Response of intracellular carbohydrates to a NaCl shock in *Rhizobium leguminosarum* biovar *trifolii* TA-1 and *Rhizobium meliloti* SU-47

MICHAËL W. BREEDVELD,* COR DIJKEMA, LUDOVICUS P. T. M. ZEVENHUIZEN
and ALEXANDER J. B. ZEHNDER†

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

(Received 13 April 1993; revised 12 July 1993; accepted 27 July 1993)

The dynamic response of cellular carbohydrates to a NaCl shock in *Rhizobium leguminosarum* biovar *trifolii* TA-1 (0.25 M-NaCl) and *Rhizobium meliloti* SU-47 (0.4 M-NaCl) grown in NaCl-free medium was investigated in non-growing cell cultures and in cell suspensions, using *in vivo* NMR. After transferring cells grown in a NaCl-free medium to a glutamic-acid-free medium containing mannitol and NaCl, both strains immediately responded to the increased osmotic pressure by augmenting the cellular trehalose content of the cell. Without mannitol in the medium trehalose synthesis was slower, but clearly detectable. Its synthesis paralleled the breakdown of the reserve materials glycogen and poly- β -hydroxybutyric acid (PHB). NMR experiments with 25-fold-concentrated cell suspensions using $^{13}\text{C}_1$ -mannitol as substrate revealed that 15–20% of the trehalose synthesized was derived from mannitol, but 80–85% was from other sources. Trehalose was mainly formed from the internal pool of glycogen and/or PHB, whether mannitol was present or not, and reached 135 and 280 μg (mg cell protein) $^{-1}$ in the strains TA-1 and SU-47, respectively. At low osmolarity, intracellular trehalose was metabolized by strains TA-1 and SU-47. Intracellularly accumulated phosphoglycerol-substituted and neutral cyclic (1,2)- β -glucans of SU-47 cells grown in the absence of NaCl were neither degraded nor excreted after exposure to NaCl. Strain TA-1, which only makes neutral cyclic (1,2)- β -glucans, continued to synthesize and excrete cyclic (1,2)- β -glucans after exposure to NaCl. By using *in vivo* ^{31}P -NMR, a sharp peak at 1.34 p.p.m. was present in cell suspensions of strain SU-47. This peak, representing glycerol-1-phosphate-substituted cyclic glucans, was absent in strain TA-1.

Introduction

Rhizobia, like other non-halophilic Gram-negative bacteria, are able to adapt to differences in osmotic pressure between the cytoplasm and the environment. The processes involved in adaptation take place in different compartments of the cells, depending on the osmotic pressure of the environment. In media with low osmotic pressure (≤ 3 atm) the organisms accumulate low- M_r carbohydrates in their periplasm, as for the membrane-derived oligosaccharides in *Escherichia coli* or the cyclic (1,2)- β -glucans in *Rhizobium* and *Agrobacterium* spp., for example. Their synthesis is repressed when the medium

has a high osmotic pressure (Kennedy & Rumley, 1988; Miller *et al.*, 1986, 1988; Zorreguieta *et al.*, 1990).

Cells of *E. coli* or *Rhizobium meliloti* respond to increased environmental osmotic pressure (5–20 atm) by import of potassium ions and concomitant synthesis and accumulation of glutamic acid in their cytoplasm; this is followed in a later growth stage by the formation of trehalose as a compatible solute (Csonka, 1989; Le Rudulier & Bernard, 1986; Welsh *et al.*, 1991). Inhibition of growth by NaCl could be alleviated by adding exogenous proline betaine, an osmo-protectant not synthesized by *Rhizobium*. In the presence of NaCl, proline betaine accumulates in the cells, while without NaCl in the medium it is metabolized by the cells (Gloux & Le Rudulier, 1989). Actively growing *R. meliloti* cells accumulate 0.5 μmol glutamic acid (mg protein) $^{-1}$ in media containing 0.4 M-NaCl (Botsford & Lewis, 1990) and 0.2 μmol (mg protein) $^{-1}$ of the dipeptide *N*-acetylglutaminylglutamine amide (Tombras Smith & Smith, 1989). Strains of *Agrobacterium tumefaciens* accumulate both glutamic acid and the novel disac-

* Author for correspondence. Present address: Department of Food Science, 101 Borland Laboratory, The Pennsylvania State University, University Park, PA 16802, USA. Tel. +1 814 863 7784; fax +1 814 863 6132; e-mail MWB6@psuvm.psu.edu.

† Present address: EAWAG/ETH, CH-8600 Dübendorf, Switzerland.

Abbreviations: bv, biovar; PHB, poly- β -hydroxybutyric acid.

charide β -fructofuranosyl α -mannopyranoside, or mannosucrose (Tombras Smith *et al.*, 1990).

Recently, it was suggested that trehalose in micro-organisms has a function in protecting against various stress conditions rather than solely being a reserve material for carbon and energy (Van Laere, 1989; Wiemken, 1990; Zevenhuizen, 1991). *Rhizobia* accumulate trehalose in small amounts of up to 25 nmol (mg protein)⁻¹ when the osmotic pressure of the medium is low (Streeter, 1985). Under microaerophilic conditions (1% O₂), the trehalose content increases between 10- and 20-fold, reaching up to 250 nmol trehalose (mg protein)⁻¹ (Hoelzle & Streeter, 1990). To compensate for the difference in osmotic pressure across the cytoplasmic membrane, *R. meliloti* cells accumulate up to 850 nmol trehalose (mg protein)⁻¹ in the cytoplasm when grown in medium containing 200–800 mM-NaCl (Breedveld *et al.*, 1990b).

We have investigated the ability of members of the *Rhizobiaceae* to grow in medium at high osmotic pressures, and their tolerance towards several ionic and non-ionic osmolytes (Breedveld *et al.*, 1990b, 1991). Two model organisms were used, *Rhizobium leguminosarum* bv. *trifolii* TA-1, which grows in media containing up to 350 mM-NaCl, and *R. meliloti* SU-47, which can grow at concentrations of up to 1 M-NaCl. The two strains were found to differ in their (1,2)- β -glucan synthesis. In the presence of 250 mM-NaCl, strain TA-1 synthesized and continuously excreted neutral cyclic (1,2)- β -glucans into the medium (Breedveld *et al.*, 1991). In strain SU-47, the formation of both anionic and neutral (1,2)- β -glucan formation was repressed in medium containing 400 mM-NaCl (Breedveld *et al.*, 1990b). At osmotic pressures of 14–20 atm trehalose accumulated, while glycogen synthesis was fully repressed in both strains. In the present study we have investigated the dynamics of intracellular carbohydrate production in non-growing cells of *Rhizobium* upon exposure to NaCl.

Methods

Organisms and cultivation. *R. leguminosarum* bv. *trifolii* TA-1 and *R. meliloti* SU-47 were obtained from the culture collection of the Department of Microbiology, Wageningen, The Netherlands. Pre-cultures were prepared by inoculating the organism into standard medium with the following composition: 5 g mannitol l⁻¹, 1 g glutamic acid l⁻¹ and mineral salts and vitamins, having a total osmotic pressure of 1.3 atm (Breedveld *et al.*, 1990b). The pH was adjusted to 7.0 with NaOH. All media were autoclaved at 121 °C for 25 min. Cells from the late exponential phase were inoculated (1%, v/v) into 3 litre Erlenmeyer flasks containing 500 ml medium. The flasks were incubated on a rotary shaker (200 r.p.m.) for 72 h at 25 °C. Cells and culture supernatant were separated by centrifugation at 35000 g for 30 min in sterile tubes. For the exposure to NaCl, cells obtained from 1 litre of a batch culture (500 mg cellular protein) were washed and resuspended in 50 ml washing buffer (standard medium without glutamic acid and mannitol). This cell suspension was transferred into

a 2 litre fermenter with 1 litre of standard medium containing 250 mM-NaCl (strain TA-1) or 400 mM-NaCl (strain SU-47), but no glutamic acid. The fermenter was used with an airflow of 1 litre air min⁻¹ per litre of culture and the impeller speed set at 600 r.p.m. as described previously (Breedveld *et al.*, 1990a); 80 ml samples were taken at different time intervals. In all experiments, cells were separated from the medium by centrifugation for 30 min at 35000 g. Both pellet and supernatant were used to quantify the different oligo- and polysaccharides.

Cell pellet. For the determination of low-*M_r* carbohydrates, cell pellets were extracted with 75% (v/v) ethanol at 70 °C for 30 min, after which the mixture was centrifuged. In the 75% ethanolic supernatant, the total low-*M_r* carbohydrate fraction (containing cyclic glucans and/or trehalose) was quantified as total hexoses. For the determination of glycogen, the ethanol-extracted cells of *R. meliloti* SU-47, which do not produce capsules, were incubated in 1 M-HCl at 100 °C for 2 h in order to hydrolyse the glycogen fraction. The hydrolysate was centrifuged, after which glycogen was measured in the supernatant and expressed as mg glucose equivalents per litre of culture. In the case of *R. leguminosarum* bv. *trifolii* TA-1, ethanol-extracted cells were first suspended in 1 M-NaOH for 15 min at 70 °C to solubilize capsular polysaccharides; the cells were then centrifuged and glycogen was determined in the ethanol- and alkaline-extracted cell pellet after hydrolysis in HCl.

Supernatant. High-*M_r* polysaccharides were separated from the low-*M_r* carbohydrates by precipitation with 3 vols ethanol. The ethanolic supernatant contained cyclic glucans which were quantified as described earlier (Breedveld *et al.*, 1990a).

Analytical methods. Hexoses were measured by the anthrone-sulphuric acid method (Trevelyan & Harrison, 1952). Mannitol was determined by periodate oxidation-chromotropic acid colorimetric method (Burton, 1957). Total cellular protein was determined by the Lowry method, using bovine serum albumin as the standard. PHB determinations were performed according to the method of Braune *et al.* (1978), in which PHB was measured by GLC with benzoic acid as the standard and expressed as mg PHB per litre of culture.

Low-*M_r* cellular carbohydrates were separated by gel-permeation chromatography using a column of Ultrogel Aca 202 (25 × 2.5 cm). Fractions of 2.5 ml were collected and hexoses were measured. Charged glucans, neutral glucans and trehalose were found in fractions 10–13, 20–24 and 27–30 respectively (Breedveld *et al.*, 1990b). Peak fractions were analysed by methylation analysis (Harris *et al.*, 1984).

In vivo NMR. Cell pellets were washed with 50 mM-phosphate buffer pH 7.0 or 50 mM-Tris/HCl pH 7.8. To 10 ml of 25-fold-concentrated cell suspensions [12.5 mg protein (ml buffer)⁻¹] of strains SU-47 and TA-1 grown in standard medium for 72 h, 1 ml D₂O was added together with 91.8% ¹³C₁-labelled mannitol (Merck, Sharp and Dohme) or unlabelled mannitol (³¹P-NMR); the suspensions were continuously sparged with O₂ (1 litre h⁻¹). CaCO₃ (10 g l⁻¹) was added to prevent acidification of the suspension. Fourier transform NMR ¹³C and ³¹P spectra were obtained using a Bruker CXP-300 spectrometer equipped with a 20 mm internal diameter ¹³C/³¹P switchable probe, operating either at 75.46 MHz (¹³C) or 121.47 MHz (³¹P). The WALTZ pulse sequence was used to decouple protons during recording of the ¹³C-NMR spectra, the temperature being kept close to 25 °C. Spectra of 1 h, covering a spectral width of 20000 Hz (a line broadening of 5 Hz was applied), were obtained after Fourier transformation of blocks of 7200 FIDs that were sequentially stored on disk in 8 K data points, using a 60 ° pulse and a pulse delay of 0.5 s. The ³¹P-NMR spectra, covering a spectral width of 10000 Hz, were recorded using a 60 ° pulse and a pulse delay of 0.5 s. A line broadening of 20 Hz for whole cells and 3 Hz for cell extracts was applied prior to Fourier transformation. WALTZ decoupling was only used in the case of cell extracts that were measured at a temperature kept close to 4 °C. ¹³C-NMR chemical shifts

were referenced to the mannitol C₁-resonance at 64.3 p.p.m., whereas ³¹P-NMR chemical shifts were referenced to glycerophosphorylcholine at 0.49 p.p.m.

Results and Discussion

Dynamics of cellular carbohydrate metabolism after exposure to NaCl

The low-*M_r* cellular carbohydrate fraction of strain TA-1, which was exposed to 0.25 M-NaCl, consisted of two peaks after separation by gel chromatography. These peaks eluted at the same positions as neutral cyclic (1,2)- β -glucans and trehalose (Breedveld *et al.*, 1991) (Table 1). Methylation analysis of the cellular glucan fraction, which remained constant during the experiment, yielded

Table 1. *Cellular carbohydrates in R. leguminosarum* bv. *trifolii* TA-1 after exposure to NaCl

Cells were pregrown in NaCl-free medium, then resuspended in a glutamic-acid-free medium containing 5 g mannitol l⁻¹ and 250 mM-NaCl

Time (h)	Low <i>M_r</i> *			Glycogen [μ g (mg protein) ⁻¹]
	Total	Glucan	Trehalose	
0†	80	80	0	390
6	100	70	30	145
24	125	75	50	50
48	150	75	75	20
120	220	85	135	10

* Low-*M_r* carbohydrates in μ g glucose equivalents (mg protein)⁻¹. The low-*M_r* fraction was separated by gel chromatography into a cyclic glucan fraction and a trehalose fraction.

† The data at *t* = 0 are from the TA-1 cells grown for 72 h in the absence of NaCl.

Table 2. *Cellular carbohydrates in R. meliloti* SU-47 after exposure to NaCl

Cells were pregrown in NaCl-free medium, then resuspended in a glutamic-acid-free medium containing 5 g mannitol l⁻¹ and 400 mM-NaCl

Time (h)	Low M_r^*				Glycogen [$\mu\text{g (mg protein)}^{-1}$]
	Total	Glucan		Trehalose III	
		I	II		
0†	250	80	165	3	450
1	270	70	150	50	340
2	300	70	140	90	270
3	320	70	150	100	240
6	360	70	160	130	210
30	410	60	140	210	120
80	495	70	140	280	80

* Low-*M_r* carbohydrates in μ g glucose equivalents (mg protein)⁻¹. The low-*M_r* fraction was separated by gel chromatography into a charged cyclic glucan fraction (I), a neutral cyclic glucan fraction (II) and a trehalose fraction.

† The data at *t* = 0 are from the SU-47 cells grown for 72 h in the absence of NaCl.

1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-glucitol as the sole methylated sugar, confirming the cyclic glucan structure with (1,2)-linkages. The second peak, which increased with time, revealed exclusively 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, confirming the (1,1)-structure of trehalose (α -D-glucopyranosyl- α -D-glucopyranoside).

When strain SU-47 was exposed to 0.4 M-NaCl three peaks could be distinguished after separation of the low-*M_r* cellular carbohydrate fraction by gel chromatography. The total internal cyclic (1,2)- β -glucan pool, comprising both the anionic (peak I, Table 2) and neutral cyclic glucans (peak II, Table 2), remained constant during the experiment (80 h). Trehalose (peak III, Table 2) synthesis occurred faster and reached a higher level than in strain TA-1.

The external carbon source (5.0 g mannitol l⁻¹) was partially utilized; after 120 h 1.5 g (strain TA-1) or 3.1 g mannitol l⁻¹ (strain SU-47) was still present. Glycogen was degraded almost completely (Tables 1 and 2). The total cell protein remained constant, indicating that no cell lysis had occurred after exposure to NaCl. The TA-1 cells excreted up to 450 mg of cyclic (1,2)- β -glucans per litre after 120 h of incubation, in agreement with the osmotically induced glucan excretion reported earlier (Breedveld *et al.*, 1991). Strain SU-47 did not excrete cyclic glucans (not shown), nor were they degraded after exposure to NaCl.

When no external mannitol and glutamic acid were present, trehalose synthesis was still observed in both strains (not shown). This synthesis could only take place at the expense of the reserve materials present, since no other carbon sources were available. Indeed, in SU-47 cells the decrease of PHB [from 410 to 240 μ g (mg cell protein)⁻¹] and glycogen [from 630 to 320 μ g (mg cell protein)⁻¹] paralleled the synthesis of trehalose [210 μ g (mg cell protein)⁻¹] during the course of the experiment (52 h). The onset of trehalose synthesis was much slower in the absence than in the presence of mannitol. Furthermore, the cellular concentration of neutral (strains TA-1 and SU-47) and anionic (SU-47) cyclic β -(1,2)-glucans remained constant during the incubation period.

In vivo ¹³C-NMR

NMR experiments were performed to obtain more insight into the dynamics of trehalose synthesis and breakdown of reserve materials. Aerated cell suspensions of the strains *R. meliloti* SU-47 and *R. leguminosarum* bv. *trifolii* TA-1 were first incubated in the absence of NaCl and ¹³C₁-mannitol. The intracellular concentration of cyclic (1,2)- β -glucans in the cell suspension of strain SU-47 was 260 μ g (mg cell protein)⁻¹ or 3 mg (ml sus-

Fig. 1. *In vivo* ^{13}C -NMR spectra of aerated cell suspensions of *R. meliloti* SU-47. (a) Incubation of cells in phosphate buffer in the absence of mannitol, showing all six resonances of cyclic (1,2)- β -glucans (G_1 - G_6) and the mobile CH_3 -resonances of PHB. * Most likely reflects the broad resonances of endogenous high- M_r polysaccharides and/or PHB compounds. G-P, glycerol-phosphate. (b) Incubation of cells in Tris buffer (B) in the presence of $^{13}\text{C}_1$ -mannitol (M), before ($t = -1$ h) and after ($t = 1-5$ h) the exposure to NaCl, showing the synthesis of trehalose (T). A, C_2 -glutamic acid. Note the overlap of the C_6 -resonances of glucan and trehalose.

pension) $^{-1}$. The resonances of C_1 - C_6 of the cyclic (1,2)- β -glucans at 102.9, 83.3, 76.5, 69.8, 77.2 and 61.7 p.p.m. (Fig. 1a) could be easily assigned (Zevenhuizen *et al.*, 1990). Resonances of low intensity at 63.7 and 67.7 p.p.m. could be assigned to the C_3 - and C_1 -carbons of the glycerol-phosphate substitutions being present on part of the C_6 -glucoses of the anionic cyclic glucans in *R. meliloti* (Zevenhuizen *et al.*, 1990). The C_2 -resonance of the glycerol-phosphate substitution at 71.9 p.p.m. was less resolved.

The carbon resonances of the cyclic glucans in cell suspensions of strain SU-47 were well resolved, reflecting the high intrinsic flexibility of the cyclic (1,2)- β -glucans. The cellular cyclic glucan concentration in TA-1 cells [0.8 mg (ml suspension) $^{-1}$] is much lower than in strain SU-47 and its resonances were inherently less visible in the spectra of TA-1 cells before exposure to NaCl. It is however clear that under no circumstances were cyclic glucans degraded, not even at high NaCl concentrations (see below), at which their function is dispensable (Dylan *et al.*, 1990; Miller *et al.*, 1986; Zorreguieta *et al.*, 1990).

The broad resonance around 20 p.p.m. that was observed in suspensions of both strain SU-47 (Fig. 1a) and TA-1 (not shown) was most likely due to the CH_3 -carbon atoms in a mobilized PHB fraction (Claassen *et al.*, 1986). This peak diminished upon prolonged in-

cubation, both in the presence or absence of mannitol and/or NaCl, indicating breakdown of PHB.

Exposure to NaCl

$^{13}\text{C}_1$ -Mannitol at 4.5 g l^{-1} and NaCl at a concentration of 0.4 M (strain SU-47) or 0.25 M (strain TA-1) were added to suspensions of cells grown in the absence of NaCl. Low amounts of ^{13}C -enriched glutamate were measured in both strains after exposure to NaCl (Figs 1b and 2). Its C_2 - (55.6 p.p.m.) and C_3 -carbons (28.0 p.p.m.; not shown) had half the intensity of the C_4 -carbon (34.4 p.p.m.; not shown), indicating that the carbon atoms were derived from $^{13}\text{C}_1$ -mannitol after entering the Krebs cycle (Claassen *et al.*, 1986).

Cyclic β -(1,2)-glucan synthesis by strain TA-1 was observed after exposure to NaCl (Fig. 2). It can be assumed that the glucans were predominantly formed from the substrate mannitol and not from the reserve materials because of the following: (i) in strain TA-1 grown at high NaCl concentrations the synthesis of glycogen and PHB was strongly repressed (Breedveld *et al.*, 1991); and (ii) in the presence of $^{13}\text{C}_1$ -mannitol in NaCl-exposed strain TA-1 cells, only the $^{13}\text{C}_1$ - and $^{13}\text{C}_6$ -resonances of the glucose residues of the cyclic (1,2)- β -glucans were enhanced (Fig. 2).

Fig. 2. *In vivo* ^{13}C -NMR of aerated cell suspensions of *R. leguminosarum* bv. *trifolii* TA-1. Cells in phosphate buffer were incubated in the presence of $^{13}\text{C}_1$ -mannitol (M). The spectra show the appearance of trehalose (T) and cyclic glucans (G) after ($t = 1$ –15 h) exposure to NaCl. For other abbreviations and remarks see Fig. 1.

Resonances at 94.1 and 61.6 p.p.m. were a clear indication for C_1 and C_6 of trehalose (Figs 1*b* and 2). The highest incorporation of mannitol-derived label in trehalose was obtained after 5–9 h. Trehalose levels remained constant thereafter, even after all mannitol had disappeared (not shown). Trehalose formed a major fraction in the low- M_r carbohydrate pool of cell suspensions of both strains SU-47 and TA-1 incubated for 20 h in the NaCl-containing buffer (not shown), as was shown for the NaCl-exposed cultures (Tables 1 and 2). Therefore, synthesis of trehalose could not be explained by sole conversion from the enriched $^{13}\text{C}_1$ -

mannitol. Indeed, at 72.1, 73.5, 70.5 and 73.1 p.p.m. the four remaining natural abundant resonances of equal amplitude belonging to the C_2 – C_5 -carbon atoms of trehalose (Meikle *et al.*, 1991) could easily be detected in both strains (Figs 1*b* and 2). Since the natural abundance of ^{13}C is 1.1%, calculations indicate that about 15–20% of the trehalose was derived from the external mannitol and 80–85% from the internal carbon-reserve pool, e.g. from glycogen and PHB. These polymers contain few mobile carbon chains and do not show well-resolved resonances in the NMR spectra, except for the PHB- CH_3 resonance. To summarize, in non-growing cells of both strains: (i) trehalose synthesis occurred immediately after exposure to NaCl; and (ii) at least 80% of the trehalose was synthesized from the internal pool of reserve glycogen and PHB, whether the external carbon source mannitol was present or not. It remains unclear whether synthesis of trehalose is directly coupled to degradation of one of the reserve materials. However, because of structural resemblance, we suggest that trehalose [containing the α -(1,1)-bond] is most likely formed from glycogen [α -(1,4)-glucan] and not from the structurally unrelated PHB.

Strain SU-47 cells which were incubated for 20 h in Tris buffer in the presence of 0.4 M-NaCl and $^{13}\text{C}_1$ -mannitol contained partially $\text{C}_{1,6}$ - ^{13}C -enriched trehalose. Trehalose was immediately broken down when these cells were transferred to NaCl-free buffer without external carbon (Fig. 3). It has been proposed that trehalose functions as a general protectant against adverse conditions like desiccation, and osmotic and temperature stress, rather than as a reserve material (Van Laere, 1989; Wiemken, 1990; Zevenhuizen, 1991). Therefore, the breakdown of trehalose in cells in the

Fig. 3. *In vivo* ^{13}C -NMR spectra of aerated C_1, C_6 - ^{13}C trehalose-accumulated *R. meliloti* SU-47 cells in NaCl-free Tris buffer. Note the overlap of the Tris buffer (B) and C_6 -resonance of trehalose and cyclic glucans at $t = 1$ h. For other abbreviations, see Fig. 1.

Fig. 4. ^{31}P -NMR spectra (0.5 h) of aerated cell suspensions of *R. leguminosarum* bv. *trifolii* TA-1 (a) and *R. meliloti* SU-47 (b) in Tris buffer. The resonance at 1.34 p.p.m. represents the phosphoglycerol substitution (G-P) in the cyclic glucans of strain SU-47. HP, hexose-phosphates; P_i , cytosolic inorganic phosphate; NAD, phosphates present in NAD(P)/NAD(P)(H); α , β , and γ , phosphates present in ATP.

absence of NaCl is a clear indication that its function is dispensable in a low osmolarity environment.

In vivo ^{31}P -NMR

The phosphoglycerol-substituted cyclic (1,2)- β -glucans of strain SU-47 were investigated with ^{31}P -NMR. In ^{31}P -spectra of aerated cell suspensions, differences between strains SU-47 and TA-1 could be clearly observed, strain TA-1 showing much lower levels than strain SU-47. Since the cells were suspended in Tris buffer, no external phosphate was present. The spectra of strain SU-47 cells showed the resonances of alkaline cytosolic inorganic P_i (2.91 p.p.m.) besides hexose-phosphates (around 4.9 p.p.m.), co-factors NAD(P)/NAD(P)(H) (-10.7), ATP (-5.0 , -10.0 , and -18.6 p.p.m. belonging to γ , α and β ATP) and UDPG (-10.7 and -12.3 p.p.m.). In addition, the spectra of strain SU-47 revealed a sharp peak at 1.34 p.p.m. which was absent in strain TA-1 (Fig. 4). Its resonance position, being non-titratable in the physiological region, was calibrated against added glycerophosphoryl-choline (0.49 p.p.m.). The peak position and area remained unchanged during the whole incubation period of 20 h in the presence of NaCl. Glycerol-1-phosphate-substituted cyclic (1,2)- β -glucans, obtained from strain SU-47 cells after separation by gel chromatography of the low- M_r carbohydrate fraction, were added either to the cell suspensions or to the cell

extract. The amplitude of this single narrow resonance at 1.34 p.p.m. increased, but its shape remained constant. This clearly indicates that the resonance at 1.34 p.p.m. belonged to the phosphate group of the glycerol-1-phosphate-substituted cyclic (1,2)- β -glucans. Batley *et al.* (1987) observed the phosphate resonance of a purified phosphoglycerol-substituted cyclic (1,2)- β -glucan fraction of *Rhizobium* sp. NGR234 in a proton-decoupled ^{31}P spectrum as a single peak, as is found for the phosphoglycerol-substituted glucans in whole cell suspensions of strain SU-47 (Fig. 4).

The synthesis of phosphoglycerol-substituted cyclic glucans of *R. meliloti* SU-47 was strongly repressed in medium containing 0.4 M-NaCl, while synthesis of the neutral cyclic glucans was only moderately repressed (Breedveld *et al.*, 1990b). In the experiments presented here, strain SU-47 cells were grown in standard medium having a low osmotic pressure of 1.3 atm. Therefore, the concentration of cyclic glucan, being 35–40 % substituted for in stationary-phase cells grown in standard medium, had already reached its maximum value. We did not find any change in the phosphoglycerol-substituted glucan content after exposure of the SU-47 cells to NaCl, implying that no release of the substituent from the glucans had occurred under the specified conditions. Batley *et al.* (1987) discussed the possibility that glycerol-phosphorylated cyclic glucans might function as a source of phosphorus. Therefore, the glycerolphosphate group should preferentially be released from the cyclic glucan under conditions of phosphate limitation.

This investigation was carried out with the support of the Dutch National Innovation Oriented Program Carbohydrates (IOP-k). We thank Nees Slotboom for the artwork.

References

- BATLEY, M., REDMOND, J. W., DJORDJEVIC, S. P. & ROLFE, B. G. (1987). Characterization of glycerophosphorylated cyclic β -(1,2)-glucans from a fast growing *Rhizobium* species. *Biochimica et Biophysica Acta* **901**, 119–126.
- BOTSFORD, J. L. & LEWIS, T. A. (1990). Osmoregulation in *Rhizobium meliloti*: production of glutamic acid in response to osmotic stress. *Applied and Environmental Microbiology* **56**, 488–494.
- BRAUNEGG, G., SONNLEITER, B. & LAFFERTY, R. M. (1978). A rapid gas chromatographic method for the determination of poly- β -hydroxybutyric acid in microbial biomass, *European Journal of Applied Microbiology* **6**, 29–37.
- BREEDVELD, M. W., ZEVENHUIZEN, L. P. T. M. & ZEHNDER, A. J. B. (1990a). Excessive excretion of cyclic β -(1,2)-glucan by *Rhizobium trifolii* TA-1. *Applied and Environmental Microbiology* **56**, 2080–2086.
- BREEDVELD, M. W., ZEVENHUIZEN, L. P. T. M. & ZEHNDER, A. J. B. (1990b). Osmotically-induced oligo- and polysaccharide synthesis by *Rhizobium meliloti* SU-47. *Journal of General Microbiology* **136**, 2511–2519.
- BREEDVELD, M. W., ZEVENHUIZEN, L. P. T. M. & ZEHNDER, A. J. B. (1991). Osmotically-regulated trehalose accumulation and cyclic β -(1,2)-glucan excretion by *Rhizobium leguminosarum* biovar *trifolii* TA-1. *Archives of Microbiology* **156**, 501–506.
- BURTON, R. M. (1957). The determination of glycerol and dihydroxy-acetone. *Methods in Enzymology* **3**, 246–249.

- CLAASSEN, P. A. M., DIJKEMA, C., VISSER, J. & ZEHNDER, A. J. B. (1986). *In vivo* ^{13}C -NMR analysis of acetate metabolism in *Thiobacillus versutus* under denitrifying conditions. *Archives of Microbiology* **146**, 227–232.
- CSONKA, L. N. (1989). Physiological and genetic responses of bacteria to osmotic stress. *Microbiological Reviews* **53**, 121–147.
- DYLAN, T., HELINSKI, D. R. & DITTA, G. S. (1990). Hypoosmotic adaptation in *Rhizobium meliloti* requires β -(1,2)-glucan. *Journal of Bacteriology* **172**, 1400–1408.
- GLOUX, K. & LE RUDULIER, D. (1989). Transport and catabolism of proline betaine in salt-stressed *Rhizobium meliloti*. *Archives of Microbiology* **151**, 143–148.
- HARRIS, P. J., HENRY, R. J., BLAKENEY, A. B. & STONE, B. A. (1984). An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydrate Research* **127**, 59–73.
- HOELZLE, I. & STREETER, J. G. (1990). Increased accumulation of trehalose in rhizobia cultured under 1-percent oxygen. *Applied and Environmental Microbiology* **56**, 3213–3215.
- KENNEDY, E. P. & RUMLEY, M. K. (1988). Osmotic regulation of biosynthesis of membrane-derived oligosaccharides in *Escherichia coli*. *Journal of Bacteriology* **170**, 2457–2461.
- LE RUDULIER, D. & BERNARD, T. (1986). Salt tolerance in rhizobia: a possible role for betaines. *FEMS Microbiological Reviews* **39**, 67–72.
- MEIKLE, A. J., CHUDEK, J. A., REED, R. H. & GADD, G. M. (1991). Natural abundance ^{13}C -nuclear magnetic resonance spectroscopic analysis of acyclic polyol and trehalose accumulation by several yeast species in response to salt-stress. *FEMS Microbiological Letters* **82**, 163–168.
- MILLER, K. J., KENNEDY, E. P. & REINHOLD, V. N. (1986). Osmotic adaptation by Gram-negative bacteria: possible role for periplasmic oligosaccharides. *Science* **231**, 48–51.
- MILLER, K. J., GORE, R. S. & BENESI, A. J. (1988). Phosphoglycerol substituents present on the cyclic β -1,2-glucans of *Rhizobium meliloti* 1021 are derived from phosphatidylglycerol. *Journal of Bacteriology* **170**, 4569–4575.
- STREETER, J. G. (1985). Accumulation of α - α -trehalose by *Rhizobium* bacteria and bacteroids. *Journal of Bacteriology* **164**, 78–84.
- TOMBRAS SMITH, L. & SMITH, G. M. (1989). An osmoregulated dipeptide in stressed *Rhizobium meliloti*. *Journal of Bacteriology* **172**, 4714–4717.
- TOMBRAS SMITH, L., SMITH, G. M. & MADKOUR, M. A. (1990). Osmoregulation in *Agrobacterium tumefaciens*: accumulation of a novel disaccharide is controlled by osmotic strength and glycine betaine. *Journal of Bacteriology* **172**, 6849–6855.
- TREVELYAN, W. E. & HARRISON, J. S. (1952). Studies on yeast metabolism. I. Fractionation and microdetermination of cell carbohydrates. *Biochemical Journal* **50**, 298–310.
- VAN LAERE, A. (1989). Trehalose, reserve and/or stress metabolite? *FEMS Microbiological Reviews* **63**, 201–210.
- WELSH, D. T., REED, R. H. & HERBERT, R. A. (1991). The role of trehalose in the osmoadaptation of *Escherichia coli* NCIB 9484. Interaction of trehalose, K^+ and glutamate during osmoadaptation in continuous culture. *Journal of General Microbiology* **137**, 745–750.
- WIEMKEN, A. (1990). Trehalose in yeast, stress protectant rather than reserve carbohydrate. *Antonie van Leeuwenhoek* **58**, 209–217.
- ZEVENHUIZEN, L. P. T. M. (1991). Levels of trehalose and glycogen in *Arthrobacter globiformis* under conditions of nutrient starvation and osmotic stress. *Antonie van Leeuwenhoek* **61**, 61–68.
- ZEVENHUIZEN, L. P. T. M., VAN VELDHIJZEN, A. & FOKKENS, R. H. (1990). Re-examination of cellular β -(1,2)-glucans of *Rhizobiaceae*: distribution of ring sizes and degrees of glycerol-1-phosphate substitution. *Antonie van Leeuwenhoek* **57**, 173–178.
- ZORREGUIETA, A., CAVAGNAC, S., GEREMIA, R. A. & UGALDE, R. A. (1990). Osmotic regulation of β -(1,2)-glucan synthesis in members of the family *Rhizobiaceae*. *Journal of Bacteriology* **172**, 4701–4704.