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Molecular Cloning and Characterization of *cgt*, the *Brucella abortus* Cyclic β-1,2-Glucan Transporter Gene, and Its Role in Virulence

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The animal pathogen *Brucella abortus* contains a gene *cgt*, which complemented *Sinorhizobium meliloti* nodule development (*ndvA*) and *Agrobacterium tumefaciens* chromosomal virulence (*chvA*) mutants. Complemented strains recovered the presence of anionic cyclic β-1,2-glucan, motility, tumor induction in *A. tumefaciens*, and nodule occupancy in *S. meliloti*, all traits strictly associated with the presence of cyclic β-1,2-glucan in the periplasm. Nucleotide sequencing revealed that *B. abortus cgt* contains a 1,797-bp open reading frame coding for a predicted membrane protein of 599 amino acids (65.9 kDa) that is 58.5 and 59.9% identical to *S. meliloti* NdvA and *A. tumefaciens* ChvA, respectively. Additionally, *B. abortus cgt*, like *S. meliloti ndvA* and *A. tumefaciens chvA* possesses ATP-binding motifs and the ABC signature domain features of a typical ABC transporter. Characterization of Cgt was carried out by the construction of null mutants in *B. abortus* 2308 and S19 backgrounds. Both mutants do not transport cyclic β-1,2-glucan to the periplasm, as shown by the absence of anionic cyclic glucan, and they display reduced virulence in mice and defective intracellular multiplication in HeLa cells. These results suggest that cyclic β-1,2-glucan must be transported into the periplasmatic space to exert its action as a virulence factor.

Brucella spp. are facultative intracellular gram-negative bacteria pathogenic for a variety of mammalian species including humans. They cause a chronic infectious disease known as brucellosis, a major zoonosis in several countries (12). Six *Brucella* spp. with different host specificities have been described previously (19, 46). *Brucella abortus* is the etiological agent of bovine brucellosis and can also infect humans, causing undulant fever.

Brucella, *Agrobacterium*, and *Rhizobium* belong, according to 16S rRNA sequences, to the α-2 subgroup of the *Proteobacteria* (28). Complete-genome sequencing reveals similarities in transport, metabolic capabilities, and genome structure with these soil- and plant-associated bacteria. Extensive gene synteny between chromosome 1 and the genome of the plant symbiont *Mesorhizobium loti* emphasizes the similarity between this animal pathogen with plant pathogens and symbionts. A limited repertoire of genes homologous to known bacterial virulence factors were identified (13, 31, 44). Several studies revealed common themes between animal and plant pathogens and endosymbionts. For example the *Brucella* two-component regulatory system BvrS-BvrR (39) is highly similar to the two-component regulatory systems ChvG-ChvI of *Agrobacterium tumefaciens* (9) and ExoS-ChvI of *Sinorhizobium meliloti* (10). These two-component regulatory systems are equivalent to the *Salmonella* PhoP-PhoQ (40) and the *Bordetella bronchiseptica* BvgA-BvgS systems (42). These two-component sensory systems are involved in controlling virulence or, in the case of *Rhizobium*, nodule invasion; in *B. abortus bvrS-bvrR*, mutants also display reduced invasiveness and virulence (32, 39).

The *Brucella virB* operon was recently identified (29, 38); it is highly homologous to the *A. tumefaciens virB* operon. The *B. abortus virB10* mutant lost the ability to multiply in HeLa cells and was not recovered from the spleens of infected BALB/c mice (38). The same results were obtained with a *Brucella suis virB10* mutant (29), thus demonstrating that in *Brucella*, as in *Agrobacterium*, the *virB* operon is involved in virulence.

A highly conserved *B. abortus* homologue of the *S. meliloti bacA* gene, which encodes a putative cytoplasmic membrane transport protein required for symbiosis, was identified (26). The *B. abortus bacA* mutant shows decreased survival in macrophages and reduced virulence in BALB/c mice (26).

Brucella, like *Agrobacterium* and *Rhizobium*, produces cyclic β-1,2-glucans (45). *chvB* in *A. tumefaciens* and *ndvB* in *S. meliloti* are the genes coding for the cyclic β-1,2-glucan synthetase (*cgs*) (50). The biosynthesis of cyclic β-1,2-glucan proceeds in *Brucella* by the same mechanism as in *Rhizobium* and *Agrobacterium* (7). Cgs acts as an intermediate during the synthesis of the cyclic β-1,2-glucan (21). Cyclic glucan is required for effective nodule invasion and symbiotic nitrogen fixation in *S. meliloti* and for crown gall tumor induction in *A. tumefaciens* (4). *Agrobacterium* cyclic β-1,2-glucan mutants have several altered cell surface properties, including loss of motility due to a defective assembly of flagella and increased sensitivity to certain antibiotics and detergents (4). *B. abortus cgs* codes for the cyclic β-1,2-glucan synthetase (21). *B. abortus cgs* mutants showed reduced survival in BALB/c mouse spleen tissues and impeded intracellular multiplication, indicating that, as in *Rhizobium* and *Agrobacterium*, cyclic glucan is required for effective host interaction (6). Moreover, *Agrobacterium* or *Rhizobium* cyclic β-1,2-glucan mutants can be complemented by the

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Brucella cgs gene, indicating that their functions are highly conserved (6, 21).

The presence of cyclic β-1,2-glucan in the periplasmic space is also required for effective *S. meliloti* nodule invasion (17, 41) and *A. tumefaciens* crown gall tumor induction (15, 22). Two chromosomal homologue loci, *ndvA* in *S. meliloti* and *chvA* in *A. tumefaciens*, code for cyclic β-1,2-glucan transport genes (*cgt*). *ndvA* and *chvA* genes are interchangeable, and mutations in one gene can be complemented by the other, indicating that their functions are highly conserved (15). *S. meliloti* NdvA and *A. tumefaciens* ChvA are membrane proteins with homology to bacterial ATP-binding transporters of the ABC transporter superfamily (22, 41). ABC transporters utilize the energy of ATP hydrolysis to transport a wide variety of molecules across cellular membranes. These molecular pumps are found in all phyla and form a large protein family (37). The amino acid sequence of the ATPase domain contains the characteristic Walker A and B motifs involved in ATP binding (48). However, the intervening sequence between these two motifs is usually longer than in other ATP-binding enzymes, and the unique signature motif LSGGQ absent in other ATPases is always present.

Once cyclic β-1,2-glucan is transported into the periplasm, a variety of nonglycosidic substituents (glycerol phosphate, succinate, and/or methyl malonate) are added, leading to the accumulation of periplasmic anionic cyclic β-1,2-glucans (5, 8, 22). *S. meliloti ndvA* and *A. tumefaciens chvA* mutants have less than 15% of the wild-type levels of anionic periplasmic cyclic β-1,2-glucan (41). The arrangement of *ndvA* and *ndvB* in the *S. meliloti* chromosome is similar to that of *chvA* and *chvB* in *A. tumefaciens*, with the two loci being adjacent to each other and transcribed in a convergent fashion (15, 17).

In this report we describe the isolation of the *B. abortus* cyclic β-1,2-glucan transporter gene (*cgt*). *B. abortus cgt* complemented the phenotypes associated with *A. tumefaciens chvA* and *S. meliloti ndvA* mutations. On the other hand, *B. abortus cgt* mutants do not accumulate anionic cyclic β-1,2-glucan and have reduced virulence in mice and defective intracellular multiplication in HeLa and J774 cells. These results suggest that, as in *Agrobacterium* and *Rhizobium*, *B. abortus* cyclic β-1,2-glucan must be transported into the periplasm to exert its action.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *A. tumefaciens*, *Escherichia coli*, and *S. meliloti* strains were grown on tryptone-yeast extract (50), Luria broth (35), and yeast extract-mannitol medium (23), respectively. *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: carbenicillin, 100 μg/ml; kanamycin, 50 μg/ml; ampicillin, 100 μg/ml. Merodiploid strains for complementation analysis were obtained by tri- and biparental mating as described previously (14). Motility assays were carried out in GYM medium (16) (0.35% agar) for 3 days at 28°C. The absence of smooth-to-rough dissociation was checked by testing the sensitivity of smooth-specific phages (Tb, Wb, and Iz) (1)

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

Cloning and DNA sequencing. The putative *B. abortus cgt* gene was amplified from *B. abortus* S2308 genomic DNA by PCR with primers *cgt*-fw (5′-CTCGC CCGCATCCACAATCT-3′) and *cgt*-rev (5′-CCGCACCCAAGCCATTTTTC-3′), designed according to the sequence of a *B. abortus* gene highly homologue to *A. tumefaciens chvA* (complete genome of *B. abortus* strain 2308) (unpublished data).

The amplified products were ligated to pBluescript II KS(+) (Stratagene, La Jolla, Calif.) and digested with EcoRV by following manufacturer's instructions. The resulting plasmid, containing a 2.3-kb fragment, was named pBK*cgt*. DNA sequencing was carried out by the dideoxy method with an automated model 373 DNA sequencer (Perkin-Elmer Applied Biosystems Division, Foster City, Calif.) according to the manufacturer's instructions.

TLC of cyclic β-1,2-glucan. Cells from cultures grown for 48 h were harvested by centrifugation at 10,000 × *g* for 10 min. Cyclic β-1,2-glucans were extracted from cell pellets with ethanol (70% ethanol, 1 h at 37°C). Ethanolic extracts were centrifuged in an Eppendorf centrifuge, and supernatants were dried in a speed-vac centrifuge. Extracted glucans were dissolved in 70% ethanol and submitted to thin-layer chromatography (TLC) as described previously (7). TLC plates were developed by spraying with 5% sulfuric acid in ethanol and heating for 5 min at 120°C.

Nodulation and virulence test. Alfalfa seeds were surface sterilized with concentrated sulfuric acid for 30 s and washed several times with sterile distilled water until total removal of the acid. Seeds were germinated on wet filter paper in petri dishes. Two-day-old seedlings were planted in autoclaved modified Leonard jars filled with Jensen's N-free solution (47). Seedlings were dipped into 2-day-old cultures immediately before planting. After 4 weeks, plants were removed and strains were isolated from nodules as described previously (20). Virulence assays were carried out on *Kalanchoe* leaves as previously described (18).

Construction of *B. abortus* β-1,2-glucan transporter mutant. An NcoI fragment (300 bp) of *cgt* was digested from pBK*cgt*. The deleted plasmid was blunt ended with T4 DNA polymerase (New England BioLabs) and ligated to a 1.3-kb HincII fragment containing a kanamycin resistance cassette (30). The recombinant plasmid was electroporated into *B. abortus* S19 and *B. abortus* S2308 strains, where it is incapable of autonomous replication. Transconjugants were selected in BB agar with kanamycin (50 μg/ml). Double crossover events were selected by streaking colonies in duplicate in BB agar with kanamycin (50 μg/ml) and BB agar with ampicillin (100 μg/ml). Kanamycin-resistant, ampicillin-sensitive clones were selected as possible double recombinants. Putative double recombinants were confirmed by colony PCR with primers 5′-TCAGCAATGTTTCG GTGG-3′ and 5′-GAACGGCGGCTGACGGTG-3′. Genetic complementation of *cgt* mutants was carried out with plasmids pBB4*cgt* and pBB4522, containing a wild-type copy of the *B. abortus* and *A. tumefaciens cgt* genes, respectively. Plasmids pBB4*cgt* and pBB4522 were introduced in the mutants by biparental mating with *E. coli* S17.1 as the donor strain (6, 11).

Cell culture and infection assay. HeLa cells were cultured at 37°C in 5% CO_2 atmosphere in minimal essential medium (Gibco, Paisley, Scotland), supplemented with 2 mM glutamine and 5% fetal calf serum without antibiotic (cell culture medium). Infection of cells with different *Brucella* strains was performed at a multiplicity of infection of 100 as previously described (32, 38). 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) supplemented with 5% fetal bovine serum (Gibco) and the multiplicity of infection was 50.

Virulence in mice. Nine-week-old female BALB/c mice were injected intraperitoneally with 0.2 ml of a suspension containing the appropriate number of viable *Brucella* organisms. Stock cultures were grown for 48 h on BB plates, and cells were suspended in sterile phosphate-buffered saline and adjusted turbidimetrically to the selected concentration. The exact bacterial concentration was calculated retrospectively by viable count. At selected postinfection times, groups of 5 mice were killed by cervical dislocation and spleens were homogenized in 1 ml of phosphate-buffered saline, serially diluted, and plated in triplicate on BB plates with the appropriate antibiotic (27).

Statistical analysis. Differences between the means of experimental groups were analyzed by using the Student *t* test. Differences were considered significant at P values of <0.05.

Nucleotide sequence accession number. The sequence of the *B. abortus* cyclic β-1,2-glucan transporter gene (*cgt*) has been assigned GenBank accession number AY237159.

RESULTS

Identification and cloning of *B. abortus* ABC transporter gene. A *B. abortus* 1,797-bp open reading frame highly similar to *A. tumefaciens chvA* was identified by genome sequencing of

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Phenotype or genotype[a]	Reference or source
Strains		
E. coli K-12 DH5αF′IQ	F′ϕ*80dlaczΔM15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_K^+ m_K^+) *phoA supF44* λ$^-$ *thi-1 gyrA96 relA1*/F′ *proAB*$^+$ LacI^{q}ZΔ*M15 zzf*:: Tn*5*(Km^r)	49
S. meliloti		
102 F34	Wild type	14
LI1	*ndvA*, deficient in the secretion and modification of cyclic β-1,2-glucan, Km^r	41
LI1(PBB4*cgt*)	*ndvA* with plasmid PBB4*cgt*; Km^r Amp^r	This study
A. tumefaciens		
A348	Wild type, Vir$^+$, pTiA6NC	18
A1011	Vir$^-$, Km^r, Tn*5* chromosomal in *chvB* region	15
ME104	Vir$^-$, Cb^r, Tn*3*::HoHo1 in *chvA* region	15, 22
ME104(pBB2*cgt*)	Vir$^+$, Cb^r, Km^r, ME104(pBB2*cgt*)	This study
ME104(pCD522)	Vir$^+$, Cb^r, Km^r, ME104(pCD522)	This study
B. abortus		
2308	Virulent, field isolated; wild type, Nal^r, erythritol resistant	36
Cgs08	*B. abortus* 2308 *cgs* mutant, Tn*3*-HoHo1 chromosome; Amp^r	6
Cgt08	*B. abortus* 2308 *cgt* mutant, *cgt*::Km^r	This study
Cgt08(pBB4*cgt*)	*B. abortus* 2308 *cgt* mutant with plasmid PBB4*cgt*, Km^r Amp^r	This study
Cgt08(pBB4522)	*B. abortus* 2308 *cgt* mutant with plasmid pBB4522, Km^r Amp^r	This study
S19	Vaccine strain, Nal^r, erythritol sensitive, naturally occuring derivate of *B. abortus* 2308	36
Cgs19	*B. abortus* S19 *cgs* mutant, Tn*3*-HoHo1 chromosome, Amp^r	21
Cgt19	*B. abortus* S19 *cgt* mutant, *cgt*::Km^r	This study
Cgt19(pBB4*cgt*)	*B. abortus* S19 *cgt* mutant with plasmid PBB4*cgt*, Km^r Amp^r	This study
Cgt19(pBB4522)	*B. abortus* S19 *cgt* mutant with plasmid pBB4522, Km^r Amp^r	This study
Plasmids		
pBBR1MCS-4	Broad-host-range cloning vector (Amp^r)	25
pBBR1MCS-2	Broad-host-range cloning vector (Km^r)	25
pBB4*cgt*	pBBR1MCS-4 containing *B. abortus cgt* gene, Amp^r	This study
pBB2*cgt*	pBBR1MCS-2 containing *B. abortus cgt* gene, Km^r	This study
pBB4522	pBBR1MCS-4 containing *A. tumefaciens* SalI *chvA* gene, Amp^r	This study
pCD522	pVK102 *A. tumefaciens chvA* Km^r	15
pBK*cgt*	pbluescript KS II(+) containing *B. abortus cgt* gene, Amp^r	This study

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

B. abortus strain 2308 (unpublished data). The gene is highly conserved in *Brucella melitensis* and *B. suis* (13, 31). A set of two primers (5′-CTCGCCCGCATCCACAATCT-3′ and 5′-CCGCACCCAAGCCATTTTTC-3′) was used for PCR amplification of the complete *B. abortus* ABC transporter gene. Amplified products were cloned in pBluescript II KS(+) and sequenced as described in Materials and Methods. Analysis of the sequence revealed that the gene codes for a predicted protein with 599 amino acid residues that is 58.5 and 59.9% identical to *S. meliloti* NdvA and *A. tumefaciens* ChvA, respectively (Fig. 1). Accordingly, the gene was named *B. abortus cgt*, for cyclic glucan transporter.

The putative *B. abortus* Cgt has all the conserved features of a typical ABC transporter, such as the Walker site A (GXXGXGKS/T), the Walker site B (hhhhD), (where h is a hydrophobic amino acid residue), and the ABC signature (LSGGERQR) (Fig. 1). Kyte-Doolittle analysis predicts that *Brucella* Cgt may be a membrane protein with 6 membrane-spanning segments very similar at the N-terminal region to those of ChvA and NdvA. Sequence analysis of the regions upstream and downstream of the *B. abortus cgt* gene revealed no significant homology to the *B. abortus cgs* gene, which was surprising since, in *A. tumefaciens* and *S. meliloti*, the two loci *chvA*/*chvB* and *ndvA*/*ndvB*, respectively, are adjacent to each other. Complete genome sequencing of the *B. abortus*, *B. melitensis*, and *B. suis* genomes revealed that the *cgs* and *cgt* genes are both located in chromosome 1 but separated by 857 kb, thus suggesting that *cgs* and *cgt* were either acquired independently or have suffered a severe rearrangement in the *Brucella* genome.

Functional characterization of *B. abortus cgt*. *S. meliloti ndvA* and *A. tumefaciens chvA* mutants were used as heterologous backgrounds to study function and expression of *B. abortus cgt*. Two plasmids, pBB4*cgt* and pBB2*cgt* (see Materials and Methods and Table 2), were introduced by mating in *S. meliloti* LI1 *ndvA* (41) and *A. tumefaciens* ME104 *chvA* mutants (15, 22), respectively.

As shown in Table 2 and Fig. 2A, *B. abortus cgt* restored the formation of normal nodules by the *S. meliloti* LI1 *ndvA* mutant as judged by the induction of pink cylindrical nodules and the healthy aspect of plants inoculated with the *S. meliloti*

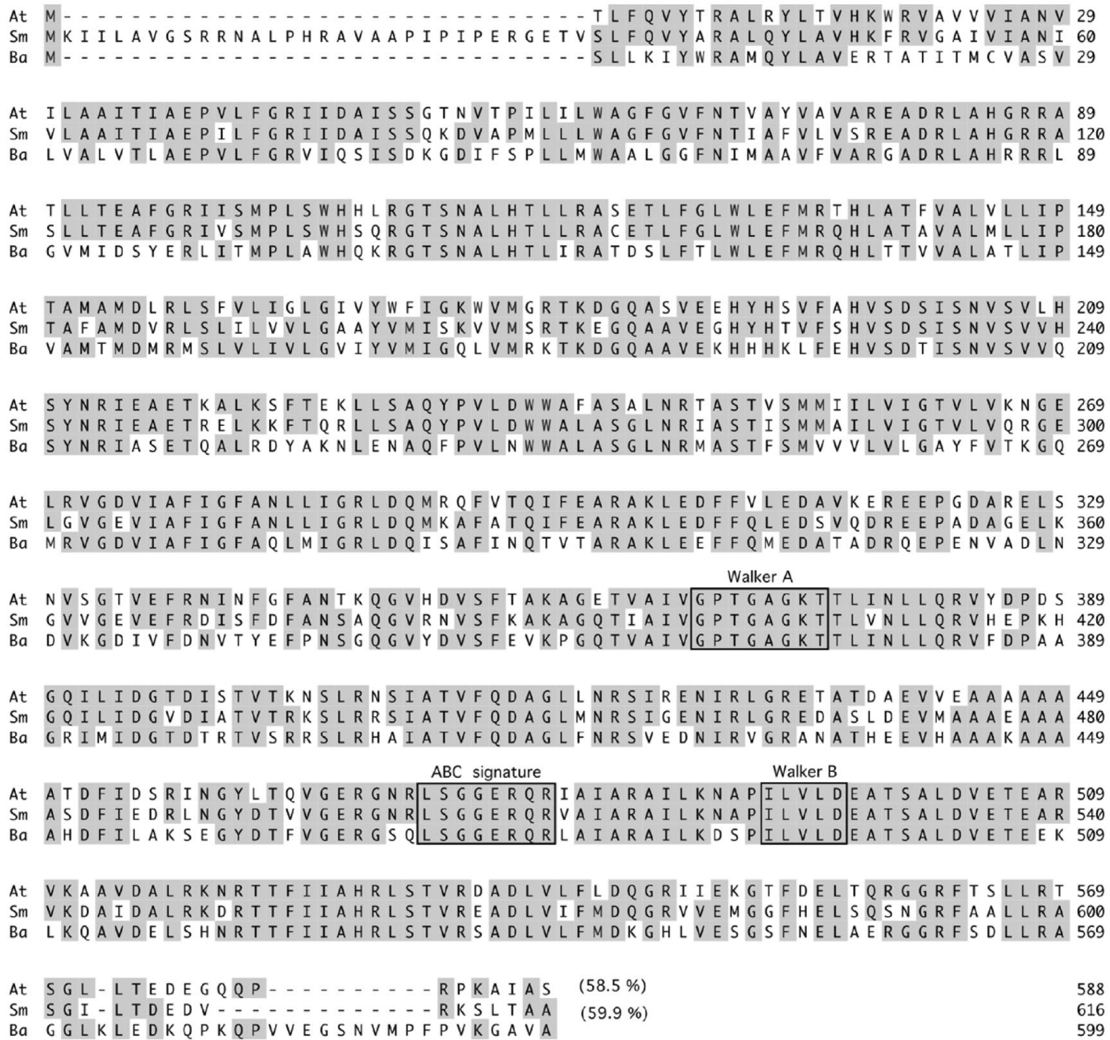


FIG. 1. Comparison of the *B. abortus* (Ba) Cgt cyclic β-1,2-glucan transporter protein with the *A. tumefaciens* (At) ChvA and *S. meliloti* (Sm) NdvA proteins. Conserved amino acids are indicated by gray boxes. Three typical ABC transporter motifs, Walker A and B and the SGG(Q) ABC signature, are indicated by boxes. The percentages of identity with the amino acid sequence of the ABC transporter protein (Cgt) from *B. abortus* are indicated in parentheses. The alignment was performed with the MegAlign program.

LI1(pBB4*cgt*) strain after 1 month. In contrast, nodules formed by the *S. meliloti* LI1 *ndvA* mutant were round and white and plants remained blanched and stunted, indicating lack of nitrogen fixation. Moreover, wild-type *S. meliloti* 102F34 and LI1(pBB4*cgt*) strains were recovered from 4-week-old nodules, whereas no bacteria were recovered from nodules induced by the LI1 mutant.

On the other hand, the resulting recombinant strain *A. tumefaciens* ME104(pBB2*cgt*) recovered the capacity to form tumors on *Kalanchoe* leaves at wild-type levels (Table 2 and Fig. 2B). In contrast, tumors formed by the *A. tumefaciens* ME104 *chvA* mutant were smaller than those formed by the wild-type strain, indicating reduced virulence. No tumor was formed by the *A. tumefaciens* A1011 *chvB* mutant (Fig. 2B). These results demonstrated that *B. abortus cgt* restores the normal plant interaction of *S. meliloti ndvA* and *A. tumefaciens chvA* mutants.

A variety of pleiotropic phenotypes linked to the synthesis and secretion of cyclic β-1,2-glucan were described previously (4) *A. tumefaciens* ME104 *chvA* and *S. meliloti* LI1 *ndvA* mutants are nonmotile due to defective flagellum assembly (4). Plasmids pBB2*cgt* and pBB4*cgt* restored to wild-type level the motility of *chvA* and *ndvA* mutants, respectively, thus indicating that *B. abortus* Cgt restored the correct assembly of flagella in these backgrounds.

Cyclic β-1,2-glucan transport. It has been described that once cyclic β-1,2-glucans are transported into the periplasm, they are replaced by nonglycosidic anionic substituents (4, 5). To study whether *B. abortus cgt* restores in *A. tumefaciens* and *S. meliloti* the transport of cyclic β-1,2-glucan into the periplasmic space, cyclic β-1,2-glucans were extracted from strains *A. tumefaciens* ME104(pBB2*cgt*) and *S. meliloti* LI1(pBB4*cgt*) and the presence of anionic glucans was analyzed by TLC as described in Materials and Methods. As shown in Fig. 3A and B and Table 2, the *B. abortus cgt* gene restored the accumulation of anionic glucan to wild-type levels in both mutants, thus

TABLE 2. Complementation of *Sinorhizobium* and *Agrobacterium* mutants

Strain	Motility[a]	Virulence[b]	Nitrogen fixation[c]	Anionic glucan[d]
S. meliloti				
F34 (wild type)	++	ND	++	++
LI1 (ndvA)	−	ND	−	−
LI1(pBB4*cgt*)	++	ND	++	++
A. tumefaciens				
A348 (wild type)	++	++	ND	++
ME104 (chvA)	+	+	ND	+
ME104(pBB2*cgt*)	++	++	ND	++

[a] Motility on 0.35% agar medium was studied as described in Materials and Methods. ++, motile; +, decreased motility; −, nonmotile.

[b] Virulence was determined on *Kalanchoe* leaves as described previously (18). ++, wild-type tumor size; +, smaller tumor size (Fig. 2B); ND, not determined.

[c] Nitrogen fixation was assessed visually by plant color, nodulation, production of leghaemoglobin in nodules, and eventual death of plants as a result of chlorosis. −, yellowish plant color, round white nodules; ++, green plant color, pink cylindrical nodules.

[d] The presence of anionic glucan was determined by glucan extraction and characterization by TLC (Fig. 3). ++, accumulation of anionic glucans in the wild-type strain; +, less accumulation of anionic glucans than the wild-type strain; −, no accumulation of anionic glucans.

indicating that Cgt is indeed a cyclic β-1,2-glucan transporter. As shown in Fig. 3B, the *A. tumefaciens chvA* mutant has strongly reduced the amount of anionic glucans as well as the degree of polymerization of neutral glucans. Both effects where reverted to the wild-type phenotype by pBB2*cgt* and pCD522 plasmids.

Characterization of *B. abortus cgt* mutants. *B. abortus cgt* null mutant strains, *B. abortus* 2308 *cgt*::Km and *B. abortus* S19 *cgt*::Km, were obtained as described in Materials and Methods. The absence of smooth-to-rough dissociation of the mutants was demonstrated by studying sensitivity to three different phages that are known to lyse smooth strains (phages Tb, Wb, and Iq) and resistance to one phage that lyses rough strains (phage Rc) (data not shown).

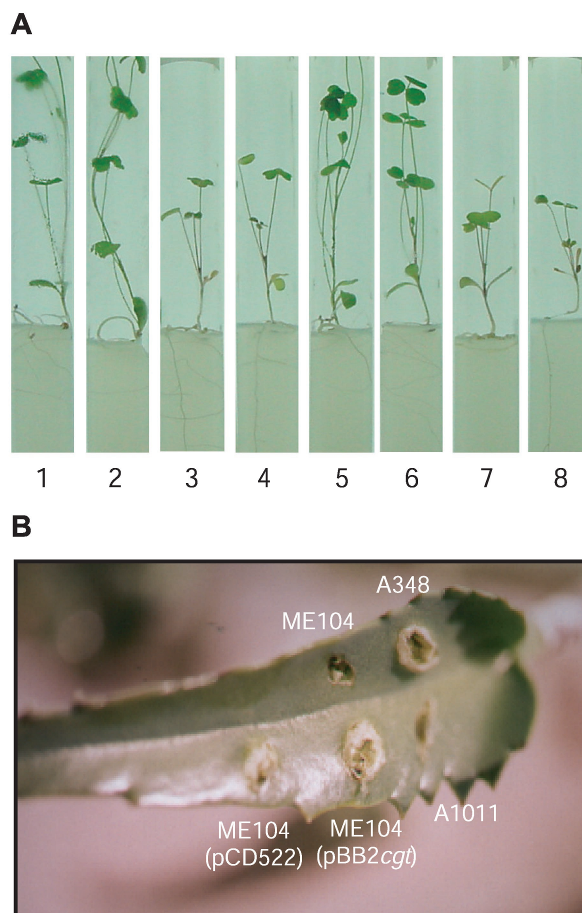


FIG. 2. Virulence and nodulation complementation assays. (A) Alfalfa plants inoculated with different strains of *S. meliloti*. (1 and 2) F34; (3 and 4) LI1; (5 and 6) LI1 (pBB4*cgt*); (7 and 8) noninoculated control. (B) Kalanchoe leaf inoculated with different strains of *A. tumefaciens.* The virulence and nodulation assays were carried out as described in Materials and Methods. Photos were taken 4 weeks postinoculation in both assays.

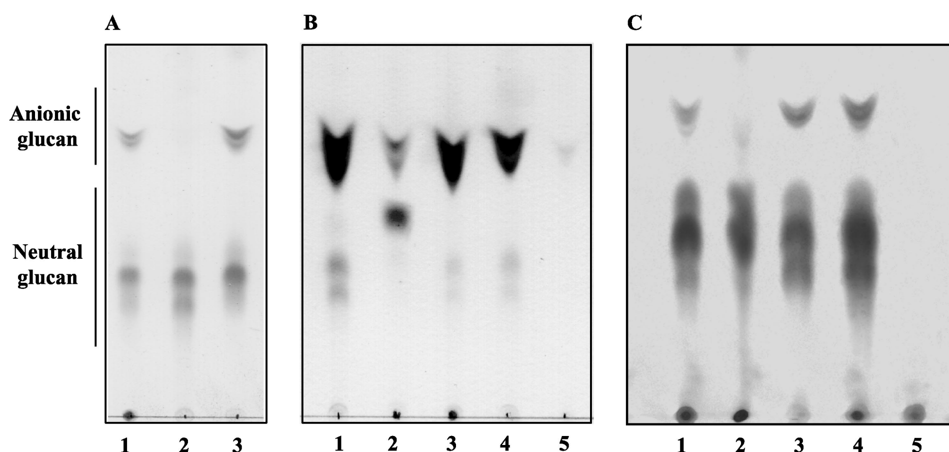
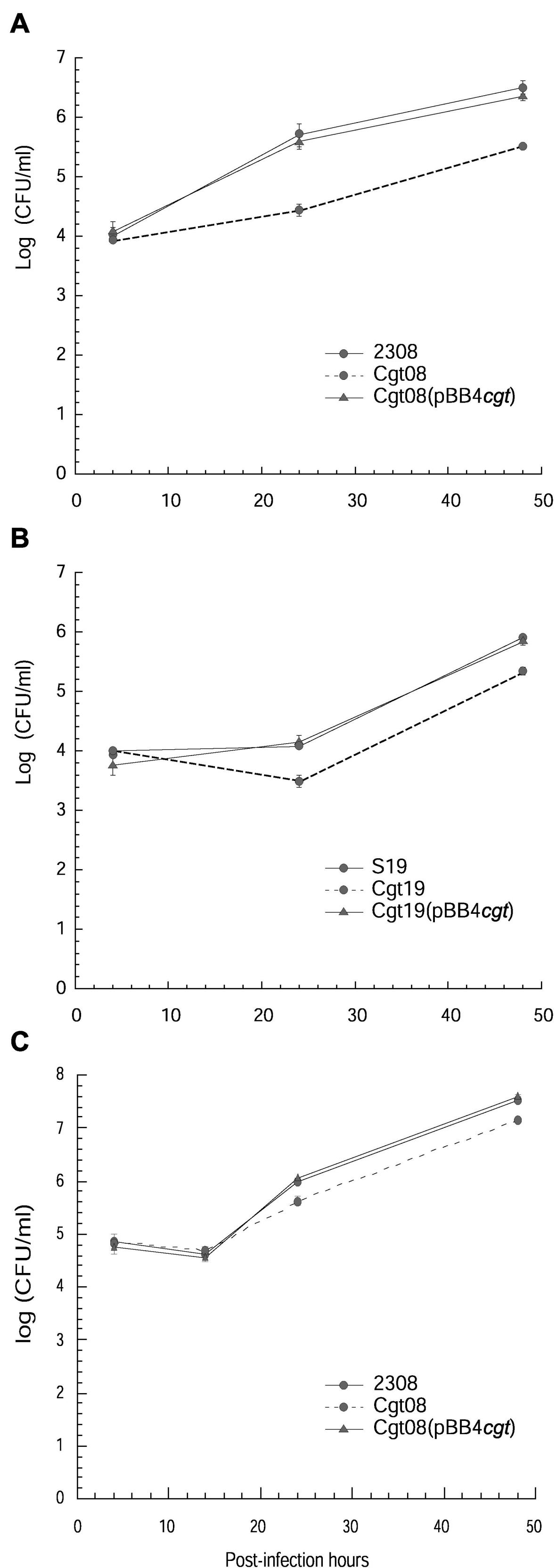


FIG. 3. TLC of cyclic β-1,2-glucans formed by *S. meliloti*, *A. tumefaciens*, and *B. abortus* strains. Total cellular glucans of *Sinorhizobium*, *Agrobacterium*, and *B. abortus* strains were extracted and subjected to TLC as described in Materials and Methods. (A) *S. meliloti*. Lane 1, wild-type 102F34; lane 2, *ndvA* LI1; lane 3, LI1(pBB4*cgt*). (B) *A. tumefaciens*. Lane 1, wild-type A348; lane 2, ME104 mutant; lane 3, ME104(pBB2*cgt*); lane 4, ME104(pCD522); lane 5, *chvB* mutant. (C) *B. abortus*. Lane 1, wild-type 2308; lane 2, Cgt08 mutant; lane 3, Cgt08(pBB4*cgt*); lane 4, Cgt08(pBB4522); lane 5, Cgs08 mutant.



Glucan extraction and characterization by TLC. Localization of cyclic β-1,2-glucan in the periplasm was studied indirectly by TLC determination of the presence of anionic glucans. As shown in Fig. 3C, *B. abortus* 2308 *cgt*::Km does not accumulate anionic glucans; the same result was obtained with *B. abortus* S19 *cgt*::Km (data not shown). It is shown that plasmid pBB4*cgt*, containing the *B. abortus cgt*, and plasmid pBB4522, containing *A. tumefaciens chvA*, restored the presence of anionic glucans to the wild-type level. These results demonstrate that *cgt* is the *B. abortus* cyclic glucan transporter gene and that the phenotype can also be complemented by the *A. tumefaciens* gene, thus *cgt* and *chvA* are fully interchangeable.

Intracellular multiplication of *B. abortus cgt* mutants. The importance of *B. abortus cgt* in invasion and intracellular survival was evaluated in HeLa and J774 cells as described in Materials and Methods. In HeLa cells, as shown in Fig. 4A and B, the number of intracellular bacteria recovered 4 h postinfection displayed no significant difference between the wild-type and *cgt* mutants, thus indicating that *cgt* does not have any apparent role during invasion. However, both *B. abortus* 2308 *cgt*::Km and *B. abortus* S19 *cgt*::Km mutants showed reduce intracellular multiplication at 24 and 48 h postinfection. J774 cells were infected with 2308 and the 2308 *cgt* mutant (Fig. 4C). Both strains invade the cells to the same extent and were equally recovered at 14 h postinfection. However, intracellular multiplication at 24 and 48 h were significantly reduced. Intracellular multiplication was restored to wild-type levels after complementation of the mutants with plasmid pBB4*cgt*. The intracellular multiplication of *cgt* mutants is similar to that described for *cgs* mutants (6), thus suggesting that cyclic glucan must be transported into the periplasm to exert its action.

Persistence of the *B. abortus cgt* mutants in mice. To assess the possible role of cyclic β-1,2-glucan transporter in the virulence of *B. abortus*, experimental infections in mice were carried out as described in Materials and Methods. Groups of 5 mice were injected intraperitoneally with 10^4 CFU of *B. abortus* wild type and *cgt* mutants. *B. abortus* 2308 and S19 *cgs* mutants were also used for comparison. At different postinoculation times, mice were sacrificed, spleens were weighed, and the numbers of CFU/spleen were determined. As shown in Fig. 5A, no significant difference was observed at 4 weeks postinfection between the wild-type 2308 strain and the corresponding *cgt* and *cgs* mutants. However, at 8 weeks postinfection, the numbers of CFU recovered from the spleens of mice infected with *B. abortus* 2308 *cgt* and *B. abortus* 2308 *cgs* mutants were 1.3 log and 1.5 log lower than those of the wild-type parental strain, respectively. Plasmid pBB4*cgt* restored spleen recovery of *cgt* mutants to the wild-type level, thus suggesting

FIG. 4. Intracellular replication of different strains of *B. abortus*. Monolayers of HeLa (A and B) and J774 (C) cells were inoculated with 10^7 and 5.10^6 CFU of bacteria, respectively. After 1 h of incubation at 37°C, cells were washed as described in Materials and Methods and streptomycin and gentamicin were added. Numbers of CFU were determined at the indicated times. Values are the means ± standard deviations of the results from one independent experiment out of two performed in duplicate; the *P* value is <0.05). Statistical analysis was performed with a *t* test.

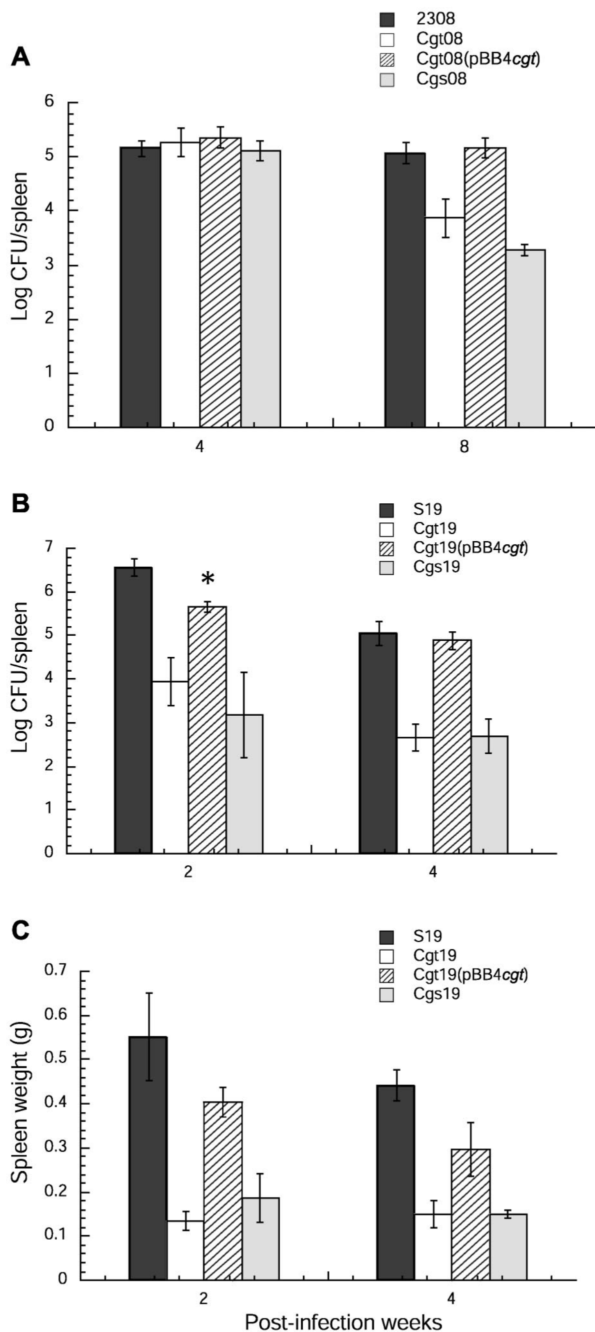


FIG. 5. Brucella persistence in spleens of mice inoculated with different strains of *B. abortus* 2308 and S19. Mice were inoculated intraperitoneally with 10^4 CFU of *B. abortus*. At the indicated times postinfection, 5 mice per group were killed and their spleens were removed. The numbers of CFU in spleen tissues were determined as indicated in Materials and Methods. (A) *B. abortus* 2308 strains: recovery of viable bacteria from spleens at 4 or 8 weeks postinfection. (B) *B. abortus* S19 strains: recovery of viable bacteria from spleens at 2 or 4 weeks postinfection. (C) *B. abortus* S19 strains: weight of spleens of infected mice at 2 or 4 weeks postinfection. Values are expressed as means ± standard errors of the means ($n = 5$); the P value is <0.05. Statistical analysis was performed with a t test. *, mice inoculated with the *B. abortus cgt* mutant complemented with plasmid pBB4522 showed values not statistically different from those observed with the same strain complemented with pBB4*cgt*.

that *cgt* is required for full virulence. The similar behavior observed between *cgt* and *cgs* mutants suggests that cyclic glucans must be located in the periplasm to exert its action.

A more drastic effect on virulence reduction was observed with a *cgt* mutant of the partially attenuated strain S19. As shown in Fig. 5B, 2 and 4 weeks after infection the numbers of CFU recovered from the spleens of mice infected with the *B. abortus* S19 *cgt* mutant were 2.6 log and 2.4 log lower than that of the wild-type parental S19 strain, respectively. Plasmid pBB4*cgt* restored spleen recovery of the *cgt* mutant to wild-type levels. A similar result was observed with plasmid pBB4522 harboring the *A. tumefaciens chvA* gene at 2 weeks postinfection (Fig. 5B). A significant reduction of spleen weight was also observed with the *B. abortus* S19 *cgt* mutant (Fig. 5C), thus suggesting a strong inhibition of the inflammatory response of strain S19. This effect was also abolished by plasmid pBB4*cgt*. These results were similar to those obtained with *cgs* mutants that do not produce cyclic β-1,2-glucan (6), thus supporting the hypothesis that cyclic β-1,2-glucan plays a role during *Brucella* host interaction and that to exert its action it must be exported into the periplasm.

DISCUSSION

The role of cyclic glucans in bacterium-host interaction was extensively studied in *Agrobacterium* and *Rhizobium.* Synthesis and transport mutants have some associated pleiotropic phenotypes, for example, defective flagellum assembly (4), defective plant attachment (33), decreased temperature stability of the virB10 protein (2), all traits that may affect bacterium-host interaction. It was also described that the accumulation of cyclic glucan changes with the osmolarity of the media (4), thus suggesting that cyclic glucan may be involved in sensing environmental changes, and thus, its localization in the periplasm is predictable. However, it must be remarked that although *B. abortus* cyclic glucan is located in the periplasmic space (7), the protein involved in its transport has not been characterized.

ABC transporters are a major class of cellular translocation machinery in all bacterial species (37, 43). In this study, we have identified, sequenced, and disrupted a *B. abortus* ABC transporter that exhibits high homology to the cyclic β-1,2-glucan transporters of *A. tumefaciens* ChvA (60% identity) (15) and *S. meliloti* NdvA (59% identity) (17). Accordingly, the gene was name *B. abortus cgt*, for cyclic glucan transporter.

Computer analysis of the predicted amino acid sequence revealed the presence of Walker and ABC signatures found in all ABC transporters in the C-terminal domain of *Brucella* Cgt.

Contrary to what happens in *S. meliloti* and *A. tumefaciens*, in which cyclic β-1,2-glucan transporter (*cgt*) and cyclic β-1,2-glucan synthase (*cgs*) are contiguous and convergently transcribed, in *Brucella*, *cgs* and *cgt* genes are separated by 857 kb. This suggests that *Brucella cgs* and *cgt* were either acquired independently or have suffered a severe genome rearrangement during evolution.

Cross-complementation studies revealed that this *B. abortus* ABC transporter gene restores cyclic β-1,2-glucan transport of *S. meliloti ndvA* and *A. tumefaciens chvA* mutants. Conversely, the *A. tumefaciens chvA* gene complemented glucan transport of the *B. abortus* ABC transporter mutant. Thus, we concluded

that this gene codes for the *Brucella* cyclic β-1,2-glucan transporter.

B. abortus cgt mutants accumulate nonsubstituted neutral forms of cellular cyclic β-1,2-glucan. The accumulation of nonsubstituted cyclic β-1,2-glucan may be due either to a defect of the enzymes or substrates required for the modifying reaction or to a defect in the transport of cyclic β-1,2-glucan to the periplasmic space. It was well established that cyclic β-1,2-glucan modifying reactions take place in the periplasmatic space of the bacteria (3–5). Cross-complementation of *Brucella cgt* mutants with the *A. tumefaciens* cyclic β-1,2-glucan transporter *chvA* gene demonstrated that the absence of anionic cyclic β-1,2-glucan in *B. abortus cgt* mutants is due to the absence of cyclic β-1,2-glucan transport into the periplasm.

A large number of genes with high homology to ABC transporters were identified in the genome of brucellae; however, this is the first report in which the role of a *B. abortus* ABC transporter was assigned. Recently, an ABC transporter homologue to *S. meliloti* ExsA was identified and characterized in *B. abortus. exsA* is critical for full *B. abortus* virulence (34); however, the substrate transported by ExsA was not identified. Mutants in the *Brucella* ATP-binding genes *bapA* and *bapB* (24) and in a *B. abortus* ABC transporter mapping in chromosome 1 have no effect on cyclic β-1,2-glucan transport (M. Roset, unpublished data), thus indicating the high specificity of ABC transporters.

In a previous work, it was described that the absence of cyclic β-1,2-glucan reduced the virulence of *B. abortus* in mice and impeded normal intracellular multiplication in HeLa cells (6). Cgs, the enzyme responsible for cyclic β-1,2-glucan synthesis, is a 300-kDa inner membrane protein. All the *cgs* mutants studied so far were obtained by transposon insertion; thus, the lack of cyclic glucan was always accompanied by the lack of the 300-kDa inner membrane protein. Accordingly, the observed phenotype may be due to the lack of cyclic glucan, the lack of the 300-kDa inner membrane protein, or the lack of both.

In this study, we have obtained a *B. abortus* cyclic β-1,2-glucan transport mutant. The mutant lacks the ABC-type transport protein Cgt but contains an intact Cgs and accumulates cytoplasmic nonsubstituted neutral cyclic β-1,2-glucan. Interestingly, *cgt* mutants have reduced virulence in mice and defective intracellular multiplication in HeLa and J774 cells, a phenotype identical to that described for *cgs* null mutants. These results suggest that the presence of cyclic glucan in the periplasmic space is required in *B. abortus* for appropriate host interaction and full expression of virulence.

Further work is required to determine whether cyclic β-1,2-glucan is an extracellular signal recognized by the host or whether the presence of the glucan in the periplasmic space stabilizes and/or promotes the correct folding of other membrane proteins required for successful bacterium-host interaction.

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