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Effect of Phosphate Limitation on Synthesis of Periplasmic Cyclic β-(1,2)-Glucans

MICHAËL W. BREEDVELD,[1]† ALAN J. BENESI,[2] MARIA L. MARCO,[1] AND KAREN J. MILLER[1,3,4]*

Departments of Food Science[1] and Chemistry[2] and Graduate Programs in Plant Physiology[3] and Genetics,[4] The Pennsylvania State University, University Park, Pennsylvania 16802

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Rhizobium meliloti **and** ***Agrobacterium tumefaciens*** **synthesize periplasmic cyclic β-(1,2)-glucans during adaptation to hypoosmotic environments. It also appears that these glucans provide important functions during the interactions of these bacteria with plant hosts. A large fraction of these glucans may become modified with anionic substituents such as phosphoglycerol or succinic acid; however, the role(s) of these substituents is unknown. In this study, we show that growth of these bacteria in phosphate-limited media leads to a dramatic reduction in the levels of phosphoglycerol substituents present on the periplasmic cyclic β-(1,2)-glucans. Under these growth conditions,** ***R. meliloti*** **1021 was found to synthesize anionic cyclic β-(1,2)-glucans containing only succinic acid substituents. Similar results were obtained with** ***R. meliloti*** **7154 (an** ***exoH*** **mutant which lacks the ability to succinylate its high-molecular-weight exopolysaccharide), revealing that succinylation of the cyclic β-(1,2)-glucans is mediated by an enzyme system distinct from that involved in the succinylation of exopolysaccharide. In contrast, when** ***A. tumefaciens*** **C58 was grown in a phosphate-limited medium, it was found to synthesize only neutral cyclic β-(1,2)-glucans.**

A variety of gram-negative bacteria have been shown to synthesize low-molecular-weight periplasmic glucans (1, 30; see reference 6 for a review). In *Escherichia coli*, these glucans are called membrane-derived oligosaccharides, and their structure consists of a linear β-(1,2)-linked backbone with β-(1,6)-linked branches (27). In *Agrobacterium* and *Rhizobium* species, cyclic β-glucans containing glucose residues linked solely by β-(1,2)-glycosidic bonds are synthesized (6). In addition to their periplasmic localization, the membrane-derived oligosaccharides and the cyclic β-(1,2)-glucans have been shown to share several properties: (i) a β-(1,2)-linked backbone, (ii) intermediate size, (iii) osmoregulated biosynthesis (with the highest levels of synthesis occurring during growth at low osmolarity), and (iv) the presence of nonsugar substituents (6, 26–28).

It appears that the membrane-derived oligosaccharides of *E. coli* and the cyclic β-(1,2)-glucans of *Agrobacterium tumefaciens* and *Rhizobium meliloti* are major periplasmic solutes when cells are grown in media of low osmotic strength (6, 27). Under these growth conditions, these glucans also become highly modified with anionic substituents such as *sn*-1-phosphoglycerol (derived from the head group of phosphatidylglycerol) and succinate (3, 22, 27, 33–35, 42). It is generally believed that the periplasmic β-glucans play an important role during hypoosmotic adaptation by functioning as fixed osmolytes in the periplasm (6, 26, 27). Anionic β-glucans would be expected to be the most effective form of periplasmic solute because the counterions of these glucans should also contribute to periplasmic osmolarity (6). However, the importance of the anionic substituents has not been established. In addition to their role(s) during hypoosmotic adaptation, the cyclic β-(1,2)-glucans of *Rhizobium* and *Agrobacterium* species are believed to provide functions during plant infection (15, 17). The precise roles of the cyclic β-glucans during the infection process have yet to be established; however, Swart et al. (40) have recently provided evidence that the osmoadaptive functions of these molecules are important during plant infection.

In addition to the osmotic strength of the growth medium, other cultural conditions, such as growth temperature and carbon source, have been shown to influence cyclic β-glucan synthesis in a variety of *Rhizobium* species (10, 18). In the present study, we have examined the effect of phosphate limitation on cyclic β-(1,2)-glucan synthesis in *R. meliloti* and *A. tumefaciens*. These studies were prompted by the finding that the majority of phospholipid turnover in these bacteria results from the transfer of phosphoglycerol substituents from phosphatidylglycerol to the cyclic β-(1,2)-glucans (33). The results of the present study reveal that both *R. meliloti* and *A. tumefaciens* continue to synthesize cyclic β-(1,2)-glucans during growth under severe phosphate limitation; however, phosphoglycerol substituents are no longer transferred to the cyclic β-(1,2)-glucan backbone. Although *R. meliloti* was found to compensate for the lack of phosphoglycerol substituents by synthesizing cyclic β-(1,2)-glucans containing elevated levels of succinyl substituents, *A. tumefaciens* was found to synthesize only neutral cyclic β-(1,2)-glucans under these growth conditions.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are described in Table 1. All strains were grown in standard or phosphate-limited medium. Standard medium (pH 7.0) contained 6.5 mM glutamic acid, 27 mM mannitol, 5.5 mM K_2HPO_4, 25 mM Tris, 10 mM KCl, 0.8 mM $MgSO_4$, 0.2 mM $CaCl_2$, and other mineral salts and vitamins as previously described (11). Phosphate-limited medium contained K_2HPO_4 at a concentration between 10 and 150 μM, with all other components identical to that in the standard medium. Phosphate-limited cultures were inoculated (1%, vol/vol) with precultures grown in medium containing 100 μM phosphate. Precultures were supplemented with 200 μg of streptomycin or 10 μg of neomycin per ml when appropriate. Cultures were grown at 30°C on a rotary shaker.

Measurement of growth, EPS production, and cellular glucan production. The effect of phosphate concentration on growth and polysaccharide production was determined 72 h after inoculation of cultures. Cells and culture supernatant were separated by centrifugation at 25,000 × *g* for 30 min. Cell pellets were resus-

* Corresponding author. Mailing address: 105 Borland Lab, Department of Food Science, The Pennsylvania State University, University Park, PA 16802. Phone: (814) 863-2954. Fax: (814) 863-6132. Electronic mail address: KMILLER@PSUPEN.PSU.EDU.

† Present address: Department of Microbiology, Wageningen Agricultural University, 6703 CT Wageningen, The Netherlands.

TABLE 1. Bacterial strains

Bacterial strain	Description	Reference
R. meliloti 1021	Derivative of wild-type strain SU-47; Str^r	33
R. meliloti 7154	*exoH*::Tn*5* mutant of strain 1021; Neo^r Str^r	29
A. tumefaciens C58	Wild type	34

pended in water to 20% of the original culture volume. Total cellular protein was determined according to Lowry et al. (31) after pretreatment of cell suspensions with 1 M NaOH at 100°C for 30 min. For quantification of cellular cyclic glucans, cell suspensions were extracted with 75% ethanol for 30 min at 70°C; this was followed by centrifugation. The hexose content of the alcoholic supernatant (as determined by the phenol-sulfuric acid method [13]) could be taken as a measure of the cellular cyclic glucan content under the low-osmotic-strength growth conditions employed (11). Total extracellular carbohydrates in the culture supernatant were assayed by the phenol-sulfuric acid method. High-molecular-weight exopolysaccharide (EPS) was precipitated with 3 volumes of ethanol, as previously described (11). For large-scale isolation of EPS, culture supernatants were concentrated to 20% of their original volumes prior to precipitation with ethanol.

Large-scale isolation of cell-associated cyclic β-(1,2)-glucans. Precultures (5 ml) were inoculated into 500 ml of standard medium or phosphate-limited medium, and cells were grown to an optical density at 650 nm of 0.8 to 1.2. Cells were then separated from the culture supernatant by centrifugation at 4°C for 25 min at 20,000 × *g*. Cell pellets were extracted with 75% ethanol (total volume, 15 ml) at 70°C for 30 min. After centrifugation, the alcoholic supernatant was concentrated under vacuum and then chromatographed on a Sephadex G-50 column as previously described (33). Column fractions containing cyclic β-(1,2)-glucans were pooled, concentrated, and desalted, using a Sephadex G-15 column (33). Finally, neutral and anionic cyclic β-(1,2)-glucan subfractions were separated by chromatography on DEAE-cellulose, using a gradient of 0 to 300 mM KCl in 10 mM Tris-HCl (pH 8.4) containing 7% (vol/vol) propanol (33).

TLC. Cultures (5 ml) were grown for 48 h in standard or phosphate-limited medium. After centrifugation at 3,500 × *g* for 10 min, cells were resuspended in 1 ml of water and recentrifuged. Cell pellets were extracted with 200 μl of 75% ethanol (vol/vol) at 70°C for 30 min. After centrifugation, the alcoholic supernatant was collected and concentrated under vacuum. Finally, the residue was dissolved in 20 μl of water. Aliquots (5 μl) were spotted onto aluminum-backed silica gel 60 thin-layer chromatography (TLC) plates (EM Industries, Gibbstown, N.J.) and were chromatographed with ethanol-butanol-water (5:5:4). For visualization of the cellular glucans, the TLC plates were sprayed with 5% H_2SO_4 in methanol and heated at 120°C for 10 min. Some samples were pretreated with 0.1 M NaOH at 37°C for 30 min prior to spotting onto TLC plates in order to selectively remove succinic acid substituents.

Viscosity measurements. The relative viscosity of culture supernatants was measured at 25°C, using a Rheometrics Fluid Spectrometer (model RFS II; Rheometrics, Piscataway, N.J.) with a shear rate of 10 s^{-1} and a concentric cylinder geometry (16.5-mm internal diameter, 17.0-mm external diameter, and 13.0-mm length).

Compositional analysis. The succinate content of cyclic β-(1,2)-glucan and EPS preparations was determined enzymatically by the succinyl-coenzyme A synthetase method (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) after treatment of the samples with 0.1 M NaOH at 37°C for 30 min (samples were neutralized with HCl prior to the enzyme analysis). The phosphorus content of cyclic β-(1,2)-glucan preparations was determined spectrophotometrically after digestion of the samples with magnesium nitrate (33).

NMR. All nuclear magnetic resonance (NMR) experiments were performed at 500.13 (^1H) and 125.78 (^{13}C) MHz on a Bruker AM-500 spectrometer operating in the quadrature mode at 298°K. ^1H and ^{13}C spectra were referenced indirectly to sodium-2,3-dimethyl-2-silapentane-5-sulfonate. Long-range two-dimensional ^1H-^{13}C correlation spectra were acquired with the heteronuclear multiple bond correlation experiment described by Summers et al. (39). Glucan and EPS samples were dissolved in 99.9% D_2O at a concentration of 2 to 5 mg/ml. EPS preparations were redissolved with the aid of a single (1-s) sonicating burst with a microtip probe (Vibra-cell, model 250W; Sonics and Materials, Inc., Danbury, Conn.).

RESULTS

Effect of phosphate limitation on cell growth and EPS production by *R. meliloti* 1021. To investigate the effect of phosphate limitation on cell growth, *R. meliloti* 1021 was grown in media containing a wide range of phosphate concentrations (10 μM to 5.5 mM). Table 2 shows that phosphate became limiting for cell protein production at concentrations below 150 μM.

TABLE 2. Effect of growth medium phosphate concentration on cyclic glucan and polysaccharide production by *R. meliloti* 1021[a]

K_2HPO_4 (μM)[b]	Cellular protein (mg/liter)	Cell-associated cyclic glucans (g/g of protein)[c]	Extracellular carbohydrate (g/g of protein)[d]	Relative viscosity
10	55	0.13	3.82	9
20	120	0.16	4.25	21
30	195	0.20	3.95	29
50	280	0.19	4.11	61
100	410	0.18	3.29	90
150	460	0.21	0.37	5
5,500	465	0.21	0.34	2

[a] Cells were grown for 72 h in standard medium or phosphate-limited medium. Each measurement was performed at least in duplicate, and values for protein concentration, cyclic glucan content, extracellular carbohydrate, and relative viscosity differed by ≤10%.

[b] Phosphate concentration of the growth medium.

[c] Cell-associated cyclic β-(1,2)-glucan content is expressed as grams of glucose equivalent per gram of cellular protein.

[d] Total carbohydrate in the culture supernatant was determined by the phenol-sulfuric acid method (13) and is expressed as grams of glucose equivalent per gram of cellular protein. In phosphate-limited cultures (10 to 100 μM phosphate), ≥95% of the total extracellular carbohydrate was precipitable with 75% ethanol. In cultures containing 150 or 5,500 μM phosphate, 35 to 40% of the total extracellular carbohydrate was precipitable with 75% ethanol.

Growth in low-phosphate medium (<150 μM) also had a dramatic effect on the viscosity of the culture, which was shown to be 4 to 45 times higher than that measured in the standard medium (Table 2). Furthermore, analysis of extracellular carbohydrate levels in these cultures revealed that phosphate-limited cells produced approximately 10-fold-higher levels of EPS when values were normalized for total cellular protein (Table 2). This result is consistent with a previous report by Zhan et al. (43), who reported that *R. meliloti* produces highly mucoid colonies when grown on an agar medium containing limiting phosphate concentrations. These researchers have also shown that the EPS produced under phosphate-limiting conditions is quite different in structure from that produced in the presence of high phosphate concentrations. Instead of producing succinoglycan, these cultures produce an alternative EPS, termed EPSb or EPSII, which consists of a disaccharide repeating unit containing acetyl and pyruvyl substituents (43, 44).

Succinate is the principal substituent on the cyclic β-(1,2)-glucans of *R. meliloti* 1021 grown under phosphate-limiting conditions. In contrast to EPS production, cellular levels of cyclic β-(1,2)-glucans synthesized by *R. meliloti* 1021 remained relatively constant when cells were grown in media containing a wide range of phosphate concentrations (Table 2). To further characterize the cyclic β-(1,2)-glucans produced by cells grown under phosphate-limiting conditions, large-scale preparations were isolated from 1-liter cultures. The DEAE-cellulose profiles of cyclic β-(1,2)-glucan preparations obtained from cultures grown in the presence of 5.5 mM phosphate and 100 μM phosphate are shown in Fig. 1A and B, respectively. Both preparations contained neutral and anionic cyclic β-(1,2)-glucan subfractions, although the relative amounts of these subfractions were different.

The presence of anionic cyclic β-(1,2)-glucans in cultures grown under phosphate-limiting conditions was also revealed by TLC analyses (Fig. 2A). Furthermore, *R. meliloti* 1021 was shown to synthesize high levels of anionic cyclic β-(1,2)-glucans even when grown under extreme phosphate-limiting conditions

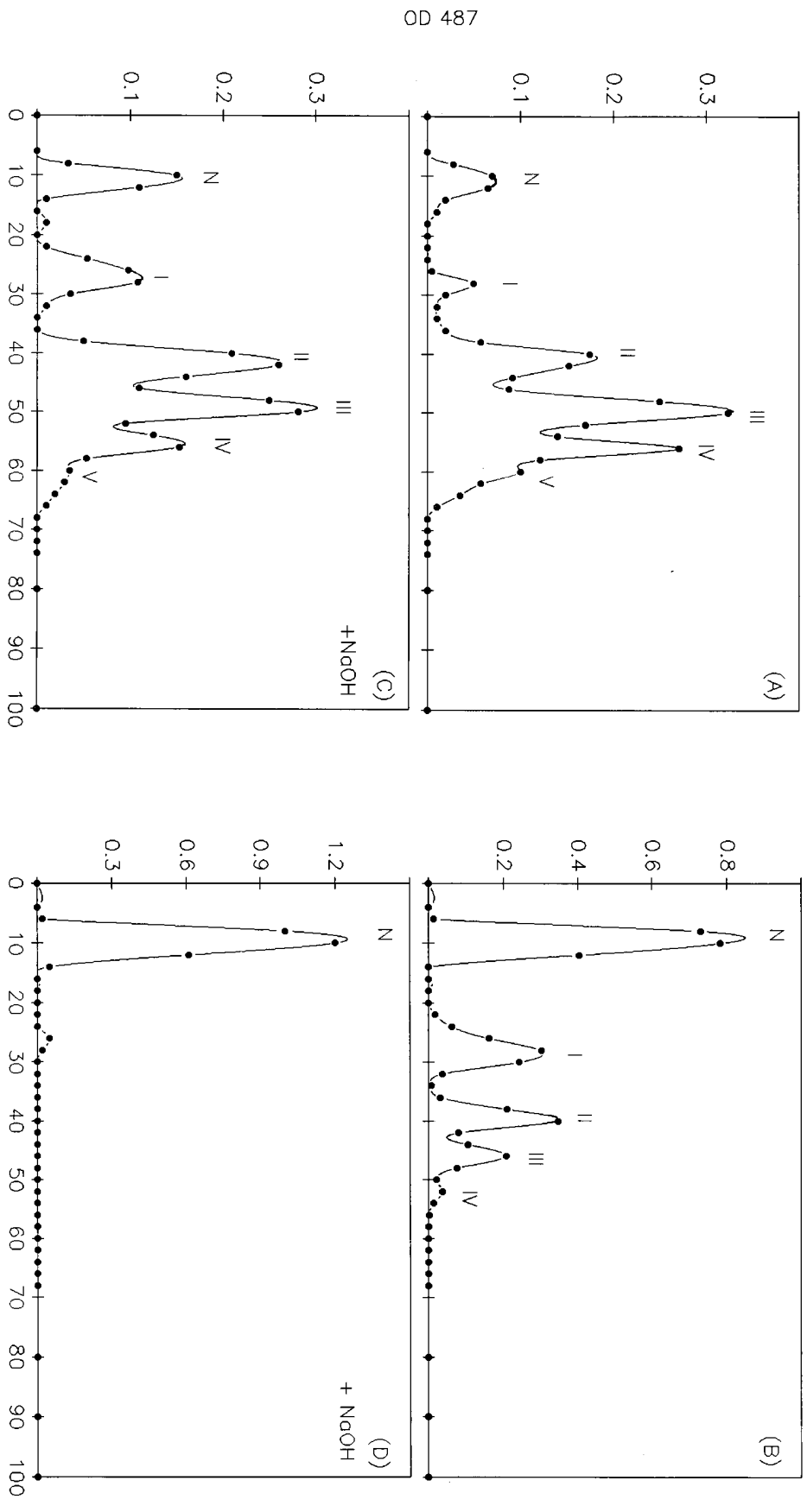

FIG. 1. DEAE-cellulose anion-exchange column chromatography profiles of the cell-associated cyclic β-(1,2)-glucans from *R. meliloti* 1021. (A, C) Cultures were grown in standard medium containing 5.5 mM phosphate. (B, D) Cultures were grown in phosphate-limited medium containing 100 μM phosphate. Samples were either directly applied onto the column (A, B) or first subjected to mild alkali treatment (C, D) in order to remove succinic acid substituents. The dimensions of the column were 1 by 17 cm. Fractions (2 ml) were collected, and portions were assayed for total carbohydrate by the phenol-sulfuric acid method (13). N, neutral cyclic β-(1,2)-glucans; I to V, anionic cyclic β-(1,2)-glucan subfractions. OD_{487}, optical density at 487 nm.

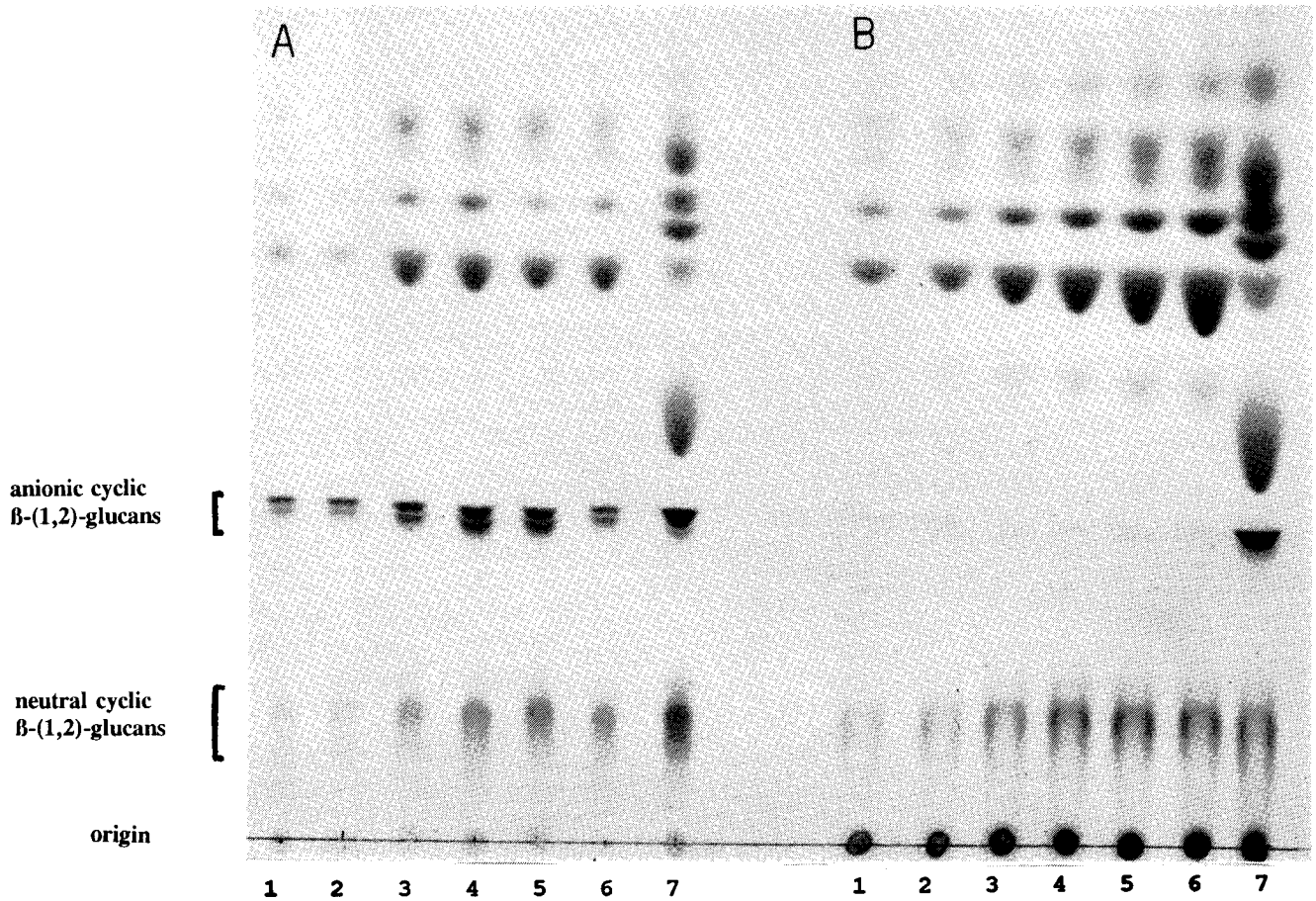

FIG. 2. TLC analysis of ethanolic cell extracts of *R. meliloti* 1021. Cells were grown in medium containing the following phosphate concentrations: 10 μM (lane 1), 20 μM (lane 2), 30 μM (lane 3), 50 μM (lane 4), 100 μM (lane 5), 150 μM (lane 6), and 5.5 mM (lane 7). Extracts were either directly applied to TLC plates (A) or first subjected to mild alkali treatment (B) in order to remove succinic acid substituents. The positions of the neutral cyclic β-(1,2)-glucans and anionic cyclic β-(1,2)-glucans (containing phosphoglycerol and/or succinic acid substituents) are indicated.

(e.g., 10 μM [Fig. 2A]). Although TLC and DEAE-cellulose chromatography revealed that *R. meliloti* synthesizes high levels of anionic cyclic β-(1,2)-glucans when grown in phosphate-limited media, these analyses also revealed that the anionic glucans synthesized under these conditions are quite different in character from those produced during growth in standard medium. Treatment of these anionic cyclic β-(1,2)-glucan preparations with mild alkali (0.1 M NaOH at 37°C for 30 min) resulted in the complete conversion of these molecules to neutral cyclic β-(1,2)-glucans (Fig. 1D and Fig. 2B). In contrast, the TLC and DEAE-cellulose profiles of cyclic β-(1,2)-glucan preparations derived from cells grown in standard medium were only slightly altered by this treatment (Fig. 1C and Fig. 2).

The sensitivity to mild alkali treatment indicated that the anionic cyclic β-(1,2)-glucans synthesized by *R. meliloti* during phosphate-limited growth did not contain phosphoglycerol substituents [since these substituents are not released from the cyclic β-(1,2)-glucans by mild alkali treatment (33)]. This was, in fact, confirmed by phosphorus analyses which revealed that the anionic cyclic β-(1,2)-glucans isolated from phosphate-limited cultures contained about 10% of the phosphate found in anionic cyclic β-(1,2)-glucans isolated from cultures grown in standard medium (Table 3). Because *sn*-1-phosphoglycerol has previously been shown to be the predominant substituent on the anionic cyclic β-(1,2)-glucans of *R. meliloti* (33), it was surprising that the anionic cyclic β-(1,2)-glucans isolated from phosphate-limited cultures lacked this substituent. However, Miller et al. (33) previously reported the presence of mild alkali-labile succinate substituents on the anionic cyclic β-(1,2)-glucans of *R. meliloti.*

The above results suggested that *R. meliloti* 1021 synthesizes

TABLE 3. Compositional analysis of anionic cyclic β-(1,2)-glucan preparations

Bacterial strain	K_2HPO_4 (μM)	Level of substituent [mol/mol of β-(1,2)-glucan][a]	
		Phosphoryl	Succinyl
R. meliloti 1021	5,500	1.9	0.4
R. meliloti 1021	100	0.2	1.5
R. meliloti 7154 (*exoH*)	100	0	1.1
A. tumefaciens C58	5,500	1.1	0

[a] The levels of substituents are expressed as moles per mole of anionic cyclic β-(1,2)-glucan backbone, assuming an average size of 20 glucose residues per glucan molecule. Glucose content was determined by the phenol-sulfuric acid method (13). Each measurement was performed in duplicate, and values differed by ≤6%. Detection limits for phosphoglycerol and succinyl substituents were 0.01 and 0.03 mol per mol of anionic cyclic β-(1,2)-glucan backbone, respectively.

anionic cyclic β-(1,2)-glucans containing predominantly succinate substituents when cells are grown in phosphate-limited media. This was confirmed by compositional and NMR analyses. As shown in Table 3, succinate substituents were detected in anionic cyclic β-(1,2)-glucan preparations derived from cells grown in both standard medium and phosphate-limited medium; however, the levels of succinate substituents were over fourfold greater in the latter. Furthermore, two prominent triplets between 2.4 and 2.7 ppm in the ^{1}H NMR spectrum of the anionic cyclic β-(1,2)-glucans isolated from phosphate-limited cultures (Fig. 3B) also revealed the presence of high levels of succinyl substituents. That these triplets correspond to the methylene protons of succinate was unequivocally confirmed by long-range two-dimensional ^{13}C-^{1}H NMR correlation experiments (data not shown). Consistent with the compositional analyses, these triplets were weaker in the spectrum of the anionic cyclic β-(1,2)-glucans isolated from cultures grown in standard medium (Fig. 3A).

Distinct enzyme systems in *R. meliloti* 1021 are responsible for the addition of succinyl substituents to the cyclic β-(1,2)-glucans and succinoglycan. Leigh et al. (29) demonstrated that the EPS synthesized by *exoH* mutants of *R. meliloti* is identical to that produced by wild-type cells, except that it lacks succinyl substituents. More recently, Becker et al. (4) and Glucksmann et al. (19) have sequenced the *exoH* gene of *R. meliloti* and have shown that the predicted protein shares 24% amino acid identity with the 14-kDa hydrophobic subunit of the succinate dehydrogenase of *E. coli*. On the basis of this homology, it has been proposed that ExoH directly utilizes succinyl-coenzyme A during succinoglycan biosynthesis (19). It was therefore of interest to examine whether or not an *exoH* mutant of *R. meliloti* retained the capacity to synthesize cyclic β-(1,2)-glucans containing succinyl substituents.

Initial experiments utilized TLC to examine cyclic β-(1,2)-glucan preparations from cultures of *R. meliloti* 7154 (*exoH*) grown in phosphate-limited media. These experiments revealed the presence of mild alkali-labile substituents similar to those found on the anionic cyclic β-(1,2)-glucans produced by *R. meliloti* 1021 (data not shown). To confirm the presence of succinyl substituents, cyclic β-(1,2)-glucans were isolated from a 1-liter culture of strain 7154 grown in medium containing 100 μM K_2HPO_4. Both compositional (Table 3) and NMR (Fig. 3C) analyses confirmed the presence of succinyl substituents on the anionic cyclic β-(1,2)-glucans of this *exoH* mutant. Furthermore, the level of succinyl modification was found to be similar to that present on the anionic cyclic β-(1,2)-glucans of strain 1021 grown under phosphate limitation. Additional experiments (NMR analysis and succinyl-coenzyme A synthetase assays) confirmed that the EPS synthesized by *R. meliloti* 7154 lacked succinyl substituents (data not shown). Thus, it may be concluded that the product of the *exoH* gene is not involved in the succinylation of the cyclic β-(1,2)-glucans.

***A. tumefaciens* synthesizes only neutral cyclic β-(1,2)-glucans when grown in phosphate-limited medium.** In order to examine the effects of phosphate limitation on the cyclic β-(1,2)-glucans synthesized by *A. tumefaciens*, strain C58 was cultured in standard medium containing 5.5 mM phosphate or phosphate-limited medium containing 100 μM phosphate. When grown in standard medium, both neutral and anionic cyclic β-(1,2)-glucan subfractions were readily detected upon analysis by DEAE-cellulose chromatography (Fig. 4A), consistent with previous results (34, 35). No succinate could be detected in these preparations (Table 3), confirming that phosphoglycerol is the only anionic substituent present on the cyclic β-(1,2)-glucans of *A. tumefaciens* C58 (35).

In contrast to *R. meliloti*, however, only neutral cyclic β-(1,2)-glucans were detected when *A. tumefaciens* C58 was grown in phosphate-limited medium (Fig. 4B). This result was confirmed by compositional analysis in which neither phosphate nor succinate substituents could be detected in these preparations (not shown). Thus, although *A. tumefaciens* synthesizes succinoglycan with essentially the same structure as that reported for *R. meliloti* (2, 21), this bacterium apparently lacks the ability to modify its cyclic β-(1,2)-glucans with succinyl substituents. This result, therefore, provides further evidence that the succinyl modification of the cyclic β-(1,2)-glucans in *R. meliloti* is mediated by an enzyme system that is distinct from that which functions during succinoglycan biosynthesis.

EPS biosynthesis by *A. tumefaciens* is greatly reduced during phosphate-limited growth. Both *A. tumefaciens* and *R. meliloti* produce succinoglycan in media containing 5.5 mM phosphate (11). However, in contrast to *R. meliloti* (Table 2), cultures of *A. tumefaciens* were found to be very viscous under these growth conditions (Table 4). Furthermore, when cultures were grown under phosphate-limiting conditions (100 μM phosphate), EPS production was found to be dramatically reduced (Table 4). This result suggests that *A. tumefaciens* does not synthesize the second EPS (EPSb) under conditions of phosphate limitation. It is noted, however, that large quantities of a gel-like material were found to be associated with *A. tumefaciens* cell pellets when cultures were grown under phosphate limitation. The nature of this gel-like material was not investigated further. However, this material was not curdlan [a high-molecular-weight β-(1,3)-glucan shown to be produced by several *A. tumefaciens* strains (20)] because it did not react with the color dye aniline blue (20).

DISCUSSION

Although closely related within the *Rhizobiaceae* family, *R. meliloti* and *A. tumefaciens* have dramatically different interactions with higher plants. *R. meliloti* is noted for its symbiotic interaction with alfalfa, which leads to the formation of nitrogen-fixing root nodules. In contrast, the infection of dicotyledonous plants by *A. tumefaciens* leads to the formation of tumors. In spite of this striking difference in plant infection behavior, *R. meliloti* and *A. tumefaciens* have been shown to synthesize certain classes of cell surface carbohydrates that are essentially identical in structure. These cell surface carbohydrates include succinoglycan and periplasmic cyclic β-(1,2)-glucans. The succinoglycan produced by *R. meliloti* and *A. tumefaciens* consists of an octasaccharide repeating unit which contains acetate, succinate, and pyruvate substituents (2, 22, 37). The periplasmic cyclic β-(1,2)-glucans produced by both bacteria are similar in size and contain *sn*-1-phosphoglycerol substituents. In addition to their possible roles during plant infection (15, 17), the cyclic β-(1,2)-glucans have been shown to function during hypoosmotic adaptation of the free-living forms of *A. tumefaciens* and *R. meliloti* (12, 16).

In the present study, we have shown that the levels of phosphoglycerol substituents on the periplasmic cyclic β-(1,2)-glucans of both *R. meliloti* and *A. tumefaciens* are dramatically reduced when cells are grown under phosphate-limiting conditions. *R. meliloti* apparently compensates for the loss of this anionic substituent by adding higher levels of succinyl substituents. It is noted that preliminary experiments have revealed a similar response by *E. coli* K-12 during membrane-derived oligosaccharide biosynthesis (8). Specifically, when *E. coli* K-12 was grown under phosphate-limiting conditions, the levels of succinic acid substituents were found to increase approximately twofold, while the levels of phosphoryl substituents

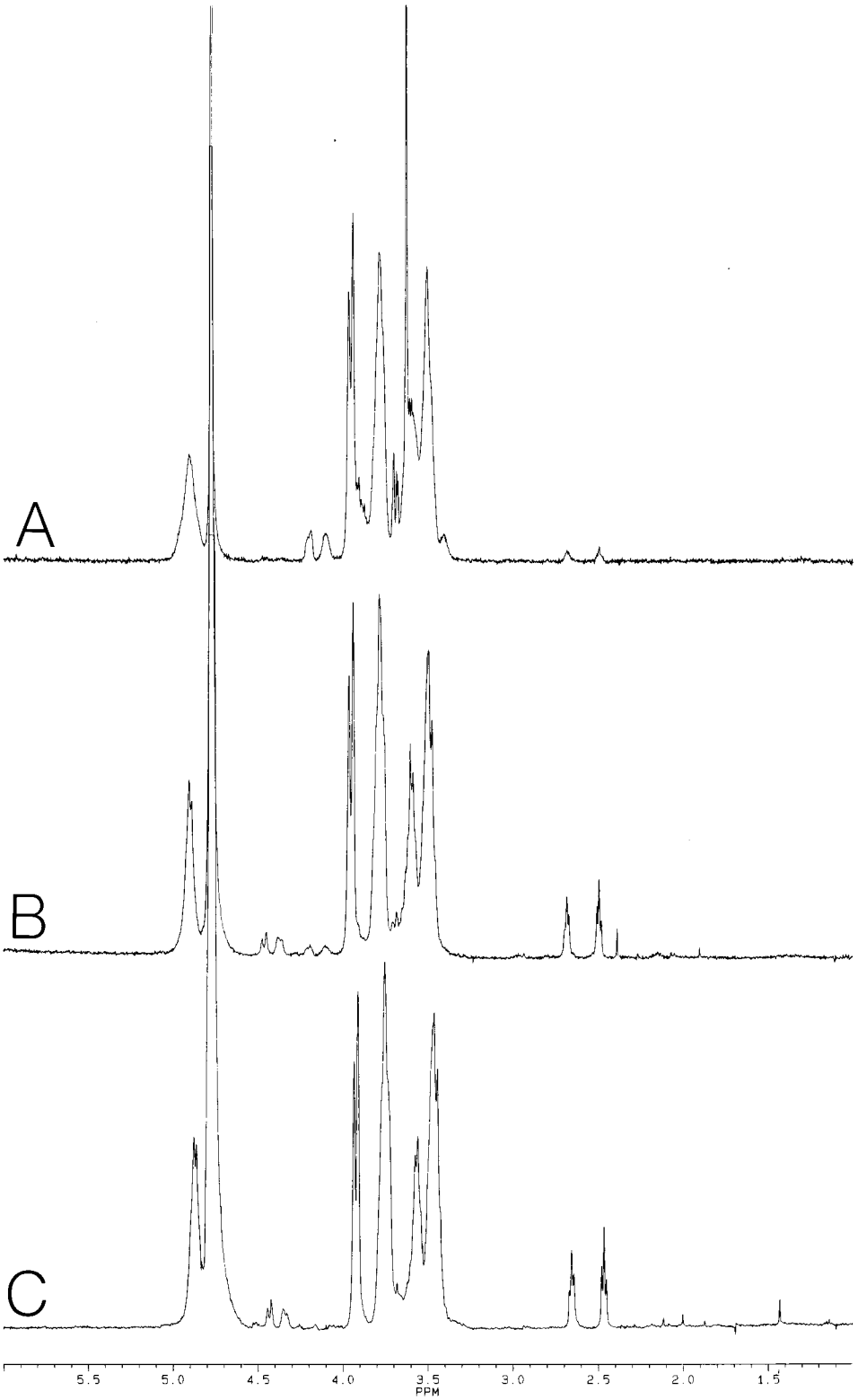

FIG. 3. ^{1}H NMR spectra of anionic cyclic β-(1,2)-glucan preparations from *R. meliloti*. (A) Strain 1021 grown in standard medium containing 5.5 mM phosphate. (B) Strain 1021 grown in phosphate-limited medium containing 100 μM phosphate. (C) Strain 7154 (*exoH*) grown in phosphate-limited medium containing 100 μM phosphate. The two triplets between 2.4 and 2.7 ppm correspond to the methylene protons of the succinic acid substituents. The resonances at 4.90, 3.55, 3.82, 3.44, and 3.48 ppm correspond to the H_1 to H_5 resonances of the glucose residues, and the resonances at 3.78 and 3.90 ppm correspond to the two H_6 resonances of the glucose residues. In panel A, the resonance at 3.58 ppm is derived from Tris, which apparently copurified with this sample. The resonance at 4.78 ppm represents H_2O.

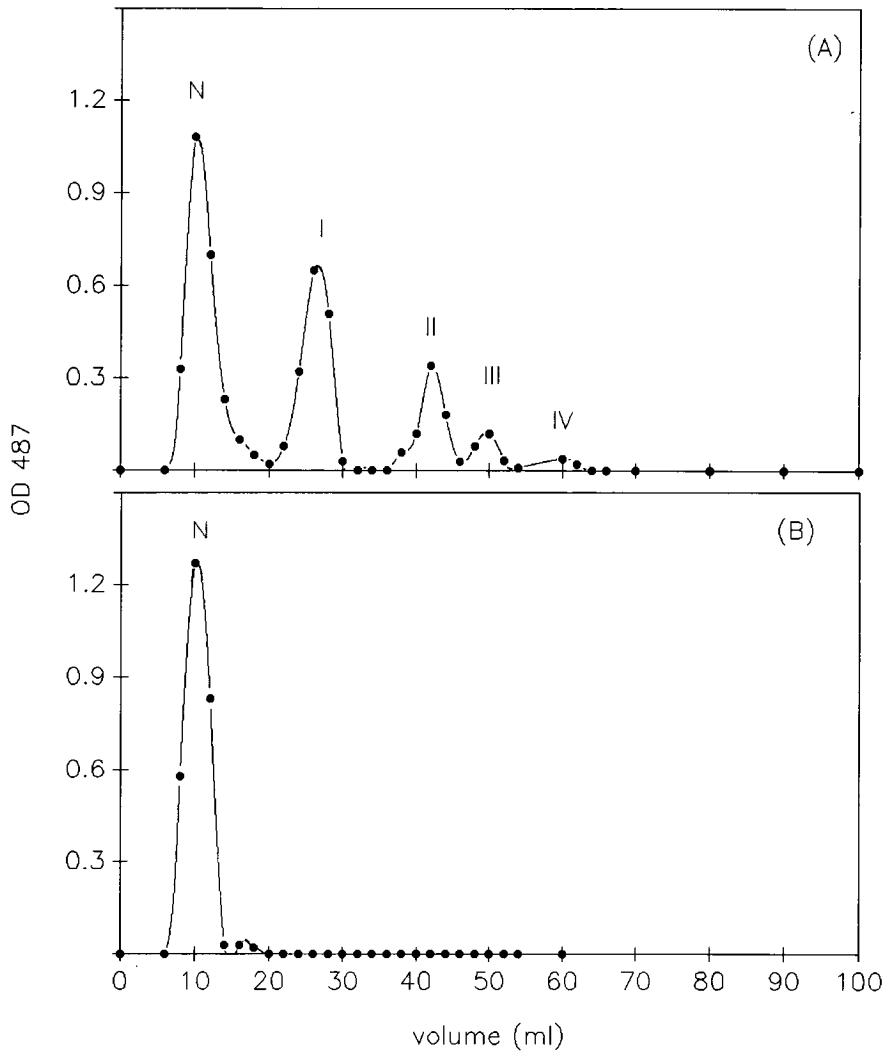

FIG. 4. DEAE-cellulose anion-exchange column chromatography profiles of the cyclic β-(1,2)-glucans of *A. tumefaciens* C-58. (A) Cells were grown in standard medium containing 5.5 mM phosphate. (B) Cells were grown in phosphate-limited medium containing 100 μM phosphate. Neither sample was subjected to mild alkali treatment. For additional information, see the legend to Fig. 1. OD_{487}, optical density at 487 nm.

were found to be greatly reduced (e.g., 30% of the level found in cultures grown in the presence of 5.5 mM phosphate).

Very little is known concerning the enzyme systems which mediate the transfer of *sn*-1-phosphoglycerol and succinic acid substituents to the cyclic β-(1,2)-glucans. However, the present study reveals that the product of the *exoH* gene is not involved in the addition of succinyl substituents. Therefore, it may be concluded that succinylation of the cyclic β-(1,2)-glucans is mediated by a system that is distinct from that which functions during succinoglycan biosynthesis. Furthermore, it may be concluded that neither succinoglycan nor the octasaccharide repeating unit of succinoglycan serves as the source of succinyl substituents for the cyclic β-(1,2)-glucans.

TABLE 4. Effect of growth medium phosphate concentration on polysaccharide production by *A. tumefaciens* C58[a]

K_2HPO_4 concn (μM)[b]	Cellular protein (mg/liter)	Extracellular carbohydrate (g/g of protein)[c]	Relative viscosity
100	410	0.49	3
5,500	450	3.55	170

[a] Cells were grown for 72 h in standard medium or phosphate-limited medium. Each measurement was performed in duplicate, and values for protein concentration, extracellular carbohydrate, and relative viscosity differed by ≤8%.

[b] Phosphate concentration of the growth medium.

[c] Total carbohydrate in the culture supernatant was determined by the phenol-sulfuric acid method (13) and is expressed as grams of glucose equivalent per gram of cellular protein.

Recently, we have shown that the transfer of phosphoglycerol substituents to the cyclic β-(1,2)-glucans occurs in the periplasmic compartment (7). Furthermore, we have shown that *ndvA* mutants [defective in the ability to transport cyclic β-(1,2)-glucans across the cytoplasmic membrane] accumulate neutral, unsubstituted cyclic β-(1,2)-glucans within the cytoplasm (9). It would therefore seem likely that the transfer of succinyl substituents to the cyclic β-(1,2)-glucans also occurs within the periplasmic compartment.

Although *A. tumefaciens* was found to no longer transfer phosphoglycerol substituents to its cyclic β-(1,2)-glucans when grown in a phosphate-limited medium, no succinyl substituents

were detected on these molecules. Instead, only neutral cyclic β-(1,2)-glucans were synthesized when cells were grown under these conditions. The difference in the abilities of *R. meliloti* and *A. tumefaciens* to succinylate their cyclic β-(1,2)-glucans is intriguing since several other aspects of cyclic β-(1,2)-glucan structure and biosynthesis are remarkably similar in the two bacteria. For example, the biosynthesis of these glucans is strictly osmoregulated in both *A. tumefaciens* and *R. meliloti*, and the glucans of these bacteria are the same size, contain similar levels of *sn*-1-phosphoglycerol substituents, and accumulate to similar levels within the periplasmic compartment (11, 12, 16, 34, 45). Furthermore, the genes involved in cyclic β-(1,2)-glucan biosynthesis and transport (thus far identified) have been shown to be functionally interchangeable between *A. tumefaciens* and *R. meliloti* (17). It is possible that the presence of succinyl groups on the cyclic β-(1,2)-glucans of *R. meliloti* reflects a specific requirement for these substituents during different stages of legume nodulation. In this regard, we note that *exoH* mutants of *R. meliloti* are defective for nodule invasion, strongly suggesting that the presence of succinyl substituents on the EPS is required for successful symbiosis (29).

In the present study we have shown that phosphate limitation leads to alterations in the structure of the cyclic β-(1,2)-glucans produced by *R. meliloti* and *A. tumefaciens*. Previous studies have revealed that the biosynthesis of other rhizobial cell surface carbohydrates such as EPS (43) and lipopolysaccharide (41), as well as the excretion of lipooligosaccharides (32), is also strongly influenced by extracellular phosphate concentrations. All of these phenomena may have profound effects on the infection of the plant host. There have been conflicting reports concerning the effect of phosphate limitation on legume nodulation by *Rhizobium* species. For example, it has been suggested that phosphate limitation affects nodulation directly (e.g., metabolic activity of the nodule [5, 24]), whereas other studies have suggested that phosphate limitation has an indirect effect on nodulation because growth of the host plant becomes inhibited (23, 25, 38). Interestingly, phosphate concentrations have been shown to be much higher within nodules than in roots or shoots (38), and it has been concluded that higher phosphate concentrations within nodules are required for optimal nitrogen fixation (36). It remains to be determined, however, if the high level of phosphorus found within nodules is associated with the plant tissue or bacteroids (36). Israel (23) has suggested that phosphorus limitation has a specific effect on the early stages of nodule development. Whether or not this results from effects on the bacterial symbiont remains to be determined. In a recent study, De Weger et al. (14) reported the use of phosphate reporter strains of *Pseudomonas putida* to study the levels of available phosphate within the rhizosphere. Perhaps such a reporter system could be developed to study phosphorus availability during root nodulation in various rhizobial legume systems.

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