Osmotically induced oligo- and polysaccharide synthesis by *Rhizobium meliloti* SU-47

M. W. BREEDVELD,* L. P. T. M. ZEVENHUIZEN and A. J. B. ZEHNDER

Department of Microbiology, Agricultural University, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands

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In standard liquid medium containing 5 g mannitol l⁻¹ and 1 g glutamic acid l⁻¹, *Rhizobium meliloti* SU-47 cells accumulated 350 mg cyclic 1,2- β -glucans (g protein)⁻¹. The cyclic glucans were 36% glycerol-1-phosphate-substituted and 64% were uncharged. In the same medium with 10 g mannitol l⁻¹, repeating units of succinoglycan (1110 mg l⁻¹) were found as extracellular carbohydrates, and only low amounts of the succinoglycan polymer (up to 300 mg l⁻¹) were excreted. By raising the osmotic pressure of the medium by the addition of NaCl or other ionic and non-ionic osmolytes, succinoglycan production could be stimulated: up to 2.4 g l⁻¹ at 0.2 M-NaCl was produced at the expense of the repeating units. Above 0.2 M-NaCl growth was slowed down, and succinoglycan excretion diminished. At 1 M-NaCl growth stopped completely. In standard medium containing 0.6 M-NaCl the amount of cellular cyclic 1,2- β -glucans was lowered to 150 mg (g protein)⁻¹ out of which the glycerol-1-phosphate-substituted glucan fraction was reduced to 15%. Instead, high amounts of oligosaccharides were synthesized as osmoprotectants, with trehalose as the major component [up to 200 mg (g protein)⁻¹]. Glycogen synthesis was completely suppressed at this salt concentration, while poly β -hydroxybutyric acid synthesis was unaffected.

Introduction

Cyclic 1,2- β -glucans of rhizobia are considered to function as osmoregulators in order to equilibrate the osmotic balance between the cytoplasm and the periplasm, in particular at low osmotic values of the medium (Miller *et al.*, 1986). They are localized in the periplasmic space (Abe *et al.*, 1982). Cyclic 1,2- β -glucans were isolated for the first time from the extracellular fluid of an *Agrobacterium tumefaciens* culture (McIntire *et al.*, 1942). Since then, several authors have found 1,2- β -glucans in the cell (Zevenhuizen and Scholten-Koerselman, 1979; Miller *et al.*, 1986) or in the medium (Amemura *et al.*, 1983, 1985; Zevenhuizen, 1986) of a number of *Rhizobium* and *Agrobacterium* species.

Cellular concentrations of 1,2- β -glucans follow a distinct pattern depending on the species and the growth phase. In mannitol-glutamic acid-salts medium of low osmotic value cells of *R. leguminosarum*, biovars *viciae*, *trifolii* and *phaseoli* build up concentrations of 1,2- β -

glucans to a maximum of 100 mg (g dry cell wt)⁻¹ at the end of the exponential phase. Their concentrations are reduced by more than 50% during the stationary phase, during which capsular polysaccharide (CPS) is deposited around the cells. In contrast *R. meliloti*, which forms no capsules, produces up to 200 mg 1,2- β -glucans (g dry cell wt)⁻¹. During the stationary phase, this concentration remains constant (Zevenhuizen, 1981; Zevenhuizen & Van Neerven, 1983).

At high osmotic values of the medium the synthesis of cyclic glucans is repressed in *Agrobacterium tumefaciens* (Miller *et al.*, 1986). Several authors have reported the formation of compatible solutes in the cytoplasm of cells grown under conditions of high osmotic pressure (Le Rudulier & Bernard, 1986; Czonka, 1989). At concentrations up to 1.3% NaCl growth of *R. meliloti* is not affected, while growth of *R. leguminosarum* biovar *trifolii* is impaired at 0.4% NaCl (Vincent, 1974).

In this paper we report how the osmotic values of the medium affect oligo- and polysaccharide synthesis by *Rhizobium meliloti* strain SU-47. Comparison is made with other *R. meliloti* and *A. tumefaciens* strains. A role for cellular and capsular carbohydrates in osmoregulation is also discussed.

Abbreviations: CPS, capsular polysaccharide; DP, degree of polymerization; EPS extracellular polysaccharide; PHB, poly β -hydroxybutyric acid.

Methods

Organisms and cultivation. The following strains were obtained from the culture collection of the Department of Microbiology, Wageningen, The Netherlands: *Rhizobium meliloti* strains SU-47, SU-255, and SU-256; *Agrobacterium tumefaciens* strains PD15, PD18 and PD51. These bacteria were maintained on agar slants of *Rhizobium* medium containing yeast extract, mannitol and mineral salts. Precultures were prepared by inoculating the organism into 'standard medium' containing 5 g mannitol and 1 g glutamic acid litre⁻¹, and mineral salts and vitamins according to Zevenhuizen (1986). The term 'production medium' is used when the mannitol concentration was 10 g l⁻¹. In the experiments on osmoregulation media were supplemented with NaCl, other electrolytes or non-electrolytes as indicated.

Cells from the late exponential phase were used as inoculum (1%, v/v). They were cultivated at 25 °C in 50 ml medium in 100 or 300 ml Erlenmeyer flasks at 25 °C. To obtain larger quantities of cultures, cells were grown in 300 ml medium in 2 litre Erlenmeyer flasks. The flasks were incubated on a rotary shaker at 200 r.p.m. Experiments with controlled pH and oxygen supply were done in a 2 litre batch fermenter with 1 litre of medium. The culture was aerated at the rate of 1 l air min⁻¹, and stirred at 500 r.p.m.

Calculation of osmotic pressure. The osmotic pressure π was calculated as $\pi = \sum n_i \cdot M_i \cdot R \cdot T$ where n = no. of moles of solute i , M = molarity of the solute i (mol l⁻¹), R = 0.0821 l atm K⁻¹ mol⁻¹, and T = 298.15 °K. Ideal behaviour of the solutions is assumed (Chang, 1977). The osmotic pressure of the production medium was calculated as 2.0 atm (1 atm = 101 325 Pa).

Analysis of the cultures. Cells and culture supernatant were separated by centrifugation at 35 000 g for 30 min. Highly viscous cultures were first diluted with distilled water prior to centrifugation. Cell pellets from 50 ml cultures were resuspended in 10 ml distilled water and briefly sonicated to obtain a homogeneous suspension.

Supernatant. The supernatant was analysed for total excreted carbohydrates by the anthrone-H₂SO₄ method (Trevelyan & Harrison, 1952). The carbohydrate concentration was expressed as glucose equivalents. Mannitol was measured by the periodate oxidation-chromotropic acid colorimetric method (Burton, 1957). Viscosity measurements were made with an Ubbelohde viscometer at 25 °C.

Excreted carbohydrate was fractionated into a low- M_r and a high- M_r fraction by precipitating the high- M_r fraction with 3 vols ethanol. The precipitated succinoglycan was redissolved in water, and the sugar content was measured by the anthrone-H₂SO₄ assay. Repeating units of succinoglycan which remained in the ethanolic supernatant were also quantified by the anthrone-H₂SO₄ assay. The extracellular low- M_r carbohydrates of this fraction were separated according to charge on a DEAE-Sepharose anion-exchange column (3 × 25 cm, Pharmacia-LKB), and were eluted with distilled water followed by a linear KCl gradient (0–0.5 M). Fractions (5 ml) were collected, and checked for hexose content by the anthrone method. Peak fractions were desalted by gel-filtration over a Bio-Gel P2 column. The amount of succinate found within the collected peak fractions after methanolysis was determined as methylsuccinate on a capillary Wax 57-CB column (Chrompack) connected to a flame ionization detector in a Kipp Analytica 8200 gas chromatograph at 100 °C (Zevenhuizen & Van Neerven, 1983).

Cell pellet. Fractionation into high- M_r and low- M_r cellular carbohydrates was as follows. Cell suspensions of *A. tumefaciens* were extracted with 1 vol. 2 M-NaOH for 15 min at 70 °C. After centrifugation, the NaOH extract was decanted. Ethanol (1 vol.) was added to this extract, which precipitated CPS [curdian, a linear 1,3- β -glucan (Hisamatsu *et al.*, 1978)]. The precipitate was collected by centrifugation, and

redissolved in 1 M-NaOH. Curdian was quantified by the anthrone method and expressed as glucose equivalents. In the ethanolic extract, containing cyclic 1,2- β -glucans and oligosaccharides, total carbohydrate was measured by the anthrone assay. In the case of *R. meliloti* strains, which do not produce CPS, cell suspensions were extracted with 3 vols ethanol for 30 min at 70 °C, followed by centrifugation and resuspension of the extracted cells in water. The hexose content of this suspension was determined by the anthrone method and could be taken as a measure of the glycogen content of the cells (Zevenhuizen, 1981). In the ethanolic extract, low- M_r carbohydrates (cyclic glucans and oligosaccharides) were present. These were separated after evaporation of the ethanol on a column of Ultrogel AcA-202 (2.5 × 37 cm; M_r range 1000–15 000; Pharmacia-LKB) with water as the eluent. Fractions of about 2.5 ml were collected and measured for hexose content.

TLC was done out on silica gel plates (Merck) with the solvent [n-butanol/ethanol/water (5:5:4, by vol.)] ascending the plates (Amemura *et al.*, 1985). Cell protein was measured according to the Lowry method with BSA as standard. PHB was determined after methanolysis of whole cell suspensions with GLC on a capillary Wax 57-CB column at 100 °C by the method of Braunegg *et al.* (1978).

Sugar composition and linkage types of isolated poly- and oligosaccharides. Separation of neutral sugar components was done after hydrolysis and conversion of the liberated sugars into alditol acetates by GLC (Blakeney *et al.*, 1983). Glycosidic linkages were determined after methylation analysis according to Harris *et al.* (1984). With both GLC experiments a capillary Sil 43-CB column (Chrompack) was used at 210 °C. HPLC of glucans and oligosaccharides was done according to Breedveld *et al.* (1990). ¹H-NMR was done with a Bruker Ac-200E apparatus with 4,4'-dimethyl-4-silapentane sulphonate (DDS) as internal standard.

Results

Influence of NaCl and other osmolytes on extracellular and cellular carbohydrate production by R. meliloti and A. tumefaciens

A difference in succinoglycan production between *A. tumefaciens* and *R. meliloti* strains was observed (Table 1). The *A. tumefaciens* strains PD15, PD18 and PD51 formed highly viscous cultures in production medium without and with added NaCl. With *R. meliloti* strains SU-47, SU-255 and SU-256 the major part (70–80%) of the extracellular carbohydrate produced in the NaCl-free medium was of low- M_r . By adding NaCl to the medium the proportion of high- M_r succinoglycan was greatly enhanced. Succinoglycan production was paralleled by an increase in viscosity of the medium (data not shown).

The low content of low- M_r cellular carbohydrates (1,2- β -glucans) and the high curdian content of *A. tumefaciens* cells in media not supplemented with NaCl was in contrast to the higher amounts of 1,2- β -glucans of *R. meliloti* cells, which did not produce capsules. At increasing NaCl concentrations, large amounts of oligosaccharides were synthesized, functioning probably as compatible osmoregulators in the cytosol of the cells (see below for *R. meliloti* SU-47).

Table 1. *NaCl-induced osmotic effects on extracellular and cellular carbohydrates of A. tumefaciens and R. meliloti strains*

Cultures were incubated in production medium (with NaCl added as indicated) for 14 d on a rotary shaker (200 r.p.m.) at 25 °C. Carbohydrate contents are expressed as mg glucose equivalents per litre of culture. The data shown here are representative of two experiments between which values varied not more than 5%.

Strain	Supernatant		Cell pellet		CPS§
	High- M_r^*	Low- $M_r^\dagger$	Protein (mg l ⁻¹)	Low- $M_r^\ddagger$	
<i>(a) A. tumefaciens</i>					
Strain PD15	2600	40	425	30	980
+ 0.2 M-NaCl	2100	50	435	60	880
+ 0.6 M-NaCl	770	340	360	140	60
Strain PD18	2400	450	450	45	760
+ 0.2 M-NaCl	2100	400	440	105	1400
+ 0.6 M-NaCl	875	240	390	180	30
Strain PD51	3000	210	430	50	340
+ 0.2 M-NaCl	2940	180	450	70	270
+ 0.6 M-NaCl	680	620	330	180	30
<i>(b) R. meliloti</i>					
Strain SU-47	240	1100	455	160	—
+ 0.2 M-NaCl	2350	450	470	190	—
+ 0.6 M-NaCl	750	320	455	180	—
Strain SU-255	350	800	540	240	—
+ 0.2 M-NaCl	1290	280	550	245	—
+ 0.6 M-NaCl	540	385	360	190	—
Strain SU-256	470	165	510	250	—
+ 0.2 M-NaCl	945	10	530	230	—
+ 0.6 M-NaCl	400	85	380	220	—

* High- M_r carbohydrates in supernatant (succinoglycan) quantified as mg glucose equivalents per litre of culture.

† Low- M_r carbohydrates in supernatant quantified as mg glucose equivalents per litre of culture.

‡ Low- M_r carbohydrates in pellet (cyclic glucans + oligosaccharides) quantified as mg glucose equivalents per litre of culture.

§ Curdian quantified as mg glucose equivalents per litre of culture; absent in *R. meliloti*.

The production of succinoglycan in media supplemented with 0.6 M- as compared to 0.2 M-NaCl was lowered in all strains tested. Curdian production in *Agrobacterium* was strongly reduced in these media. None of the strains were able to grow in the presence of 1 M-NaCl.

To distinguish between an ion-specific (Na^+ , Cl^-) and a general osmotic effect on succinoglycan production by *R. meliloti* SU-47 various ions and non-ionic osmolytes were tested (Table 2). Initial experiments showed that 0.2 M-NaCl was the optimal NaCl concentration for succinoglycan production by strain SU-47. With divalent ions (CaCl_2 , MgCl_2 , MgSO_4 and Na_2SO_4) much lower concentrations (25 mM) were needed for an even higher production on succinoglycan and a higher viscosity of the cultures. Stimulation of succinoglycan production and concomitant rise in viscosity occurred also with 0.2 M-KCl and high concentrations of mannitol. Sucrose

(0.5 M) also stimulated succinoglycan production as could be seen from a rise in viscosity (because sucrose interfered with the anthrone assay, results were not shown).

Osmotic effects on excretion of extracellular carbohydrates of R. meliloti SU-47 at different incubation times

R. meliloti SU-47 was cultivated in the presence or absence of 0.2 M-NaCl in a well-aerated batch-fermenter (Fig. 1). Hardly any differences in growth rate and substrate uptake were observed. In both cases, synthesis of extracellular carbohydrates occurred mainly in the stationary phase. Because of the high carbon/nitrogen ratio of the medium not all the mannitol was consumed. The total amount of carbohydrate material excreted was higher in the NaCl-supplemented medium, and, as a

Fig. 1. Growth (as protein; Δ), production of exocellular carbohydrates (∇) and mannitol consumption ($\square$) by *R. meliloti* SU-47 at 25 °C as a function of time in production medium without (a) and with (b) 0.2 M-NaCl.

Table 2. *Osmotically induced cellular and extracellular carbohydrate synthesis by R. meliloti SU-47*

Cultures were incubated for 14 d at 25 °C on a rotary shaker (200 r.p.m.) in production medium (with additions as indicated). The data shown here are representative of two experiments between which values varied not more than 10%.

Addition	Osmotic pressure (atm)*	Supernatant			Cell pellet	
		Viscosity (cP)	High- M_r †	Low- M_r ‡	Low- M_r §	Protein (mg l ⁻¹)
None	2.0	1.5	240	1100	160	455
200 mM-NaCl	11.8	30	2330	470	190	470
200 mM-KCl	11.8	45	2280	290	140	445
25 mM-Na ₂ SO ₄	3.8	75	2870	660	155	420
25 mM-MgCl ₂	3.8	92	2990	120	210	450
25 mM-CaCl ₂	3.8	ND	2400	130	205	455
500 mM-mannitol	14.2	80	2700	700	180	395

ND, Not determined; the culture had to be diluted prior to centrifugation because of high viscosity.

* $\pi = \sum n_i \cdot M_i \cdot R \cdot T$ with π = calculated osmotic pressure, n = no. of moles of solute, M = molarity of the solutes, R = gas constant and T = absolute temperature (see Methods). (1 atm = 101 325 Pa.)

† High M_r succinoglycan quantified as mg glucose equivalents per litre of culture.

‡ Low M_r extracellular carbohydrates quantified as mg glucose equivalents per litre of culture.

§ Low M_r cellular carbohydrates quantified as mg glucose equivalents per litre of culture.

consequence, the viscosity (28 cP at 0.2 M-NaCl vs 1.5 cP without added NaCl) also. Although in Erlenmeyer flasks oxygen limitation might have occurred, the distribution of both high- and low- M_r carbohydrates was very much comparable to that shown in Table 1. As the pH in NaCl-supplemented media often fell below 5.5 during incubation an experiment was set up in a batch fermenter with the pH set at 7.0. No differences from the data in Fig. 1 were observed.

Succinoglycan production was also enhanced by solely elevating the concentrations of medium components. In

a medium with 40 g mannitol, 8 g glutamic acid and 8 times the concentration of salts and vitamins (8 $\times$ GMS medium), 7.8 g succinoglycan per litre of medium (95% of the total extracellular carbohydrate fraction) was produced by a biomass of 4 g protein (Fig. 2).

Identification of extracellular carbohydrates

Both the high- and low- M_r carbohydrate fractions showed glucose:galactose ratios of 6.5–7.2:1 as determined by component analysis. This agrees with succino-

Fig. 2. Growth, succinoglycan production and mannitol consumption by *R. meliloti* SU-47 at 25 °C as a function of time in a medium containing (l^{-1}) 40 g mannitol and 8 g glutamic acid. Symbols, as Fig. 1.

glycan having a glucose:galactose ratio of 7:1. To exclude interference from excreted cyclic glucans, the low- M_r carbohydrate fraction of *R. meliloti* SU-47 cultures grown in the presence or absence of 0.2 M-NaCl was chromatographed with a DEAE-Sephacrose anion-exchange column. In the experiment without added NaCl, three peaks were obtained. They corresponded to the repeat-units of succinoglycan having 0 (peak I, 5%), 1 (II, 35%) and 2 (III, 60%) succinate substituents per repeat-unit, respectively. With 0.2 M-NaCl, peak I could not be detected while peak II was predominant (70%). In neither case were cyclic glucans detected.

Influence of NaCl on cellular carbohydrates of R. meliloti strain SU-47

R. meliloti SU-47 was grown in standard medium containing 0 or 0.6 M-NaCl at 25 °C, and analysed at different time intervals (Table 3). When inoculating cells from salt-free medium into standard medium containing 0.6 M-NaCl, maximal cell yield was obtained after 4 d instead of 2 d. Most striking was the absence of glycogen at 0.6 M-NaCl. Without added NaCl, glycogen accumulation occurred within 2 d. Thereafter the glycogen disappeared gradually. PHB synthesis was unaffected by NaCl. At both 0 and 0.6 M-NaCl, a large fraction of cellular low- M_r carbohydrates was present, which remained constant during the whole incubation period (see below).

Identification of low- M_r cellular carbohydrates

The low- M_r carbohydrate fractions from cells of *R. meliloti* SU-47 grown at 0 and 0.6 M-NaCl were separated on a gel-filtration column (Fig. 3*a, b*). With cells grown in the absence of added NaCl almost all carbohydrates detected were glucans (Fig. 3), both charged (peak I) and uncharged (peak II). With NaCl-grown cells, a low- M_r oligosaccharide fraction became predominant (peak III), whereas the total amount of glucans was lowered, especially the charged glucans (Table 4).

Peak fractions were collected and freeze-dried. Component analysis of all peaks revealed exclusively glucose as the sugar constituent. Peak fractions I, II and III were further separated by TLC on silica gel. Peak II displayed

Table 3. Growth (as cell protein), cellular carbohydrates and PHB formation in *R. meliloti* SU-47 grown in the presence or absence of 0.6 M-NaCl during the course of incubation

Cells were incubated at 25 °C on a rotary shaker (200 r.p.m.) in standard medium. The data shown here are representative of at least three experiments.

	Time (d)	High- M_r * (mg l^{-1})	Low- M_r † (mg l^{-1})	Protein (mg l^{-1})	PHB (mg l^{-1})
No NaCl	2	340	125	510	75
	4	280	150	500	225
	7	250	170	475	250
	10	170	170	480	180
0.6 M-NaCl	2	10	0	240	0
	4	30	210	475	185
	7	35	215	425	215
	10	35	225	425	180

* High- M_r cellular carbohydrates (glycogen) in mg glucose equivalents per litre of culture.

† Low- M_r cellular carbohydrates (cyclic glucans and oligosaccharides) in mg glucose equivalents per litre of culture.

Fig. 3. Gel chromatography on Ultrogel AcA-202 of low- M_r cellular carbohydrates of *R. meliloti* SU-47. Cells were cultivated at 25 °C in standard medium without (a) and with (b) 0.6 M-NaCl.

Table 4. Distribution of the cellular low- M_r fractions (cyclic glucans and oligosaccharides) of *R. meliloti* SU-47 grown at different NaCl concentrations

Peak fractions I, II and III were obtained after gel-permeation chromatography (Fig. 3). Values are expressed relative to the total low- M_r fraction (100%).

NaCl concn (M)	Percentage of total low- M_r cellular fraction:			Total*
	I	II	III	
0	35	61	4	160
0.2	24	45	31	190
0.4	10	39	51	155
0.6	6	34	60	160

* Total cellular low- M_r carbohydrates in mg glucose equivalents per litre of culture.

a pattern of bands of neutral cyclic 1,2- β -glucans of DPs 19–25 (Zevenhuizen *et al.*, 1990). Peak I gave a pattern of closely situated bands that moved at faster rates. These bands have been identified as glycerol-1-phosphate-substituted cyclic glucans (Batley *et al.*, 1987; Zevenhuizen *et al.*, 1990). On methylation analysis of peak I and II the principal linkage was found to be 1,2- since only 3,4,6-tri-*O*-methyl-D-glucose was detected. With HPLC, ring cycles with DPs of 17–29 could be found.

Peak III consisted of gluco-oligosaccharides with DPs of 2–6. The DP values were determined by comparing the R_F values of the gluco-oligosaccharides with those of a series of sophoro-oligosaccharides. These were obtained by partial hydrolysis of 1,2- β -glucans and analysed by HPLC and TLC (results not shown). Methylation analysis of peak III fractions from cells cultivated at various salt concentrations yielded 2,3,4,6-

Fig. 4. ^1H -NMR spectra of peak fraction III (a) taken from the experiment in Table 4 (0.4 M-NaCl) compared with that of α , α -trehalose (b). Concentration 20 mg D_2O ml^{-1} .

tetra-*O*-methyl-D-glucose and 3,4,6-tri-*O*-methyl-D-glucose in the ratios 95:5 at 0.6 M-NaCl, 84:16 at 0.4 M-NaCl, and 62:38 at 0.2 M-NaCl. These data show that the 1,1- bond was predominant in all cases tested, indicating a trehalose structure. Some 1,2- bonds were also present in the higher oligosaccharides, with a maximum at 0.2 M-NaCl. Peak III fractions were subjected to ^1H -NMR with trehalose as a reference. The

spectra for the oligosaccharide fraction of peak III from cells grown at 0.4 M-NaCl and pure trehalose (α -D-glucopyranosyl α -D-glucopyranoside) were identical (Fig. 4). Most characteristic is the resonance at 5.20 p.p.m. ($J_1-J_2 = 3.3$ Hz) as a result of the α -anomeric protons (Usui *et al.*, 1974). As in α,α -trehalose the two glucose residues are identical, only one doublet being observed. Some very weak signals in the region between 5.0–5.5 could be observed as well, possibly due to anomeric protons of glucose in the 1,2- α -configuration.

Discussion

Osmoregulation at low osmolarity

Cyclic glucans of *Agrobacterium* and *Rhizobium* are localized in the periplasmic space, have an intermediate M_r of about 3500, and contain a 1,2- β -glucan backbone. Because of their structural resemblance to the so-called membrane-derived oligosaccharides (MDO) of *E. coli* (Schulman & Kennedy, 1979), the regulation of the biosynthesis of cyclic glucans is comparable (Miller *et al.*, 1986). At low osmotic values of the medium a net flow of water into the cytosol takes place leading to an increase of turgor pressure, and therefore it was supposed that the periplasm tends to remain iso-osmotic with the cytoplasm (Stock *et al.*, 1977). The neutral and especially the anionic periplasmic cyclic glucans may be very well suited as a fixed osmolyte for increasing the turgor pressure in the periplasm against the cytoplasmic membrane (Miller *et al.*, 1986, 1987).

R. leguminosarum cells (all biovars) build up considerable amounts of 1,2- β -glucans during the exponential growth phase, but during the stationary phase their glucan content falls. At this point the cells became embedded in a CPS-matrix (Zevenhuizen, 1981, 1984). It appears that strains of *R. leguminosarum* grown in standard medium combine a low 1,2- β -glucan concentration with a high deposition of CPS as is the case with *A. tumefaciens* cells. However *R. meliloti* cells, which do not form capsules, usually have constantly high 1,2- β -glucan concentrations (Table 1). Therefore, we suggest that both properties – constant high periplasmic 1,2- β -glucan concentration and the presence of capsular material – can be mechanisms for cells of the *Rhizobiaceae* to maintain their integrity in standard medium of low osmolarity.

Capsular polysaccharides could very well function to counteract the turgor pressure of the cells against the outer membrane. Studies on isolated curdland of *A. tumefaciens* and of CPS of *R. leguminosarum* biovar *trifolii* TA-1 have shown that these polymers form strong gels in water (gel strength about 500 g cm⁻² of a 1% CPS solution; Zevenhuizen, 1984). These gelling matrices

may act mechanically against the turgor pressure of the cells *in vivo* also.

At certain critical conditions of osmolarity and of temperature, when cells of *R. leguminosarum* biovars *viciae* and *trifolii* are unable to form CPS and can form small amounts of EPS only, continuous 1,2- β -glucan excretion into the medium takes place in amounts up to several grams per litre (Breedveld *et al.*, 1990). It thus seems that in biovars of *R. leguminosarum*, which are osmotically and temperature sensitive, 1,2- β -glucan synthesis is the first action undertaken by the cells in an effort to maintain cell integrity. *R. meliloti* cells, which are osmotically much more tolerant, are not deregulated in such a way, but can maintain a high glucan concentration within their cells at low osmolarity.

The ring size distribution of the cyclic glucans from *R. meliloti* SU-47, as determined by HPLC, is clearly different from that of *R. leguminosarum* biovar *trifolii* TA-1 (Breedveld *et al.*, 1990), namely DPs 17–29 and 17–25, respectively. The proportion of the glucans which were glycerol-phosphorylated was 40% in *R. meliloti* SU-47 while substituted glucans were not detected, or formed a minor fraction, in *R. leguminosarum* biovars (Zevenhuizen *et al.*, 1990).

Osmoregulation at high osmolarity

When *A. tumefaciens* C58 was grown in yeast extract-mannitol medium supplemented with 0.4 M-NaCl, the amount of both the neutral and the anionic cyclic glucan fraction was strikingly lower in comparison to the situation without added NaCl (Miller *et al.*, 1986). No osmoregulatory function can be ascribed for periplasmic 1,2- β -glucans in media with high NaCl concentrations. However, the neutral cyclic glucans were still synthesized by *R. meliloti* SU-47, although at a lower level.

In media containing NaCl, a considerable increase of oligosaccharides was observed, with DP = 2 [trehalose, up to 200 mg (g protein)⁻¹] as the principal one, and with minor amounts of oligomers of DPs 4–6. Their accumulation at high osmotic values of the medium is most likely to function as osmoregulators for the cytoplasm of the cell. Because of the presence of 1,2- and 1,1- linkages in these latter oligosaccharides they are probably identical with the three koji-oligosaccharides (containing 1,2- α -bonds and a terminal 1,1- α -bond) of DPs 4–6 from cultures of *R. meliloti* J7017 studied by Hisamatsu *et al.* (1985). These authors detected the non-reducing oligosaccharides after ethanol extraction of whole cell cultures without reporting the origin (cellular or extracellular) of the molecules.

Trehalose was found in small quantities, varying from 1–20 mg (g dry wt)⁻¹ within the cytoplasm of cells of *Rhizobium* spp. grown in media of low osmolarity (Streeter, 1985). The high cellular low- M_r carbohydrate

content detected in *A. tumefaciens* cells grown at 0.4 M NaCl by Miller *et al.* (1986) was probably due to trehalose as osmoprotectant. Recently, Dylan *et al.* (1990) showed that *R. meliloti* 102F34 mutants defective in 1,2- β -glucan accumulation were impaired in their growth rate under low-osmotic conditions as compared to the wild-type. Their growth was restored by raising the osmolarity of the medium, at which point a cellular low- M_r oligosaccharide appeared, apparently trehalose. As is shown in these examples, the cellular low- M_r carbohydrate fraction did not consist solely of cyclic glucans. We found evidence that the majority of the cellular low- M_r carbohydrate fraction in NaCl-supplemented media consisted of trehalose.

It is important to keep in mind that the nature of the medium and the growth phase can influence the amount of trehalose synthesized. In carbon-rich media as used in our study high concentrations of various carbohydrates can be obtained. In nitrogen-rich media and at other growth phases different osmoregulatory mechanisms may take place, like glycine, proline and betaine accumulation (Le Rudulier & Bernard, 1986; Czonka, 1989).

EPS and osmoregulation

Osmotically induced changes in extracellular polysaccharide production have been reported for a *Pseudomonas aeruginosa* strain (Berry *et al.*, 1989). With *A. tumefaciens* strains, and with *A. radiobacter* NCIB 11883 (Linton *et al.*, 1987), succinoglycan was produced in media of low osmotic strength. The production of 2–4 g succinoglycan (g protein)⁻¹ by *R. meliloti* SU-47 was comparable to the data reported for *R. meliloti* J7017 and *R. leguminosarum* biovar *trifolii* J60 by Amemura *et al.* (1983). Although these authors found acidic oligosaccharides and succinoglycan in addition to cyclic glucans in the medium, we did not find extracellular cyclic glucans for strain SU-47.

Many reports claim strong correlations between functional genes needed for succinoglycan biosynthesis and the ability of *R. meliloti* to invade root hairs (Leigh *et al.*, 1985; Zhan *et al.*, 1989) or to induce the formation of nodules (Long *et al.*, 1988). The differences in the relative production of high- and low- M_r extracellular carbohydrates depending on the environmental osmolarity, as was found for different *R. meliloti* strains, may be related to this phenomenon.

It has been shown that growth of *R. meliloti* NSI was inhibited by various salts depending on the specific ion rather than the increase in osmolarity (Botsford, 1984). Our report shows that succinoglycan production by *R. meliloti* appeared to be enhanced by increasing osmotic pressure. With increasing succinoglycan production viscosity rose, but the relatively small differences in

production of EPS by *R. meliloti* SU-47 could not explain the large differences in viscosity, especially with the divalent ions tested. This last phenomenon is probably an effect of a different conformation of the polysaccharide in the presence of divalent ions. This was found for the Ca-form of succinoglycan from *Alcaligenes faecalis* var. *myxogenes* 10C3 (Harada & Amemura, 1981) and for the acidic EPSs from *R. leguminosarum* (Morris *et al.*, 1989).

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