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MONOMER SEQUENCE AND ACETYLATION PATTERN IN SOME BACTERIAL ALGINATES

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ABSTRACT

The sequential structures and acetylation patterns of alginates from several strains of *Azotobacter vinelandii* and *Pseudomonas* species, including *P. aeruginosa, P. putida, P. fluorescens,* and *P. mendocina,* have been studied by ^{1}H-n.m.r. spectroscopy. *O*-Acetyl groups were exclusively associated with the D-mannuronic acid residues and the degree of acetylation varied in the range 4–57%, depending upon the proportion of this acid in the polymer. ^{1}H-N.m.r. spectroscopy of a naturally occurring and an artifically acetylated D-mannuronan made it possible to determine the degrees of acetylation at O-2, O-3, and O-2,3. The most conspicuous difference between alginates from *A. vinelandii* and the four *Pseudomonas* species was the complete absence of consecutive L-guluronic acid residues in the latter.

INTRODUCTION

Alginates from brown algae are linear, (1→4)-linked copolymers of β-D-mannuronic acid (M) and its C-5 epimer, α-L-guluronic acid (G). The uronic acid residues are arranged in the chains as homopolymeric blocks of M or G interspaced with sequences in which there is a high degree of alternation[1]. The selectivity for cations and, hence, the gel-forming properties of the polymer are correlated with the content of G-blocks[2]. Alginate-like polymers are also produced as exocellular material by several bacteria[3]. Linker and Jones[4] isolated an exopolysaccharide from a mucoid mutant of *P. aeruginosa.* This bacterium, which is a secondary pathogen in patients with cystic fibrosis[5], produces a partially acetylated alginate containing variable amounts of M and G. A similar polymer, produced by the soil bacterium *A. vinelandii*[6], was shown to have a block-copolymeric structure similar to that of the algal product[7]. Recently, alginate production has been reported in three non-pathogenic species of *Pseudomonas*[8], including *P. mendocina, P. putida,* and *P. fluorescens.*

O-Acetyl groups invariably seem to be present in bacterial alginates and are the most characteristic feature distinguishing them from the algal polymers. In the alginate of *A. vinelandii,* they are associated with the M-residues[9,10] in the

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M-blocks, and one of their functions is to protect these units from conversion into G-residues by mannuronan C-5-epimerase[10]. Since the acetylation is an intracellular process, whereas epimerisation occurs exocellullarly, the organism is provided with a means of controlling the composition and, hence, the physical properties of its exopolymers.

The function of the *O*-acetyl groups in *Pseudomonas* alginates is not well understood, due to a lack of information about their distribution and the epimerase system in these bacteria. We now report on the sequential structure and the distribution of *O*-acetyl groups in alginate produced by various strains of the five known alginate-producing species.

EXPERIMENTAL

Materials. — The bacterial cultures were *A. vinelandii* AE-IV, *A. vinelandii* TL, *P. aeruginosa* DE 127 (provided by Dr. D. S. Feingold, Pittsburgh), *P. mendocina* NCIB 10541 (provided by The National Collections of Industrial and Marine Bacteria, Ltd., Aberdeen), and *Klebsiella aerogenes* type 25 (provided by Dr. J. R. Turvey, Bangor).

The cultural medium for *A. vinelandii* contained D-glucose (20 g), K_2HPO_4 (1 g), $MgSO_4 \cdot 7\ H_2O$ (200 mg), $FeSO_4 \cdot 7\ H_2O$ (50 mg), $NaMoO_4 \cdot 2\ H_2O$ (5 mg), NH_4OAc (2.3 g), and $CaCl_2 \cdot 2\ H_2O$ (50 mg) per litre[7]. For *Pseudomonas* species, the medium *A* used for culture maintenance consisted of sodium D-gluconate (20 g), monosodium glutamate (20 g), Na_2HPO_4 (3 g), and $MgSO_4 \cdot 7\ H_2O$ (0.3 g) diluted to one litre. Medium *B* consisted of D-glucose (20 g), yeast extract (Difco, 6 g), $(NH_4)_2SO_4$ (0.6 g), Na_2HPO_4 (2 g), and $MgSO_4 \cdot 7\ H_2O$ (0.3 g)[11] per litre. Medium *B* containing 2% of agar was used for plate culture.

Alginates isolated from *P. putida* NCIB 10007 and *P. fluorescens* NCIB 10525 were kindly donated by Dr. I. Sutherland (Edinburgh). One sample of alginate from *A. vinelandii,* having a very low content of transition diads, was provided by Dr. S. Paoletti (Trieste). Alginate from *A. vinelandii* was isolated[12] from liquid cultures. Alginates were also prepared from cultures of *P. aeruginosa* DE 27 and from mucoid strains of *P. mendocina* NCIB 10541. The latter were isolated by selecting for carbenicillin resistance[13]. The cells were subcultured in medium *A* for 48 h at 30° on a gyratory shaker, stored at 4°, and then grown on agar plates (medium *B*) at 22° for 48 h. The cells and exopolymer were scraped from the surfaces of 5 plates with a glass rod, and stirred for 1 h with 0.1M sodium chloride containing 0.01M EDTA (200 mL). The suspension was then centrifuged and the polymer-containing supernatant solution was decanted off. This extraction procedure was repeated once. The combined supernatant solutions were filtered through a 0.8-μm membrane filter and dialysed against distilled water for 48 h. The polymer was then precipitated by adding 1 vol. of 2-propanol in the presence of 0.2% of sodium chloride.

Long M-blocks were prepared from *A. vinelandii* alginate of extreme block

composition[10] by degradation with a specific poly-L-guluronate lyase isolated from *K. aerogenes*[14], and the resulting oligomers were fractionated by gel-permeation chromatography[10].

Methods. — Deacetylation was carried out in 0.1M NaOH at 25° for 20 min. Acetylation of a partially degraded mannuronan (100 mg) having d.p. 50 was carried out after dissolution of the sodium salt in distilled water and precipitation by adding 0.1M HCl to pH 3.0. The alginic acid was collected by centrifugation and partly dried, and a suspension in pyridine–acetic acid anhydride (100 mL, 1:1) was kept at 22° for 16 h. The product was precipitated with acetone, collected by filtration, washed with acetone and ether, and dried.

Samples for n.m.r. spectroscopy were prepared as described previously[15] and the 400-MHz ^{1}H-n.m.r. spectra were recorded at 92° with a Bruker WM-400 spectrometer. The chemical shifts were expressed in p.p.m. downfield from the signal for sodium 3-(trimethylsilyl)propanesulfonate. The spectra of the acetylated samples were also recorded after deacetylation. The ^{13}C-n.m.r. spectra were recorded at 25 MHz with a JEOL FX-100 spectrometer[16]. The monomeric composition and the diad and triad frequencies were determined as described previously[15–17].

The content of acetyl groups was determined from the 400-MHz ^{1}H-n.m.r. spectra by comparing the intensities (I) of the acetyl protons with those of the uronic acid corrected for the contribution of HOD, *i.e.*, $(I_{OAc/3} \times 100\%)/I_{total/5} - I_{HDO})$. Thus, an acetyl content of 100% would signify a polymer with one acetyl group on each monomer unit. Since each monomer unit has two hydroxyl groups available for substitution, the value for a fully acetylated alginate would be 200%. The homonuclear shift-correlated, two-dimensional ^{1}H-n.m.r. spectra (COSY-90) were recorded at 400 MHz with a Bruker WM-400 spectrometer[18].

RESULTS AND DISCUSSION

The ^{1}H-n.m.r. spectra of acetylated alginate containing both monomers were complex due to the overlapping of the resonances for the "M" and "G" units in the high-field region. Since the acetyl groups always seemed to be associated with the M residues in the polymer[9,10], an acetylated homopolymer of mannuronic acid was investigated. A highly viscous, partially acetylated D-mannuronan was prepared from *P. aeruginosa* DE 27, grown at low temperature on agar plates. No resonances for G units could be detected in the 400-MHz spectrum (Fig. 1C), suggesting that the G content in the polymer was <1%.

The spectrum of a deacetylated sample of this polymer displayed four major signals when recorded at pD 7.0 (Table I, 1B). Consideration of β-D-mannuronic acid in its $^4C_1$-conformation and of spin–spin couplings led to a complete interpretation of the spectrum.

Thus, the signal for H-1 was easily identified[19] at 4.7 p.p.m. and that for H-5 by its pD-dependence as shown by acidification with DCl to pD 3.8 (Fig. 1A),

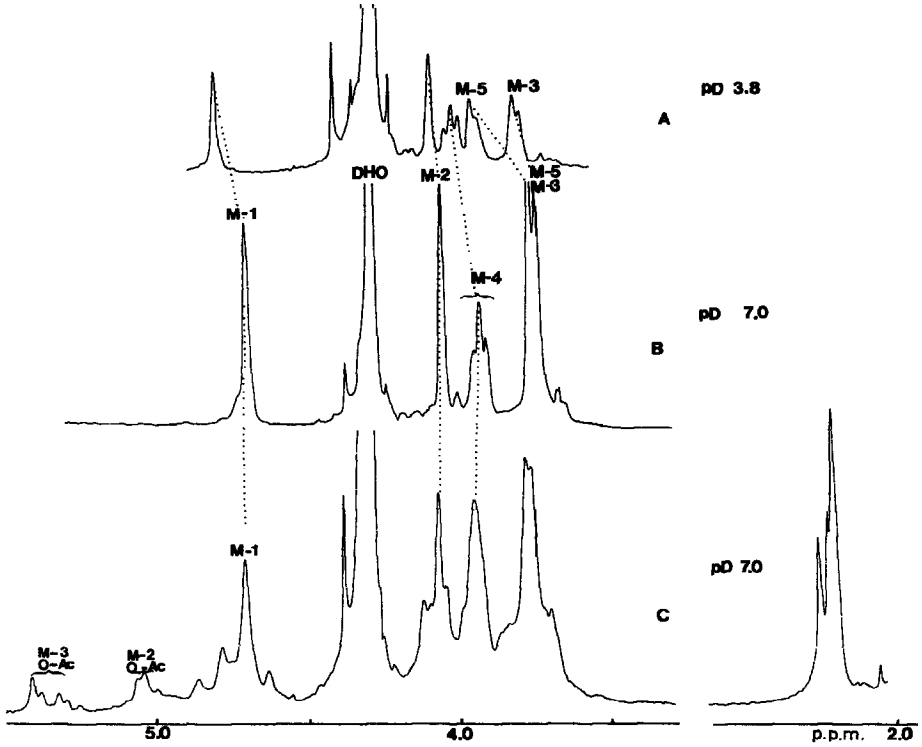

Fig. 1. 400-MHz F.t.-^{1}H-n.m.r. spectra of D-mannuronan from *P. aeruginosa*: A, deacetylated sample recorded at pD 3.8; B, deacetylated sample recorded at pD 7.0; C, native acetylated polymer (pD 7.0).

when the signal moved downfield from 3.7 to 3.9 p.p.m. The triplet at 3.9 p.p.m. (Fig. 1B) was due to H-4 which was almost equally coupled to both H-3 and H-5. The splitting of H-5 and H-3 of ~10 Hz (Fig. 1A) was due to coupling with H-4, and in accordance with their *trans*-diaxial arrangement. Irradiation of H-5 or H-3 caused the H-4 triplet to collapse into a doublet, and irradiation of H-4 caused the splitting of the signals for H-5 and H-3 to collapse. The spectra were not sufficiently resolved to determine the low $J_{1,2}$ and $J_{2,3}$ values, but these couplings were confirmed by a homonuclear shift-correlated, two-dimensional experiment (COSY-90)[18] (Fig. 2).

The spectrum (Fig. 1C) of the native acetylated D-mannuronan displays three large, partly overlapping peaks at 2.18, 2.15, and 2.13 p.p.m. for the acetyl protons, suggesting the presence of diacetylated units and at least one of the two possible mono-acetylated units. In addition to the resonance signals of the acetyl groups, the spectrum contained several peaks in the anomeric as well as in the high-field region which may be assigned to the signals of protons that were shifted downfield because of the influence of the acetyl groups. The largest changes in shift (1–2 p.p.m.)[20,21] occur for protons on the substituted carbon and smaller changes

TABLE I

CHEMICAL SHIFTS OF THE SIGNALS FOR THE PROTONS IN ACETYLATED[a] AND DEACETYLATED D-MANNURONAN[a]

	D-*Mannuronan* *pD 7.0*		D-*Mannuronan* *pD 3.8*	*Acetylated* D-*mannuronan* *pD 7.0*
M-3 AcO-3	—		—	5.36
M-3 AcO-3 (in 2,3Ac_2)				5.26
M-2 AcO-2	—		—	5.04
				5.01
M-1 AcO-2				4.82
or 3				4.74
M-1	4.67		4.76	4.67
M-2	4.04		4.08	4.08
M-4	3.91		4.00	3.91
M-3	3.75	M-3	3.79	3.73
or				
M-5	3.73	M-5	3.91	3.74
CH_3(AcO)				2.18
				2.15
				2.13

[a]Isolated from *P. aeruginosa* DE 27. [b]Solutions in D_2O at 92° (internal TPS).

(0.1–0.3 p.p.m.) are induced on neighbouring protons. The magnitude and sign of these shifts depend on the spatial orientation of the acetyl groups. The resonances at 5.38–5.34 and 5.28–5.25 p.p.m. showed splitting similar to that of H-3, suggesting that they represented the H-3 signals with O-3 acetylated. This was confirmed by the COSY-90 spectrum (Fig. 3), which demonstrated the coupling between these signals and that of H-4. Since there were two signals for H-3, it was likely that one of them was due to H-3 with both O-2 and O-3 acetylated. In order to confirm this interpretation, and to identify the two peaks, alginates with a very high content of *O*-acetyl were examined, including a chemically acetylated, M-rich alginate (Fig. 4). With increasing content of *O*-acetyl, the intensity of peak II increased relative to that of peak I, suggesting that I represented H-3 in 3-Ac-ManA and II H-3 in 2,3-Ac_2ManA. The content of acetyl groups estimated from the intensities of the H-3 signals of the acetylated residues accounted for 14% of a total acetyl content of 37% (Table III), indicating that 2-Ac-ManA units were also present in this polymer. The resonance at 5 p.p.m. (Fig. 1C, peak III) is believed to have been due to H-2 with O-2 acetylated. The sum of the intensities of the resonances I–III corresponded approximately to one third of the acetyl signals. The signals for H-2 with O-2 acetylated were not so well resolved and were overlapped slightly by a peak which, in the COSY-90 experiment, was coupled to H-2. Three additional peaks in the anomeric region of the spectrum probably arose from H-1 in acetylated residues. In alginates containing L-guluronic acid, peak III will be overlapped by signals for H-1 from that unit and the fraction of acetylation at C-2 was estimated as the total AcO − AcO-3.

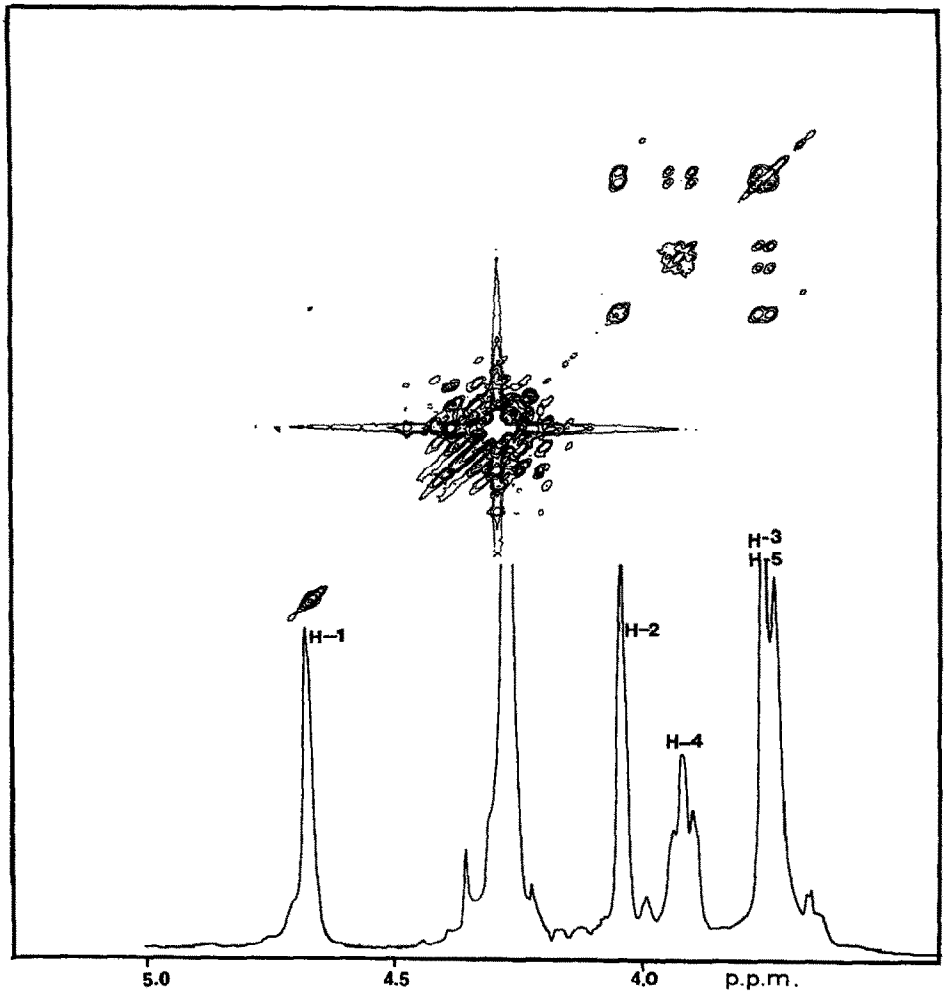

Fig. 2. 400-MHz ^{1}H-Shift-correlated 2D-n.m.r. spectrum of D-mannuronan in D_2O. The projection of the spectrum is plotted at the bottom.

The 400-MHz n.m.r. data for alginates isolated from various sources, given in Fig. 5 and in Tables II and III, clearly demonstrate the correlation between the content of acetyl groups and the monomeric composition of the polymer. In the alginate isolated from *A. vinelandii* IV which contained 93% of G, only 4% of acetyl groups was found, whereas alginate containing 55% of M had 22% of acetyl groups. An even higher degree of acetylation (~1 acetyl group for every 2 M-units) was found in M-rich blocks obtained from *A. vinelandii* alginate by digestion with a poly-G-lyase followed by gel-permeation chromatography[10]. The correlation between the content of acetyl groups and mannuronic acid residues was also

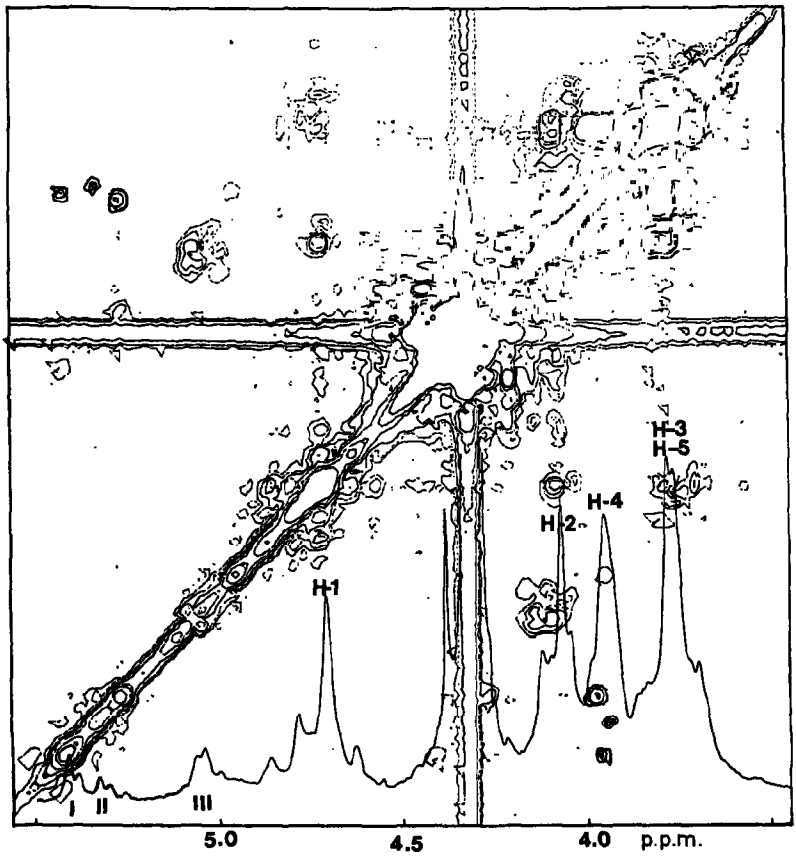

Fig. 3. 400-MHz ^{1}H-Shift-correlated 2D-n.m.r. spectrum of a native acetylated D-mannuronan acid from *P. aeruginosa*. The projection of the spectrum is plotted at the bottom.

observed in the alginates from *Pseudomonas* species. A high content of acetyl groups was found in the D-mannuronan from *P. aeruginosa* DE27. A low content of guluronic acid and a correspondingly high content of acetyl groups was also found in the alginate from *P. mendocina*, whereas, in the polymers produced by *P. putida* and *P. fluorescens*, a higher content of guluronic acid (37–40%) and a low content of acetyl groups (3–4%) were found.

The patterns of acetylation in the alginates are shown in Table III. Acetylation at position 2 was more extensive than at position 3. In highly acetylated samples from both *Azotobacter* and *Pseudomonas*, 2,3-diacetylated units could be detected. The polymer from *P. aeruginosa* DE 27 seemed to be heterogeneous with respect to the content of acetyl groups. Fractional precipitation from an aqueous solution with solvents of decreasing polarity yielded products having increasing contents of acetyl groups. Precipitation with ethanol, 2-propanol, and acetone gave poly-M with 37, 54, and 57% of acetyl groups respectively.

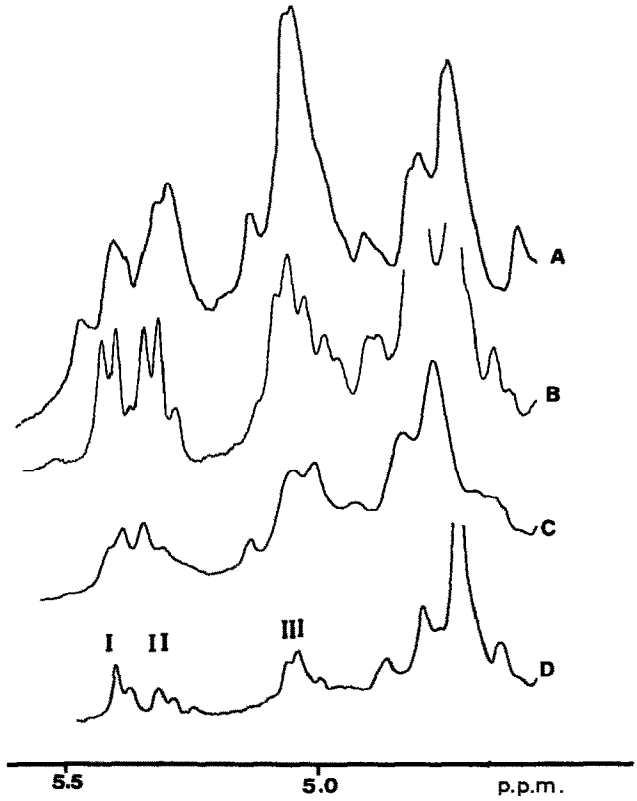

Fig. 4. ^{1}H-N.m.r. spectral region for H-2,3 in acetylated alginates: A, chemically acetylated M-rich alginate (M >95%, OAc 135%); B, D-mannuronan isolated from *P. aeruginosa* DE27 by precipitation with acetone (OAc 57%): C, acetylated M block isolated from *A. vinelandii* (OAc 52%); D, D-mannuronan from *P. aeruginosa* DE27 by precipitation with ethanol (OAc 37%).

TABLE II

COMPOSITION OF SOME BACTERIAL ALGINATES

Bacteria	F_G	F_M	F_{GG}	F_{MM}	$F_{MGM,GM}$	F_{GGG}	F_{MGM}	O-*Acetyl* (%)
P. aeruginosa DE 27	0	1.00	—	1.00	—	—	—	37–57
P. mendocina 10541	0.26	0.74	0	0.48	0.26	—	0.26	30
P. putida 1007	0.37	0.63	0	0.26	0.37	—	0.40	4
P. fluorescens 10255	0.40	0.60	0	0.20	0.40	—	0.40	3
Azotobacter vinelandii TL	0.45	0.55	0.42 (0.20	0.52 0.30	0.03 0.24	0.41 0.09[a])	0.02	22
A. vinelandii IV	0.94	0.06	0.93	0.04	0.01			4

[a]Bernoullian distribution.

TABLE III

ACETYLATION PATTERNS IN SOME BACTERIAL ALGINATES

Bacteria	F_G	F_M	F_{GG}	$F_{MG,GM}$	F_{MM}	*Total (%)*	*C-2 (%)*	*C-3 (%)*	*2,3-Ac₂ (%)*
A. vinelandii I	0.67	0.33	0.54	0.13	0.20	21	13	8	3
A. vinelandii II	0.05	0.95	—	—	—	52	30	22	11
P. aeruginosa I	0.00	1.0	0	0	1.0	37	23	14	5
P. aeruginosa[a] II	0.00	1.0	0	0	1.0	57	34	23	11

[a]Precipitated with acetone.

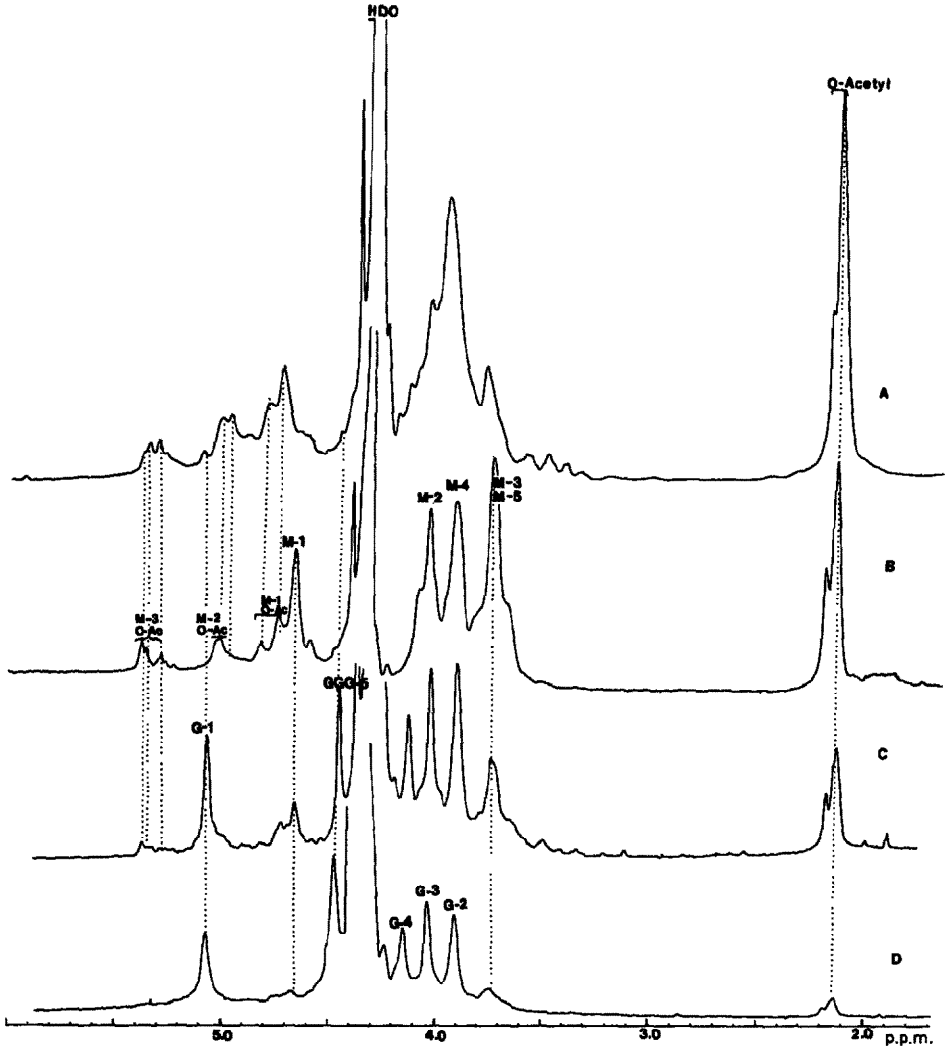

Fig. 5. 400-MHz F.t.-^{1}H-n.m.r. spectra of bacterial alginates: A, alginate enriched in mannuronic acid (M 95%) from *A. vinelandii*; B, poly-M isolated from *P. aeruginosa* DE27; C, alginate from *A. vinelandii* (G 67%; OAc 11%); D, "G"-rich alginate (G 94%) from *A. vinelandii* AE-IV (OAc 4%).

The most obvious structural difference between the alginate from *A. vinelandii* and those produced by the four *Pseudomonas* species was the complete absence of G-blocks in the latter. This was demonstrated by the 400-MHz ^{1}H-n.m.r. spectrum shown in Fig. 6 where no resonance signal for GG-5 could be detected, and where $I_{MGM} = I_{MG} = I_G$, indicating that, in these polymers, all of the guluronic acid residues were involved in heteroglycosidic linkages. Even in alginates containing almost 50% of guluronic acid, no GG diad could be detected on the basis of a highly resolved n.m.r. spectrum[22], suggesting a complete alternating structure. Similar results have been reported for several strains of *P. aeruginosa*[23]. Thus, it seems that the *Pseudomonas* epimerase system is unable to generate G-blocks and as such is very different from the epimerase in *A. vinelandii*. In the latter bacterium, the D-mannuronan C-5-epimerase attacks the substrate either in a random manner, producing a polymer with a near-Bernoullian distribution of the four diad frequencies, or, under certain conditions, by a multiple-attack mechanism[24], generating long G-blocks[25].

The polymers from *P. putida* and *P. fluorescens* containing M and G in the ratio 60:40 were analysed by ^{13}C-n.m.r. spectroscopy to see if the structure MMGMMGMMG preponderated, as in some algal alginates[26] having high contents of heteropolymeric sequences. It would be expected that $F_{MMM} = 0$ and $F_{MMG} = F_{MG} = F_G = 0.40$. The M-centered triad frequencies were calculated from the spectra, and the results (Table IV) clearly demonstrated the presence of long sequences of strictly alternating structure, together with some M-blocks.

Alginates from *A. vinelandii* exhibit all the physical properties noted for algal polymers, and as such they represent a potential source for industrial production.

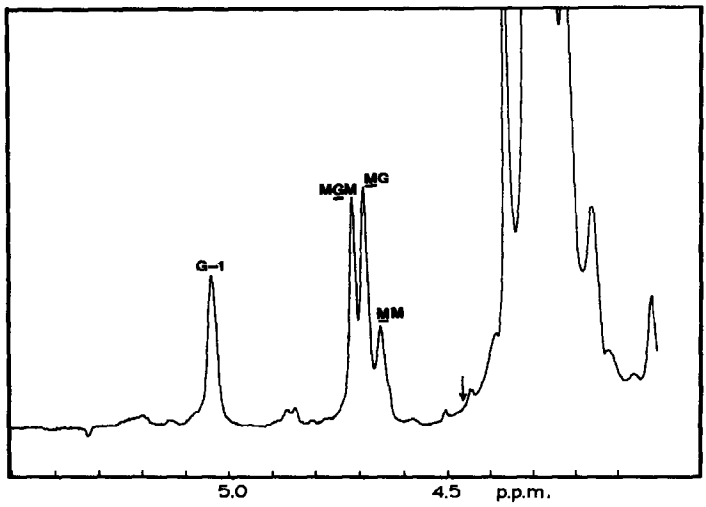

Fig. 6. 400-MHz F.t.-^{1}H-n.m.r. spectrum of alginate from *P. fluorescens* 10255. The arrow indicates the chemical shift of GG-5 H-1 resonance.

TABLE IV

COMPLETE TRIAD FREQUENCIES OF ALGINATE FROM *P. fluorescens* AND *P. putida* BASED ON 400-MHz ^{1}H-N.M.R. AND ^{13}C-N.M.R. DATA

	F_G	F_M	F_{MG}	F_{MM}	F_{GG}	F_{MGM}	F_{GMG}	F_{GMM}	F_{MMM}	$\bar{N}_{M>1}$
P. fluorescens	0.40	0.60	0.40 (0.24)	0.20 (0.36)	0.00 (0.16)	0.40 (0.14)	0.34 0.096	0.06 (0.14)	0.14 (0.21)[a]	3.3
P. putida	0.37	0.63	0.37	0.26	0.00	0.37	0.32	0.06	0.20	4.3

[a]Bernoullian distribution.

This is not so for the alginates from *Pseudomonas* species, since they seem to lack diaxially linked, contiguous G-residues, which are the main structural feature contributing to gel-formation[27].

Thus, in the alginates from *A. vinelandii* and *Pseudomonas* spp., the acetyl groups are associated with the M residues, although it cannot be ruled out that acetylated guluronic acid residues exist. Except for the inhibitory effect of the acetyl groups on mannuronan C-5-epimerisation in *A. vinelandii*[10], and on alginate lyase[28] in some strains of *P. aeruginosa*, the function of the acetyl groups is not well understood. Generally, the *O*-acetyl groups will have an effect on the solution properties of polymers[29], and a study of the influence of these groups on the gel-forming and water-binding properties of the bacterial alginates will be presented elsewhere.

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