Periplasmic cyclic 1,2-β-glucan in *Brucella* spp. is not osmoregulated

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INTRODUCTION

The family *Rhizobiaceae* includes the genera *Agrobacterium* and *Rhizobium*. Bacteria from both genera synthesize cyclic 1,2-β-glucans (York *et al.*, 1978; Zevenhuizen & Neerven, 1983; Hisamatsu *et al.*, 1983). Many roles have been assigned to these polysaccharides. For instance, several studies have shown that in the family *Rhizobiaceae* the biosynthesis of cyclic β-glucans is osmotically regulated and thus a role for the polysaccharides in hypo-osmotic adaptation has been suggested (Miller *et al.*, 1985; Dylan *et al.*, 1986; Geremia *et al.*, 1987; Iión de Iannino & Ugalde, 1989). Genes *chvA* and *chvB* encode 235 and 75 kDa inner-membrane proteins involved in the synthesis and secretion, respectively, of cyclic 1,2-β-glucans (Zorreguieta & Ugalde, 1986; Iión de Iannino & Ugalde, 1989; Cangelosi *et al.*, 1989). Mutants in the *ndvB* locus induce the formation of pseudonodules in alfalfa (Dylan *et al.*, 1986; Geremia *et al.*, 1987). In *Rhizobium* and *Agrobacterium*, cyclic 1,2-β-glucan synthesis proceeds through the formation of a protein intermediate (Zorreguieta & Ugalde, 1986). Glucose is transferred from UDP-Glc to an unidentified amino acid of the above-mentioned 235 kDa inner-membrane protein. After elongation the glucan is cyclized, released from the protein and secreted into the periplasmic space (Zorreguieta & Ugalde, 1986; Iión de Iannino & Ugalde, 1989). Williamson *et al.* (1992) demonstrated that the size distribution of cyclic 1,2-β-
glucan products depends on competing elongation and cyclization reactions, thus suggesting that both processes are interrelated.

The role of cellular polysaccharides in bacteria–cell interactions and pathogenesis is well known (Finan et al., 1985; Leigh et al., 1985; Whatley et al., 1976; Abe et al., 1982; Puvanesarajah et al., 1985). Brucella spp. are animal pathogens that produce a chronic disease characterized by recurrent fever in humans, abortion in ungulates and persistence of the pathogen inside phagocyte cells of the reticuloendothelial system (Smith & Ficht, 1990). Brucella lipopolysaccharide is highly antigenic and induces a specific antibody response (Moreno et al., 1981b). Rough mutants of B. abortus are defective in the synthesis of the O polysaccharide or the core region and cases have been described in which such mutants have lost virulence (Reeves, 1995). B. ovis and B. canis isolated from infected animals are, however, pathogenic rough mutants. In Brucella interest has also been given to another polysaccharide, polysaccharide B (poly B). B. abortus-infected animals have been reported to produce antibodies that precipitate poly B, while serum from S19-vaccinated animals does not. It has been proposed that poly B might be useful in differentiating vaccinated from infected animals (Cheronovogrodzky et al., 1990; Moreno et al., 1981a).

More recently, however, Bundle et al. (1988) purified and characterized poly B and demonstrated that it was a mixture of a non-immunogenic family of cyclic 1,2-β-glucans and the O chain of the lipopolysaccharide that carried the serological reactivity.

Brucella, Rhizobium and Agrobacterium belong to a very close taxonomic group according to their 16S rRNA sequences (Triplett et al., 1994; De Lay, 1987) and individual species of all these genera have been shown to produce cyclic 1,2-β-glucans (Hisamatsu et al., 1983; Bundle et al., 1987, 1988). In Agrobacterium and Rhizobium these glucans are required for virulence and nodule invasion, respectively. In Brucella, however, the biosynthesis and the possible role in pathogenesis of this periplasmic polysaccharide have not yet been studied. No homologies of Brucella genome with chuB/chvA or ndvB/ndvA genes have been reported so far. In this paper we demonstrate that in B. ovis and B. abortus, contrary to what happens in species of the genera Agrobacterium and Rhizobium, the accumulation of periplasmic cyclic 1,2-β-glucan is not osmoregulated. However, although the biosynthesis of the polysaccharide proceeds, as in the other mentioned species, through a glucoprotein intermediate, the cyclization reaction requires a factor(s) in the Brucella spp. examined.

**METHODS**

**Bacterial strains and growth conditions.** Brucella ovis strain REO198 and the attenuated vaccine strain B. abortus S19 were used. Both strains were obtained from the Centro Panamericano de Zoonosis, Buenos Aires, Argentina. Cells were grown at 37 °C in a rotary shaker (200 r.p.m.) in Brucella broth medium (BB, Difco). For growing B. ovis, REO198 medium was supplemented with 10% normal calf serum (BBS, Gibco BRL). For osmotic studies, 200 ml BBS medium was supplemented with 0.5 M mannitol, 0.5 M glucose or 0.25 M NaCl.

**Gel chromatography, TLC and paper electrophoresis.** Gel chromatography was carried out in Bio-Gel P4 columns (78 cm × 1.8 cm), equilibrated with 0.1 M pyridine acetate buffer, pH 5.5. Fractions of 1.5 ml were collected, radioactivity detected in a liquid scintillation counter and sugars quantified by the anthrone-sulfuric acid method (Dische, 1962). TLC was carried out with silica gel-60 plates (Merck) developed three times with 1-butanol/ethanol/water (5:5:4, by vol.) as described by Zevenhuizen et al. (1990). Paper electrophoresis was carried out as described previously by Iión de Iannino & Ugalde (1993).

**Preparation of permeabilized cells and total membranes.** Cells from 1 litre cultures grown for 48 h in 21 flasks were harvested by centrifugation at 8000 g for 15 min at 4 °C. Cells were washed with physiological solution (8.5 g NaCl l⁻¹), centrifuged in an Eppendorf centrifuge for 5 min and pellets stored at −20 °C until used. Cell pellets were resuspended in 50 mM Tris/HCl buffer, 3 mM EDTA, pH 8.0, and 2 mM PMSF at approximately 40 mg protein ml⁻¹. Permeabilized cells were prepared by adding 0.1% Nonidet P 40 to resuspended cells just before adding UDP-[¹⁴C]Glc to start the enzymic assays. Total membranes were prepared by ballistic disintegration of cells (Morion & Berman, 1982). Cells from 1 litre cultures were harvested, resuspended in 50 mM Tris/HCl buffer, 3 mM EDTA, pH 8.0, 2 mM PMSF and broken in a MSK-Braun Cell Homogenizer with 0.25–0.3 mm glass beads for one cycle of 45 s. After removing glass beads by centrifugation at 3000 g for 15 min at 5 °C, supernatants were submitted to centrifugation at 100000 g for 2 h at 5 °C. Membrane pellets were resuspended in 50 mM Tris/HCl buffer, 3 mM EDTA, pH 8.0, at approximately 40 mg protein ml⁻¹ and stored at −20 °C until used.

**Extraction of cell-associated glucans.** Cells from 200 ml cultures were harvested by centrifugation at 10000 g for 15 min. Pellets were treated with 10% trichloroacetic acid (TCA). TCA extracts were neutralized with ammonium hydroxide, concentrated and subjected to gel filtration on Bio-Gel P4 columns as described above. Acid hydrolysis, reduction with borohydride, paper chromatography and paper electrophoresis were carried out as described previously (Iión de Iannino & Ugalde, 1993) and DEAE-Sephadex chromatography was carried out also as described previously (Iión de Iannino & Ugalde, 1989).

**Osmotic shock.** The zwittergent–lysozyme extraction method was carried out as described by Stabel et al. (1994) with some modifications. Freshly harvested B. ovis strain REO198 cells were resuspended in 50 ml 0.2 M Tris/HCl buffer, pH 8.0, with 0.5 M sucrose and 0.25% N-alkyl-N,N-dimethylammonio-1-propanesulfonate SB3-16 (Sigma, equivalent to Zwittergent-316 from Calbiochem). After 5 min of incubation in an ice bath, cells were harvested by centrifugation at 3000 g for 5 min and pellets exposed to osmotic shock by addition of 45 ml cold distilled water. A solution of lysozyme (50 mg ml⁻¹, Sigma) was added immediately to the suspension to obtain a final concentration of 50 μg ml⁻¹. The cell suspension (total cellular fraction) was shaken for 15 min at 25 °C and centrifuged for 15 min at 12000 g. Supernatants (periplasmic fluid) were freeze-dried and subjected to Bio-Gel P4 chromatography. For malate dehydrogenase, assay aliquots
were withdrawn from the total cellular fraction before centrifugation, cells were disrupted by freeze-thaw and sonication, centrifuged for 30 min at 8000 g and filtered supernatants assayed for enzyme activity as described by Worthington Diagnostic Systems, Inc. (1982). The remaining pellet was treated with 10% TCA as described above and the TCA extracts were neutralized with ammonium hydroxide and subjected to Bio-Gel P4 chromatography. When osmotic shock was carried out on B. abortus S19 cells, the method described by Stabel et al. (1994) was used with no modifications.

**In vitro synthesis of 1,2-β-glucan.** In vitro synthesis with permeabilized cells or total membranes as enzyme sources was carried out as described previously (Iión de Iannino & Ugalde, 1993) with the following modifications: the incubation temperature was 37 °C and 500000 c.p.m. (10.5 GBq mmol⁻¹) UDP-[¹⁴C]Glc was used in the incubation mixtures.

**Purification and characterization of glycopeptides.** The TCA precipitates obtained as described previously (Iión de Iannino & Ugalde, 1993) were washed twice with 2 ml 5% TCA, twice with 2 ml ethyl ether and dried under a nitrogen stream. The TCA precipitates (50000 c.p.m.) were then resuspended in 100 mM Tris/HCl buffer, pH 7.5, with 10 mM CaCl₂ and incubated at 37 °C with 2 mg protease type XIV from Streptomyces griseus (Sigma) in a total volume of 1 ml. After 48 h, 1 mg protease was added and the incubations were continued for 2 weeks. TCA (10%) was added to stop the reactions and the glycopeptides were recovered from the supernatants after centrifugation for 2 min in an Eppendorf centrifuge. The supernatants were then washed several times with ethyl ether to remove TCA and evaporated under a nitrogen stream. After this extensive protease treatment most of the glycopeptides have only one amino acid attached to the reducing end of their oligosaccharides (Yamashita et al., 1978). Glycopeptides labelled with [¹³C]glucose were subjected to paper electrophoresis in 5% (v/v) formic acid and labelled cations were eluted from the papers with water and chromatographed on Bio-Gel P4 columns.

**RESULTS**

**Characterization of cellular glucans**

*B. ovis* RE0198 and *B. abortus* S19 cells were treated with 10% TCA. As shown in Fig. 1, TCA extracts from *B. ovis* and *B. abortus* contained anthrone-positive compounds that eluted from a Bio-Gel P4 column with an elution volume similar to cyclic 1,2-β-glucans formed in vitro by inner membranes of *R. meliloti* or *A. tumefaciens*. These products (fractions 10-20, Fig. 1) yielded, after total acid hydrolysis and paper chromatography with solvent A, glucose as the only monosaccharide, indicating that they were glucans. Partial acid hydrolysis and paper chromatography with solvent B yielded glucose, sophorose and a series of oligosaccharides with an increasing degree of polymerization, indicating that these products were 1,2-β-glucans. These results showed that *B. ovis* and the attenuated strain S19 of *B. abortus*, contained cellular 1,2-β-glucans. DEAE-Sephadex chromatography of *B. ovis* and *B. abortus* S19 cellular glucans showed that approximately 60% of them were recovered in the percolate and 40% eluted from the column with 50-500 mM NaCl, indicating that they produce neutral and anionic cellular 1,2-β-glucans (data not shown).

**Effect of osmolarity on glucan accumulation**

It was previously shown that *A. tumefaciens* and *R. meliloti* cells grown in media of high osmolarity (0.5 M mannitol, 0.5 M glucose or 0.25 M NaCl) displayed strong inhibition of the accumulation of cellular cyclic 1,2-β-glucans (Miller et al., 1986; Zorreguieta et al., 1996). As shown in Table 1, the accumulation of *B. ovis* cellular glucans was not reduced when cells were grown in the presence of 0.5 M mannitol or 0.5 M glucose. On the contrary, an increase was observed. When cells were grown in 0.25 M NaCl, a condition under which *Brucella* grows very poorly, inhibition was observed. However, it was not statistically significant. No inhibition of the accumulation of cellular glucan was observed when *B. abortus* S19 was grown in the presence of the 0.5 M mannitol (Table 1).

**Subcellular localization of 1,2-β-glucan**

To determine the subcellular localization of 1,2-β-glucans in *Brucella*, *B. ovis* and *B. abortus* S19 cells were
**Table 1. Effect of osmolarity on the accumulation of cellular cyclic 1,2-β-glucan in Brucella spp.**

Cells were grown in BBS or BB medium as described in Methods.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Addition</th>
<th>Cellular glucan (µmol glucose (g cell wet wt)^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. ovis</em> RE0198</td>
<td>None</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>Mannitol (0.5 M)</td>
<td>18.8</td>
</tr>
<tr>
<td></td>
<td>Glucose (0.5 M)</td>
<td>17.6</td>
</tr>
<tr>
<td></td>
<td>NaCl (0.25 M)</td>
<td>8.8</td>
</tr>
<tr>
<td><em>B. abortus</em> S19</td>
<td>None</td>
<td>22.7</td>
</tr>
<tr>
<td></td>
<td>Mannitol (0.5 M)</td>
<td>22.4</td>
</tr>
</tbody>
</table>

* Cellular glucans (µmol glucose (g cell wet wt)^{-1}) were extracted from cell pellets with 10% TCA and extracts subjected to Bio-Gel P4 chromatography. Experiments were repeated twice with a dispersion lower than 20%.

**Fig. 2. Subcellular localization of cyclic 1,2-β-glucan in *Brucella* ovis.** Cells were submitted to osmotic shock and the periplasmic fluid was recovered after centrifugation, lyophilized and dissolved in 2 ml water. The remaining pellet was treated with 2 ml 10% TCA. The periplasmic fluid (a) and the TCA extract (b) were submitted to gel chromatography. Results are expressed as µmol glucose equivalent (g cell pellet wet wt)^{-1}.

Subjected to osmotic shock. Fig. 2 shows that after such treatment cyclic glucans from *B. ovis* cells were recovered from the periplasmic fluid (Fig. 2a). The remaining cell pellet had no detectable TCA-extractable glucan (Fig. 2b). Assay of the cytoplasmic enzyme malate dehydrogenase detected 0.024 units ml^{-1} in the total cellular fraction, whereas no activity was detected in the periplasmic fluid. This indicated that the osmotic shock did not produce cellular lysis and that the 1,2-β-glucans recovered from the periplasmic fluid were not the result of leaking of cytoplasmic fluid into the periplasmic fraction. When *B. abortus* S19 cells were subjected to osmotic shock, 65% of the glucan was recovered in the periplasmic fluid; no detectable malate dehydrogenase activity was observed in this fraction.

**In vitro biosynthesis of cyclic 1,2-β-glucans**

Incubation of *B. ovis* total membranes with UDP-[14C]Glc led to the incorporation of [14C]glucose into water-soluble and TCA-insoluble compounds. Water-soluble products recovered in the percolate of DEAE-Sephadex columns were characterized as cyclic 1,2-β-glucans as described below. Total membranes incubated with UDP-[14C]Glc incorporated approximately the same amount of radioactivity into soluble and TCA-insoluble products, 12 and 10 pmol [14C]glucose min^{-1} (mg protein)^{-1}, respectively. After a chase with non-labelled UDP-Glc, TCA-insoluble products did not display the behaviour expected for intermediates, as no reduction in the amount of radioactivity was observed (Fig. 3b, lanes 3 and 4). In contrast, when permeabilized cells were used as enzymic preparation, the radioactivity accumulated in TCA-insoluble products was lower than with total membranes (4 pmol [14C]glucose min^{-1} (mg protein)^{-1}). Upon the addition of non-labelled UDP-Glc, the insoluble products behaved as intermediates as the label amount decreased (Fig. 4b). In the case of permeabilized cells, the formation of soluble glucans per mg protein was approximately three times higher than with total membranes. PAGE of TCA-insoluble compounds labelled upon incubation of *B. ovis* total membranes (Fig. 3, lanes 3 and 4) and *B. ovis* permeabilized cells (Fig. 4, lanes 6, 7 and 8) with UDP-[14C]Glc revealed the presence of proteins with apparent molecular masses indistinguishable from *A. tumefaciens* or *R. fredii* 235 kDa 1,2-β-glucan intermediate proteins (Fig. 3, lanes 1, 2, 5 and 6). It can be observed that after the addition of non-labelled UDP-Glc, labelled proteins formed by membranes of *A. tumefaciens* or *R. fredii* (Fig. 3, lanes 1, 2 and 5, 6) or permeabilized cells of *B. ovis* (Fig. 4, lanes 6, 7 and 8) and *B. abortus* (Fig. 4, lanes 3, 4 and 5) behaved as intermediates, with a rapid reduction in radioactivity. Fig. 3 shows that *B. ovis* membranes contained, besides the 235 kDa protein, at least two other labelled proteins having lower molecular masses. They probably represent products of proteolytic degradation since their relative amounts changed in different membrane preparations. After the addition of non-labelled UDP-Glc (2 mM), an increment of the apparent molecular mass became apparent (Fig. 3b, lanes 3 and 4, and Fig. 6b inset). These results suggested that the reaction of cyclization, that under normal circumstances releases cyclic glucan from the intermediate protein, proceeded less efficiently *in vitro* with membranes; moreover it seems to be completely blocked upon chasing with 2 mM UDP-Glc. It may be assumed that this defective cyclization led, upon addition of 2 mM UDP-Glc, to uncontrolled elongation and conse-
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Fig. 3. PAGE of membranes incubated with UDP-[^14C]Glc. Total membranes (0.2 mg protein) of *B. ovis* strain RE0198 (lanes 3 and 4) or inner membranes (0.2 mg protein) of *A. tumefaciens* A348 (lanes 1 and 2) or *R. fredii* USDA191 (lanes 5 and 6) were incubated with UDP-[^14C]Glc, the reactions were stopped by the addition of 10% TCA and the precipitates were subjected to gel electrophoresis. Proteins were stained with Coomassie blue (a) and radioactivity, detected by fluorography (b). For the chase experiment (even-numbered lanes), 2 mM non-radioactive UDP-Glc was added after a 10 min incubation and the reactions were stopped after 30 min. Numbers on the right indicate the molecular masses of standards.

Fig. 4. PAGE of total membranes and permeabilized cells incubated with UDP-[^14C]Glc. Lanes: 1 and 2, inner membranes (0.2 mg protein) of *A. tumefaciens* A348; 3, 4 and 5, permeabilized cells (0.2 mg protein) of *B. abortus* S19; 6, 7 and 8 permeabilized cells (0.2 mg protein) of *B. ovis* RE0198; 1, 3 and 6, 10 min incubation with UDP-[^14C]Glc; 2, 4 and 7, chase experiment in which, after 10 min incubation with UDP-[^14C]Glc, 2 mM non-radioactive UDP-Glc was added and the incubations were continued for 30 min; 5 and 8, chase experiment with 20 mM UDP-Glc. Proteins were stained with Coomassie blue (a) and radioactivity, detected by fluorography (b).

Corresponding to the accumulation of protein intermediates with larger than normal linear oligosaccharides that cannot be cyclized (see below). The kinetics of incorporation of [^14C]glucose into soluble glucan and intermediate protein by permeabilized cells are shown in Fig. 5.
Characterization of glycopeptides

Total membranes of *B. ovis* were incubated at 37 °C with UDP-[14C]Glc and after 15 min half of the total incubation was inactivated with 10% TCA; non-labelled UDP-Glc (2 mM) was then added and the rest of the reaction mixture was further incubated for 20 min. One-tenth aliquots (200 µg protein) of pulse and chase samples were subjected to PAGE (Fig. 6b, inset); the rest of the samples were subjected to extensive proteolysis to generate pulse and chase glycopeptides which were purified by paper electrophoresis. Glycopeptides were eluted from the paper strips with water and subjected to Bio-Gel P4 chromatography to estimate their degree of polymerization. The pulse incubation with 0.02 mM UDP-[14C]Glc yielded glycopeptides that eluted from the Bio-Gel P4 columns with elution volumes slightly higher than *B. ovis* 1,2-β-glucans obtained in vitro (Fig. 6a and c). After a chase with 2 mM UDP-Glc, glycopeptides appeared in the void volumes of the Bio-Gel P4 columns (Fig. 6b), indicating that they had a higher degree of polymerization. PAGE of pulse and chase TCA-insoluble products revealed the presence of [14C]glucose-labelled proteins with similar amounts of radioactivity. The chase-derived species showed an increment in their apparent molecular masses (Fig. 6b inset, lane 2). Pulse and chase experiments carried out with *B. abortus* total membranes showed similar behaviour (data not shown). It was thus concluded that *Brucella* membranes do not control the elongation reaction when a relatively high concentration of UDP-Glc (2 mM) is added to the reaction mixture. As a consequence of this, intermediate proteins with oligo-
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were recovered after partial acid hydrolysis and paper chromatography in buffer B (Fig. 7b). To determine if these 1,2-β-glucans formed in vitro were cyclic molecules, 60,000 c.p.m. of glucan recovered from the Bio-Gel P4 column (fractions 10–20) was subjected to sodium borohydride reduction, total acid hydrolysis and paper electrophoresis. As shown in Fig. 7(c), no sorbitol was detected after paper electrophoresis with buffer C. The same result was obtained with glucans formed by total membranes of B. abortus S19. These results showed that total membranes of B. ovis and B. abortus formed cyclic 1,2-β-glucan in vitro. Cyclic 1,2-β-glucans formed in vitro eluted from a Bio-Gel P4 column at an approximately similar elution volume as the cellular glucan accumulated in vivo, suggesting that they had the same degree of polymerization (Fig. 1). TLC of cyclic 1,2-β-glucans formed in vitro by B. ovis permeabilized cells and A. tumefaciens inner membranes revealed that they had the same size distribution and degree of polymerization (Fig. 8a and b). In contrast, B. ovis total membranes formed 1,2-β-glucans in vitro with a size distribution displaced toward a higher degree of polymerization than glucans formed by permeabilized cells and similar to cyclic 1,2-β-glucans formed by R. meliloti and R. fredii (Fig. 8a, d and e).

**DISCUSSION**

Our results show that the rough strain of B. ovis REO198 and the attenuated vaccine strain B. abortus S19 contain, as described for B. melitensis (Bundle et al., 1987) and a pathogenic strain of B. abortus (Bundle et al., 1988), cyclic 1,2-β-glucans. Osmotic shock studies established that B. ovis REO198 and B. abortus S19 glucans are mainly accumulated in the periplasmic space. Since it was observed that B. ovis is more sensitive than B. abortus to the detergent–lysozyme extraction method, a modification of the original method was used to study glucan cellular localization in this strain. The results reported here suggest the presence in Brucella of a functional secretory pathway for cyclic 1,2-β-glucans as described in Agrobacterium and Rhizobium. In these species, cyclic glucans are synthesized in the cytoplasm by a membrane-bound enzyme and secreted into the periplasmic space.

The biosynthesis of cyclic glucans in Agrobacterium and Rhizobium proceeds through a novel mechanism in which a protein participates as intermediate (Zorreguieta & Ugalde, 1986). Glucose units are transferred from UDP-Glc to an inner-membrane protein with an apparent molecular mass of 235 kDa, until the 1,2-β-glucan formed has about 17–24 glucose residues. The glucan is then released from the protein as a cyclic molecule secreted into the periplasmic space by a secretory system in which NvdA or ChvA proteins participate. Indirect evidence supports the notion that initiation, elongation and cyclization reactions are catalysed by the 235 kDa protein (Altabe et al., 1990).

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**Fig. 7. Characterization of cyclic 1,2-β-glucan formed in vitro.** In vitro synthesis of cyclic 1,2-β-glucan was carried out with total membranes of B. ovis REO198. Glucan was purified by Bio-Gel P4 chromatography and subjected to total and partial acid hydrolysis and reduction with sodium borohydride. (a) Paper chromatography of total acid hydrolysis products developed with solvent A; (b) paper chromatography of partial acid electrophoresis with buffer C (2% sodium molybdate, pH 5.0). Water-soluble compounds formed after incubation of standards: Gal, galactose; Glc, glucose; Man, mannose; Sof, sophorose; Sol, sorbitol.

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**Characterization of 1,2-β-glucan formed in vitro**

Water-soluble compounds formed after incubation of total membranes with UDP-[14C]Glc eluted from a Bio-Gel P4 column as two main products (Fig. 1). The most abundant product (fractions 10–20) had approximately the same elution volume of in vivo-formed cellular 1,2-β-glucans. Compounds eluting from the column close to the total volume were not characterized. Neutral glucans from B. ovis recovered from Bio-Gel P4 columns (fractions 10–20) were subjected to total and partial hydrolysis and products subjected to paper chromatography. As shown in Fig. 7(a), [14C]glucose was the only monosaccharide recovered after total acid hydrolysis and paper chromatography with buffer A. [14C]Glucose, [14C]sophorose and a series of 14C-labelled oligosaccharides with increasing degree of polymerization were recovered after partial acid hydrolysis and paper chromatography in buffer B (Fig. 7b). To determine if these 1,2-β-glucans formed in vitro were cyclic molecules, 60,000 c.p.m. of glucan recovered from the Bio-Gel P4 column (fractions 10–20) was subjected to sodium borohydride reduction, total acid hydrolysis and paper electrophoresis. As shown in Fig. 7(c), no sorbitol was detected after paper electrophoresis with buffer C. The same result was obtained with glucans formed by total membranes of B. abortus S19. These results showed that total membranes of B. ovis and B. abortus formed cyclic 1,2-β-glucan in vitro. Cyclic 1,2-β-glucans formed in vitro eluted from a Bio-Gel P4 column at an approximately similar elution volume as the cellular glucan accumulated in vivo, suggesting that they had the same degree of polymerization (Fig. 1). TLC of cyclic 1,2-β-glucans formed in vitro by B. ovis permeabilized cells and A. tumefaciens inner membranes revealed that they had the same size distribution and degree of polymerization (Fig. 8a and b). In contrast, B. ovis total membranes formed 1,2-β-glucans in vitro with a size distribution displaced toward a higher degree of polymerization than glucans formed by permeabilized cells and similar to cyclic 1,2-β-glucans formed by R. meliloti and R. fredii (Fig. 8a, d and e).
We describe here that in Brucella spp. the biosynthesis of cyclic 1,2-β-glucans proceeds as in Agrobacterium and Rhizobium through an intermediate membrane protein with an apparent molecular mass indistinguishable from the 235 kDa protein of A. tumefaciens. The in vitro kinetic behaviour of the Brucella protein intermediate was very different to that observed with Agrobacterium or Rhizobium. In Brucella, membrane preparations displayed defective synthesis in vitro characterized by an impaired cyclization and uncontrolled elongation when a relatively high concentration of UDP-Glc (2 mM) is added to the reaction mixture. The same behaviour was observed when the Brucella 235 kDa protein was expressed in a R. meliloti ndvB mutant (Lión de Iannino et al., 1996) or a A. tumefaciens chvB mutant (N. Lión de Iannino and others, unpublished). When Nonidet P40-permeabilized cells were used as enzymic preparation, the biosynthesis proceeded more efficiently than with total membranes and cyclization was not impeded. These results suggest that a factor(s), that was lost or inactivated during the preparation of membranes, is required in Brucella for the effective regulation between elongation and cyclization reactions involved in the synthesis of cyclic 1,2-β-glucans. The degree of polymerization of cyclic glucans formed by total membranes or by permeabilized cells was different. The degree of polymerization of glucans formed by total membranes was higher than permeabilized cell glucans.

A. tumefaciens and R. meliloti cells grown in media of high osmolarity display a strong inhibition of the accumulation of cellular cyclic 1,2-β-glucans (Miller et al., 1986; Dylan et al., 1990; Zorreguieta et al., 1990). According to our results, B. ovis and B. abortus cellular cyclic 1,2-β-glucan accumulation is not inhibited by osmolarity as previously described in Agrobacterium and Rhizobium. We conclude that Brucella cyclic glucan biosynthesis is not osmoregulated. This regulation might not be important for a bacterium such as Brucella, which is poorly adapted to survive outside the host and which persists in nature inside the iso-osmotic environment of the host phagocyte cells.

The role of cyclic 1,2-β-glucan in Brucella is unknown, but the presence of this polysaccharide and its conservation in different genera of the α2 group of Proteobacteria, suggest a possible function in the interaction of bacteria with eukaryotic cells as demonstrated in Agrobacterium and Rhizobium.

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