

Accumulation of Cell-Associated $\beta(1-2)$ -Glucan in *Rhizobium meliloti* Strain GR4 in Response to Osmotic Potential

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High-osmolarity media strongly inhibit the *in vivo* accumulation of cell-associated glucans in *Agrobacterium tumefaciens*, *Rhizobium meliloti*, and *Bradyrhizobium japonicum*, as also occurs with the membrane-derived oligosaccharide (MDO) in *Escherichia coli*. However, we show here that in *Rhizobium meliloti* strain GR4 the level of cell-associated $\beta(1-2)$ -glucan is not altered by the environmental osmotic conditions.

Recently much attention has focused on the possible role of the periplasmic $\beta(1-2)$ -glucan on plant infection and legume nodulation by *Agrobacterium* and *Rhizobium* species respectively (Abe *et al.* 1982; Cangelosi *et al.* 1987; Douglas *et al.* 1985; Dylan *et al.* 1990; Geremía *et al.* 1987; Ielpi *et al.* 1990; Miller *et al.* 1986; Puvanesaraj *et al.* 1985; Zorreguieta and Ugalde 1986). Recent studies have demonstrated that *Agrobacterium* and *Rhizobium* species synthesize neutral and anionic periplasmic $\beta(1-2)$ -glucans of similar structure with phosphoglycerol as the predominant anionic substituent (Miller *et al.* 1988). *R. meliloti* cyclic glucans influence proper functioning of such important systems as motility, adaptation to low osmolarity, exopolysaccharide production, and nodule formation (Soto *et al.* 1992). The production of MDO has been shown to be inhibited by the addition of high concentration of salts or sucrose to the *E. coli* growth medium (Bohin and Kennedy 1984; Kennedy 1982). High-osmolarity media also inhibit the *in vivo* accumulation of cell-associated glucans in *A. tumefaciens*, *R. meliloti*, and *B. japonicum* (Miller *et al.* 1986; Dylan *et al.* 1990; Breedveld *et al.* 1990; Tully *et al.* 1990; Miller and Gore 1992). It has been proposed that in *R. meliloti* $\beta(1-2)$ -glucan would play a role in hypoosmotic adaptation (Dylan *et al.* 1990). Unlike *R. meliloti* strains 102F34 and SU-47, in this work, we present evidence that the level of cell-associated $\beta(1-2)$ -glucan in *R. meliloti* strain GR4 does

not change in response to osmotic potential. *R. meliloti* strain GR4 was described previously (Casadedús and Olivares 1979). Cell-associated, soluble carbohydrates were isolated from 200-ml cultures of *R. meliloti* grown for 4 days at 30° C in GYM medium containing 20 mM mannitol, added with 20 mM NaCl or 20 mM sucrose (low-osmolarity media); 400 mM NaCl or 0.5 M sucrose (high-osmolarity media) as described (Dylan *et al.* 1990; Ielpi *et al.* 1990). Under low- and high-osmolarity basal GYM, the wild-type strain GR4 exhibited similar growth rates. Isolation of cell-associated glucans was performed by two different procedures, the aqueous methanol (Miller *et al.* 1988) and with 1% trichloroacetic acid (TCA) (Miller *et al.* 1986). Each extraction was performed at least three times. Samples were applied to a column of Sephadex G50 (2.6 by 53 cm) and fractions of 1 ml were collected. Total carbohydrates were determined by the phenol method (Hanson and Phillips 1981). After the non-sugar substituents were removed, carbohydrate-containing peaks were subjected to partial acid hydrolysis and paper chromatography and compared to the pattern of *in vitro* synthesized neutral labeled $\beta(1-2)$ -glucan of *Agrobacterium* (Iñon de Iannino and Ugalde 1989; Soto *et al.* 1992). The *ndvB* mutant GR4 derivative, strain GRT21s (Soto *et al.* 1992) was also used as a negative control for $\beta(1-2)$ -glucan production. Cell cultures of *R. meliloti* GR4 grown in low-osmolarity media (20 mM NaCl) were harvested by centrifugation, and the cell-associated glucan was extracted with aqueous methanol. The extracts were chromatographed on Sephadex G-50 yielding one major hexose-containing peak (Fig. 1A) characterized by partial acid hydrolysis and paper chromatography analyses as $\beta(1-2)$ -glucan. When cells of *R. meliloti* GR4 were grown in GYM medium supplemented with 400 mM NaCl (Fig. 1B) two major hexose-containing peaks were obtained when the extract was chromatographed on Sephadex G-50. The second hexose-containing peak has been also observed for *R. meliloti* 102F34 cells grown in high-osmolarity media, apparently trehalose (Dylan *et al.* 1990). However, it was surprising that the level of the cell-associated $\beta(1-2)$ -glucan (first hexose-containing peak) in strain GR4 did not decrease when the cells were grown in high-osmolarity media (Fig. 1A,B). Dylan *et al.* (1990) reported that $\beta(1-2)$ -glucan synthesis in *R. meliloti* strain 102F34 was osmoregulated and the accumulation of these molecules was maximal when cells were cultured at low osmolarity.

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To determine whether accumulation of cell-associated glucan in *R. meliloti* GR4 occurs in a different manner to that of strain 102F34, the cell-associated glucan for the latter strain was obtained from cells grown at low and high osmolarity. As it is shown in Figure 1C and D, high-osmolarity media dramatically reduced the amount of glucans recovered from wild-type 102F34 cells (63% reduction), which is in agreement with the results published by Dylan *et al.* (1990). When instead of NaCl, we used sucrose, a nonionic, nonpermeating solute as osmolyte, the amount of the cell-associated glucan obtained from GR4 cell extracts did not decrease at high osmolarity (Fig. 2A,B). Therefore, data indicate that accumulation of cell-associated $\beta(1-2)$ glucan in *R. meliloti* GR4 is not altered in response to the external osmolarity following a different pattern to that of *R. meliloti* 102F34.

Dylan *et al.* (1990) used the TCA extraction method to analyze *R. meliloti* 102F34 $\beta(1-2)$ -glucan content in response to osmotic potential. To rule out any possible effect of the extraction method in the analysis of cell-associated $\beta(1-2)$ -glucan in response to osmotic conditions,

we also performed TCA extraction. TCA extracts were prepared from GR4 cells grown in low- and high-osmolarity media, *R. meliloti* 102F34 was also used for comparison. As is shown in Figure 3A and B, the total amount of glucans in wild-type strain GR4 was 27% lower when the cells were grown at high osmolarity. However, the glucan content in wild-type strain 102F34 grown at high osmolarity decreased up to 81% (Fig. 3C,D). These results further confirmed the different behavior on the accumulation of cell-associated glucans between both *R. meliloti* strains. It is worth noticing that total cell-associated glucans obtained by the TCA method were considerably lower than were those obtained with the methanol procedure. Thus, the latter seems to be a more effective method for glucans extraction.

The possibility was considered that extracellular cyclic $\beta(1-2)$ -glucan levels may be affected by growth medium osmolarity. When supernatants from cultured GR4 cells were analyzed for extracellular $\beta(1-2)$ -glucans as described by Zevenhuizen (1986), no significant difference was found between cultures grown in low or high osmolarity. Similar

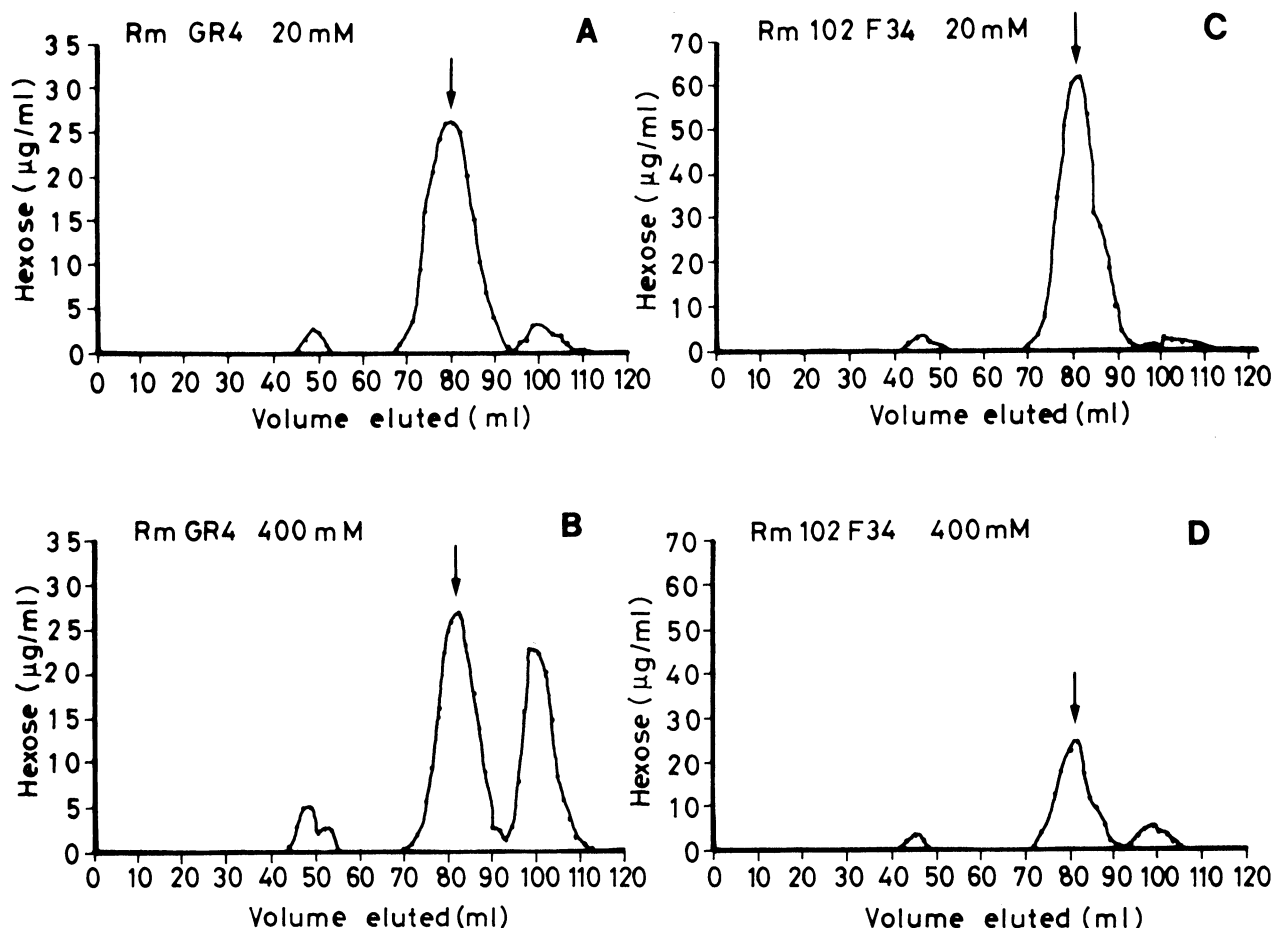


Fig. 1. Effect of extracellular osmolarity on the glucan content of *Rhizobium meliloti* strain GR4 and strain 102F34 (for comparison). Aqueous methanol extracts derived from 200-ml culture of *R. meliloti* were chromatographed on a column of Sephadex G50. The column was eluted at room temperature at a rate of 15 ml/h with 0.15 M ammonium acetate (pH 7.0) containing 7% (vol/vol) propanol. Fractions (1 ml) were collected and assayed for total carbohydrate as described in the text. Results are expressed as micrograms of hexose per milliliter of eluant and are normalized per milligram of protein. Strain GR4: **A**, Low-osmolarity media (20 mM NaCl) and, **B**, high-osmolarity media (400 mM NaCl). Strain 102F34: **C**, low-osmolarity media (20 mM NaCl) and, **D**, high-osmolarity media (400 mM NaCl). The arrows indicate the position of $\beta(1-2)$ -glucan.

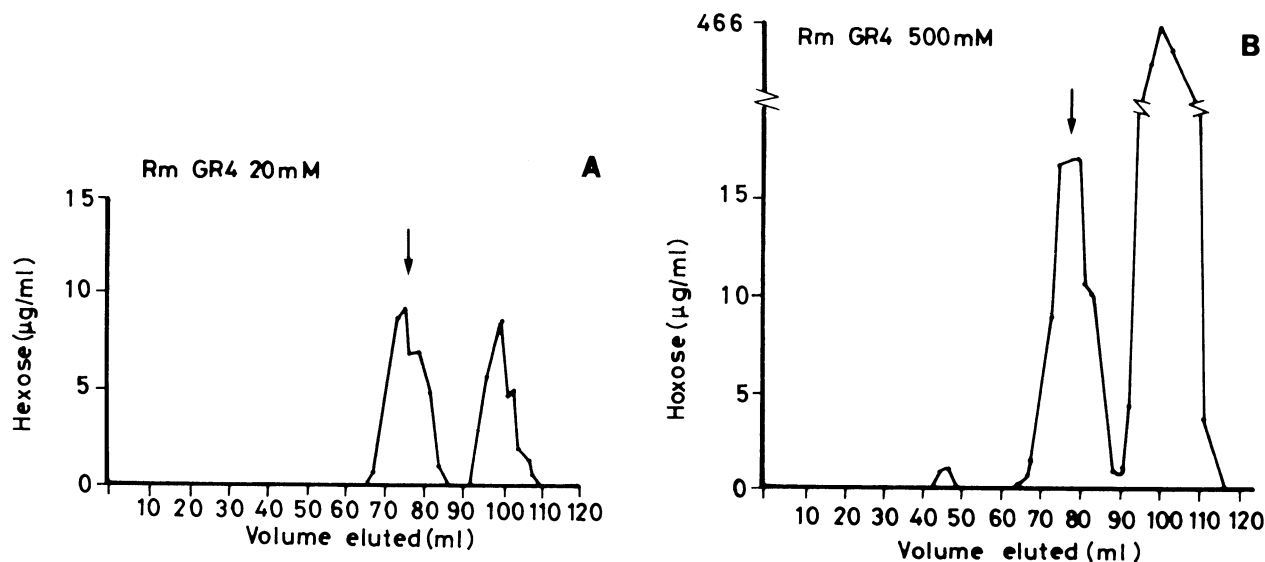


Fig. 2. Effects of extracellular osmolarity on the glucan content of *Rhizobium meliloti* strain GR4 using sucrose as osmolyte. Aqueous methanol extracts derived from a 200-ml culture of wild-type GR4 cells were chromatographed on a column of Sephadex G50. **A**, Low-osmolarity media (20 mM sucrose) and, **B**, high-osmolarity media (0.5 M sucrose). The arrows indicate the position of $\beta(1-2)$ glucan.

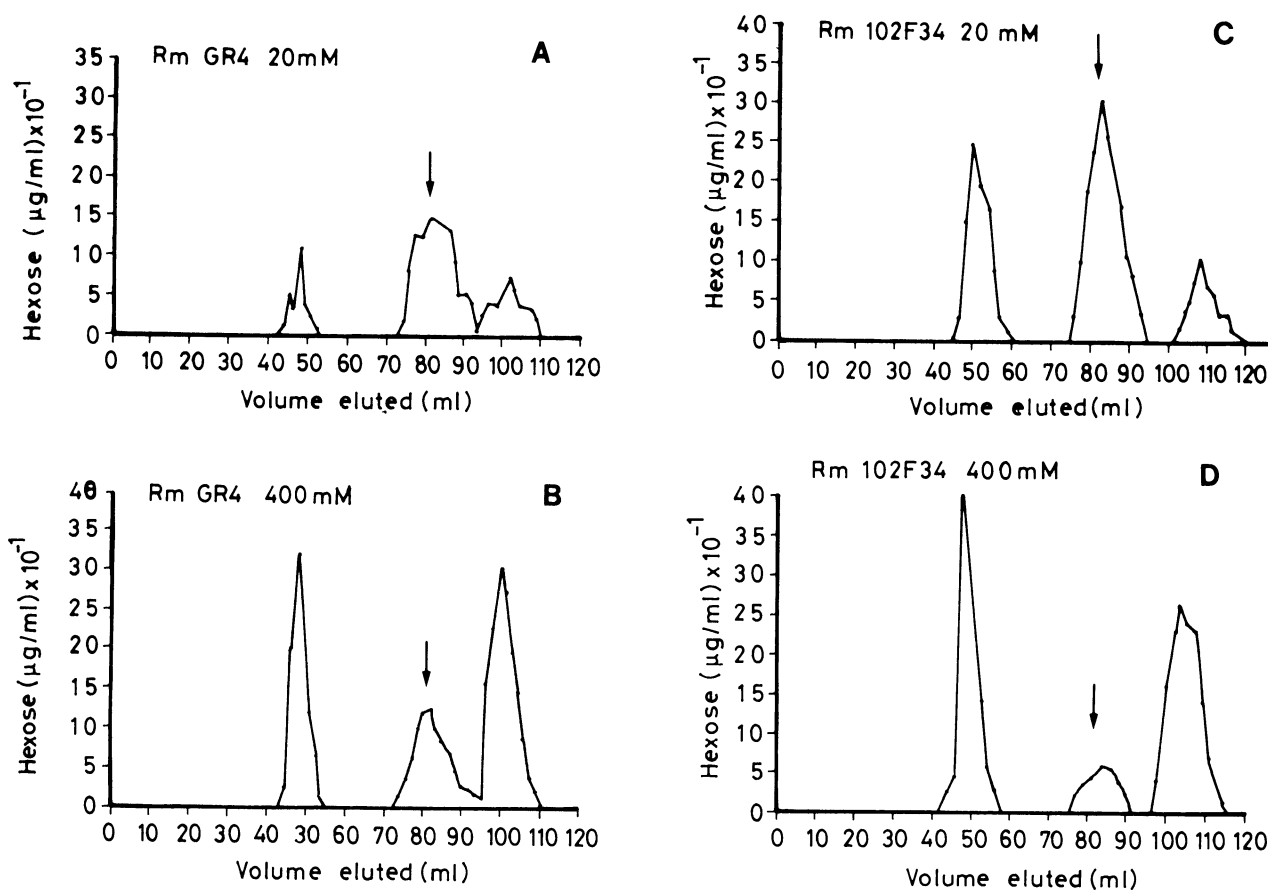


Fig. 3. Effects of extracellular osmolarity on glucan content of *Rhizobium meliloti* strain GR4 and strain 102F34 (for comparison). TCA extracts derived from a 200-ml culture of *R. meliloti* were chromatographed on a column of Sephadex G50 as described in Figure 1. Strain GR4: **A**, low-osmolarity media (20 mM NaCl) and, **B**, high-osmolarity media (400 mM NaCl). Strain 102F34: **C**, low-osmolarity media (20 mM NaCl) and, **D**, high-osmolarity media 400 mM NaCl). The arrows indicate the position of $\beta(1-2)$ -glucan.

results were reported in *A. tumefaciens* by Miller *et al.* (1986).

The results shown in this work indicate that the level of cell-associated glucan in wild-type strain GR4 does not change in response to external osmolarity. Similar results have been reported by Breeveld *et al.* (1991) in *R. leguminosarum* bv. *trifolii* TA-1, although in this strain high osmotic conditions leads to higher $\beta(1-2)$ -glucan excretion. Thus, it is possible that the mechanism of signal-transduction into the cell of the environmental osmotic conditions in *R. meliloti* strain GR4, functions in a different manner to that of *R. meliloti* strain 102F34 or even *R. leguminosarum* strain TA-1. Whether our results indicate a different and specific mechanism of osmoadaptation in *R. meliloti* GR4, or whether this behavior might be a more general feature in *Rhizobium* would require additional work.

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