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

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Biosynthesis of periplasmic cyclic 1,2- β -glucans in *Brucella ovis* strain REO198 and *B. abortus* strain S19 was found to be carried out by membrane-bound enzymes that use UDP-glucose (UDP-Glc) as donor substrate. Contrary to what happens in species of the genera *Agrobacterium* and *Rhizobium*, the accumulation of the reaction products in *Brucella* appeared not to be osmotically regulated. Incubation of permeabilized cells with UDP-[¹⁴C]Glc led to the formation of soluble neutral cyclic 1,2- β -glucans and [¹⁴C]glucose-labelled glucoproteins. PAGE of pulse-chase experiments carried out with permeabilized cells showed that the molecular mass of the labelled protein was indistinguishable from *Agrobacterium tumefaciens* A348 and *Rhizobium fredii* USDA191 glucoproteins known to be intermediates in the synthesis of cyclic glucans. *Brucella* total membrane preparations were less efficient than permeabilized cells in the formation of cyclic glucan; this was attributed to defective cyclization. Accumulation of protein intermediates having oligosaccharides of high molecular mass that were not released from the protein was observed after chase with 2 mM UDP-Glc. This defect was not observed when permeabilized cells were used as enzyme preparation, thus suggesting that in *Brucella* a factor(s) that was lost or inactivated upon the preparation of membranes was required for the effective regulation between elongation and cyclization reactions.

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

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.*, 1986; Dylan *et al.*, 1990; Breedveld *et al.*, 1990; Tully *et al.*, 1990; Cangelosi *et al.*, 1990; Zorreguieta *et al.*, 1990). In contrast, studies carried out with *R. meliloti* and *A. tumefaciens* mutants (*ndvA/ndvB* and *chvA/chvB*, respectively) provided

substantial evidence of their involvement in bacteria-plant interactions (Puvanesarajah *et al.*, 1985; Douglas *et al.*, 1985; Dylan *et al.*, 1986; Geremia *et al.*, 1987; Iñón de Iannino & Ugalde, 1989). Genes *chvB* and *chvA* encode 235 and 75 kDa inner-membrane proteins involved in the synthesis and secretion, respectively, of cyclic 1,2- β -glucans (Zorreguieta & Ugalde, 1986; Iñón 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; Iñón de Iannino & Ugalde, 1989). Williamson *et al.* (1992) demonstrated that the size distribution of cyclic 1,2- β -

Abbreviations: BBS, *Brucella* broth serum medium; TCA, trichloroacetic acid.

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 (Cherwonogrodzky *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 *chvB/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 $\times$ 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 Iñón de Iannino & Ugalde (1993).

Preparation of permeabilized cells and total membranes.

Cells from 1 litre cultures grown for 48 h in 2 l 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 (Moriyon & 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 (Iñón de Iannino & Ugalde, 1993) and DEAE-Sephadex chromatography was carried out also as described previously (Iñón 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 μ g 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 (Iñón de Iannino & Ugalde, 1993) with the following modifications: the incubation temperature was 37 °C and 500 000 c.p.m. ($10.5 \text{ GBq mmol}^{-1}$) UDP-[^{14}C]Glc was used in the incubation mixtures.

Purification and characterization of glycopeptides. The TCA precipitates obtained as described previously (Iñón 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 (50 000 c.p.m.) were then resuspended in 100 mM Tris/HCl buffer, pH 7.5, with 10 mM CaCl_2 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 [^{14}C]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 REO198 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

Fig. 1. Bio-Gel P4 chromatography of *B. ovis* REO198 (a) and *B. abortus* S19 (b) 1,2- β -glucans. ●, 1,2- β -glucans formed *in vitro*; ○, 1,2- β -glucans accumulated *in vivo*; V_0 , void volume; V_t , total volume; I and II, elution positions of cyclic 1,2- β -glucans formed *in vitro* by *R. meliloti* and *A. tumefaciens*, respectively. The Bio-Gel P4 column (78 cm $\times$ 1.8 cm) was eluted with pyridine acetate buffer, pH 5.5, fractions of 1.5 ml were collected and carbohydrates and radioactivity were determined.

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.*, 1990). 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.

Strain	Addition	Cellular glucan*
<i>B. ovis</i> REO198	None	10.7
	Mannitol (0.5 M)	18.8
	Glucose (0.5 M)	17.6
	NaCl (0.25 M)	8.8
<i>B. abortus</i> S19	None	22.7
	Mannitol (0.5 M)	22.4

* Cellular glucans [$\mu\text{mol glucose (g cell wet wt)}^{-1}$] 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 $\mu\text{mol glucose equivalent (g cell pellet wet wt)}^{-1}$.

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- ^{14}C Glc led to the incorporation of ^{14}C 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- ^{14}C Glc incorporated approximately the same amount of radioactivity into soluble and TCA-insoluble products, 12 and 10 $\text{pmol } ^{14}\text{Cglucose min}^{-1} (\text{mg protein})^{-1}$, 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 $\text{pmol } ^{14}\text{Cglucose min}^{-1} (\text{mg protein})^{-1}$ }. 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- ^{14}C 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-

Fig. 3. PAGE of membranes incubated with UDP-[^{14}C]Glc. Total membranes (0.2 mg protein) of *B. ovnis* strain REO198 (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-[^{14}C]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-[^{14}C]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. ovnis* REO198; 1, 3 and 6, 10 min incubation with UDP-[^{14}C]Glc; 2, 4 and 7, chase experiment in which, after 10 min incubation with UDP-[^{14}C]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).

quently to the accumulation of protein intermediates with larger than normal linear oligosaccharides that cannot be cyclized (see below). The kinetics of in-

corporation of [^{14}C]glucose into soluble glucan and intermediate protein by permeabilized cells are shown in Fig. 5.

Fig. 5. 1,2- β -Glucan synthesis by permeabilized cells of *B. ovis*. Permeabilized cells of *B. ovis* REO198 were incubated with UDP-[14 C]Glc. The graphs (●) show incorporation of [14 C]glucose into 1,2- β -glucan (a) and into 1,2- β -glucan protein intermediate (b). Incorporation of [14 C]glucose after addition of 2 mM non-radioactive UDP-Glc is indicated by the dashed line (○). The arrows indicate the time of addition of non-radioactive UDP-Glc.

Characterization of glycopeptides

Total membranes of *B. ovis* were incubated at 37 °C with UDP-[14 C]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 μ g 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-[14 C]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 [14 C]glucose-labelled proteins with similar amounts of

Fig. 6. Bio-Gel P4 chromatography of *B. ovis* REO198 [14 C]glucose-labelled glycopeptides. [14 C]Glucose-labelled peptides were obtained and purified as described in Methods. Total membranes were incubated with UDP-[14 C]Glc for 10 min (pulse-glycopeptides, a); after 10 min incubation with UDP-[14 C]Glc, 2 mM non-radioactive UDP-Glc was added and the incubation was continued for 30 min (chase-glycopeptides, b). (c) Cyclic 1,2- β -glucan obtained *in vitro* from total membranes of *B. ovis* REO198. The inset in (b) shows PAGE of TCA precipitates of pulse (lane 1) and pulse-chase samples (lane 2); the arrow indicates the position of the 235 kDa intermediate protein of *A. tumefaciens* A 348 and the asterisks indicate degradation products of the intermediate protein.

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-

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 hydrolysis products developed with solvent B; (c) paper electrophoresis with buffer C (2% sodium molybdate, pH 5.0). Standards: Gal, galactose; Glc, glucose; Man, mannose; Sof, sophorose; Sol, sorbitol.

saccharides having a high degree of polymerization are accumulated, resulting in impeded cyclization.

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

Water-soluble compounds formed after incubation of total membranes with UDP-[14 C]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), [14 C]glucose was the only monosaccharide recovered after total acid hydrolysis and paper chromatography with buffer A. [14 C]Glucose, [14 C]sophorose and a series of 14 C-labelled oligo-saccharides 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, 60000 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 zwittergent-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 NdvA 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).

Fig. 8. TLC of cyclic 1,2- β -glucans obtained *in vitro*. Lanes: A, inner membranes of *A. tumefaciens*; B, permeabilized cells of *B. ovis*; C, total membranes of *B. ovis*; D and E, inner membranes of *R. meliloti* and *R. fredii*, respectively. (a) Autoradiograph; (b) densitometric scanning of autoradiographs. The autoradiograph of the plate was scanned using a Gilford Response II spectrophotometer with an autoradiograph holder accessory at 500 nm.

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 (Iñón de Iannino *et al.*, 1996) or a *A. tumefaciens* *chvB* mutant (N. Iñón 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 $\alpha 2$ group of *Proteobacteriaceae*, suggest a possible function in the interaction of bacteria with eukaryotic cells as demonstrated in *Agrobacterium* and *Rhizobium*.

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