S.S. Stivala, V. Crescenzi, and I.C.M. Dea (Eds.), *Industrial Polysaccharides*, Gordon and Breach, New York, 1987, pp 301–311.

[10] M. Dentini, V. Crescenzi, M. Fidanza, and T. Coviello, *Macromolecules*, 22 (1989) 954–959.

[11] D. Marion and K. Wüthrich, *Biochem. Biophys. Res. Commun.*, 113 (1983) 967–974.

[12] U. Piantini, O.W. Sørensen, and R.R. Ernst, *J. Am. Chem. Soc.*, 104 (1982) 6800–6801.

[13] M. Rance, O.W. Sørensen, G. Bodenhausen, G. Wagner, R.R. Ernst, and K. Wüthrich, *Biochem. Biophys. Res. Commun.*, 117 (1983) 479–485.

[14] A. Bax and D. Davis, *J. Magn. Reson.*, 65 (1985) 355–360.

[15] A. Bax, *J. Magn. Reson.*, 53 (1983) 517–520.

[16] A. Bax and S. Subramanian, *J. Magn. Reson.*, 67 (1986) 565–569.

[17] H. Kessler, H. Oschkinat, C. Griesinger, and W. Bermel, *J. Magn. Reson.*, 70 (1986) 106–133.

[18] H. Kessler, U. Anders, G. Gemmecker, and S. Steuernagel, *J. Magn. Reson.*, 85 (1989) 1–14.

[19] P.J. Garegg, P.-E. Jansson, F. Lindh, J. Lönngren, I. Kvarström, and W. Nimmich, *Carbohydr. Res.*, 78 (1980) 127–132.

[20] S. Miertuš, L. Navarini, and A. Cesàro, *Carbohydr. Res.*, 257 (1994) 227–238.

[21] G. Gravanis, M. Milas, M. Rinaudo, and A.J. Clarke-Sturman, *Int. J. Biol. Macromol.*, 12 (1990) 195–200.

[22] K. Bock, J.Ø. Duus, B. Norman, and S. Pedersen, *Carbohydr. Res.*, 211 (1991) 219–233.

[23] M. Chmielewski, A. Banaszek, and A. Zamojski, *Carbohydr. Res.*, 83 (1980) 3–7.

[24] E. Altman, J.-R. Brisson, D.R. Bundle, and M.B. Perry, *Biochem. Cell. Biol.*, 65 (1987) 876–889.