Osmotic regulation of cyclic 1,2- β -glucan synthesis

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In contrast to what happens in *Agrobacterium tumefaciens* and *Rhizobium meliloti*, synthesis of periplasmic cyclic 1,2- β -glucan in *Brucella* spp. was not inhibited when bacteria were grown in media of high osmolarity. Studies performed with crude membrane preparations showed that cyclic 1,2- β -glucan synthetase of *Brucella* spp. was not inhibited by 0.5 M KCl or potassium glutamate; concentrations that completely inhibit the osmosensitive enzymes of *A. tumefaciens* A348 or *R. meliloti* 102F34, respectively encoded by the *chvB* or *ndvB* genes. The *Brucella abortus* cyclic 1,2- β -glucan synthetase gene (*cgs*) was introduced into *A. tumefaciens* A1011 *chvB* and *R. meliloti* GRT21s *ndvB* mutants. Synthesis of cyclic 1,2- β -glucan by the recombinant strains was not inhibited when grown in media of high osmolarity (0.25 M NaCl or 0.5 M mannitol). On the other hand, when the *A. tumefaciens* cyclic 1,2- β -glucan synthetase gene was introduced into the *R. meliloti* GRT21s *ndvB* mutant, the recombinant strain displayed marked inhibition of cyclic 1,2- β -glucan synthesis when grown in high-osmolarity media. However, the same gene introduced into a *B. abortus* *cgs* mutant background resulted in no inhibition of glucan synthesis at high osmolarity. *In vitro* studies with crude membranes isolated from recombinant strains revealed that *Brucella* cyclic 1,2- β -glucan synthetase was not inhibited by high concentrations of KCl or potassium glutamate even when expressed in *Agrobacterium* or *Rhizobium* backgrounds. It was concluded that the lack of effect of high osmolarity on 1,2- β -glucan synthesis in *Brucella* is due to two convergent mechanisms: a) the presence of a cyclic 1,2- β -glucan synthetase that is not affected by concentrations of solutes such as KCl or potassium glutamate and b) either the possible accumulation of compatible solutes that might protect the enzyme from the inhibition by potassium glutamate or the accumulation of other osmolytes that do not affect the 1,2- β -glucan synthetase.

Keywords: cyclic 1,2- β -glucan, osmoregulation, *Brucella*, *Agrobacterium*

INTRODUCTION

Agrobacterium tumefaciens chromosomal virulence genes *chvB* and *chvA* are required for synthesis and secretion of cyclic 1,2- β -glucans (Douglas *et al.*, 1985; Puvanesarajah *et al.*, 1985). *chvB* encodes the inner-membrane-bound cyclic 1,2- β -glucan synthetase and *chvA* encodes a 75 kDa inner-membrane protein required for secretion of cyclic 1,2- β -glucan (Zorreguieta & Ugalde, 1986; Iñón de Iannino & Ugalde, 1989). In *Rhizobium* spp. the *ndvB* and *ndvA* genes, homologous to *A. tumefaciens* *chvB* and *chvA*, are required for 1,2- β -glucan synthesis and secretion respectively (Dylan *et al.*,

1986; Geremía *et al.*, 1987). *ndvB* and *chvB* mutants are defective in nodule invasion and virulence, and show impaired growth in low osmolarity medium (Zorreguieta *et al.*, 1990; Dylan *et al.*, 1990; Cangelosi *et al.*, 1990). The cyclic 1,2- β -glucan synthetase enzyme contains all the enzymic activities required for synthesis of cyclic 1,2- β -glucan, i.e. initiation, elongation and cyclization (Altabe *et al.*, 1990; Castro *et al.*, 1996); the glucan-linked synthetase is, therefore, an intermediate in glucan synthesis (Zorreguieta & Ugalde, 1986).

Osmotic regulation of *Rhizobium meliloti* and *A. tumefaciens* cyclic 1,2- β -glucan synthesis is similar to

that of membrane-derived oligosaccharides (MDOs) in *Escherichia coli* (Breedveld & Miller, 1994). High osmolarity was found to inhibit the *in vivo* accumulation of cellular 1,2- β -glucans in *Agrobacterium* and *Rhizobium* or MDO in *E. coli* (Breedveld & Miller, 1994). Exceptions have been reported with respect to osmoregulation. In several strains of *R. leguminosarum* and in *R. meliloti* strain GR4, the levels of cell-associated cyclic 1,2- β -glucans were independent of osmolarity of the growth medium (Breedveld *et al.*, 1991; Soto *et al.*, 1993).

It was demonstrated that high osmolarity has no effect on gene expression of cyclic 1,2- β -glucan synthetase, since membranes prepared from cells grown at high osmolarity displayed normal glucan synthesis activity *in vitro* (Zorreguieta *et al.*, 1990). Osmotic regulation of periplasmic glucan biosynthesis by *Agrobacterium* and *Rhizobium* appeared to occur at a post-translational level, as with the osmotic regulation of biosynthesis of MDOs in *E. coli* (Rumley *et al.*, 1992). *A. tumefaciens* and *R. meliloti* 1,2- β -glucan synthesis is inhibited *in vitro* by KCl and potassium glutamate.

Bacteria respond to changes of osmolarity of the environment by varying the intracellular concentration of specific solutes. The accumulation of K⁺, followed by glutamate, is a primary response to osmotic upshift in *E. coli* and other bacteria in minimal salt media. Increase of the intracellular concentration of potassium glutamate allows adaptation of the cell to environments of moderately high osmolarities. At high osmolarity, bacteria replace K⁺ ions with high intracellular concentrations of one or more compatible solutes. These include polyols such as trehalose, amino acids such as proline and methylamines such as glycine betaine (Lucht & Bremer, 1994). *Rhizobium* and *Agrobacterium* cytoplasmic concentrations of K⁺ and glutamate also increase in response to increases of osmolarity of the growth media (Miller & Wood 1996).

Brucella is a pathogenic bacterium that causes a chronic disease in mammals, including humans (Smith & Ficht, 1990). *Brucella*, like *Agrobacterium* and *Rhizobium*, forms cyclic 1,2- β -glucans (Bundle *et al.*, 1987, 1988). As described in *Agrobacterium* and *Rhizobium*, the biosynthesis of cyclic 1,2- β -glucans in *Brucella* spp. proceeds through a membrane-bound 1,2- β -glucan synthetase, with the formation of protein-bound intermediates. A characteristic of *Brucella* cyclic 1,2- β -glucan synthesis is that high osmolarity of the growth medium has no effect on cellular cyclic 1,2- β -glucan accumulation (Briones *et al.*, 1997). Recently, the *B. abortus* S19 cyclic 1,2- β -glucan synthetase (*cgs*) gene was cloned, characterized and sequenced. The gene complemented *A. tumefaciens chvB* and *R. meliloti ndvB* mutants, restoring virulence, nodule development, motility and biosynthesis of cyclic 1,2- β -glucan (Iñón de Iannino *et al.*, 1998). It was also determined that a *B. abortus cgs* mutant displayed a reduced ability to survive in the spleen of mice, thus suggesting that, as in *Agrobacterium*, cyclic 1,2- β -glucans may behave as virulence factors (Iñón de Iannino *et al.*, 1998).

In this communication we characterized osmoregulation of the synthesis of cyclic 1,2- β -glucan by *Brucella* spp., *Agrobacterium*, *Rhizobium* and various recombinant strains harbouring *Brucella* or *Agrobacterium* cyclic 1,2- β -glucan synthetase genes.

METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids used are listed in Table 1. *A. tumefaciens* A348 wild-type strain, *A. tumefaciens chvB* mutant A1011 and plasmid pCD523 (Douglas *et al.*, 1985) were provided by Dr E. Nester (Dept Microbiology, Washington University). *R. meliloti* GR4 and GRT21s *ndvB* mutant (Geremía *et al.*, 1987), and cosmid pBA19, containing a 19 kb *B. abortus* DNA fragment (Iñón de Iannino *et al.*, 1998) have been described previously. *A. tumefaciens* strains were grown on tryptone–yeast extract (TY) medium (0.5% tryptone, 0.3% yeast extract). *R. meliloti* strains were grown on mannitol–yeast extract (AMA) medium (0.5 g K₂HPO₄ l⁻¹, 0.2 g MgSO₄·7H₂O l⁻¹, 0.2 g NaCl l⁻¹, 1 g yeast extract l⁻¹, 10 g mannitol l⁻¹). *Brucella* strains were grown in *Brucella* broth medium (BB; Difco). Kanamycin or tetracycline were added when required to a final concentration of 100 µg ml⁻¹ and 10 µg ml⁻¹, respectively. When indicated, medium was supplemented with 0.25 M NaCl or 0.5 M mannitol.

DNA techniques and gene manipulations. *B. abortus* S19 *cgs* was cloned as described previously (Iñón de Iannino *et al.*, 1998). *R. meliloti ndvB* mutant GRT21s and *A. tumefaciens chvB* mutant A1011 were complemented with cosmid pBA19 as described previously (Iñón de Iannino *et al.*, 1998). *R. meliloti* GRT21s(pCD523) was obtained by using the triparental system described by Ditta *et al.* (1980). Mating was carried out by patching similar numbers of donor cells [*E. coli* HB101(pCD523)], recipient cells (*R. meliloti* GRT21s) and helper cells [*E. coli* HB101(pRK2013)]. Transconjugants were selected on AB media containing the appropriate antibiotics. *B. abortus* BAI129(pCD523) was obtained by a biparental system. *E. coli* S17.1 carrying pCD523 was used as the donor. Transconjugants were selected on BB agar medium containing 5 µg nalidixic acid ml⁻¹, 100 µg ampicillin ml⁻¹, 100 µg kanamycin ml⁻¹ and 10 µg tetracycline ml⁻¹.

Isolation of cyclic 1,2- β -glucan from cells and osmotic regulation studies. Cells from 100 ml cultures were harvested by centrifugation at 10000 g for 10 min. Cell pellets were extracted with 10% TCA for 30 min at room temperature. The TCA extracts were neutralized with ammonium hydroxide, concentrated and subjected to gel filtration on Bio-Gel P4 columns (78 × 1.8 cm; Bio-Rad). The columns were eluted with 0.1 M pyridine–acetate buffer (pH 5.5). Fractions of 1.5 ml were collected and carbohydrates determined by the anthrone–sulfuric acid method (Dische, 1962). DEAE-Sephadex chromatography was carried out as described previously (Iñón de Iannino & Ugalde, 1989). Studies on the effect of osmolarity on cellular-glucan accumulation were carried out by growing the cells to stationary phase with 0.5 M mannitol or 0.25 M NaCl. Cells were harvested, cellular glucans extracted with TCA and subjected to Bio-Gel P4 chromatography as described above.

***In vitro* synthesis of cyclic 1,2- β -glucans.** *In vitro* synthesis of 1,2- β -glucan was studied with *Brucella*, *Rhizobium* or *Agrobacterium* total or inner membranes as described previously (Iñón de Iannino & Ugalde 1989; Briones *et al.*, 1997). Membranes (0.1 mg protein) were incubated with 0.2 µCi UDP-[¹⁴C]glucose (specific activity 300 mCi mmol⁻¹) at 28 °C

Table 1. Bacteria and plasmids used in this study

Nal^r, resistant to nalidixic acid; Amp^r, resistant to ampicillin; Tc^r, resistant to tetracycline; Km^r, resistant to kanamycin.

Strain or plasmid	Phenotype or genotype	Reference
Strains		
<i>B. abortus</i> S19	Vaccine strain, Nal ^r , spontaneous derivative of <i>B. abortus</i> 2308	Iñón de Iannino <i>et al.</i> (1998)
<i>B. abortus</i> BAI129	<i>B. abortus</i> S19 <i>cgs</i> mutant, Tn3HoHo1 integrated into chromosomal <i>cgs</i> gene, Amp ^r	Iñón de Iannino <i>et al.</i> (1998)
<i>A. tumefaciens</i> A348	Wild type strain, Vir ⁺	Douglas <i>et al.</i> (1985)
<i>A. tumefaciens</i> A1011	<i>chvB</i> mutant, Tn5 integrated into chromosomal <i>chvB</i> gene, Vir ⁻ , Km ^r	Douglas <i>et al.</i> (1985)
<i>R. meliloti</i> GR4	Wild-type	Geremía <i>et al.</i> (1987)
<i>R. meliloti</i> 102F34	Wild-type	Dylan <i>et al.</i> (1986)
<i>R. meliloti</i> GRT21s	<i>ndvB</i> mutant	Geremía <i>et al.</i> (1987)
Plasmids		
pCD523	pLAFR1 containing the <i>A. tumefaciens chvB</i> gene, Tc ^r	Douglas <i>et al.</i> (1985)
pBA19	pVK102 containing the <i>B. abortus cgs</i> gene, Tc ^r	Iñón de Iannino <i>et al.</i> (1998)

(*Agrobacterium* or *Rhizobium* membranes) or at 37 °C (*Brucella* membranes). Formation of cyclic 1,2- β -glucan and the presence of the inner-membrane-protein intermediate were estimated as described previously (Iñón de Iannino & Ugalde 1989). The enzymic activity was expressed as pmol [¹⁴C]-glucose accumulated in the intermediate protein (10% TCA-insoluble products) per mg protein. When indicated, different concentrations of KCl or potassium glutamate were added. The basal activity was the level of activity in the standard assay mixture with no additional salts.

RESULTS

Effect of KCl on *in vitro* cyclic 1,2- β -glucan synthesis

All the evidence accumulated so far supports the interpretation that the effect of high osmolarity on *Agrobacterium* and *Rhizobium* cyclic 1,2- β -glucan accumulation is due to a direct inhibition of the cyclic 1,2- β -glucan synthetase enzymic activity and not to an effect on *cgs* gene expression. Increasing the osmolarity of the growth medium led to an increase in the ionic strength of the cytosol, mainly due to an uptake of potassium ions (Miller & Wood, 1996). Inhibition of the cyclic 1,2- β -glucan synthetase by high ionic strength might be the mechanism by which its activity is reduced when *Agrobacterium* and *Rhizobium* cells are grown in a medium of high osmolarity (Zorreguieta *et al.*, 1990; Ingram-Smith & Miller, 1998).

To determine if the lack of effect of high osmolarity on *Brucella* cyclic 1,2- β -glucan is due to an insensitivity of the *Brucella* cyclic 1,2- β -glucan synthetase to high cytoplasmatic ionic strength, the effect of KCl on the *in vitro* cyclic 1,2- β -glucan synthetase activity was studied. The effect of different concentrations of KCl on the incorporation of [¹⁴C]glucose into TCA-insoluble pro-

Table 2. Effect of KCl on the incorporation of radioactivity into 1,2- β -glucan protein intermediates by membranes of different strains

Results are expressed as a percentage of the incorporation obtained when the reaction was carried out without the addition of KCl. The enzymic activities of non-treated reactions (100%) were 204 pmol glucose h⁻¹ (mg protein)⁻¹ for *A. tumefaciens* A348; 288 for *R. meliloti* 102F34; 300 for *B. abortus* S19; 276 for *R. meliloti* GR4; 900 for *A. tumefaciens* A1011(pBA19) and 510 for *R. meliloti* GRT21s(pBA19).

Strain	KCl (mM)				
	0	50	100	250	500
<i>A. tumefaciens</i> A348	100	61	51	40	34
<i>R. meliloti</i> 102F34	100	69	56	38	35
<i>B. abortus</i> S19	100	95	105	106	110
<i>R. meliloti</i> GR4	100	98	92	82	70
<i>A. tumefaciens</i> A1011(pBA19)	100	105	110	108	94
<i>R. meliloti</i> GRT21s(pBA19)	100	97	98	96	78

tein intermediates by membranes from different sources incubated with UDP-[¹⁴C]glucose is shown in Table 2. This assay quantified the formation of protein-glucosyl intermediates during initiation, the first of three reactions that take place during the synthesis of cyclic glucans. It can be seen that an inhibition of approximately 50% was obtained when 0.1 M KCl was added to membranes of *A. tumefaciens* A348 and *R. meliloti* 102F34, two strains in which the accumulation of cyclic

1,2- β -glucan is osmoregulated. On the other hand, at that KCl concentration, membranes of *R. meliloti* GR4 and *B. abortus* S19, in which synthesis *in vivo* is not inhibited by the osmolarity of the growth media, showed an inhibition of less than 8 %. The same behaviour was observed with *B. abortus* 2308 and *Brucella ovis* REO198 (data not shown). It can be observed that in *A. tumefaciens* A1011 *chvB* and *R. meliloti* GRT21s *ndvB* mutants complemented with *B. abortus* *cgs*, glucan synthesis was not inhibited by 0.25 M KCl, thus showing the same behaviour observed with *B. abortus* membranes.

Effect of potassium glutamate on *in vitro* synthesis

Immediately after an increase in external osmolarity, water is released from cells, reducing turgor and inducing shrinking. Under these conditions, K⁺ is accumulated in order to restore turgor. The uptake of K⁺ is the first response to osmotic stress, and glutamate is the most common counter-ion of K⁺. The effect of potassium glutamate on the incorporation of [¹⁴C]-glucose into glucan-protein intermediates (initiation reaction) was studied and results very similar to those observed with KCl were obtained (data not shown).

To characterize the products accumulated in the TCA-insoluble fraction, SDS-PAGE was carried out. We demonstrated previously that the only protein that became labelled with [¹⁴C]glucose after incubation of membranes with UDP-[¹⁴C]glucose is the cyclic 1,2- β -glucan synthetase (Iñón de Iannino *et al.*, 1998). No protein was labelled when the incubation was carried out with membranes isolated from a cyclic 1,2- β -glucan synthetase null mutant. As shown in Fig. 1, 0.5 M potassium glutamate inhibited the incorporation of [¹⁴C]glucose into the protein intermediate by *A. tumefaciens* A348 and by *R. meliloti* 102F34 (Fig. 1a, b). In contrast, the incorporation of [¹⁴C]glucose into protein intermediates was not inhibited by 0.5 M potassium glutamate when *B. abortus* S19 or *R. meliloti* GR4 membranes were used as the enzyme source (Fig. 1c, d). Membranes of *A. tumefaciens* A1011 *chvB* and *R. meliloti* GRT21s *ndvB* mutants complemented with the *B. abortus* S19 *cgs* gene were not inhibited by 0.5 M potassium glutamate (Fig. 1e, f). On the other hand, membranes of *R. meliloti* GRT21s *ndvB* or *B. abortus* BAI129 *cgs* mutants complemented with the *A. tumefaciens* cyclic 1,2- β -glucan synthetase were inhibited by 0.5 M potassium glutamate (Fig. 1g, h).

These results showed that *Brucella* cyclic 1,2- β -glucan synthetase activity is not affected by concentrations as high as 0.5 M KCl or potassium glutamate. Both compounds strongly inhibited the *in vitro* activity of *Rhizobium* and *Agrobacterium* cyclic 1,2- β -glucan synthetase at similar concentrations. These results suggest that the lack of inhibition of the *Brucella* enzyme is due to an intrinsic characteristic of the enzyme, the activity of which is not affected by concentrations of solutes that are known to be accumulated inside the cells upon growth in high-osmolarity media.

Fig. 1. Effect of potassium glutamate on the incorporation of [¹⁴C]glucose into glucan-protein intermediates of different strains. Total membranes (0.2 mg protein) of different strains were incubated with UDP-[¹⁴C]glucose as described in Methods, with or without the addition of 0.5 M potassium glutamate. Reactions were stopped by the addition of 10 % TCA and the precipitates subjected to SDS-PAGE. Radioactivity was detected by autoradiography. (a), *A. tumefaciens* A348; (b) *R. meliloti* 102F34; (c), *B. abortus* S19; (d), *R. meliloti* GR4; (e), *A. tumefaciens* A1011(pBA19); (f), *R. meliloti* GRT21s(pBA19); (g), *R. meliloti* GRT21s(pCD523); (h), *B. abortus* BAI129(pCD523).

Effect of glycine betaine on *A. tumefaciens* glucosyl transferase activity

The effect of the osmoprotectant glycine betaine to reverse the inhibitory effect of potassium glutamate on the *A. tumefaciens* glucosyltransferase activity was studied as described in Methods. The addition of 0.1 M potassium glutamate to the reaction mixture inhibited the glucosyl transferase activity by 45 %. However, the addition of 1 M glycine betaine prevented this inhibition, restoring the glucosyltransferase activity to 96 %. We observed an inhibition of 60 % when 0.5 M potassium glutamate was added; this inhibition was only partially reversed to 44 % inhibition of enzyme activity when 1 M glycine betaine was added [100 % enzymic activity corresponded to 210 pmol glucose h⁻¹ (mg protein)⁻¹]. These results demonstrated that the intracellular accumulation of glycine betaine may prevent inhibition of the glucosyltransferase activity by potassium glutamate accumulated intracellularly under osmotically stressful conditions.

Effect of osmolarity on cellular cyclic 1,2- β -glucan accumulation

Various strains were grown in media of low or high osmolarity, as indicated under Methods. TCA extracts obtained from stationary-phase cultures were subjected

Fig. 2. Bio-Gel P4 chromatography of cyclic 1,2- β -glucans accumulated *in vivo* by different strains in cells grown in medium of low and high osmolarity. Cells from 100 ml cultures were harvested and extracted with 10% TCA. Extracts were subjected to Bio-Gel P4 chromatography. Fractions were collected and carbohydrates measured as indicated in Methods. Carbohydrates were expressed as mg glucose equivalents (g wet weight)⁻¹. (a), *A. tumefaciens* A348; (b), *A. tumefaciens* A1011(pBA19); (c), *B. abortus* S19; (d), *B. abortus* BAI129(pCD523); (e), *R. meliloti* GR4; (f), *R. meliloti* GRT21s(pCD523); (g), *R. meliloti* GRT21s(pBA19). (a), (b), (e), (f) and (g) contained 0.25 M NaCl; (c) and (d) contained 0.5 M mannitol. ●, control; ○, with 0.25 M NaCl or 0.5 M mannitol.

to Bio-Gel P4 chromatography. Products recovered between fractions 5 and 18 (Fig. 2) were pooled and characterized as cyclic 1,2- β -glucans by TLC as described by Iñón de Iannino *et al.* (1998). Total accumulation of cellular cyclic 1,2- β -glucan [mg glucose equivalents (g cellular wet weight)⁻¹] was calculated from the total amount of glucan recovered from fractions 5 to 18 of Bio-Gel P4 columns. The addition of 0.25 M NaCl to the growth media abolished accumulation of cellular glucans by *A. tumefaciens* A348 wild-type strain (fractions 8 to 18 of Fig. 2a). Cosmid pBA19, containing the complete *B. abortus* *cgs* gene, restored synthesis of cyclic 1,2- β -glucan in *R. meliloti* GRT21s *ndvB* and *A. tumefaciens* A1011 *chvB* mutants (Iñón de Iannino *et al.*, 1998). It is shown in Fig. 2(b, g), that the accumulation of cellular cyclic 1,2- β -glucan by *A. tumefaciens* A1011 *chvB* or *R. meliloti* GRT21s *ndvB* mutants complemented with *B. abortus* *cgs*, was not inhibited by the presence of 0.25 M NaCl in the growth medium. Similar results were obtained when these complemented strains were grown in medium containing 0.5 M mannitol (data not shown). Although increasing the osmolarity had no effect on the total

amount of cellular glucan accumulated by *R. meliloti* GRT21s(pBA19) strain, when cells were grown in media containing 0.25 M NaCl, glucans were recovered from the Bio-Gel P4 column at an elution volume slightly higher than glucans recovered from the cells grown in medium without salt (Fig. 2g). The same results were obtained with the *R. meliloti* GR4 wild-type strain (Fig. 2e). As described below, differences in Bio-Gel P4 elution were caused by differences in glucan substitution. Glucans accumulated by the *A. tumefaciens* *chvB* mutant complemented with *Brucella* *cgs* grown in medium with 0.25 M NaCl eluted from the Bio-Gel P4 column at the same elution volume as those obtained from cells grown without NaCl (Fig. 2b). The addition of 0.5 M mannitol to *B. abortus* S19 cultures had no effect on the accumulation of cellular glucans recovered from cells growing at high or low osmolarity (Fig. 2c). The same results were obtained with the *Brucella* *cgs* mutant harbouring the *A. tumefaciens* *chvB* gene (Fig. 2d). On the other hand, the *R. meliloti* GRT21s *ndvB* mutant complemented with the *Agrobacterium* cyclic 1,2- β -glucan synthetase gene, displayed strong inhibition when grown in high-osmolarity medium (Fig. 2f).

Fig. 3. DEAE-Sephadex chromatography of cellular glucans accumulated by different strains. Glucans were recovered from Bio-Gel P4 columns (fractions 5 to 18 of Fig. 2) and subjected to DEAE-Sephadex chromatography. Columns (0.25 × 3 cm) were eluted with 1.5 ml water (fractions 1–3), 1.5 ml 0.25 M NaCl (fractions 4–6) and 0.5 M NaCl (fractions 7–9). Fractions of 0.5 ml were collected and carbohydrates measured by the anthrone-sulfuric acid method (Dische *et al.*, 1962). (a), *R. meliloti* GR4 grown in AMA medium; (b), *R. meliloti* GRT21s(pBA19) grown in AMA medium; (c), *R. meliloti* GRT21s(pBA19) grown in TY medium; (d), *A. tumefaciens* A348 grown in TY medium; (e), *A. tumefaciens* A1011(pBA19) grown in TY medium; (f), *A. tumefaciens* A1011(pBA19) grown in TY medium with 0.25 M NaCl.

Osmolarity affects the substitution of *R. meliloti* cellular glucans

Cellular glucans accumulated by *R. meliloti* GR4 or *R. meliloti* GRT21s complemented with the *Brucella* cyclic 1,2- β -glucan synthetase grown in AMA medium supplemented or not with 0.25 M NaCl (fractions 5 to 18 of Fig. 2e, g) were recovered from the Bio-Gel P4 column and subjected to DEAE-Sephadex chromatography. As shown in Fig. 3, glucans recovered from cells grown without the addition of NaCl eluted from the DEAE-Sephadex column with 250 mM NaCl (fractions 4 to 6 of Fig. 3a, b), thus indicating that most of cellular glucans accumulated under this growth condition were modified with anionic substituents. However, glucans recovered from cells grown with 0.25 M NaCl eluted

from the DEAE-Sephadex column with water (fractions 1 to 3 of Fig. 3c), thus indicating that most of them were non-substituted neutral glucans. An *A. tumefaciens* *chvB* mutant complemented with the *B. abortus* *cgs* gene produced, when grown in medium with 0.25 M NaCl, glucans with the same net charge as control cells (Fig. 3d–f), thus indicating that in the *Agrobacterium* background high osmolarity had no effect on glucan substitution.

DISCUSSION

We studied the effect of high osmolarity of the growth media on the accumulation of cyclic 1,2- β -glucan in *Agrobacterium*, *Rhizobium* and *Brucella*. It is known

that K⁺ and glutamate are solutes accumulated in the cytoplasm when bacteria are exposed to high osmolarity, in order to restore cell turgor (Csonka, 1991). The absence of inhibition by these solutes of cyclic 1,2- β -glucan synthetase activity might explain in part the osmoresistant accumulation of glucan at high osmolarity by *Brucella* and *R. meliloti* strain GR4.

Synthesis of cyclic glucans by *A. tumefaciens* and *R. meliloti* complemented with *Brucella* cyclic 1,2- β -glucan synthetase was not inhibited by high osmolarity of growth media. Since this is the behaviour observed with *Brucella* spp., we concluded that the enzyme is responsible for the lack of inhibition at high osmolarity. *Brucella* cyclic 1,2- β -glucan synthetase in the *Agrobacterium* or *Rhizobium* background was not inhibited by 0.5 M KCl or potassium glutamate, thus indicating that the *Brucella* enzyme is insensitive to inhibition by those endocellular osmolytes accumulated under osmotic stress. Since the *in vivo* accumulation of cyclic glucan by the recombinant *B. abortus* BAI129(pCD523) strain (*B. abortus* BAI129 *cgs* mutant complemented with the *A. tumefaciens* cyclic 1,2- β -glucan synthetase gene) was not inhibited by the high osmolarity of the medium, but the enzyme was inhibited *in vitro* by KCl or potassium glutamate, we may postulate the following hypothesis: I) cyclic 1,2- β -glucan synthesis is not regulated by the osmotic inhibition of the activity of the cyclic 1,2- β -glucan synthetase or II) *Brucella* may have additional mechanisms that prevent inhibition of cyclic 1,2- β -glucan synthesis under osmotically stressful conditions, in addition to possessing an enzyme resistant to these osmolytes. These additional mechanisms may be the ability of *Brucella* to accumulate different osmolytes or osmoprotective solutes, such as glycine betaine. Interestingly we have found that *B. abortus* has three genes highly homologous to *proV*, *proW* and *proX* involved in the transport of glycine betaine (D. O. Sanchez, R. Zandomeni & S. Cravero, unpublished). Glycine betaine reversion of the inhibitory effect of potassium glutamate on *A. tumefaciens* 1,2- β -glucan synthetase activity, which has been described by Ingram-Smith & Miller (1998) in *R. meliloti*, may be the mechanism operative in *Brucella*. Either one, or both, of these two possibilities (lack of glucan synthetase inhibition by salts or/and accumulation of protective osmolytes) may be responsible for the lack of inhibition of cyclic glucan accumulation under high osmolarity by a *Brucella* mutant expressing the *A. tumefaciens* enzyme.

We have shown the synthesis of anionic cyclic 1,2- β -glucans by *R. meliloti* GR4. Although synthesis of cyclic 1,2- β -glucans was not osmoregulated, the anionic substitution of cyclic 1,2- β -glucans was inhibited by high osmolarity, indicating that they are independent processes. This behaviour is different from that observed in *Agrobacterium*, in which high osmolarity has no effect on anionic substitution of cyclic glucans synthesized by the osmoresistant *Brucella* enzyme. These results are consistent with earlier studies, which showed that synthesis of glycerophosphorylated cyclic glucans in *R. meliloti* 1021 was inhibited in cells grown at high

osmolarity (Breedveld & Miller, 1995; Wang *et al.*, 1999).

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