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Characterisation of glycerophosphorylated cyclic β-1,2-glucans from a fast-growing *Rhizobium* species

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A family of cyclic β-1,2-glucans, substituted with *sn*-glycerol-1-phosphate at the C6 position of some of the glucose residues, has been found in the culture supernatant of the fast-growing, broad host range *Rhizobium* species, NGR234. The dynamic behaviour of the molecules, studied by ^{13}C nuclear magnetic resonance spectroscopy, showed that they are disc-shaped with significant, but limited internal motion.

Introduction

Characteristic cyclic β-1,2-glucans are found in the periplasmic space of bacteria from the *Rhizobiaceae* family [1–3]. Their function is presently unknown, but it has been suggested [3–5] that they are involved in the special interaction between these bacteria and plants. The glucans are cyclic homopolymers of 17 to 24 β-D-glucopyranose units. Although early reports [3] suggested additional substituents or branching, it was later established that this was not the case [6].

In a study of the polysaccharides produced by transposon-induced mutants of the fast-growing, broad host range *Rhizobium* species, NGR234, we have found a family of cyclic glucans substituted with phosphoglycerol. The number of substituents per molecule ranges from zero to four. Many mutants produced little or no extracellular polysaccharide (Muc^-), but all synthesised the substituted cyclic glucans.

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Materials and Methods

Bacteria. Mutants deficient in polysaccharide production were induced in ANU280, a rifampicin and streptomycin-resistant derivative of the New Guinea isolate, NGR234 [7] by transposon Tn5 and selected on the basis of altered colony morphology as described earlier [8]. Cells were grown by shake culture for four days in defined medium with D-mannitol as the carbon source [9].

Isolation and purification of polysaccharides. Cells and extracellular polysaccharide were separated from material of lower molecular weight using an Amicon DC10L hollow-fibre filtration system fitted with a 0.1 μm cut-off filter (Amicon H5MP01-43). The cells were then separated from the polysaccharide by centrifugation at $9000 \times g$ for 10 min. The filtrate was passed through a H10P3-20 hollow-fibre filter, which retains material with a nominal molecular weight of 3000. Cyclic glucans and other oligosaccharides retained by the filter were extensively washed to remove monosaccharides, excess mannitol and salts, and freeze-dried.

The oligosaccharide fraction was separated on

a DEAE-Sephadex A25 column using a salt gradient as described previously [9]. The eluent was monitored for hexose [10] and uronic acid [11] and the fractions thus isolated were rechromatographed on Bio-Gel P-2 at pH 3.3 [9]. The component sugars were identified using paper chromatography as described previously [9].

Nuclear magnetic resonance. NMR spectra were recorded for ^2H_2O solutions at 20 °C using a Varian XL-200 spectrometer. Relaxation times were measured with the inversion-recovery and Carr-Purcell pulse sequences and quantitative carbon spectra were obtained using gated decoupling and delays of 9-times the longest T_1 value. Evidence for chemical exchange was sought by selective inversion of single carbon resonances by a train of short pulses [12], followed by a variable delay and a non-selective 90 ° observation pulse.

For ^{31}P spectra, EDTA was added to a concentration of 10 mM, in order to reduce the possibility of paramagnetic broadening by impurities.

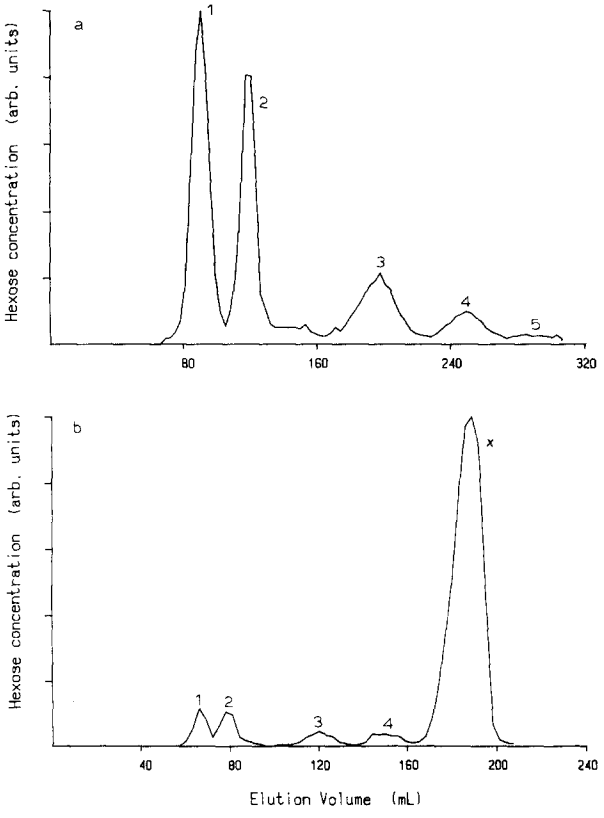

Fig. 1. Elution profiles on DEAE-Sephadex A25 for oligosaccharides from the culture supernatant from (a) strain ANU2838 and (b) ANU280, the parent strain. Different columns were used in each case. Peaks marked 1 to 5 contained cyclic glucans bearing 0 to 4 phosphoglycerol substituents and the peak marked × contained acidic nonasaccharide.

Results

Identification of phosphoglycerol-substituted glucans

The oligosaccharides isolated from the culture

TABLE I

PRODUCTION OF OLIGOSACCHARIDES BY MUTANTS OF *RHIZOBIUM* sp. NGR234 WITH ALTERED POLYSACCHARIDE SYNTHESIS

The production of cyclic glucans bearing 0 to 4 phosphoglycerol substituents is expressed as mg oligosaccharide per g cells, dry weight.

Strain	Acidic nonasaccharide	Cyclic glucans				
		0	1	2	3	4
280 [a]	230	13	13	8	8	–
2801	54	11	14	12	9	–
2807	0	70	100	60	40	20
2812	120	10	25	15	8	–
2814	26	4	7	6	7	–
2816	66	4	10	13	7	–
2817	88	5	17	17	15	–
2820	0	14	5	3	2	1
2822	0	7	15	14	8	3
2823	0	12	14	11	4	<1
2825	64	44	39	12	7	–
2826	0	32	57	37	8	6
2827	62	5	7	4	4	–
2831	0	9	9	9	9	5
2833	17	8	15	20	20	8
2838	0	150	110	70	30	5
2840	0	35	33	12	12	4
2842	0	4	7	16	9	2
2844	0	2	7	14	13	6
2847	0	14	19	15	8	1
2849	80	2	21	18	14	–
2851	2	11	14	11	5	–
2854	0	14	25	21	16	5
2856	120	33	38	19	14	–
2866	54	3	2	1	<1	–
2875	0	1	6	7	6	3
2877	0	2	11	13	7	3
2885	2	11	11	7	<1	<1
2893	55	1	1	3	8	–
2894	360	40	20	5	5	–
2895	430	90	28	8	10	–

[a] Parent strain.

supernatant from the parent strain, ANU280, contained a large amount of an acidic nonasaccharide (peak × in Fig. 1b), which is the repeating unit of the extracellular polysaccharide. Its structure, determined by ^{13}C-NMR spectroscopy of purified hydrolysis fragments, has been reported elsewhere [9]. Mutants of ANU280 produced various amounts of the nonasaccharide, and several, like ANU2807, produced none (Table I). All, however, synthesised a family of oligosaccharides that eluted as a series of peaks from an ion-exchange column (Fig. 1a). The only sugar present was glucose, and all, except the first member of the series, contained phosphate, as shown by ^{31}P-NMR.

The proportions of the substituted glucans varied considerably between strains, as shown in Table I. Growth conditions were nominally the same in all cases, but it is possible that variations in the growth rate of individual strains affected the results, as it is known that the production of cyclic glucans varies with growth phase [13].

The compounds were identified by ^{13}C-NMR spectroscopy as cyclic glucans substituted with glycerol phosphate at C-6 of one or more of the glucose rings. The spectra of material from chromatographic peaks 2 and 4 are shown in Fig. 2. The relative intensities of peaks assigned to the glycerol substituents increased as the acidity of the molecule increased.

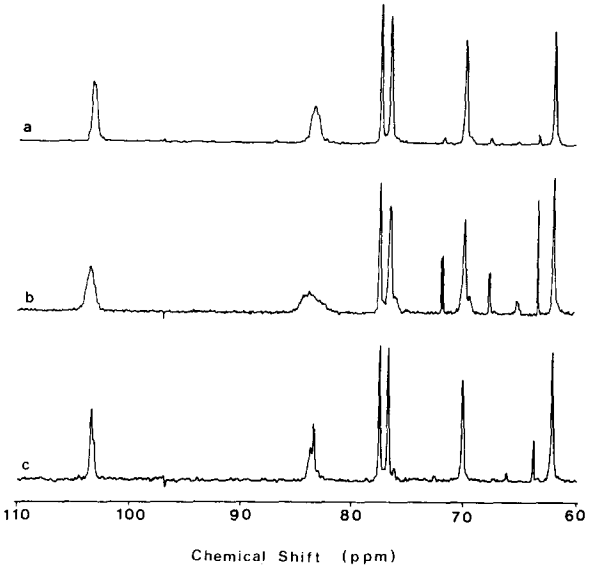

Fig. 2. ^{13}C-NMR spectra of purified cyclic glucans from ANU2807. (a) Material from peak 2 from DEAE-Sephadex A25 (see Fig. 1 for peak numbering), (b) material from peak 4 and (c) material from peak 4 after overnight treatment at pH 12, 80 °C.

The strongest signals are those for carbons in the glucopyranose rings and their chemical shifts agree well with values reported for model compounds [14]. The C-1, C-2 and C-4 resonances showed partially resolved fine structure, as reported previously for unsubstituted molecules [6]. The cyclic nature of the glucans was demonstrated by the absence of reducing terminal C-1 peaks at 92.4 and 95.1 ppm [14]. There would have been no difficulty in observing these peaks had they been present.

The peaks at 67.7, 71.9 and 63.3 ppm were assigned to C-1, C-2 and C-3 of *sn*-glycerol-1-phosphate residues. The chemical shifts for C-2 and C-3 agreed well with those reported for C-1 and C-2 of *sn*-glycerol-3-phosphate and methylene carbons in the polyglycerophosphate chain of lipoteichoic acid [15]. The number of protons attached to each carbon was confirmed by means of the DEPT pulse sequence [16] and further support for the assignments was provided by the observation of ^{31}P-induced splitting of the C-1 and C-2 resonances.

Phosphate substitution resulted in shifts of the glucose C-4, C-5 and C-6 resonances. The C-6 peak in substituted glucose residues was found at 65.1 ppm, in agreement with the spectra of similarly substituted molecules [15,17], and the C-4 resonance is displaced 0.5 ppm upfield from its unsubstituted position. The close proximity of the C-3 and C-5 signals precluded their unambiguous assignment in the spectrum of the unsubstituted cyclic glucan [6]. Phosphorylation, however, resulted in a decrease in the intensity of the peak at 77.5 ppm, which can be assigned as the C-5 resonance in unsubstituted glucose rings. The peak at 76.7 ppm is, therefore, that for C-3. The small peak corresponding to C-5 in substituted rings was observed at 76.1 ppm. Integration of spectra obtained under quantitative conditions yielded peak areas that agreed with these assignments to within a few percent.

As the number of phosphoglycerol substituents per molecule increased, broadening of the C-1 and C2 resonances occurred and the structure seen in

Fig. 2a could no longer be resolved. After removal of the substituents at pH 12, 80 °C, overnight in the NMR tube, the spectrum resembled that for unsubstituted cyclic glucan (Fig. 2c). There was no evidence of ring opening.

The width of the C-1 and C-2 signals was due to heterogeneity of the chemical environment of these nuclei. The width of individual signals within the peaks, as determined by T_2 measurements, was only 4.8 Hz. When one of the C-3 resonances in the spectrum of an unsubstituted cyclic glucan was selectively inverted, as shown in Fig. 3, no transfer of intensity to the other C-3 signal was observed during the relaxation time for the nuclear spins. The two signals are, therefore, unable to interconvert any faster than 10 times per second and are probably due to carbons in different molecules, and not to different conformations of the same molecule. Our observations are consistent with the proposal made by Dell et al. [6] that chemical shift differences occur as the result of different average angles for the glucose rings. The differences may be the result of variations in ring size or local distortions caused by the substituents.

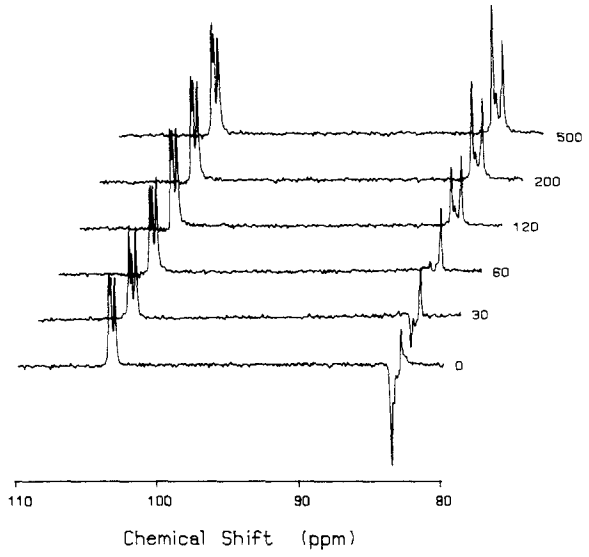

Fig. 3. Selective inversion of one of the C-3 resonances in the ^{13}C-NMR spectrum of unphosphorylated cyclic glucan isolated from *R. trifolii*, ANU843. The pulse sequence used was $(\pi)_{\text{selective}}$–D–$(\pi/2)_{\text{non-selective}}$–observe. Values for the variable delay, *D*, are recorded in ms beside each spectrum.

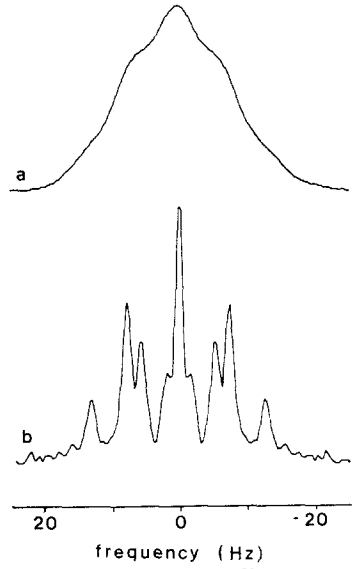

Fig. 4. Proton splitting pattern in the ^{31}P spectrum of purified cyclic glucan (peak 4 from DEAE-Sephadex A25) from ANU 2807. (a) Normal 1D proton-coupled ^{31}P spectrum. (b) Projection onto the J_{PH} axis of the heteronuclear 2D-J ^{31}P spectrum.

Rotamer populations

The proton-decoupled ^{31}P spectrum contained a single peak at 0.91 ppm relative to external phosphoric acid in a spherical container [18] and the chemical shift was independent of pH between 3 and 10. The proton-coupled signal appeared, initially, to be a broad quintet consistent with coupling to four protons. To improve the resolu-

TABLE II

CONFORMER POPULATIONS FOR ROTATION AROUND C–O BONDS ADJACENT TO PHOSPHATE GROUP

	$^3J_{PC}$ (Hz)	$^3J_{PH}$ (Hz)	population of *trans* rotamer [a]
Glycerol C-1	7.9	7.4 [b]	0.49
Glucose C-6	–	5.4 [b]	0.68

[a] The *trans* rotamer is defined as that in which the C–C bond is *trans* to the P–O bond. The populations were calculated using the equations

$^3J_{PC} = 2.0(1 - P_{III}) + 14.0\ P_{III}$

$^3J_{PH} = 12.5(1 - P_{III}) + 2.1\ P_{III}$

[b] The assignment of the two $^3J_{PH}$ values was made so as to obtain the best agreement with the rotational population calculated from $^3J_{PC}$.

tion, a two-dimensional heteronuclear *J*-resolved spectrum [19] was recorded. As shown in Fig. 4, the ^{31}P resonance is a triplet of triplets, resulting from different coupling constants to the protons on the glucose C-6 and glycerol C-1. The average rotational conformation is, therefore, different on either side of the phosphate [20]. The proportion of time spent in each rotamer was calculated from both the $^3J_{PC}$ and $^3J_{PH}$ coupling constants (Table II).

The proportion of time spent in the *trans* rotamer is considerably greater than 1/3, suggesting that the glycerol chain extends away from the molecule, into the solution. The preference for the *trans* rotamer is greater for glucose C-6, where steric hindrance is expected to be more severe. A similar picture of the behaviour of the glycerol residue is provided by the relaxation behaviour of carbon nuclei, which is discussed below.

Molecular shape

While the cyclic β-1,2-glucans are expected to have limited flexibility [21], no direct evidence of their shape or stiffness was previously available. The molecules are, fortuitously, the right size for obtaining such information from the magnetic resonance relaxation behaviour. Relaxation times and nuclear Overhauser enhancement factors for molecules with two phosphoglycerol residues are reported in Table III. The T_1 values are identical for carbons 1 to 5 in the glucose rings, but increase as one proceeds outwards along the glycerol chain from C-6. The same trends are seen in the T_2 and nuclear Overhauser enhancement values.

TABLE III

RELAXATION TIMES AND NUCLEAR OVERHAUSER ENHANCEMENT FACTORS FOR ^{13}C RESONANCES OF CYCLIC GLUCANS

N is the number of protons attached to the carbon. The figures in parenthesis are the experimental uncertainties in the last place.

Carbon number	NT_1 (s)	NT_2 (s)	η
Glucose C-1	0.107 (5)	0.065 (5)	0.45
Glucose C-2	0.098 (7)	0.066 (7)	0.56
Glucose C-3	0.105 (6)	0.068 (3)	0.50
Glucose C-4	0.107 (4)	0.066 (6)	0.50
Glucose C-5	0.106 (3)	0.071 (1)	0.53
Glucose C-6	0.132 (4)	0.080 (6)	0.74
Glycerol C-1	0.42 (6)	0.26 (6)	1.59
Glycerol C-2	0.30 (8)	0.26 (2)	1.36
Glycerol C-3	0.50 (12)	0.40 (2)	1.72

TABLE IV

OBSERVED AND CALCULATED RELAXATION PARAMETERS FOR ^{13}C NUCLEI AT C-1–C-5 OF THE GLUCOSE RINGS IN CYCLIC GLUCANS

	T_1 (s)	T_2 (s)	η
Observed	0.105	0.067	0.51
Calculated for rigid sphere 1.02 nm in diameter	0.092	0.080	0.90
Calculated for rigid ellipsoid with 7.3:1 axial ratio	0.080	0.048	0.29
Calculated for rigid ellipsoid with internal motion $S = 0.81$, $\tau_e = 1.7 \cdot 10^{-10}$ s	0.105	0.067	0.51

If the molecule were completely flexible, and had a spherical, randomly-coiled structure, its average radius, calculated from the density of glucose [22], would be 1.02 nm. The relaxation behaviour expected for such a molecule [23,24] is similar in magnitude, but different in detail from the observed behaviour (Table IV). T_1 is longer than predicted, suggesting that the molecule is disc-shaped, resulting in slower rotational diffusion.

Lipari and Szabo [24] have pointed out that the unique information about fast internal motions can be completely specified by two, model-independent, quantities, an order parameter, *S*, and an effective correlation time, τ_e. These quantities, and the correlation time for overall rotation of the molecule, τ_m, were adjusted until agreement was obtained between observed and calculated values for T_1, T_2 and η.

The mathematical expressions for T_1, T_2 and η for non-spherical molecules and for molecules with internal motion are the same [24,25], so that in general, it may be impossible to separate the two contributions. In the present case, however, the contribution of anisotropic rotation to τ_e can be shown to be negligible.

The values obtained for τ_m, τ_e and S were $(3.1 \pm 0.5) \cdot 10^{-9}$ s, $(1.7 \pm 0.4) \cdot 10^{-10}$ s and 0.81 $\pm$ 0.01, respectively. Assuming the molecule to be an oblate ellipsoid [25], the rate of rotational

diffusion about an axis in the plane of the disc can be calculated from τ_m, while diffusion about the unique axis of the molecule will contribute to τ_e. Again taking the molecular density to be the same as that for glucose, an axial ratio of 7.3 : 1 was calculated from τ_m, giving molecular dimensions of $2.0 \times 2.0 \times 0.27$ nm. The diameter of a fully extended ring in which the oxygens on C-1 and C-2 of each glucose are coplanar, was estimated to be 2.8 nm from standardised atomic co-ordinates for β-glucose [26].

One of the properties of an oblate ellipsoid is that the length of the short axis has a relatively small effect on the rate of rotational diffusion [25] about the axis perpendicular to the plane of the disc. The calculated rate for an axial ratio of 7.3 : 1 was 87% of the rate calculated for diffusion about an axis in the plane of the disc. Anisotropic rotational diffusion, therefore, contributes little to the value of τ_e. Furthermore, if the centre of the molecule is occupied by water, the effect on the above calculations would be to increase the estimate thickness of the disc, which would cause an insignificant change in the other calculated quantities.

Further interpretation of the order parameter and correlation time for internal motion requires a specific model. The most appropriate for the present situation is one in which the axis of the CH bonds, i.e. the normal to the plane of the glucose rings, diffuses freely within a cone [25]. The observed values for S and τ_e are consistent with a cone of semi-angle 30°, and a diffusion constant, D, of $1.1 \cdot 10^9\ s^{-1}$.

The increase of T_1 for carbons further along the glycerol chain and the approach of η towards its limiting value of 1.98 demonstrate that carbons in the glycerol chains rotate rapidly. The most mobile atom is C-3 of the glycerol, so the substituent extends freely into the solution and is not involved in intramolecular interactions.

Ring size

Peaks in the elution profile of the glucans from a DEAE-Sephadex column were well separated. Each peak, therefore, contained molecules with the same, integral, number of phosphoglycerol residues. The average number of glucose rings per molecule, as estimated by integration of quantitative carbon NMR spectra, alternates as the number of substituents increases, as shown in Table V. This size alternation was confirmed by the elution position of rechromatographed fractions from a Bio-Gel P-2 column.

Discussion

The isolation of Muc$^-$ mutants has enabled us to demonstrate the presence, in culture supernatants, of phosphorylated cyclic glucans. Hollow-fibre filtration greatly assisted the separation and purification of the oligosaccharides and permitted the avoidance of procedures that remove the acidic glucans [4].

Although we found phosphorylated cyclic glucans only in the broad host-range fast-growing *Rhizobium* species, NGR234, and were able to detect only the unsubstituted glucan in oligosac-

TABLE V

PROPERTIES OF THE CYCLIC GLUCANS FROM ANU2807

Number of phosphoglycerol residues per molecule	0	1	2	3	4
Average number of glucose residues per molecule (±2)	–	19	26	20	23
Elution position from Bio-Gel P-2, K_{av} [a]	0.91	0.94	0.96	0.93	0.97
Width of C-1 resonance (FWHM in Hz)	18	20	31	35	50
Width of C-2 resonance (FWHM in Hz)	20	43	65	85	135

[a] Defined as $(V_e - V_0)/(V_t - V_0)$ [38].

charides from *Rhizobium trifolii* ANU843 (results not shown), it is probable that they will be found in other species. Kennedy has recently reported [27] the isolation, from *Agrobacterium tumefaciens,* of oligosaccharides with the same chromatographic properties as the substituted glucans and purified acidic oligosaccharides from *Alcaligenes* and *Agrobacterium* species contained both succinoglycan and cyclic glucans [2].

Kennedy has pointed out [27] the remarkable similarity between the chemical structures of the cyclic glucans and the membrane-derived oligosaccharides from *Escherichia coli.* The identification of phosphoglycerol substituents on the former makes the similarity even more striking. The only remaining differences are that membrane-derived oligosaccharide molecules are smaller, branched and acyclic. It may be that the relative stiffness of the cyclic glucan structure and the branching of membrane-derived oligosaccharide play similar roles in confining the molecules to the periplasmic space.

The biological function of the cyclic glucans is likely to remain a source of continuing speculation. Evidence has been presented that they affect infection thread and nodule formation in white clover [5] and are one of the components required for virulence of *A. tumefaciens* [4]. We find no clear correlation between the proportions of the different glucans and the pattern of infectability of our Muc^- strains [8], and strains producing amounts of the cyclic glucans similar to (ANU2802) or greater than (ANU2807) those for the parent, have distinctly different host ranges. Further evidence for the role of cyclic glucans in infection is clearly required.

Kennedy has demonstrated that production of both membrane-derived oligosaccharide by *E. coli* [28] and cyclic glucans by *A. tumefaciens* [27] is stimulated by conditions of low osmolarity. These observations, together with the amount of material synthesised, provide strong arguments supporting osmoregulation as the main function of both compounds, despite the observations that (i) mutants lacking membrane-derived oligosaccharide [29] or cyclic glucans [2] are not seriously debilitated and (ii) production of membrane-derived oligosaccharide is probably not under transcriptional control [30]. Some strains of *E. coli* have been reported to produce less membrane-derived oligosaccharide under conditions of low osmolarity [31].

While osmoregulation remains the best supported hypothesis, the identification of phosphoglycerol substituents on the glucans permits a new line of speculation concerning the role of these compounds. A great deal is known about the synthesis of membrane-derived oligosaccharide and the source of the phosphoglycerol moiety [29]. Synthesis of membrane-derived oligosaccharide accounts for almost all the phosphatidylglycerol turnover in *E. coli* [32].

In the Gram-positive organism, *Bacillus subtilis,* the major receptor for glycerolphosphate from phospholipid is lipoteichoic acid [33]. The biological function for lipoteichoic acid, also, is presently unknown, but it is unlikely to be an osmoregulator. We have recently shown [34] that it is unlikely to bind divalent cations – another favoured hypothesis. It has been proposed that lipoteichoic acid functions as a reserve phosphate source [35], and *B. subtilis* does produce teichoic acid hydrolases under a variety of conditions [36] and can use cell wall teichoic acid when starved of phosphorus [35]. All three substances, cyclic glucans, membrane-derived oligosaccharide and lipoteichoic acid, may function as sources of phosphoglycerol. Their location in the periplasm or outside the cell may be an example of regulation of hydrolysis by compartmentalisation [37].

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