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Cyclic (1 → 2)-β-D-glucans (cyclosophorans) produced by *Agrobacterium* and *Rhizobium* species

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ABSTRACT

Neutral and acidic cyclic (1 → 2)-β-D-glucans (cyclosophorans), obtained from culture filtrates and cells of *Agrobacterium* and *Rhizobium*, are synthesised on the cell surface and then secreted. Eight cyclosophorans with dp 17–24 were isolated; all of the strains of *Agrobacterium* showed almost the same distribution pattern, whereas there were three other distribution patterns for the strains of *Rhizobium*.

INTRODUCTION

Agrobacterium species are phytopathogenic towards many dicotyledonous plants, and *Rhizobium* species can form nodules on, and fix nitrogen in, the roots of leguminous plants with host-selective symbiosis. These bacterial genera are closely related and secrete similar saccharides. *Agrobacterium* and *R. meliloti* secrete polysaccharides that contain D-glucose and D-galactose[1–3], and *R. phaseoli*, *R. trifolii*, and *R. leguminosarum* secrete polysaccharides that contain D-glucose, D-galactose, and D-glucuronic acid[1,4,5]. Another characteristic is the production of cyclic (1 → 2)-β-D-glucans, first found in culture filtrates of a crown-gall organism by McIntire et al.[6] and called crown-gall polysaccharide[7]. Strains of both *Agrobacterium* and *Rhizobium* produce cyclic (1 → 2)-β-D-glucans[8–10] (cyclosophorans). The structures of the cyclic (1 → 2)-β-D-glucans produced by *Agrobacterium* and *Rhizobium* are now reported and acidic cyclosophorans are described.

EXPERIMENTAL

General methods.—PC of the (1 → 2)-β-D-glucans was conducted on Toyo No. 50 paper by the descending method, using 1:1:1 1-butanol–pyridine–water and detection with the sodium metaperiodate–silver nitrate reagent[11].

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HPLC of the cyclic (1 → 2)-β-D-glucans was performed on columns of μBondapak carbohydrate (Waters Associates), using 16 : 9 acetonitrile–water at 2.0 mL/min, or Hibar LiChrosorb RP-18 (Merck), using 1 : 19 MeOH–water at 1.0 mL/min. The elutions were monitored with a refractive index detector.

Cyclic (1 → 2)-β-D-glucan (2.5 mg) was hydrolysed in 0.1 M trifluoroacetic acid (1 mL) at 100° for 60–150 min. HPLC of the partial hydrolysate was effected on a column of Finepak SIL NH_2-10 (Japan Spectroscopy, Tokyo), using 11 : 9 acetonitrile–water at 1.0 mL/min.

^{13}C-NMR spectra (50.10 MHz) of solutions (2–5%) in D_2O were recorded at room temperature in the pulsed Fourier-transform mode with complete proton-decoupling. Chemical shifts are expressed in ppm downfield from that of Me_4Si, using 1,4-dioxane (67.40 ppm) as the internal standard.

Organisms.—Agrobacterium radiobacter IFO 12607, IFO 12664, IFO 12665, IFO 13127, IFO 13256, IFO 13532, and IFO 13533, *A. rhizogenes* IFO 13259, *A. tumefaciens* IFO 3058, *Rhizobium meliloti* IFO 13336, and *R. trifolii* IFO 13337 were obtained from the Institute for Fermentation (Osaka). *A. radiobacter* IFO 12665b and IFO 13127b, which were spontaneous mutants that form large amounts of curdlan, were isolated as blue colonies on Aniline Blue plates[12]. *A. radiobacter* A1-5 strain[13], a mutant which showed a high production of cyclic (1 → 2)-β-D-glucans, was obtained by treatment of *A. radiobacter* IFO 12665b with nitrosoguanidine. *R. meliloti* J7017 was obtained from Dr. Y. Maruyama, *R. trifolii* 4S, *R. phaseoli* AHU 1133, *R. trifolii* AHU 1134, and *R. lupini* KLU from Dr. S. Higashi, and *R. leguminosarum* 303 from Dr. S. Tsuru.

Preparation of (1 → 2)-β-D-glucan, the octasaccharide repeating unit of the acidic polysaccharide, the acidic polysaccharide, and (1 → 3)-β-D-glucan (curdlan).—Synthetic medium (pH 7.0) contained 4 g of D-glucose, 150 mg of $(NH_4)_2HPO_4$, 100 mg of KH_2PO_4, 50 mg of $MgSO_4 \cdot 7H_2O$, 1 mg each of NaCl, $CaCl_2$, $MnCl_2 \cdot 4H_2O$, and $FeCl_3 \cdot 6H_2O$, 7 μg of $ZnCl_2 \cdot 7H_2O$, 5 μg of $CuSO_4 \cdot 5H_2O$, 2 μg of $Na_2MoO_4 \cdot 2H_2O$, 1 μg of H_3BO_3, 20 μg of thiamine, 2 μg of biotin, and 0.5 g of $CaCO_3$ per 100 mL. The cultures were shaken reciprocally at 120 strokes/min at 30° for 6 days, then centrifuged at 56 000*g* for 30 min. Each supernatant solution was mixed with EtOH (2 vol) and centrifuged at 10 000*g* for 30 min in order to precipitate the acidic polysaccharides. The supernatant solution was concentrated to a small volume, diluted with ethanol (2 vol), and centrifuged. The supernatant solution was diluted with EtOH (4 vol) and centrifuged at 10 000*g* for 30 min in order to precipitate the low molecular weight fraction that contained cyclic (1 → 2)-β-D-glucan and acidic oligosaccharide. The salts were removed on a column of Sephadex G-10 and the saccharides were applied to a column (3 × 12 cm) of DEAE-cellulose equilibrated with mM KCl. The (1 → 2)-β-D-glucan was eluted with mM KCl and the acidic oligosaccharides were eluted with a linear gradient of 1–100 mM KCl. The precipitates that contained cells and $CaCO_3$ were treated with M HCl to remove $CaCO_3$. For *Agrobacterium*, the cells were homogenised with the appropriate amounts of 0.5 M NaOH and centrifuged at

37000*g* for 30 min. The supernatant solution was neutralised with 2 M HCl in order to precipitate the water-insoluble (1 → 3)-β-D-glucan (curdlan).

RESULTS

Isolation of (1 → 2)-β-D-glucan.—Methylation analysis of the low molecular weight fraction from *A. radiobacter* IFO 12664 gave several minor peaks of methylated sugars (Fig. 1) in addition to a large peak for acetylated 3,4,6-tri-*O*-methyl-D-glucitol indicative of (1 → 2)-linked residues. A study[14] of the repeating unit of the extracellular water-soluble polysaccharide which accumulated in cultures indicated that the products in the minor peaks in Fig. 1 were derived from an acidic oligosaccharide. The (1 → 2)-β-D-glucan and the acidic oligosaccharide were separated on a column of DEAE-cellulose (Fig. 2). Methylation analyses of the products in peaks **1**–**4** (Table I) indicated that **1** contained a (1 → 2)-β-D-glucan, **3** and **4** contained octasaccharide repeating units composed of D-glucose, D-galactose, pyruvic acid, and succinic acid[15], and **2** was a mixture of the octasaccharide containing one mol of pyruvic acid and acidic cyclosophorans[16].

Cyclic (1 → 2)-β-D-glucans.—PC (Fig. 3) showed that the (1 → 2)-β-D-glucans produced by eleven strains of *Agrobacterium* and eight strains of *Rhizobium* could be classified on the basis of four kinds of separation pattern. Thus, those of all of the strains of *Agrobacterium* gave almost the same pattern (2 in Fig. 3) and those of *Rhizobium* gave three other patterns (1, 3, and 4). Preparative PC was used to isolate component **a** from the (1 → 2)-β-D-glucan of *R. trifolii* AHU 1134, and components **b** and **c** from that of *A. radiobacter* IFO 12665 (see Fig. 3). The components **a**–**c** were purified by rechromatography and their ^{13}C NMR spectra, together with that of the native (1 → 2)-β-D-glucan of *A. radiobacter* IFO 12665, are shown in Fig. 4. The spectrum of **a** (Fig. 4A) contained only six signals indicative of a homogeneous cyclic glucan. However, that of **b** (Fig. 4B) contained two signals for C-1 and C-2, and that of **c** (Fig. 4C) contained two signals for C-2,

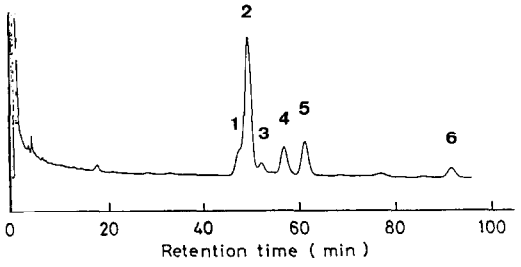

Fig. 1. GLC of methylated sugars derived from the low molecular weight fraction of *A. radiobacter* IFO 12664 on a column of 0.3% of OV275–0.4% of GEXF1150: **1**, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol; **2**, 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-glucitol; **3**, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol; **4**, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol; **5**, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol; and **6**, 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol.

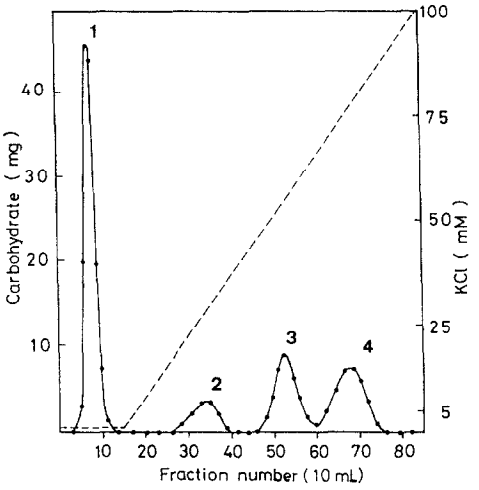

Fig. 2. Chromatography on DEAE-cellulose of the low molecular weight fraction of *A. radiobacter* IFO 12664: a sample (330 mg) was applied to a column (3×12 cm) equilibrated with mM KCl, and eluted with mM KCl (150 mL) and then with a linear gradient (700 mL) of 1–100 mM KCl (- - - - - -). Fractions (10 mL) were analysed for carbohydrate by the phenol–sulfuric acid method: **1**, neutral cyclosophoran; **2**, a mixture of acidic cyclosophoran and acidic octasaccharide containing one mol of pyruvic acid; **3**, acidic octasaccharide containing one mol of pyruvic acid and one mol of succinic acid; **4**, acidic octasaccharide containing one mol of pyruvic acid and two mol of succinic acid.

indicative of mixtures. The spectrum (Fig. 4D) of the native (1 → 2)-β-D-glucan also indicated a mixture.

HPLC (Fig. 5) of the native (1 → 2)-β-D-glucan revealed eight components corresponding to cyclo-oligosaccharides with dp 17–24, respectively. The dp values were obtained by HPLC of partial hydrolysates on Finepak SIL NH_2-10. The elution profiles of the partial hydrolysates of the smallest (**1** in Fig. 5) and the largest cyclosophoran (**8** in Fig. 5) shown in Fig. 6 are representative. The last peaks could be recognised as corresponding to dp 17 and 24, respectively, when counted from that of D-glucose. From these results, the dp of the eight cyclosophorans were determined unambiguously as 17–24, respectively[9,17]. Cyclomaltooctaose was eluted before malto-octaose in HPLC with amino columns[18].

TABLE I

Methylation analysis of **1–4** (Fig. 2) obtained by chromatography on DEAE-cellulose of the low molecular weight fraction from *A. radiobacter* IFO 12664

Product	Methylated sugar (mol ratio)						
	2,3,4,6-Glc	2,4,6-Glc	3,4,6-Glc	2,4,6-Gal	2,3,4-Glc	2,3,6-Glc	2,3-Glc
1	0	0	100	0	0	0	0
2	0	19.4	23.2	9.7	16.9	21.4	9.4
3	0	25.4	0	11.6	23.1	27.4	12.5
4	0	23.5	0	11.4	23.8	28.8	12.5

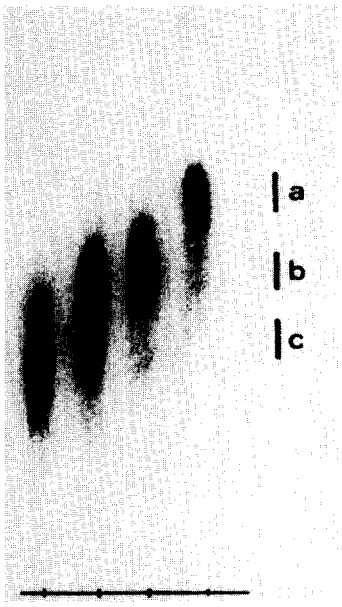

Fig. 3. PC of (1 → 2)-β-D-glucans: **1**, *R. meliloti* J7017 and *R. meliloti* IFO 13336; **2**, all strains of *Agrobacterium*; **3**, *R. trifolii* IFO 13337, *R. trifolii* 4S, and *R. leguminosarum* 303; **4**, *R. trifolii* AHU 1134 and *R. phaseoli* AHU 1133. The solid bars indicate the section extracted with water to give the products **a**–**c**.

Likewise, the cyclic (1 → 2)-β-D-glucans were eluted before the corresponding linear oligosaccharides in the partial hydrolysates. The positive-ion FAB-mass spectra[10] of the methylated cyclic (1 → 2)-β-D-glucans **1**–**8** (Fig. 5) contained signals that indicated dp of 17–24, respectively. The ^{13}C-NMR spectra of (1 → 2)-β-D-glucans of *A. tumefaciens* and *A. radiobacter* contained[19] major and minor signals for C-1 and C-2 (Table II), and the differences in the chemical shifts for the eight cyclosophorans may reflect conformational restraints arising from the cyclic structures.

Time course of the production of cyclic (1 → 2)-β-D-glucans.—The time course of the formation of extracellular saccharides by *A. radiobacter* IFO 12664, which produces no curdlan, is shown in Fig. 7. Secretion of the acidic polysaccharide increased markedly in the middle logarithmic phase; however, the production of cyclic (1 → 2)-β-D-glucans and the octasaccharide repeating unit increased in the late logarithmic and stationary phases. These results suggested that these low molecular weight materials were synthesised on the cell surface (or in the periplasmic space) and then secreted.

Cell-surface cyclic (1 → 2)-β-D-glucans.—(1 → 2)-β-D-glucans were also obtained from the surface of *Agrobacterium* and *Rhizobium* cells by sucrose-osmotic-shock

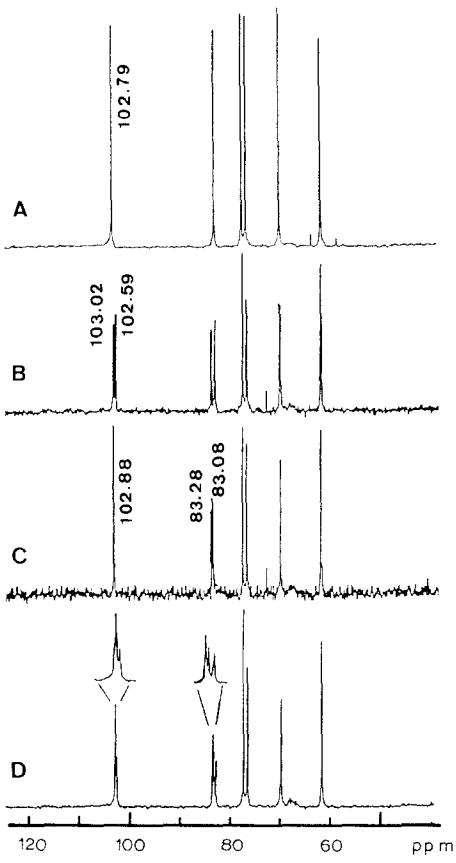

Fig. 4. ^{13}C-NMR spectra of the purified components from Fig. 3, namely, of **a** (A), **b** (B), and **c** (C), and the (1 → 2)-β-D-glucan of *A. radiobacter* IFO 12664 (D).

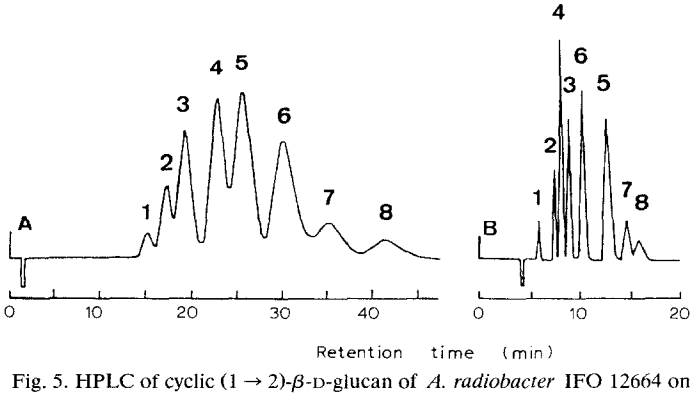

Fig. 5. HPLC of cyclic (1 → 2)-β-D-glucan of *A. radiobacter* IFO 12664 on A, μBondapak carbohydrate; and B, Hibar LiChrosorb RP-18; peaks **1–8** correspond to components with dp 17–24, respectively.

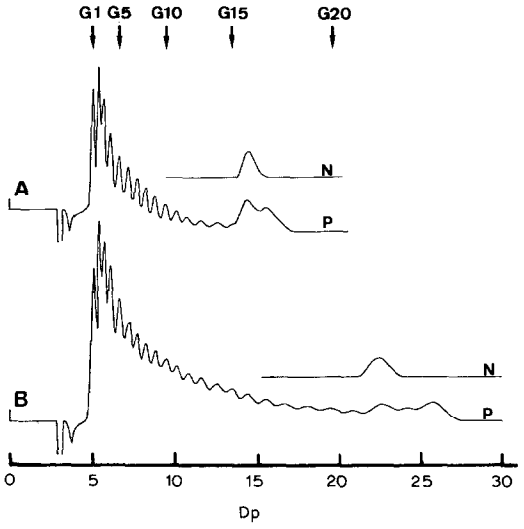

Fig. 6. HPLC of the smallest (A) and the largest (B) cyclosophorans (N) isolated, and their partial hydrolysates (P), on Finepak SIL NH_2-10: G1 indicates the position of D-glucose; and G5–G20, those of the respective D-gluco-oligosaccharides.

treatment[20] or extraction with hot aqueous 75% ethanol[21]. Strains that produce no octasaccharide were tested for the production of cell-surface cyclic (1 → 2)-β-D-glucans (see Table III). It was found that the proportions of cell-surface and extracellular cyclic (1 → 2)-β-D-glucans were similar.

Substituted cyclic (1 → 2)-β-D-glucans.—As the mutant A1–5 did not produce acidic polysaccharide and oligosaccharides, the greater part of the low molecular weight fraction was thought to be cyclic (1 → 2)-β-D-glucan; however, ~ 10% of total saccharides was always lost on the DEAE-cellulose used to remove yellow pigments. After eluting the cyclic (1 → 2)-β-D-glucan from the column with water or mM KCl, elution with 100 mM KCl yielded material, methylation analysis of which gave only 3,4,6-tri-*O*-methyl-D-glucose but which also gave a positive colori-

TABLE II

^{13}C-NMR chemical shifts for solutions of cyclosophorans in D_2O

Dp	Mol wt [a]	C-1	C-2	C-3	C-4	C-5	C-6
17	2755.7	102.79	82.64	76.28	69.71	77.18	61.50
18	2917.8	102.50	82.76	76.31	69.74	77.06	61.56
19	3079.9	103.02	83.34	76.28	69.56	77.18	61.53
20	3242.0	102.59	82.61	76.39	69.79	77.15	61.59
21	3404.1	102.88	83.28	76.31	69.62	77.15	61.56
22	3566.2	102.88	83.08	76.36	69.68	77.18	61.59
23	3728.3	102.67	83.05	76.33	69.68	77.12	61.59
24	3890.4	102.99	83.40	76.33	69.62	77.18	61.59

[a] 162.1 × dp.

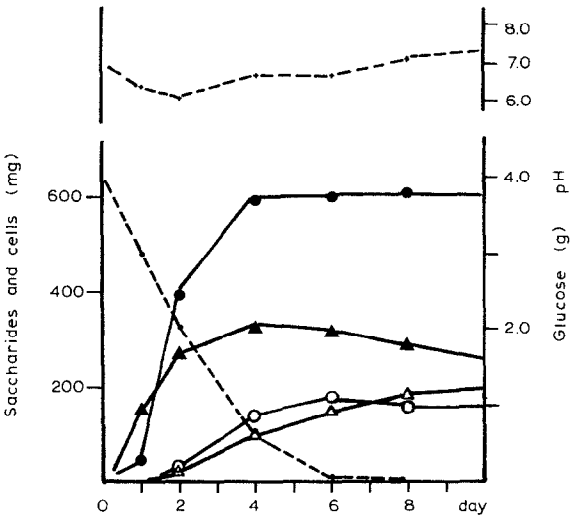

Fig. 7. Time course of the production of cyclic (1 → 2)-β-D-glucan (○———○), octasaccharide repeating unit (△———△), acidic polysaccharide (●———●), and cells (▲———▲) in the culture (100 mL) of *A. radiobacter* IFO 12664: glucose, •– – –•; pH, + – – –+.

TABLE III

Production of cyclic (1 → 2)-β-D-glucan, octasaccharide repeating unit of acidic polysaccharide, acidic polysaccharide, and curdlan by *Agrobacterium* and *Rhizobium*

Strain	Cyclic (1 → 2)-β-D-glucans		Octasaccharide repeating-unit (mg/100 mL)	Acidic polysaccharide (mg/100 mL)	Curdlan (mg/100 mL)
	EC [a] (AC) [b] (mg/100 mL)	CS [c] (AC) [b] (mg/100 mL)			
A. radiobacter					
IFO 12607	198		0	72	510
IFO 12664	176		155	610	0
IFO 12665	190		73	670	14
IFO 12665b	263 (25)	170 (19)	0	9	1100
IFO 13127	229		98	400	80
IFO 13127b	175 (22)	146 (20)	0	72	790
IFO 13256	102		64	133	128
IFO 13532	6		11	315	0
IFO 13533	6		12	1200	0
A. rhizogenes					
IFO 13259	255		0	122	480
A. tumefaciens					
IFO 3058	58		11	178	0
R. meliloti J7017	40		43	220	0
R. meliloti IFO 13336	11		123	840	0
R. trifolii IFO 13337	33		0	150	0
R. trifolii 4S	34 (3.5)	12 (1.0)	0	240	0
R. trifolii AHU 1134	25		155	560	0
R. leguminosarum 303	15		0	220	0
R. phaseoli AHU 1133	35 (2.8)	12 (1.0)	0	270	0
R. lupini KLU	12		53	800	0

[a] EC, extracellular cyclic (1 → 2)-β-D-glucan; [b] AC, acidic cyclic (1 → 2)-β-D-glucan; [c] CS, cell-surface cyclic (1 → 2)-β-D-glucan.

metric assay for acyl groups[22]. Deacylation of the material at pH 12 gave products with an elution profile in HPLC almost the same as that of the cyclic (1 → 2)-β-D-glucan. The acyl groups in the acidic cyclosophorans were half esters of methylmalonic acid and/or succinic acid[16]. Some acidic cyclosophorans were also obtained (see Table III). Cyclic (1 → 2)-β-D-glucans containing phosphoglycerol residues have been reported[23,24].

Production of saccharides by Agrobacterium *and* Rhizobium.—The production of cyclic (1 → 2)-β-D-glucans, the octasaccharide repeating unit of the acidic polysaccharide, the acidic polysaccharide, and (1 → 3)-β-D-glucan (curdlan) of eleven strains of *Agrobacterium* and eight strains of *Rhizobium* is summarised in Table III.

DISCUSSION

Some strains of *Acetobacter*[25] and *Xanthomonas*[26] produce linear sophoro-oligosaccharides with 6–42 D-glucose residues and a cyclic (1 → 2)-β-D-glucan of dp 16 with one 6-linked D-glucosyl residue, and linear sophoro-oligosaccharides with dp 8–20, respectively. *Escherichia coli*[26,27] and *Klebsiella pneumoniae*[26] produce branched sophoro-oligosaccharides. On the other hand, the labeled cyclic (1 → 2)-β-D-glucans synthesised from UDP-D-[^{14}C]glucose by cell-free extracts of *A. radiobacter* IFO 12665b and *R. phaseoli* AHU 1133 were mainly cyclosophoro-oligosaccharides with dp 18–20 and dp 17, respectively, and no sophoro-oligosaccharide with dp < 17 could be detected[28]. Thus, *Agrobacterium* and *Rhizobium* appear to produce only cyclic (1 → 2)-β-D-glucans as cell-surface (or periplasmic) oligosaccharides.

^{13}C-NMR spectroscopy showed one resonance for each carbon in a pure cyclosophoran, but large differences in the chemical shifts of the C-1 and C-2 signals within the series. Hence, since cyclosophorans are flexible molecules, each must have its own particular conformation, depending on the dp. The chemical shifts of the resonances of C-4 of maltotriose and amylose or of cyclomaltopentaose and cyclomaltoheptaose with fixed conformations are similar[29]. It was estimated that the inside diameters (approximately maximum values) and the depth of cyclosophorans were 10–15 and 15 Å, respectively.

As cyclic (1 → 2)-β-D-glucans are produced by legume-symbiotic *Rhizobium* and phytopathogenic *Agrobacterium*, their biological function is of interest in the context of the interaction of plants and bacteria. Cyclic (1 → 2)-β-D-glucan, prepared from *R. trifolii* 4S by sucrose-osmotic-shock treatment, promoted infection threads and nodule formation in the root hair of white clover, and cyclomalto-octaose promoted nodule formation[20]. Several avirulent mutants of *A. tumefaciens* with a reduced ability to attach to plants did not produce cyclic (1 → 2)-β-D-glucan[30], and mutants of *R. meliloti*, which formed small, empty nodules on alfalfa roots, were defective in the production of cyclic (1 → 2)-β-D-glucan[31]. Phytopathogenic *Xanthomonas* species also produce cyclic β-glucans[25]. Thus, the

macrocyclic structure may be necessary for the biological effects of these compounds in plants. On the other hand, the biosynthesis of cyclic (1 → 2)-β-D-glucans of *Agrobacterium* and *Rhizobium* is regulated by osmotic pressure[32,33], as with the membrane-derived oligosaccharide of *E. coli*[34].

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