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Cyclic glucans produced by *Agrobacterium tumefaciens* are substituted with *sn*-1-phosphoglycerol residues

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In a previous study (Miller, K.J., Kennedy, E.P. and Reinhold, V.N. (1986) Science 231, 48–51) it was reported that the biosynthesis of periplasmic cyclic β-1,2-glucans by *Agrobacterium tumefaciens* is strictly osmoregulated in a pattern closely similar to that found for the membrane-derived oligosaccharides of *Escherichia coli* (Kennedy, E.P. (1982) Proc. Natl. Acad. Sci. USA 79, 1092–1095). In addition to the well-characterized neutral cyclic glucan, the periplasmic glucans were found to contain an anionic component not previously reported. Biosynthesis of the anionic component is osmotically regulated in a manner indistinguishable from that of the neutral cyclic β-1,2-glucan. We now find that the anionic component consists of cyclic β-1,2-glucans substituted with one or more *sn*-1-phosphoglycerol residues. The presence of *sn*-1-phosphoglycerol residues represents an additional, striking similarity to the membrane-derived oligosaccharides of *E. coli*.

Introduction

The surface polysaccharides of the two closely-related Gram-negative genera of soil bacteria, *Agrobacterium* and *Rhizobium*, have received much attention because of their possible involvement in cell-signaling essential to the infection of specific plant hosts [1,2]. These surface polysaccharides include extracellular and capsular anionic polysaccharides, lipopolysaccharides, and cyclic glucans. The synthesis of such cyclic glucans appears to be unique to species of *Agrobacterium* and *Rhizobium*.

In a previous paper [3], we reported that the biosynthesis of cyclic β-1,2-glucans by *Agrobacterium tumefaciens* is subject to strict osmotic regulation in a fashion closely similar to that observed for the biosynthesis of the membrane-derived oligosaccharides of *Escherichia coli* [4]. In addition, several other similarities between the cyclic β-1,2-glucans of *Agrobacterium* and the membrane-derived oligosaccharides of *E. coli* were noted [3]. These include the following: (1) intermediate size, (2) glucose as the sole sugar, (3) a β-1,2-linked backbone, and (4) periplasmic localization. These results suggest that the function of periplasmic oligosaccharides in the adaptation to growth in a medium of low osmolarity, although still obscure, is a fundamental one because it is found in bacteria as widely different ecologically as the enteric and soil bacteria.

During the course of our analysis of periplasmic oligosaccharides of *A. tumefaciens*, we found that anionic cell-associated glucans are also present at levels comparable to that of the neutral

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cyclic β-1,2-glucans [3]. The synthesis of these anionic glucans was also osmoregulated in a fashion exactly parallel to that of the well-characterized neutral cyclic glucans. Anionic derivatives of the neutral cyclic β-1,2-glucans, however, appear not to have been described previously. We now report that these anionic oligosaccharides are indeed forms of the cyclic β-1,2-glucans, substituted with variable amounts of *sn*-1-phosphoglycerol in phosphodiester linkage. The substitution with *sn*-1-phosphoglycerol is a further striking similarity to the membrane-derived oligosaccharides of *E. coli*, which are also substituted with *sn*-1-phosphoglycerol moieties [5]. In *E. coli*, the *sn*-1-phosphoglycerol residues are derived from the hydrophilic head group of phosphatidylglycerol [6,7].

Materials and Methods

Preparation of anionic glucans F2, F3, and F4. Cells of *Agrobacterium tumefaciens* C58 were grown in 25 liters of YM medium, and cell-associated oligosaccharides were extracted with 1% (w/v) trichloroacetic acid as previously described [3]. The extracts were neutralized, concentrated, and fractionated by gel filtration chromatography on Sephadex G50 followed by ion exchange chromatography on DEAE-cellulose as previously described [3]. Fractions F2 and F3 were eluted from DEAE-cellulose at KCl concentrations of 10 mM and 60 mM, respectively, as shown previously [3]. In addition, a third anionic fraction F4, eluting at 90 mM KCl, was also detected in these preparations.

Analysis of anionic glucans F2, F3, and F4. Total carbohydrate was determined by the phenol method [8]. Glucose content was determined by the glucose oxidase method (Sigma Chemical Co. St. Louis, MO) after samples were first hydrolyzed for 4 h in 1.0 M HCl at 100 °C. Galactose content was determined with galactose oxidase as described by Hjelm and De Verdier [9] after hydrolysis for 4 h in 1.0 M HCl at 100 °C. Pyruvate content was determined by the lactate dehydrogenase method as described by Czok and Lamprecht [10] after mild acid hydrolysis at pH 2.0 (in dilute HCl) for 90 min at 100 °C. Total phosphorus was determined as orthophosphate by the method of Chen et al. [11] after digestion with magnesium nitrate by the method of Ames et al. [12]. Phosphomonoester content was determined as orthophosphate after treatment with pure *E. coli* alkaline phosphatase, specific for monoesters, as described by Kennedy et al. [5]. Succinate was determined by the succinate thiokinase method (Boehringer Mannheim Biochemicals, Indianapolis, IN) after samples were first treated with 0.1 M NaOH for 30 min at 37 °C.

Characterization of products of alkaline hydrolysis of F2, F3, and F4. Samples of anionic glucans F2, F3, and F4 were hydrolyzed in 0.5 M NaOH at 100 °C for 80 min. Under these conditions, phosphoglycerol, if present in phosphodiester linkage, should be released, as shown previously for the membrane-derived oligosaccharides of *E. coli* [5]. After hydrolysis, samples were cooled, diluted with water, and neutralized with CG50 cation exchange resin [5]. The neutralized hydrolysate was then analyzed for total phosphorus content and for phosphomonoester content by treatment with alkaline phosphatase [5]. After the treatment with alkaline phosphatase, the glycerol liberated was determined by the glycerokinase method with [γ-^{32}P]ATP as previously described [5].

Fast atom bombardment mass spectrometry. Mass spectra were recorded on a VG-ZAB-SE double focusing instrument (VG Analytical Ltd., Manchester, U.K.) which was operated at 8 kV accelerating voltage. Approx. 100 μg of oligosaccharide sample were dissolved in 50 μl of distilled water and aliquots of these solutions, containing 3–10 μg, were loaded by syringe into a liquid matrix of monothioglycerol (3-mercapto-1,2-propanediol). The matrix coated a stainless steel target which was attached to a direct insertion probe. Samples were desorbed by bombardment with a neutral xenon beam (operating parameters: source pressure = $9 \cdot 10^{-6}$ Torr Xe, FAB tube voltage = 6–8 kV, tube current = 2.0 mA).

Linkage analysis by gas chromatography. A sample of anionic glucan F2 was hydrolyzed in alkali as described above in order to remove phosphoglycerol substituents. The sample was then desalted on a column of Sephadex G25 (5 ml bed volume) eluted with 7% (v/v) 1-propanol and further analyzed by gas chromatography after permethylation, reductive cleavage, and acetylation as previously described [3].

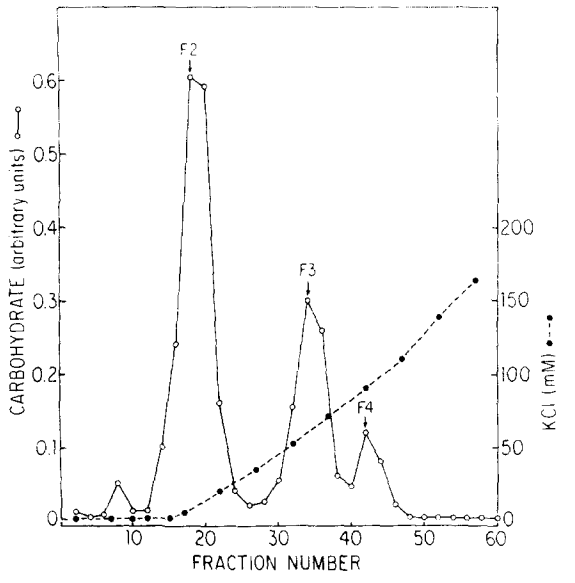


Fig. 1. Separation of anionic glucans F2, F3, and F4 by DEAE-cellulose chromatography. Cells of *Agrobacterium tumefaciens* C58, derived from 25 liters of culture in YM medium, were extracted with 1% trichloroacetic acid as described in Materials and Methods. The neutralized extracts were fractionated on a Sephadex G50 column yielding approx. 330 mg (glucose equivalent) of total oligosaccharide. The oligosaccharide material was further fractionated on a DEAE-cellulose column (2 cm×16.5 cm) that was first eluted with 2 column volumes of 10 mM Tris-HCl (pH 7.4) in 7% (v/v) 1-propanol. The neutral cyclic β-1,2-glucan was collected in this eluate and comprised approx. 45% of the total oligosaccharide material. The column was next eluted with a linear gradient beginning with 10 mM Tris-HCl (pH 7.4) in 7% (v/v) 1-propanol and ending with 250 mM KCl in 10 mM Tris-HCl (pH 7.4) in 7% (v/v) 1-propanol in a total volume of 200 ml. Fractions (3 ml) were collected at a flow rate of 25 ml per hour. Three anionic peaks were eluted at 10 mM KCl, 60 mM KCl and 90 mM KCl. These three peaks are designated F2, F3, and F4, respectively. An aliquot from each fraction was removed for total carbohydrate determination by the phenol method [8]. Results are expressed as the relative carbohydrate content of each fraction. The numbering of fractions begins with the application of the gradient to the column.

TABLE I

ANALYSIS OF ANIONIC GLUCANS F2, F3, AND F4 FROM *AGROBACTERIUM TUMEFACIENS*

Anionic glucans F2, F3, and F4 (Fig. 1) were prepared as described in the text and analyzed as described in Materials and Methods.

Constituent (μmol)	Anionic glucan		
	F2	F3	F4
Total carbohydrate (glucose equivalent)	518.4	259.7	65.8
Total glucose	467.1	246.4	63.7
Total galactose	0	0	0
Total pyruvate	0	0	0
Total succinate	0.6	0.6	0.2
Total phosphorus	16.7	19.8	8.9
Phosphate monoester	0	0	0.2
Phosphate : glucose (mole ratio)	0.036	0.080	0.140

Results

Anionic cell-associated oligosaccharides of Agrobacterium tumefaciens contain glucose as the only sugar

Upon DEAE-cellulose chromatography, the cell-associated, anionic oligosaccharides from *Agrobacterium tumefaciens* were similar to those previously reported [3] as shown in Fig. 1. Two major anionic fractions, F2 and F3, represented approx. 61% and 32%, respectively, of the total anionic oligosaccharide material. A third more anionic fraction, F4, representing approx. 7% of the total anionic oligosaccharide material, was also detected (Fig. 1). Analysis revealed that glucose accounted for at least 90% of the total carbohydrate of fractions F2, F3, and F4 (Table I). Because the recovery of glucose standards after similar strong acid hydrolysis was approx. 87%, it is probable that glucose accounts for the total carbohydrate of each fraction. Analysis by the galactose oxidase method did not reveal detectable galactose in acid hydrolysates of any of the three fractions (Table I). Neither pyruvate nor significant amounts of succinate, characteristic components of the acidic extracellular polysaccharides produced by this bacterium [13,14], were detected in any of the anionic oligosaccharide fractions.

Phosphoglycerol accounts for the total phosphorus present in anionic glucans F2, F3, and F4

Total phosphorus determinations indicated that phosphorus was present in all three fractions and that the ratio of phosphorus to glucose was progressively higher for the three fractions F2 (0.036), F3 (0.08), and F4 (0.14) as shown in Table I. The

TABLE II

ANALYSIS OF PRODUCTS OF ALKALINE HYDROLYSIS

Aliquots of anionic glucans F2, F3, and F4 were treated with 0.5 M NaOH at 100 °C for 80 min as described in Materials and Methods, and the products were analyzed as described in the text.

Constituent (nmol)	Anionic glucan		
	F2	F3	F4
Total phosphorus	366	338	432
P_i released by alkaline phosphatase	342	348	436
Glycerol released by alkaline phosphatase	371	357	407

phosphorus was not present as phosphomonoester because treatment with alkaline phosphatase released no detectable orthophosphate from any of the three fractions (Table I). Phosphoglycerol residues in phosphodiester linkage are a highly characteristic feature of the membrane-derived oligosaccharides of *E. coli* [5], therefore, an analysis for phosphoglycerol substituents was performed. Treatment of the anionic glucans with 0.5 M NaOH at 100 °C should result in the release of any phosphoglycerol residues linked to glucose by phosphodiester bonds [5]. As shown in Table II, essentially all of the total phosphate present in fractions F2, F3, and F4 was, in fact, released as phosphomonoester by alkaline hydrolysis. Treatment of the product of alkaline hydrolysis with alkaline phosphatase released 1 mole of glycerol for each mole of P_i produced (Table II). We conclude that phosphoglycerol accounts for essentially all of the phosphorus associated with the anionic glucan fractions.

Phosphoglycerol substituents of the anionic glucans are of the sn-1 configuration

Phosphoglycerol residues in the membrane-derived oligosaccharides of *E. coli* are of the *sn*-1 configuration and are derived from the head groups of phosphatidylglycerol [6,7]. To determine the stereochemistry of the phosphoglycerol moieties on the anionic glucans of *A. tumefaciens*, phosphoglycerol was released from anionic glucan F2 by alkaline hydrolysis as described above. Approximately equal amounts of α- and β-forms of

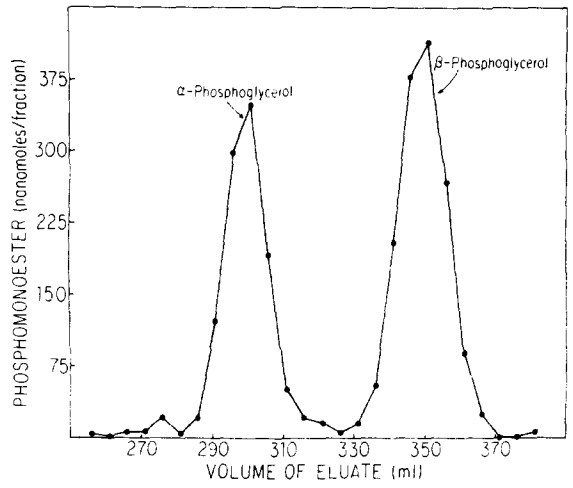


Fig. 2. Separation of α- and β-phosphoglycerol derived from anionic glucan F2 after alkaline hydrolysis. A sample of anionic glucan F2, containing 2.8 μmole total phosphoglycerol was subjected to alkaline hydrolysis and subsequently neutralized with CG50 H^+ cation exchange resin as described in the text. The products of alkaline hydrolysis (9.5 ml sample volume) were chromatographed on a Dowex-1-acetate (8% cross linked) column (1.1 cm × 40 cm) as described in the text. After sample application, the column was washed with 10 ml of 7% 1-propanol and then eluted with a gradient beginning with 0.25 M potassium acetate in 7% (v/v) 1-propanol (adjusted to pH 6.4 with acetic acid) and ending with 0.5 M potassium acetate in 7% 1-propanol at pH 6.4 in a total volume of 500 ml. Fractions (5 ml) were collected at a rate of four per hour and were assayed for phosphomonoester content using the alkaline phosphatase method as described in the text. The figure indicates the region of the chromatogram in which α- and β-phosphoglycerol were eluted.

phosphoglycerol are expected [5]. It should be noted, however, that no isomerization between *sn*-1 and *sn*-3 forms of α-phosphoglycerol will occur under such conditions. The products of alkaline hydrolysis were chromatographed on a Dowex-1-acetate column (Fig. 2) under conditions previously shown to separate the α- and β-forms of phosphoglycerol [5]. The recovery of α-phosphoglycerol was 42% and that of β-phosphoglycerol was 58% of the total phosphorus of anionic glucan F2, in good agreement with expected yields [5]. Fractions containing the α- and β-forms of phosphoglycerol were separately pooled, passed over columns of Amberlite IR 120 H^+ in order to remove potassium, concentrated by rotary evaporation, and neutralized with dilute ammonia [5]. The content of *sn*-glycero-3-phosphate was

TABLE III

ANALYSIS OF α- AND β-PHOSPHOGLYCEROL OBTAINED BY ALKALINE HYDROLYSIS OF ANIONIC GLUCAN F2.

A sample of anionic glucan F2, containing 2.8 μmole total phosphoglycerol, was subjected to alkaline hydrolysis as described in the text. The α- and β-phosphoglycerol products released by alkaline hydrolysis were separated by chromatography on Dowex-1-acetate as shown in Fig. 2. Fractions containing α-phosphoglycerol and β-phosphoglycerol were separately pooled and assayed for total phosphomonoester by the alkaline phosphatase method as described in the text. Samples were then assayed for *sn*-3-phosphoglycerol by the dehydrogenase method both before and after acid racemization as described in Materials and Methods.

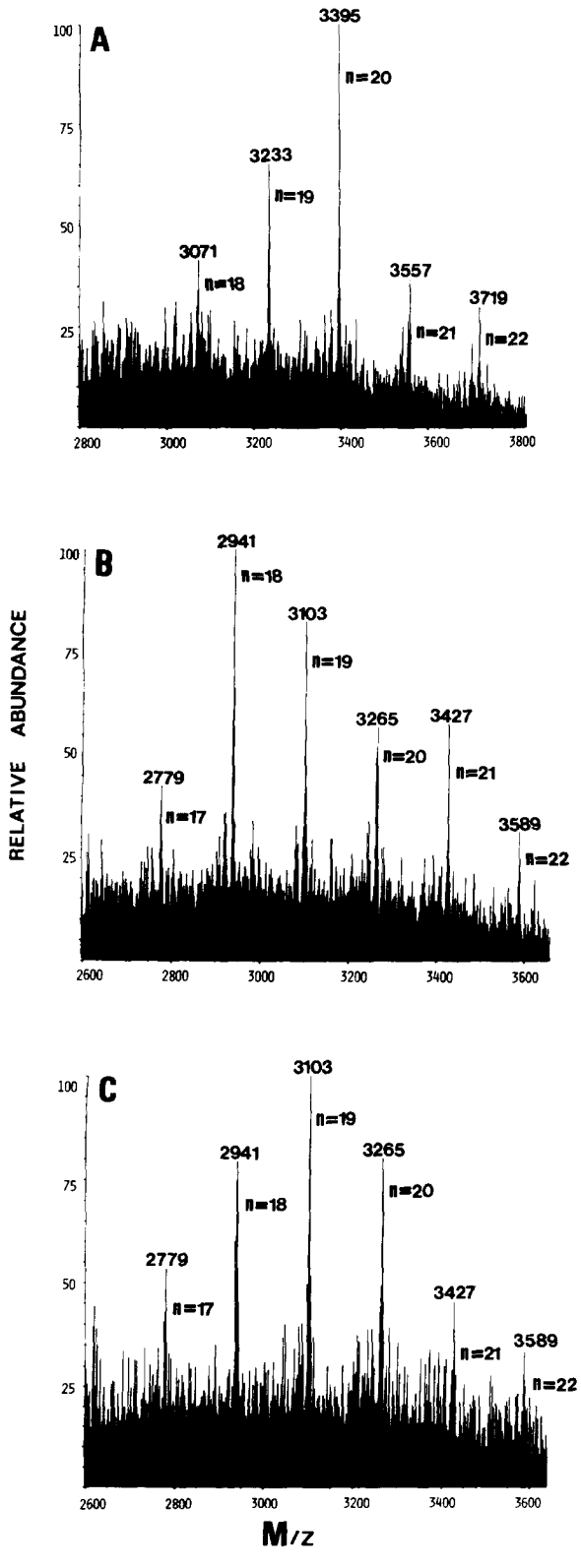
Constituent (nmol)	α-Phospho-glycerol	β-Phospho-glycerol
Total phosphomonoester	1072	1458
sn-3-Phosphoglycerol before acid racemization	0	0
sn-3-Phosphoglycerol after acid racemization	463	681

then determined by the glycerol-3-phosphate dehydrogenase method both before and after acid isomerization [5]. Acid isomerization by treatment with 1 M HCl at 100 °C for 1 h should convert both α- and β-phosphoglycerol into a mixture of *sn*-1-phosphoglycerol (45.5%), *sn*-3-phosphoglycerol (45.5%), and *sn*-2-phosphoglycerol (9.0%) as shown by Baer and Kates [15]. As Table III indicates, no *sn*-3-phosphoglycerol was detected in either the α- or β-fractions prior to isomerization. Acid isomerization, however, resulted in the generation of 43% and 47% *sn*-3-phosphoglycerol, respectively, from the α- and β-phosphoglycerol samples. The phosphoglycerol residues present as phosphodiesters in anionic glucan F2 are, thus, of the *sn*-1-phosphoglycerol configuration.

The anionic glucans possess a cyclic (1 → 2)-linked backbone with a size distribution identical to that of the neutral cyclic β-1,2-glucans

Previous analysis of the neutral cyclic β-1,2-glucans produced by *A. tumefaciens* using fast atom bombardment mass spectrometry established that these glucans were comprised of cyclic species containing 17 to 23 glucose residues per molecule [3]. This result was in good agreement with previous determinations of the size distribution of the cyclic β-1,2-glucans produced by species of both *Agrobacterium* and *Rhizobium* [13,16–18]. When a sample of anionic glucan F2 was examined directly by the fast atom bombardment technique (Fig. 3A), the molecular ion distribution was comparable to that of the neutral cyclic β-1,2-glucans (Refs. 3, 19, and Fig. 3C), yet shifted to higher mass by 154 Da (it should be noted that the molecular ion profile of Fig. 3A was obtained in the negative ion mode $[M-H]^-$, whereas the molecular ion profiles of Figs. 3B and 3C were obtained in the positive ion mode $[M+Na]^+$). This shift in mass is exactly that expected to result from the addition of one phosphoglycerol substituent per cyclic oligosaccharide backbone. A sample of anionic glucan F2 was, therefore, subjected to conditions of alkaline hydrolysis to remove phosphoglycerol substituents, and the products were directly examined by the fast atom bombardment technique. As shown in Fig. 3B, the molecular ion profile of the products of anionic glucan F2 was found to be identical to that of the neutral cyclic β-1,2-glucans (Fig. 3C). In addition, when the products of alkaline hydrolysis of anionic glucan F2 were first permethylated and then examined by the fast atom bombardment technique (data not shown), the molecular ion distribution was found to be identical to that of the permethyl-

Fig. 3. Fast atom bombardment mass spectrometry of anionic glucan F2. The molecular ion cluster for each of the oligosaccharide molecular species represents an average weight distribution ionized by either proton extraction $[M-H]^-$ or sodium addition $[M+Na]^+$. The calculated mass within each cluster exceeds the monoisotopic values by greater than one dalton at this mass because of the natural abundance. The symbol "*n*" refers to the number of glucose residues in each molecular ion species. (A) Negative ion f.a.b.-mass spectrum of anionic glucan F2, $[M-H]^-$. Prior to analysis, the sample of anionic glucan F2 was desalted by chromatography on a Sephadex G25 column (5 ml bed volume) eluted with 7% (v/v) 1-propanol. (B) Positive ion f.a.b.-mass spectrum of anionic glucan F2 after removal of phosphoglycerol substituents, $[M+Na]^+$. The sample of anionic glucan F2 was subjected to alkaline hydrolysis using 0.5 M NaOH at 100 °C for 80 min (as described in the text) and desalted on a Sephadex G-25 column prior to analysis. (C) Positive ion f.a.b.-mass spectrum of neutral cyclic β-1,2-glucan, $[M+Na]^+$. Cyclic β-1,2-glucan was prepared from cells of *Agrobacterium tumefaciens* C58 as described in Ref. 3.



ated neutral cyclic β-1,2-glucans as previously determined [3,19]. The results of the mass spectrometry analysis demonstrate that anionic glucan F2 possesses a cyclic backbone of the same size distribution as that of the neutral cyclic β-1,2-glucans. Furthermore, linkage analysis of the permethylated anionic glucan F2 revealed 2-*O*-acetyl-1,5-anhydro-3,4,6-tri-*O*-methyl-D-glucitol, indicative of a (1 → 2)-linked glucose backbone. We conclude that the oligosaccharide moieties of anionic glucan F2 are indistinguishable from the previously characterized neutral cyclic β-1,2-glucans.

Discussion

In the present study, we have demonstrated that the periplasmic cyclic β-1,2-glucans produced by *Agrobacterium tumefaciens* include anionic derivatives with one or more *sn*-1-phosphoglycerol residues in phosphodiester linkage. In the accompanying paper by Batley and co-workers [20], it is demonstrated that species of *Rhizobium* also produce similar cyclic β-1,2-glucans with *sn*-1-phosphoglycerol substituents. The presence of *sn*-1-phosphoglycerol residues is a further striking similarity of these periplasmic oligosaccharides to the membrane-derived oligosaccharides of *E. coli.* In the case of the membrane-derived oligosaccharides of *E. coli,* the *sn*-1-phosphoglycerol residues are derived from the hydrophilic head group of phosphatidylglycerol [6,7]. It is likely that phosphatidylglycerol is also the source of the *sn*-1-phosphoglycerol substituents on the cyclic β-1,2-glucans of *Agrobacterium* and *Rhizobium.* The membrane-derived oligosaccharides of *E. coli* also contain phosphoethanolamine residues that are derived from phosphatidylethanolamine [21]; such phosphoethanolamine residues have not yet been detected in the cyclic glucans of *Agrobacterium* and *Rhizobium.*

The anionic substituents of the cyclic glucans and membrane-derived oligosaccharides must augment the contribution of these oligosaccharides to the osmotic strength of the periplasmic compartment by binding cations. Further, they must contribute to the Donnan potential shown to exist across the outer membrane in *E. coli* [22] and presumed also to be present in *Rhizobium* and

Agrobacterium. The fundamental reason why such osmotic regulation of the periplasmic compartment is advantageous to the organism remains obscure. No remarkable phenotypic consequences have as yet been detected in strains of *E. coli* blocked in the production of membrane-derived oligosaccharides [23].

In addition to a possible role for the cyclic glucans in the osmotic adaptation of *Agrobacterium* and *Rhizobium*, it should be noted that other investigators have proposed that the cyclic β-1,2-glucans may contribute to the efficiency of the plant infection process [24–26]. Abe and coworkers [24,25] reported that cyclic β-1,2-glucans may enhance formation of infection threads and increase numbers of nodules in the *Rhizobium trifolii*-white clover symbiosis. Avirulent mutants of *Agrobacterium tumefaciens*, which map to the chromosomal locus *chvB*, were found by Puvanesarajah et al. [26], to be defective in the synthesis of cyclic β-1,2-glucans. When these mutants were restored to virulence by complementation with cloned DNA, the ability to produce the cyclic β-1,2-glucans was also restored. It was concluded that the cyclic glucans may be important for the pathogenic properties of *A. tumefaciens*. Now that it has been demonstrated that the cyclic glucans may become modified with substituents such as *sn*-1-phosphoglycerol, it will be of interest to examine the possible contribution of such substituents to the plant infection process.

Acknowledgements

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