

The *Brucella abortus* Cyclic β -1,2-Glucan Virulence Factor Is Substituted with O-Ester-Linked Succinyl Residues

Mara S. Roset, Andrés E. Ciocchini, Rodolfo A. Ugalde, and Nora Iñón de Iannino*

Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús (IIB-INTECH), Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de General San Martín (CONICET-UNSAM), 1650 San Martín, Buenos Aires, Argentina

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***Brucella* periplasmic cyclic β -1,2-glucan plays an important role during bacterium-host interaction. Nuclear magnetic resonance spectrometry analysis, thin-layer chromatography, and DEAE-Sephadex chromatography were used to characterize *Brucella abortus* cyclic glucan. In the present study, we report that a fraction of *B. abortus* cyclic β -1,2-glucan is substituted with succinyl residues, which confer anionic character on the cyclic β -1,2-glucan. The oligosaccharide backbone is substituted at C-6 positions with an average of two succinyl residues per glucan molecule. This O-ester-linked succinyl residue is the only substituent of *Brucella* cyclic glucan. A *B. abortus* open reading frame (BAB1_1718) homologous to *Rhodobacter sphaeroides* glucan succinyltransferase (OpgC) was identified as the gene encoding the enzyme responsible for cyclic glucan modification. This gene was named *cgm* for cyclic glucan modifier and is highly conserved in *Brucella melitensis* and *Brucella suis*. Nucleotide sequencing revealed that *B. abortus* *cgm* consists of a 1,182-bp open reading frame coding for a predicted membrane protein of 393 amino acid residues (42.7 kDa) 39% identical to *Rhodobacter sphaeroides* succinyltransferase. *cgm* null mutants in *B. abortus* strains 2308 and S19 produced neutral glucans without succinyl residues, confirming the identity of this protein as the cyclic-glucan succinyltransferase enzyme. In this study, we demonstrate that succinyl substituents of cyclic β -1,2-glucan of *B. abortus* are necessary for hypo-osmotic adaptation. On the other hand, intracellular multiplication and mouse spleen colonization are not affected in *cgm* mutants, indicating that cyclic- β -1,2-glucan succinylation is not required for virulence and suggesting that no low-osmotic stress conditions must be overcome during infection.**

Brucella spp. are gram-negative, facultative intracellular bacteria that cause a chronic zoonotic disease known worldwide as brucellosis. *Brucella abortus*, the etiological agent of bovine brucellosis, causes abortion and infertility in cattle and undulant fever in humans (17). The human disease is also caused by *Brucella melitensis*, *Brucella suis*, and *Brucella canis*. *Brucella* spp. are intracellular pathogens that invade and proliferate within host cells; virulence is associated with the ability to multiply inside professional and nonprofessional phagocytic cells (40).

Cyclic glucans appear to be important as intrinsic components of gram-negative bacterial cell envelopes (7). Beyond their common features, glucans show an unexpected structural diversity (4). The role of cyclic glucan in gram-negative bacterial cell envelopes is still controversial. Cyclic-glucan mutants have a highly pleiotropic phenotype, suggesting an overall alteration of their cell envelope properties. Mutants deficient in cyclic-glucan production were found to be attenuated or avirulent compared to their wild-type parent strains, suggesting that the cyclic glucan is an important virulence attribute for the bacteria in their interaction with eukaryotic hosts.

In different bacterial species, glucans are substituted with different nonglycosidic residues, such as phosphoglycerol, phosphoethanolamine, phosphocholine, acetyl, succinyl, and methyl-

malonyl. The degree of substitution varies among species; for example, cyclic glucans produced by the broad-host-range *Rhizobium* sp. strain GRH2 and by certain strains of *Rhizobium leguminosarum* contain no anionic substituents (30, 45). *Bradyrhizobium japonicum* cyclic β -1,6- β -1,3-glucans are neutral but substituted with the zwitterionic phosphocholine (35). On the other hand, more than 90% of *Sinorhizobium meliloti* glucan and the broad-host-range *Rhizobium* sp. strain NGR234 cyclic β -1,2-glucan fraction are anionic (3, 32).

The stage of growth also influences the degree of substitution of cyclic β -1,2-glucans; for example, Geiger et al. (23) have shown that in *S. meliloti* 1021 the conversion of neutral glucans into anionic glucans occurs mostly during the exponential phase of growth. Thus, depending on the bacterial strain and growth conditions, glucans can be nonsubstituted or substituted with neutral or anionic substituents.

Surprisingly, there is no correlation between the structure of the glucan and the nature of the substituents. Cyclic glucans of *Ralstonia solanacearum* (43) and *Xanthomonas campestris* (43, 44) and the linear glucan of *Pseudomonas syringae* (42) are devoid of substituents. Cyclic glucans of *Azospirillum brasilense* (1) are substituted only with succinyl residues, and cyclic glucans of *B. japonicum* are substituted only with phosphocholine (35). Cyclic glucan from *S. meliloti* and *Agrobacterium tumefaciens* are substituted with both succinyl and phosphoglycerol residues (7), while the cyclic glucan of *Rhodobacter sphaeroides* (41) and linear glucans of *Erwinia chrysanthemi* (15) are substituted with succinyl and acetate residues. Osmoregulated periplasmic glucans (OPG) originating from *Escherichia coli*

* Corresponding author. Mailing address: Instituto de Investigaciones Biotecnológicas, Av. Gral Paz 5445, CP1650, San Martín, Buenos Aires, Argentina. Phone: (5411) 4580 7255. Fax: (5411) 4752 9639. E-mail: norai@iib.unsam.edu.ar.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Phenotype or genotype ^a	Reference or source
Strains		
<i>E. coli</i> XL1-blue MRF	$\Delta(mcrA)183 \Delta(mcrB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F' proAB lacI^qZ\Delta M15 Tn10(Tet^r)]$	26
<i>E. coli</i> S17.1(λ pir)	λ lysogenic S17.1 derivative producing π protein for replication of plasmids carrying <i>oriR6K</i> (Nal ^s)	K. N. Timmis
<i>B. abortus</i> 2308	Virulent; field isolated; wild type; Nal ^r erythritol ^f	38
Cgm08	<i>B. abortus</i> 2308 <i>cgm</i> mutant; <i>cgm::Km^r</i>	This study
Cgm08(pBB4 <i>cgm</i>)	<i>B. abortus</i> 2308 <i>cgm</i> mutant with plasmid pBB4 <i>cgm</i> ; Km ^r Amp ^r	This study
<i>B. abortus</i> S19	Vaccine strain; Nal ^r erythritol ^s ; naturally occurring derivative of <i>B. abortus</i> 2308	38
Cgm19	<i>B. abortus</i> S19 <i>cgm</i> mutant; <i>cgm::Km^r</i>	This study
Cgm19(pBB4 <i>cgm</i>)	<i>B. abortus</i> S19 <i>cgm</i> mutant with plasmid pBB4 <i>cgm</i> ; Km ^r Amp ^r	This study
Plasmids		
pBBR1MCS-4	Broad-host-range cloning vector (Amp ^r)	28
pBB4 <i>cgm</i>	pBBR1MCS-4 containing <i>B. abortus cgm</i> gene; Amp ^r	This study
pvT <i>cgm</i>	T-easy vector containing <i>B. abortus cgm</i> gene; Amp ^r	This study

^a Amp^r, ampicillin resistance; Cb^r, carbenicillin resistance; Km^r, kanamycin resistance, Nal^r, nalidixic acid resistance.

(linear glucans) are the only ones known to be substituted with three different substituents, phosphoglycerol, phosphoethanolamine, and succinyl residues (27).

The transfer of phosphoglycerol has been shown to occur in the periplasmic compartment both in *E. coli* (5) and in *S. meliloti* (8). Succinyl transfer was also shown to occur in the periplasmic compartment in *S. meliloti* (6). In *E. coli* and *R. sphaeroides*, the genes *mdoC* and *opgC*, respectively, encode predicted membrane proteins required for wild-type succinyl modification of OPG (14, 29). *E. coli* has two phosphoglycerol transferases of glucans (I and II), one membrane bound and the second periplasmic. The *mdoB* gene encodes phosphoglycerol transferase I. In *S. meliloti*, the *cgmB* gene encodes a soluble protein required for phosphoglycerol modification of cyclic glucan (6). Nothing has been reported about the transfer of the other substituents to glucans.

Brucella spp. produce periplasmic cyclic glucans. They consist of a cyclic backbone with a degree of polymerization ranging from 17 to 25, in which all the glucose units are linked by β -1,2 linkages (11). In our laboratory, we previously cloned, sequenced, and disrupted the gene coding for the cyclic β -1,2-glucan synthetase (*cgs*) (9, 24) and cyclic β -1,2-glucan transporter (*cgt*) (36) responsible for the synthesis and transport to the periplasm of cyclic β -1,2-glucan, respectively. Both *cgs* and *cgt* mutants showed reduced survival in BALB/c mouse spleen tissues and impeded intracellular multiplication (9, 36), indicating that the presence of cyclic glucan in the periplasm is required for full *B. abortus* virulence. Furthermore, Arellano-Reynoso et al. (2) recently determined that *Brucella* cyclic glucan acts on host cell membranes at the levels of lipid rafts controlling vacuole maturation, avoiding lysosome fusion and allowing *Brucella* to reach its replicative niche.

In this report, we describe how a fraction of *B. abortus* cyclic β -1,2-glucan is substituted with O-succinyl residues, which confer on the cyclic β -1,2-glucans an anionic character. We describe the molecular and functional characterization of a new gene (*cgm*) coding for the enzyme named cyclic glucan modi-

fier that catalyzes the succinylation of *B. abortus* cyclic β -1,2-glucan.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *E. coli* strains were grown on Luria broth (LB) (37). *B. abortus* strains were grown in brucella broth (BB) (Difco Laboratories, Detroit, Mich). If necessary, media were supplemented with the appropriate antibiotics at the following concentrations: kanamycin (Km), 50 μ g/ml; ampicillin (Amp), 100 μ g/ml; and nalidixic acid (Nal), 5 μ g/ml. Merodiploid strains for complementation analysis were obtained by biparental mating as previously described (21).

All the protocols using live brucellae were performed in a biosafety level 3 laboratory facility.

Cloning and DNA sequencing. The putative *B. abortus cgm* gene was amplified from *B. abortus* S2308 genomic DNA by PCR using the primers *cgm*-fw. (5'-T CATTTCGGTGCGTGTTTT-3') and *cgm*-rev. (5'-CATAAAAAAGAGCC GCAAG-3'), designed according to the sequence of a *B. abortus* gene highly homologous to the *R. sphaeroides* *OpgC* gene (12, 14).

The amplified products were ligated to T-easy vector (Promega). The resulting plasmid containing a 1.8-kb fragment was named pvT*cgm*. DNA sequencing was carried out by the dideoxy method with an automated model 373 DNA sequencer (Perkin-Elmer Applied Biosystems Division, Foster City, CA) according to the manufacturer's instructions.

Isolation of cyclic β -1,2-glucan from cells. Cells from 100 ml of stationary-phase cultures (optical density at 600 nm [OD₆₀₀], 3.2) grown for 48 h at 37°C (250 rpm) were harvested by centrifugation at 10,000 \times g for 10 min. The pellets were extracted with ethanol (70% ethanol; 1 h at 37°C). The ethanolic extracts were centrifuged, and the supernatants were dried in a speed-vac centrifuge and subjected to gel filtration on Bio-Gel P4 columns (78 by 1.8 cm; Bio-Rad Laboratories, Richmond, Calif.). The glucans were eluted with 0.1 M pyridine-acetate buffer (pH 5.5). Fractions of 1.5 ml were collected, and carbohydrates were detected by the anthrone-sulfuric acid method (20).

For preparative purposes, 6-liter cultures of *B. abortus* S19 were extracted with 70% ethanol, and the cyclic β -1,2-glucans were subjected to Bio-Gel P4 gel filtration as described above. The glucans were eluted with acetic acid (5%). The anthrone-sulfuric-acid-positive material was collected from six columns. Fractions of a compound with elution volumes corresponding to the cyclic β -1,2-glucans were pooled and concentrated. The glucans were further purified by high-performance liquid chromatography (HPLC).

HPLC. Products recovered from the preparative Bio-Gel P4 column were subjected to HPLC on a C₁₈ ODS2 Pharmacia column (25 cm by 4 mm). After application of each sample, the column was washed with water (25 ml) and the

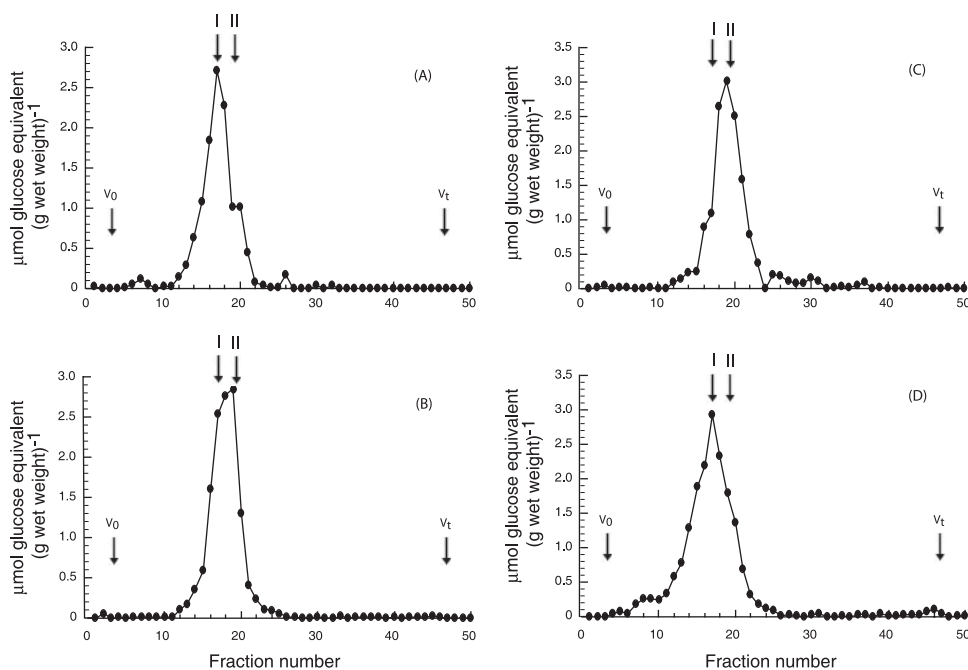


FIG. 1. Bio-Gel P4 chromatography of cyclic β -1,2-glucans accumulated in vivo by wild-type and mutant strains. Cells from 100-ml cultures were harvested and extracted with 70% ethanol. The extracts were subjected to chromatography on Bio-Gel P4 columns (78 by 1.8 cm). The glucans eluted with 0.1 M pyridine-acetate buffer (pH 5.5). Fractions of 1.5 ml were collected, and carbohydrates were expressed as μmol glucose equivalents (g wet weight^{-1}). (A) *B. abortus* 2308 cellular glucans. (B) *B. abortus* 2308 cellular glucans after mild alkali treatment (0.1 N NaOH for 30 min at 37°C). (C) *B. abortus* Cgm08 cellular glucans. (D) *B. abortus* Cgm(pBB4cgm) cellular glucans. V_0 , void volume; V_t , total volume; I, anionic glucan; II, neutral glucan.

glucans were eluted in isocratic form with 40 ml of 20% methanol at a flow rate of 0.5 ml min^{-1} at room temperature. Fractions of 1 ml were collected. The glucans eluted in fractions 4 and 5.

DEAE-Sephadex chromatography. Materials applied to DEAE-Sephadex A25 columns were batch eluted with 6 ml of water and with 6 ml each of 50, 100, 250, and 500 mM NaCl. Fractions of 1.5 ml were collected, and carbohydrates were detected by the anthrone-sulfuric acid method.

Chemical treatments. Cyclic β -1,2-glucan recovered from Bio-Gel P4 columns was successively treated with HCl (10 mM) at 100°C for 90 min and with NaOH (0.1 N) at 37°C for 30 min as previously described for removing polysaccharide nonsugar substituents (25).

TLC of cyclic β -1,2-glucan. Cells from cultures grown for 48 h were harvested by centrifugation at $10,000 \times g$ for 10 min. Cyclic β -1,2-glucans were extracted from the cell pellets with ethanol (70% ethanol; 1 h at 37°C). The ethanolic extracts were centrifuged in an Eppendorf centrifuge, and the supernatants were dried in a speed-vac centrifuge. The extracted glucans were dissolved in 70% ethanol and subjected to thin-layer chromatography (TLC) as described previously (10). The TLC plates were developed by spraying them with 5% sulfuric acid in ethanol and heating them for 10 min at 120°C.

NMR analysis. Glucans were purified by Bio-Gel P4 chromatography and HPLC on a C_{18} silica column as described previously. ^1H , ^{13}C , and ^{31}P nuclear magnetic resonance (NMR) spectroscopy was performed with a Bruker AM 500-MHz spectrometer at 30°C. The samples were dissolved in D_2O . Chemical shifts were measured relative to the external 1,4-dioxane standard.

Construction of a *B. abortus* β -1,2-glucan succinyltransferase mutant. An MscI DNA fragment (675 bp) of *cgm* was digested from pVT*cgm*. The deleted plasmid was ligated to a 1.3-kb HincII fragment containing a kanamycin resistance cassette (33). The recombinant suicide plasmid was electroporated into *B. abortus* S19 and *B. abortus* S2308 strains. Transconjugants were selected in BB agar with Km (50 $\mu\text{g/ml}$). Double-crossover events were selected by streaking colonies in duplicate on BB agar with Km (50 $\mu\text{g/ml}$) and on BB agar with Amp (100 $\mu\text{g/ml}$). Km-resistant, Amp-sensitive clones were selected as possible double recombinants. Putative double recombinants were confirmed by colony PCR using the primers *cgm-fw*, (5'-TCATTTCGTTGCGTGTTT-3') and *cgm-rev*, (5'-CATAAAAAAGAGCCGCAAG-3'). Genetic complementation of the *cgm* mutants was carried out with plasmid pBB4*cgm*, containing a wild-type copy

of the *B. abortus cgm* gene. Plasmid pBB4*cgm* was introduced into the mutants by biparental mating using *E. coli* S17.1 as the donor strain (9, 16).

Effects of osmolarity on growth in liquid and on solid media. All strains were grown in LB with 170 mM NaCl (LB-NaCl) until stationary phase. Cultures were harvested by centrifugation ($12,500 \times g$ for 5 min) and washed twice with phosphate-buffered saline (PBS), and the cells were suspended in LB or LB-NaCl at an OD_{600} of 0.90. For studies in liquid media, cell suspensions were diluted 1/100 in LB or LB-NaCl and incubated at 37°C with continuous stirring (250 rpm); samples were taken at different times, and CFU/ml were determined after appropriate dilution on BB agar plates. For studies on solid media, after being washed with PBS, the cells were suspended to an OD_{600} of 0.9 and serially diluted in PBS, and 10 μl of the dilutions was spotted on LB agar or LB agar with one of the following additions: 170 mM NaCl, 170 mM KCl, 110 mM K_2SO_4 , or 340 mM sucrose. The plates were incubated at 37°C for 5 days before being read.

Cell culture and infection assay. HeLa cells were cultured at 37°C in a 5% CO_2 atmosphere in minimal essential medium (Gibco, Paisley, Scotland) supplemented with 2 mM glutamine and 5% fetal calf serum without antibiotics (cell culture medium). Infection of cells with different *Brucella* strains was performed at a multiplicity of infection of 100 as previously described (39). The murine-macrophage-like J774 cell line was used to test phagocytic cells. Cells at 10^5 /well were infected with a bacterial suspension prepared as described above for HeLa cells except for the following changes: the culture medium was RPMI 1640 (Gibco, Paisley, Scotland) supplemented with 5% fetal bovine serum (Gibco), and the multiplicity of infection was 50.

Virulence in mice. Virulence was determined by quantitating the survival of the strains in mouse spleens at different times postinfection, as previously described (16). Groups of 9-week-old female BALB/c mice were injected intraperitoneally with 10^4 CFU of *B. abortus* 2308, *B. abortus* S19, and their isogenic mutants *B. abortus* Cgm08 and *B. abortus* Cgm19 in 0.2 ml of sterile PBS. At 2, 4, and 12 weeks postinfection, the animals were sacrificed, and the spleens were removed, weighed, homogenized in sterile 150 mM NaCl solution, serially diluted, and plated onto BB agar with the appropriate antibiotics to determine the number of CFU per spleen.

Nucleotide sequence accession number. The sequence of the *B. abortus* cyclic- β -1,2-glucan succinyltransferase gene (*cgm*) has been assigned GenBank accession number AY553720.

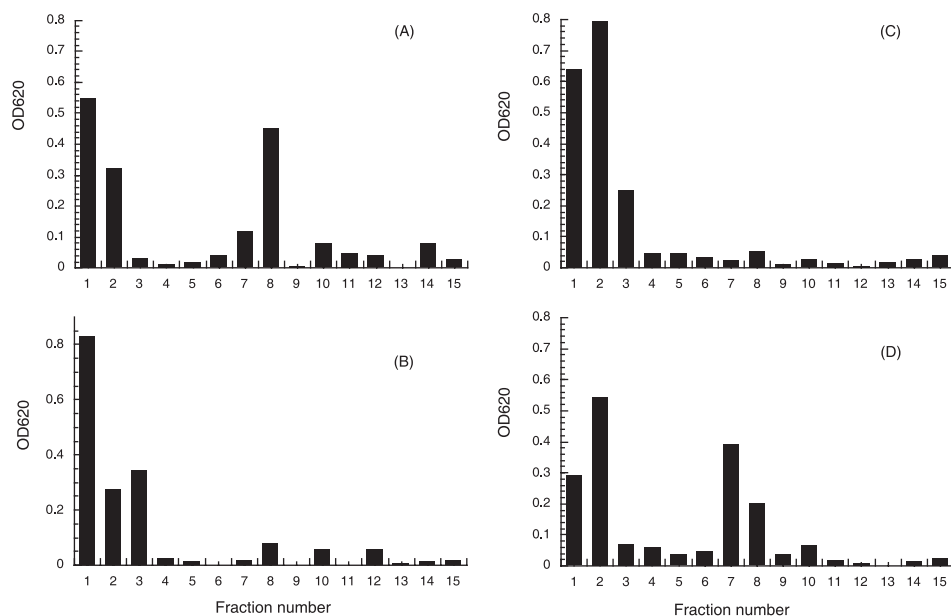


FIG. 2. DEAE-Sephadex chromatography of cellular glucans accumulated by wild-type and mutant strains. Glucans were recovered from Bio-Gel P4 columns (fractions 13 to 22) and subjected to chromatography on DEAE-Sephadex columns (0.25 by 3 cm). The glucans were eluted with 1.5 ml water (fractions 1 to 3), 1.5 ml 0.05 M NaCl (fractions 4 to 6), 1.5 ml 0.1 M NaCl (fractions 7 to 9), 1.5 ml 0.25 M NaCl (fractions 10 to 12), and 0.5 M NaCl (fractions 13 to 15). Fractions of 0.5 ml were collected, and carbohydrates were measured by the anthrone-sulfuric acid method (20). (A) *B. abortus* 2308 cellular glucans. (B) *B. abortus* 2308 cellular glucans after mild alkali treatment (0.1 N NaOH for 30 min at 37°C). (C) *B. abortus* Cgm08 cellular glucans. (D) *B. abortus* Cgm08(pBB4cgm) cellular glucans.

RESULTS

***Brucella* sp. cyclic β -1,2-glucans are partially modified with O-ester-linked anionic substituents.** Cells of *B. abortus* 2308 grown in BB medium were extracted with 70% ethanol. The elution profile from gel filtration chromatography using a Bio-Gel P4 column showed that under these conditions, one main sugar-containing compound was extracted (Fig. 1A). The elution volume of this material corresponds to the previously characterized cyclic β -1,2-glucans of *B. abortus* (10). Cellular β -1,2-glucans recovered from the Bio-Gel P4 column (fractions 13 to 22) were subjected to DEAE-Sephadex chromatography (Fig. 2). A fraction (approximately 50%) percolated through the column, indicating that the glycan was neutral, while the rest of the sample eluted as negatively charged glucans (Fig. 2A). In order to determine the nature of the linkage between cyclic β -1,2-glucans and the anionic substituent, the 70% ethanol extracts were subjected to different treatments and TLC analysis. As shown in Fig. 3, mild alkali treatment completely abolished the presence of the anionic fraction, and no effect was observed after acidic treatment. After the alkali treatment, the elution volume of the main peak was increased (Fig. 1A and B) and became neutral according to DEAE-Sephadex chromatography (Fig. 2B). Since all the anionic glucans were converted to neutral after this treatment, we concluded that *B. abortus* cyclic β -1,2-glucans contain only O-ester-linked anionic substituents. The same results were obtained with cyclic β -1,2-glucans obtained from *B. abortus* strain S19, *B. melitensis*, *B. suis*, *B. ovis*, and *B. canis*. Furthermore, the anionic cyclic glucans of *B. abortus* recovered from TLC and alkali treatment had degrees of polymerization identical to those of the neutral glucans, suggesting that there is not a

preference for a particular degree of polymerization for anionic modification (data not shown).

***B. abortus* anionic cyclic β -1,2-glucans contain succinyl substituents.** In order to determine the chemical nature of the anionic substituent of *B. abortus* cyclic β -1,2-glucan, cell-associated glucans were purified by Bio-Gel P4 column and C₁₈ silica chromatography, eluting with 20% methanol. Purified glucans in D₂O were subjected to NMR spectroscopy at room temperature using a Bruker AM-500 spectrometer. The ¹H-

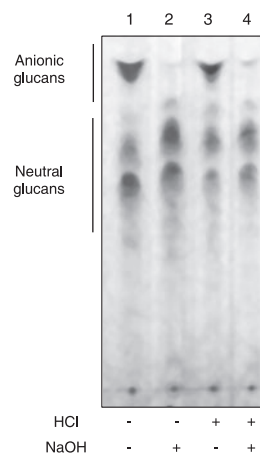


FIG. 3. Thin-layer chromatographic analysis of ethanol extracts of *B. abortus* 2308. Ethanolic extracts were either directly applied to thin-layer chromatography plates (lane 1) or first subjected to mild alkali treatment (lane 2), acidic treatment (lane 3), or both treatments (lane 4). Alkali treatment resulted in selective removal of O-ester linkages.

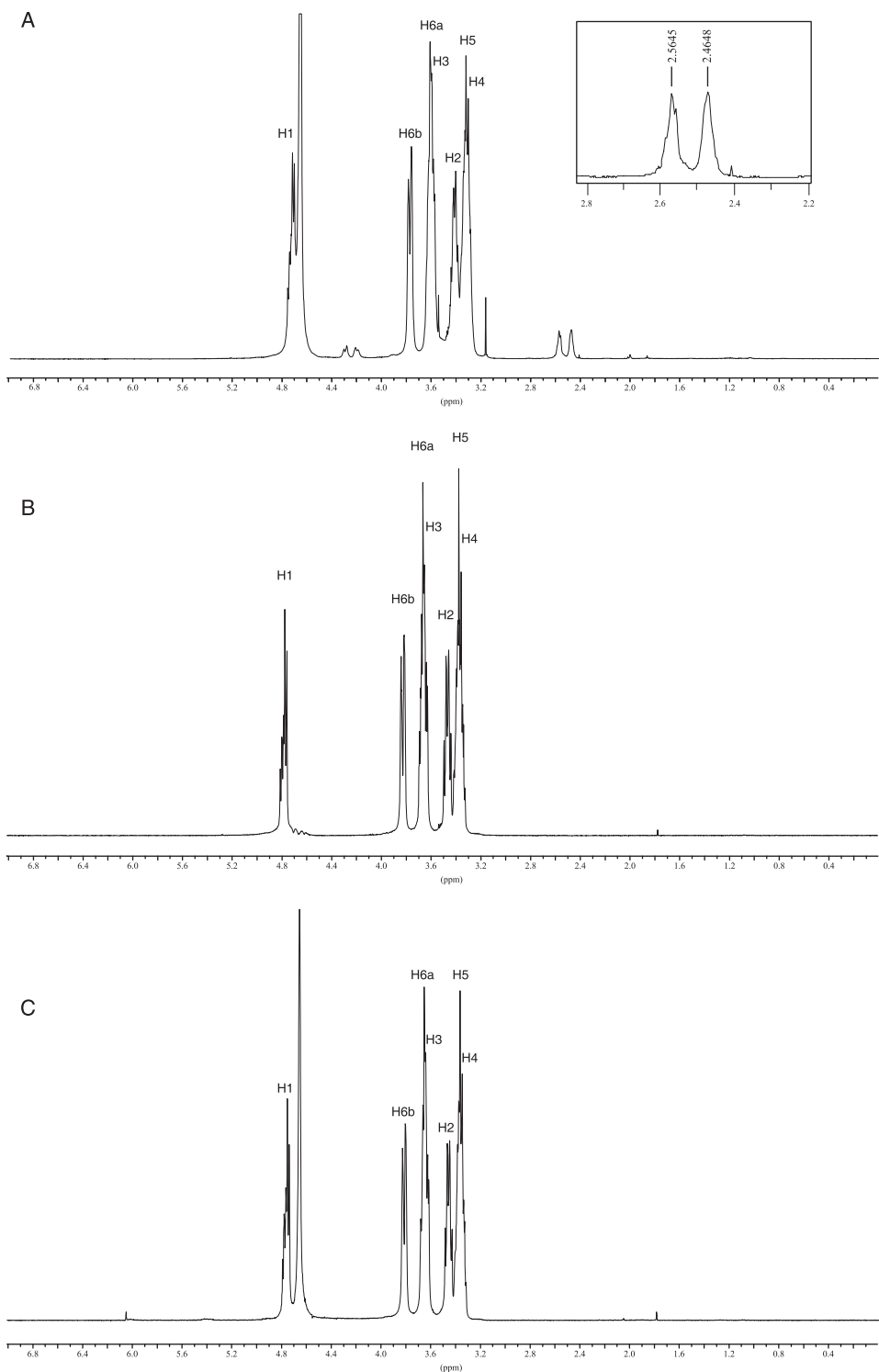


FIG. 4. ^1H -NMR spectra of purified cyclic β -1,2-glucans from *B. abortus*. (A) *B. abortus* S19. (B) *B. abortus* S19 treated with alkali. (C) *B. abortus* Cgm19. The two signals between 2.4 and 2.6 ppm correspond to the methylene protons of the succinic acid substituents. In panel A, the resonance at 3.16 is derived from methanol. The resonance at 4.7 is H_2O . Chemical shifts are expressed relative to external 1,4-dioxane.

NMR spectrum of the glycan sample prepared from the wild-type strain showed the presence of two clear and prominent signals between 2.4 and 2.6 ppm (Fig. 4A) that could be identified as succinyl residues. These signals corresponded unambiguously to the methylene protons of succinate. Furthermore, the signals between 4.25 and 4.35 ppm were attributed to H-6 and H-6', respectively, of glucose residues, with succinate linked to C-6 via an ester bond (31). In contrast, in the ^1H -

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TABLE 2. ^{13}C and ^1H chemical shifts and assignments of *B. abortus* cyclic- β -1,2-glucan resonances

Proton	Chemical shifts (ppm) ^a
C1	102.4-102.5-102.8-102.9
C-2	82.4-82.6-82.9-83.2
C-3	76.1-76.2-76.3
C-4	69.4-69.5-69.6
C-5	77.0
C-6	61.36-61.40
H-1	4.70-4.72
H-2	3.38-3.40-3.42-3.44
H-3	3.60
H-4	3.30
H-5	3.32
H-6a	3.61
H-6b	3.76-3.78

^a Chemical shifts were measured at 30°C and referenced relative to external 1% (vol/vol) 1,4-dioxane.

NMR spectrum of the glycan sample that was treated with alkali, chemical shifts between 2.4 and 2.6 and between 4.25 and 4.35 that were indicative of succinyl residues were not detected (Fig. 4B). Therefore, based on the above-mentioned evidence from NMR analysis, the wild-type cyclic glucan contains succinyl substituents. In the anomeric region of the ^1H -NMR spectra, overlapping doublets at 4.8 ppm were observed. The shift values were characteristic of β configuration of all glucose residues. Signals between 3.38 and 3.44 ppm correspond to the H-2 protons involved in β -1,2 linkage. Chemical shifts attributed to other protons in the glucan molecule are listed in Table 2. From the area of the peaks corresponding to succinyl residues, we estimated that the cyclic β -1,2-glucans of *B. abortus* have on average two succinyl residues per glucan molecule.

^{13}C -NMR analysis of the periplasmic glucans revealed a peak cluster of C-1 resonance at 102.8 and 102.9 ppm (Fig. 5A). The presence of signals with a chemical shift above 102 ppm indicates β configuration of glucose. No signals at 92 to 96 ppm, characteristic of the C-1 resonances of reducing glucose residues, could be observed. These results confirm the previous observation that the glucan molecules of *B. abortus* are cyclic and all glucose residues are β linked (11). Resonances below 60 ppm indicate nonsugar substituents. In the ^{13}C -NMR spectra, two CH_2 signals of succinate at 30.3 and 30.6 ppm were present (31). These signals were missing in extracts after mild alkali treatment, confirming that the anionic character of *B. abortus* cyclic β -1,2-glucan is determined by the presence of succinyl residues (Fig. 5B). The signals at 74.2, 72.8, 63.7, 63.2, and 60.1 ppm (Fig. 5A) could be attributed to the chemical shift of other carbons depending on substitution at C-6 with succinyl residues, since these signals were also missing after mild alkali treatment. The resonances at 82 to 83 ppm were assigned to C-2 implicated in β -1,2-linkages (Table 2). The absence of a signal at 73 to 74 ppm indicates that all C-2 carbons are involved in glycosidic bonds (18). The spectra from this analysis did not reveal chemical-shift signals indicative of substitutions by other additional components. Furthermore, phosphoryl substituents were not detected on the cyclic β -1,2-glucans of *B. abortus* by ^{31}P -NMR spectroscopy (data not shown).

Identification, cloning, and nucleotide sequence of *B. abortus* cyclic β -1,2-glucan succinyltransferase. A gene with high homology to the *opgC* gene of *Rhodobacter sphaeroides* was identified in the *B. abortus* 2308 genome (12, 14). The gene is highly conserved in *B. melitensis* and *B. suis* (19, 34). A DNA fragment of approximately 1.8 kb was cloned in the T-easy vector and sequenced. Analysis of the sequence revealed that the 1.8-kb DNA fragment contained an open reading frame of 1,182 bp coding for a protein of 393 amino acids which is 39% identical to OpgC of *R. sphaeroides* and 56% identical to a hypothetical cyclic glucan succinyltransferase of *S. meliloti*. Accordingly, the gene was named *cgm* for cyclic glucan modifier. Two inverted repeats of 8 bp (AGAGCCGCCCTTGCGGC TCT) followed by TTTTTTATG are found 157 nucleotides downstream from the stop codon of *cgm*. This could be an intrinsic transcription terminator, as no sizeable open reading frame starts before this point. Complete genome sequencing of the *B. abortus*, *B. melitensis*, and *B. suis* genomes revealed that the *cgs* (BAB1_0108), *cgt* (BAB1_1017), and *cgm* (BAB1_1718) genes—involved in synthesis, transport, and modification of cyclic β -1,2-glucans, respectively—are not contiguous in *B. abortus* chromosome 1 (12).

The *opgC* gene of *R. sphaeroides* and the *mdoC* gene of *E. coli* encode glucan succinyltransferases (14, 29). Cgm, OpgC, and MdoC have similar sizes (393, 399, and 385 amino acids, respectively) and exhibit stretches of hydrophobic amino acids over their entire lengths. For MdoC (29), 10 transmembrane segments (TMS) have been predicted by the TopPredII program (13). The same program predicted 11 TMS and 9 TMS for OpgC and Cgm, respectively. Nevertheless, no amino acid similarities between MdoC and Cgm/OpgC were found.

Cyclic β -1,2-glucans of the *cgm* mutant are not succinylated. Interstitial mutagenesis of *cgm* in *B. abortus* 2308 and S19 was carried out by deleting an MscI fragment (675 bp) of the intragenic region of *cgm* and replacing it with a kanamycin resistance cassette. The resulting Km^r mutants, named *B. abortus* Cgm08 and Cgm19, were mapped by PCR.

Cyclic β -1,2-glucans recovered from the wild type and *cgm* mutants were analyzed by TLC. As is shown in Fig. 6, lanes 2 and 5, *cgm* mutants accumulated no anionic cyclic β -1,2-glucan. As expected, when plasmid pBB4*cgm* was introduced, the mutant strains recovered the synthesis of the anionic fraction (Fig. 6, lanes 3 and 6). Alkali-treated wild-type cyclic β -1,2-glucans (Fig. 3A, lane 2) exhibit the same migration pattern as *cgm* mutants (Fig. 6, lanes 2 and 5), thus confirming that in the *cgm* mutants, cyclic glucans are not substituted with anionic residues.

Cyclic β -1,2-glucans from *B. abortus* wild-type and *cgm* mutant strains were further analyzed by Bio-Gel P4 columns and DEAE-Sephadex chromatography. Cyclic β -1,2-glucans recovered from a *cgm* mutant exhibited the same elution profile in Bio-Gel P4 and on a DEAE-Sephadex column (Fig. 1B and C and 2B and C, respectively) as alkali-treated wild type cyclic β -1,2-glucans, showing their neutral character. When the plasmid pBB4*cgm* harboring *B. abortus* *cgm* was introduced into the mutant strain, the elution profiles of the glycan by gel filtration and anion-exchange chromatography were the same as that of the glycan from the wild type (Fig. 1D and 2D).

^1H -NMR and ^{13}C -NMR analyses revealed that the cyclic β -1,2-glucan from the *cgm* mutant lacked succinyl residues,

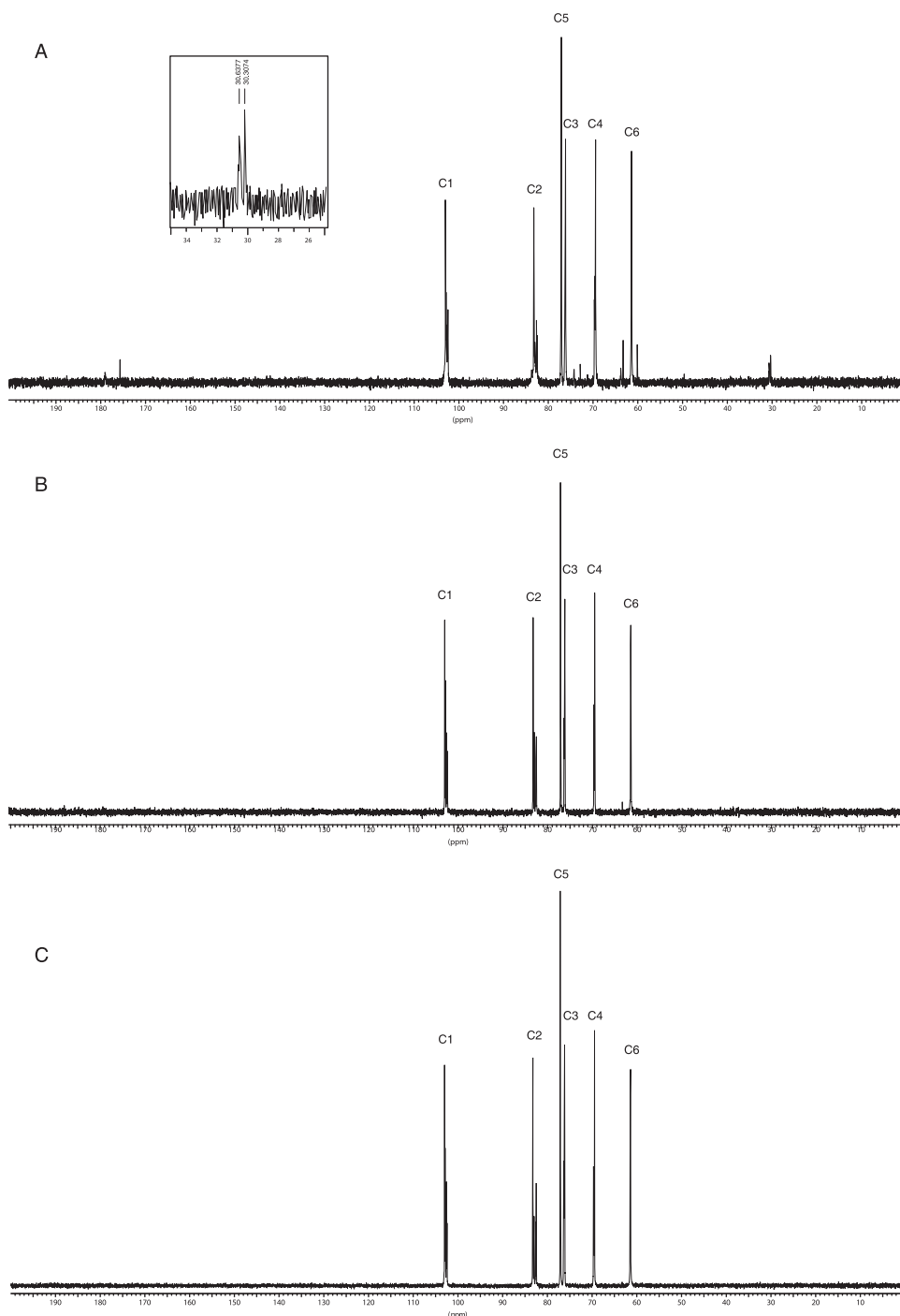


FIG. 5. ^{13}C -NMR spectra of purified cyclic β -1,2-glucans from *B. abortus*. (A) *B. abortus* S19. (B) *B. abortus* S19 treated with alkali. (C) *B. abortus* Cgm19. Chemical shifts are expressed relative to external 1,4-dioxane (δ 67.4 ppm).

since the signals between 2.4 and 2.6 ppm and between 4.25 and 4.35 ppm were not present in the ^1H -NMR spectra (Fig. 4C). The signals at 30.3 and 30.6 ppm corresponding to succinyl substituents were also absent in the ^{13}C -NMR spectra (Fig. 5C). These results confirmed that *B. abortus* *cgm* is a cyclic β -1,2-glucan succinyltransferase responsible for succinylation of cyclic β -1,2-glucan.

Moreover, as no other differences between the cyclic β -1,2-

glucan wild-type and mutant spectra existed, the mutation on the *cgm* cyclic β -1,2-glucan succinyltransferase gene did not induce other substitutions by any additional components or any other change of the glucan structure.

Succinylation of *B. abortus* cyclic β -1,2-glucan is not required for virulence in mice and intracellular multiplication but is essential for survival under hypo-osmotic conditions. The presence of a substituted form of the cyclic β -1,2-glucans

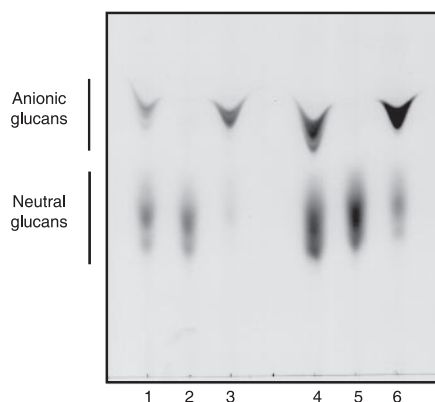


FIG. 6. TLC of cyclic β -1,2-glucans formed by *B. abortus* strains. Total cellular glucans of *B. abortus* strains were extracted and subjected to TLC as described in Materials and Methods. *B. abortus* S19 (lanes 1 to 3): lane 1, wild type; lane 2, Cgm19; lane 3, Cgm19(pBB4cgm). *B. abortus* 2308 (lanes 4 to 6): lane 4, wild-type; lane 5, Cgm08; lane 6, Cgm08(pBB4cgm).

may have significant implications for the functions of these molecules in the *Brucella* infection process. To evaluate the importance of cyclic-glucan substitution in the infectivity of *B. abortus*, intracellular survival and experimental infection in mouse assays were carried out. No significant differences were observed between the wild-type and *cgm* mutant strains on intracellular multiplication in HeLa or J774 macrophage cell lines and on clearance from the spleens of infected mice (data not shown). These results indicated that *B. abortus* cyclic β -1,2-glucan succinylation is not required for virulence. It has been widely reported that alphaproteobacterial periplasmic glucans play a central role during hypo-osmotic adaptation. In order to define the role of *B. abortus* cyclic β -1,2-glucan succinylation on hypo-osmotic adaptation, the wild-type and *cgm* mutant strains were subjected to hypo-osmotic stress conditions. As is shown in Table 3, when the *B. abortus* 2308 wild-type strain was subjected to decreasing osmotic pressure (from 391 to 109 mmol/kg), the total amount of cyclic β -1,2-glucans recovered from the cells did not change. However, the anionic/neutral-glucan ratio was significantly increased from 1.08 at normal osmolarity (391mmol/kg) to 2.42 at low osmolarity (109 mmol/kg). The addition of other salts or of a sugar, such as sucrose, restored the anionic/neutral-glucan ratio to the level obtained with LB-NaCl 170 mM. This effect was independent of the chemical nature of the solute, and thus, the increase observed in anionic glucans at 109 mmol/kg appears to be dependent solely on the decreased osmolarity of the medium. These results show that in *B. abortus*, as was suggested for *E. coli* (7), the anionic cyclic β -1,2-glucans may play a role in hypo-osmotic adaptation, being more necessary at low than at high osmolarity. Accordingly, it might be speculated that the *cgm* mutant, which is unable to produce anionic glucans, must be less efficient at surviving and or multiplying at low osmotic pressure. As is shown in Fig. 7A, the *B. abortus* *cgm* mutant did not multiply in liquid medium with an osmotic pressure of 109 mmol/kg (LB with no addition of NaCl). Growth was restored by complementation with a plasmid containing *cgm* (Fig. 7A) or by restoring the osmotic pressure to 391 mmol/kg with the

addition of 170 mM of NaCl (Fig. 7A). We concluded that modification of cyclic β -1,2-glucans with succinyl residues through the action of Cgm is required for wild-type growth at low osmotic pressure. The same effect was observed on solid LB medium (Fig. 7B), and growth was restored upon the addition of different osmolites (Fig. 7C).

DISCUSSION

Large amounts of OPG are found in the periplasmic space of *Proteobacteria*. Depending on the species considered, OPG could be modified to various extents by a variety of substituents; however, the role attributed to these substituents remains unclear. We have discovered in our laboratory that *B. abortus* produces cyclic β -1,2-glucans, which are required for full expression of virulence (2, 9, 36). As far as we know, nothing has been reported about the presence and role of nonglycosidic substituents modifying cyclic β -1,2-glucans of *Brucella* spp.

In this report, we demonstrated that *Brucella* spp. accumulated cyclic β -1,2-anionic substituted glucans. Physical evidence of the presence of anionic cyclic β -1,2-glucans with novel succinyl substituents was provided by several methods, including NMR spectroscopy, TLC, and DEAE-Sephadex anion-exchange chromatography. Our $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ analyses confirmed the previous observation that *B. abortus* glucans are cyclic, with all the glucose residues β linked (11); moreover, we identified in both profiles signals corresponding to succinyl residues. We estimated that *B. abortus* cyclic β -1,2-glucans are modified on average with two succinyl residues per molecule of glucan at the C-6 positions of the glucose residues (Fig. 8). The signals corresponding to succinyl residues were no longer present after mild alkali treatment, indicating that they are O-ester linked. Moreover, since after this treatment all glucan molecules became neutral and no other signals were detected in the $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra, it can be concluded that the anionic character is solely determined by the presence of succinyl residues. On the other hand, mild acidic treatment did not alter TLC migration of the anionic

TABLE 3. Osmolarity affects the substitution of *B. abortus* cyclic β -1,2-glucan^a

Solute (mM)	Osmolarity (mmol/kg) ^b	Total glucans ^c	Neutral glucans ^d	Anionic glucans ^e	Anionic/neutral glucans
0 NaCl	109	19.05	29.28	70.72	2.42
170 NaCl	391	20.23	48.13	51.87	1.08
170 KCl	387	19.41	48.21	51.79	1.07
110 K ₂ SO ₄	338	19.20	48.32	51.68	1.07
340 sucrose	441	20.15	49.01	50.99	1.04

^a *B. abortus* strain 2308 was cultured in 100 ml of LB or LB with the addition of 170 mM NaCl, 170 mM KCl, 110 mM K₂SO₄, and 340 mM sucrose at 37°C for 48 h. The cell pellets were extracted with 70% ethanol, and the neutral- and anionic-cyclic- β -1,2-glucan contents were determined after Bio-Gel P4 chromatography and DEAE-Sephadex chromatography as described in Materials and Methods.

^b The osmolarity of the medium was measured on a Vapro-Vapor Pressure Osmometer (model 5520; WESCOR).

^c The total cellular-glucan content is expressed as milligrams of glucose equivalent per liter of culture.

^d The neutral-glucan content is expressed as a percentage of the total cellular-glucan content.

^e The anionic-glucan content is expressed as a percentage of the total cellular-glucan content.

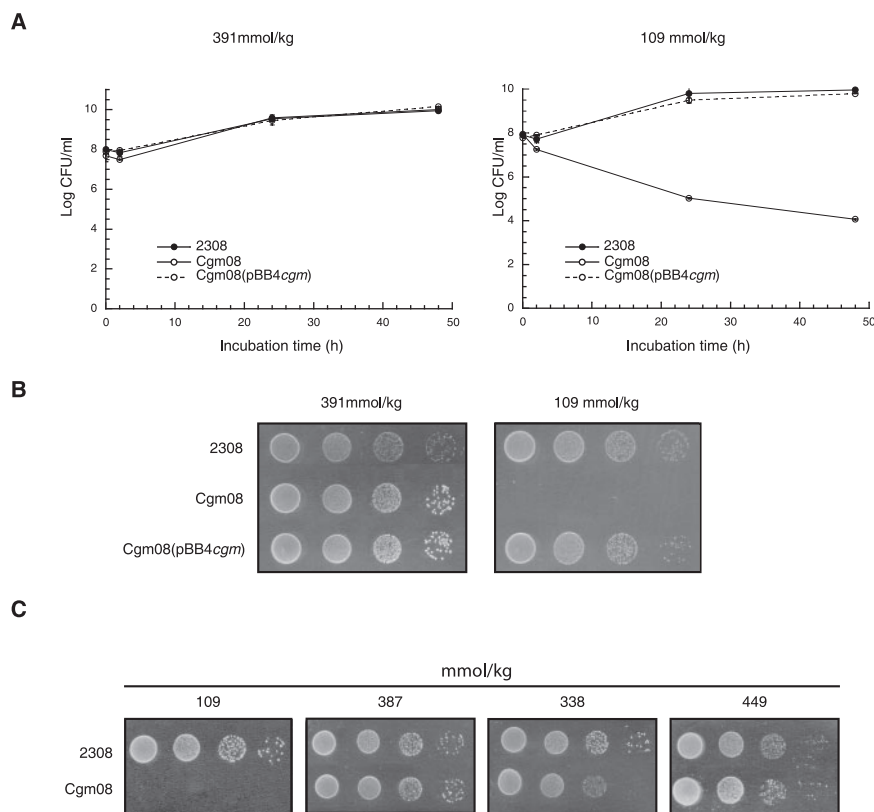


FIG. 7. Effects of osmolarity on growth of different strains of *B. abortus*. (A) Studies in liquid media. *B. abortus* 2308 wild type, Cgm08, and the complemented strain Cgm08(pBB4cgm) were grown in LB-NaCl or LB as described in Materials and Methods. The number of viable bacteria at each time point was determined by making serial dilutions in PBS and plating aliquots on brucella broth plates. (B and C) Studies on solid media. For studies on solid media, 10 μ l of the dilutions were spotted on LB agar or LB agar with the addition of 170 mM NaCl (B) or on LB agar or LB agar with one of the following additions: 170 mM KCl, 110 mM K_2SO_4 , or 340 mM sucrose (C). The plates were incubated at 37°C for 5 days before being read.

cyclic glucan, indicating that no phosphodiester-linked substituents were present. Moreover, the use of ^{31}P -NMR spectroscopy did not reveal the presence of phosphoryl substituents. These assays rule out the presence of phosphoglycerol, as was demonstrated in *S. meliloti*, *A. tumefaciens*, and *E. coli* glucans (7, 27), and zwitterionic residues, like phosphocholine or phosphoethanolamine, present in *B. japonicum* and *E. coli* glucans, respectively (27, 35).

In this report, we have also identified, sequenced, and disrupted a *B. abortus* open reading frame that codes for a cyclic-

glucan succinyltransferase that exhibits high homology to the cyclic-glucan succinyltransferase of *R. sphaeroides* (39% identity) (14). The gene was named *B. abortus cgm* for cyclic glucan modifier. Two *cgm* mutants (Cgm08 and Cgm19) were obtained from *B. abortus* 2308 and S19 strains, respectively. Analysis of cyclic glucans synthesized by *cgm* mutants has shown that they were identical to the cyclic glucans synthesized by parental strains, except for the fact that they completely lacked the succinyl modification. These results confirmed that *B. abortus cgm* encodes a cyclic β -1,2-glucan succinyltransferase responsible for succinylation of β -1,2-cyclic glucan and that succinyl residues are the only substituents of *Brucella* cyclic glucan. The mutants accumulate neutral cyclic β -1,2-glucans with a degree of polymerization identical to that of the neutral fraction synthesized by the parental wild-type strain, indicating that cyclization and substitution are independent processes.

Although other mutants of glucan substituent transferases have been described for *E. coli*, *S. meliloti*, and *R. sphaeroides*, those mutants contain increased levels of other substituents, such as phosphoethanolamine, succinyl, and acetyl substituents, respectively (6, 14, 22). *B. abortus cgm* mutants accumulated neutral cyclic β -1,2-glucans; the lack of succinyl residues did not promote any increment of other nonglycosidic substituents. Therefore, *B. abortus* is a good model that might shed light on the role that this substituent plays in the adaptation to

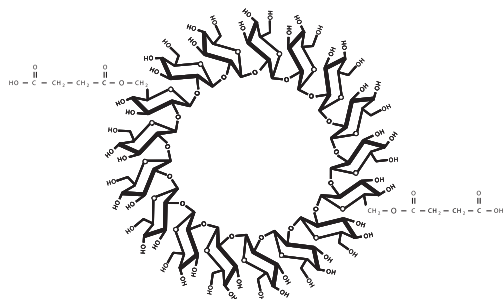


FIG. 8. Schematic representation of the structure of the succinyl-substituted cyclic β -1,2-glucan (degree of polymerization, 17) synthesized by *B. abortus*.

osmotic stress and in pathogenicity. In this report, we demonstrated that succinyl modification of *B. abortus* glucans was essential for survival under hypo-osmotic conditions; moreover, the amounts of succinylated cellular glucans changed according to the osmolarity of the growth medium, being significantly increased at low osmotic pressure. On the other hand, contrary to what happens with *B. abortus* *cgs* and *cgt* mutants (9, 36), no effect on virulence and intracellular multiplication was apparent with the *cgm* mutant, indicating that succinyl substituents on the cyclic β -1,2-glucan are not required for full expression of virulence or intracellular multiplication and also suggesting that during intracellular trafficking and host colonization, brucellae do not encounter hypo-osmotic-stress conditions. Moreover, the presence of an alkali-sensitive anionic substituent in different *Brucella* species and the fact that the *cgm* genes are identical in *B. abortus*, *B. suis*, and *B. melitensis* suggest that substitution of cyclic glucan is not a determining factor for host specificity.

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