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Genetic Characterization of a Tn*5*-Disrupted Glycosyltransferase Gene Homolog in *Brucella abortus* and Its Effect on Lipopolysaccharide Composition and Virulence

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We constructed a rough mutant of *Brucella abortus* 2308 by transposon (Tn*5*) mutagenesis. Neither whole cells nor extracted lipopolysaccharide (LPS) from this mutant, designated RA1, reacted with a *Brucella* O-side-chain-specific monoclonal antibody (MAb), Bru-38, indicating the absence of O-side-chain synthesis. Compositional analyses of LPS from strain RA1 showed reduced levels of quinovosamine and mannose relative to the levels in the parental, wild-type strain, 2308. We isolated DNA flanking the Tn*5* insertion in strain RA1 by cloning a 25-kb *Xba*I genomic fragment into pGEM-3Z to create plasmid pJM6. Allelic exchange of genomic DNA in *B. abortus* 2308 mediated by electroporation of pJM6 produced kanamycin-resistant clones that were not reactive with MAb Bru-38. Southern blot analysis of genomic DNA from these rough clones revealed Tn*5* in a 25-kb *Xba*I genomic fragment. A homology search with the deduced amino acid sequence of the open reading frame disrupted by Tn*5* revealed limited homology with various glycosyltransferases. This *B. abortus* gene has been named *wboA*. Transformation of strain RA1 with a broad-host-range plasmid bearing the wild-type *B. abortus wboA* gene resulted in the restoration of O-side-chain synthesis and the smooth phenotype. *B. abortus* RA1 was attenuated for survival in mice. However, strain RA1 persisted in mice spleens for a longer time than the *B. abortus* vaccine strain RB51, but as expected, neither strain induced antibodies specific for the O side chain.

Members of genus *Brucella* are gram-negative coccobacilli and are facultative intracellular pathogens that can cause chronic zoonotic disease. There are six well-recognized *Brucella* species which show differences in their host specificities and pathogenicities (19, 45). *Brucella abortus* is the causative agent of cattle brucellosis, but along with *Brucella melitensis*, *Brucella suis*, and *Brucella canis*, it can also cause undulant fever in humans (13). Brucellae proliferate within macrophages of the host animal and thereby successfully avoid the bactericidal effects of phagocytes. Our knowledge about all the mechanisms *Brucella* employs for its intracellular survival is limited. Recent findings indicate that brucellae replicate in phagosomes by preventing the fusion between phagosomes and lysosomes (32). In addition, several other mechanisms are thought to be operative, among which are the actions of lipopolysaccharide (LPS) (including the O side chain [8, 11, 15, 34, 35]), the structure of core LPS (1), and various other *Brucella* components (6, 7, 10, 11, 14).

Brucella organisms exhibiting a smooth phenotype are generally more virulent than those with a rough phenotype (36), with the exceptions of *B. canis* and *Brucella ovis*, which are rough but virulent in their primary host. The smooth phenotype is due to the presence of a complete LPS in the outer cell membrane, which is composed of lipid A, a core oligosaccharide, and an O-side-chain polysaccharide. LPSs of rough *Brucella* strains do not contain O side chains. Although the fine structure of *Brucella* LPS has not been elucidated, it has been reported that the lipid A region is composed of 2-amino-2-deoxy-D-glucose, *n*-tetradecanoic acid, *n*-hexadecanoic acid, 3-hydroxytetradecanoic acid, and 3-hydroxyhexa-decanoic acid (12). If we assume that most of the mannose found in the LPS is in the O-side-chain subunits, the core appears to be primarily composed of glucose (28). The amounts of glucosamine and 2-keto-3-deoxy-D-*manno*-2-octulosonic acid (KDO) have not been determined in the core region of *B. abortus* (28). The O side chain of *B. abortus* is a linear homopolymer of α-1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl subunits usually averaging between 96 and 100 subunits in length (12). In comparison, these lengths appear to be much longer than those of the O side chains of *Escherichia coli* and *Salmonella typhimurium*, which average about 40 subunits per chain (17). However, since the *B. abortus* O-side-chain subunit is a monosaccharide while those of *Salmonella* are most often pentasaccharides (47), the molecular weights of the O side chains are probably similar. In *B. abortus*, smooth LPS has been implicated in counteracting several bactericidal activities of the

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
B. abortus		
2308	Wild-type, smooth strain	G. G. Schurig
RA1	Tn*5*-induced rough mutant of 2308	This study
RB51	Rif^r rough mutant of 2308	40
E. coli DH5α	*rfbD endA1 hsdR17 supE44 thi-1* F′	Promega
Plasmids		
pGEM-3Z	Amp^r *lacZ′*	Promega
pSUP2021	Amp^r, Tn*5* (Kan^r)	41
pUC4-K	Amp^r Kan^r	Pharmacia-Biotech
pJM6	Amp^r, 25-kb *Xba*I chromosomal fragment from strain RA1 ligated into pGEM-3Z	This study
pJM63	11.6-kb *Eco*RI insert from pJM6 ligated into pGEM-3Z	This study
pRW4424	2.1-kb insert from pJM63 ligated into pUC18	R. Warren
pRW4425	5.9-kb insert from pJM63 ligated into pUC18	R. Warren

phagocytes and demonstrated to be essential for intracellular survival (34, 35). Rough strains of *B. abortus*, such as vaccine strain RB51, exhibit loss of virulence and cannot replicate within macrophages (40). Despite such an important role for the O side chain in *Brucella*, our knowledge about its biosynthesis is limited. The present study was undertaken to identify the genes encoding the essential proteins or enzymes involved in the biosynthesis of the *B. abortus* O side chain. This report describes the isolation of a rough strain of *B. abortus* by Tn*5* mutagenesis and the determination of the disrupted gene, named *wboA*, as encoding a glycosyltransferase. In addition, the LPS composition and virulence of the mutant rough strain are presented.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in these experiments are listed in Table 1. *E. coli* and *S. typhimurium* strains were grown at 37°C in either Luria-Bertani broth (27), terrific broth (43), or SOB (18). All *Brucella* strains were grown at 37°C in tryptic soy broth. Bacterial strains containing plasmids were grown in media containing appropriate antibiotics (100 μg of ampicillin per ml or 25 μg of kanamycin or chloramphenicol per ml).

Enzymes and reagents. Restriction endonucleases, calf intestinal alkaline phosphatase, T4 DNA ligase, Bluo-Gal (halogenated indolyl-β-D-galactopyranoside), and agarose were purchased from Bethesda Research Laboratories (Gaithersburg, Md.). All other chemicals were obtained from Sigma Chemical Corporation (St. Louis, Mo.). Incert and NuSieve low-melting-point agarose were purchased from FMC Bioproducts (Rockland, Maine).

Serological tests. A colony immunoblot assay was performed as previously described with a rat monoclonal antibody (MAb), Bru-38, specific for the *Brucella* O-side-chain epitopes (38, 39). Western blotting was performed according to standard procedures (4). Along with the experimental mice serum samples, MAb Bru-38 was used in the Western blot analysis. The *B. abortus* tube agglutination test was performed according to the established procedure (2).

DNA procedures. The routine molecular biologic techniques performed in this study were based on the standard procedures outlined elsewhere (4, 26). All plasmids were isolated from the bacteria by an alkaline lysis procedure (22) unless otherwise stated. Electroporation was used to introduce plasmids into *Brucella* (25). All other plasmids were introduced into *E. coli* and *Salmonella* by the $CaCl_2$ procedure (18, 26).

Southern blotting. The capillary transfer method (42) was used with 20× SSC (3 M NaCl, 0.3 M sodium citrate [pH 7.0]) as the buffer medium to transfer the DNA from the gel to a Nytran membrane (Shleicher and Schuell Inc., Keene, N.H.). The DNA was UV-crosslinked to the membrane with a Stratalinker (Stratagene Cloning Systems, La Jolla, Calif.) and allowed to dry. Nonradioactive probes were prepared and hybridized with a Genius kit and by using the accompanying procedures from Boehringer Mannheim Biochemicals (Indianapolis, Ind.).

DNA sequence analysis. Nucleotide sequence of the DNA flanking the Tn*5* insertion was determined with an Applied Biosystems Inc. (ABI) model 373A automated DNA sequencer. Fluorescence-labeled dideoxy nucleotides were incorporated into DNA with Prism thermocycling kits (ABI). Primes for DNA sequencing were prepared with an ABI model 394 automated oligonucleotide synthesizer. Contigs were assembled and aligned with the Sequencer program (GeneCodes, Madison, Wis.). DNA and protein homologies were determined with BLAST (3) programs.

Complementation. Complementation of the *wboA* mutation was accomplished by cloning the gene along with flanking DNA into the broad-host-range plasmid pBBR1MCS (24). The DNA fragment that included *wboA* was cloned by PCR with a primer pair (5′ primer, GGA TGT CGA CCA GCC CTC CAC ATC AAT AGC; 3′ primer, TTG CGG ATC CTT TAC TCG TCC GTC TCT TAC). The underlined nucleotides represent added *Sal*I and *Bam*HI restriction sites, respectively. The first four nucleotides of each primer were added to aid in restriction digestion of the PCR product. PCR mixtures contained 100 ng of *B. abortus* DNA with Hot Tub polymerase (Amersham Corp., Arlington Heights, Ill.) according to the manufacturer's directions. The reactions were carried out for 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 30 s, and polymerization at 72°C for 1 min 30 s. The generated DNA fragment was purified with a Wizard PCR purification kit (Promega, Madison, Wis.) and digested with *Sal*I and *Bam*HI, followed by ligation with similarly cleaved pBBR1MCS. Several clones containing plasmids with the expected profile were chosen for DNA sequencing to confirm the construct. One of these plasmids with the expected DNA insert was designated pAB3. Electro-competent strain RA1 was prepared by pelleting bacteria grown overnight in YENB (0.75% yeast extract, 0.8% nutrient broth) medium and by resuspending the bacteria in one-half volume of ice-cold 10% glycerol. These cells were pelleted and resuspended in 1/10 volume of ice-cold 10% glycerol. Five microliters of a 100-ng/ml concentration of pAB3 was electroporated in *B. abortus* RA1 (2.5 kV, 600 Ω, 25 mF). After 2 h of growth in SOC (18), 50 μl of cells was plated on tryptic soy broth agar supplemented with 25 μg of chloramphenicol per ml.

LPS purification. Smooth and rough *Brucella* and *Yersinia enterocolitica* O:9 LPSs were purified by a modification of the procedure described by Moreno et al. (28), as described by Inzana et al. (20). Briefly, following extraction with NaCl, the collected cells were extracted with hot, 45% aqueous phenol for 15 min and the phases were separated by centrifugation at 8,000 × *g* for 30 min at 15°C. Crude LPS in the aqueous phase was precipitated by addition of methanol, suspended in distilled water, dialyzed against water, and lyophilized (28). The crude LPS was suspended in 0.01 M phosphate-buffered saline, pH 7.2, and incubated with RNase and DNase overnight at room temperature as described previously (28). Pronase was added, and the incubation continued for 2 h at room temperature. The insoluble material was washed with distilled water until the A_{260} and A_{280} were less than 0.05, suspended in distilled water (2.5 mg/ml) containing 2.5 μl of triethylamine per ml, and stirred at room temperature for 1 h. The mixture was collected by centrifugation, the supernatant was retained, and the precipitate was extracted with triethylamine two more times. The supernatants were pooled and lyophilized. If necessary, the LPS was extracted again with hot 45% aqueous phenol, and the aqueous phase was precipitated with cold methanol, suspended in distilled water, dialyzed, and lyophilized.

Smooth LPS was extracted from acetone-killed bacteria with hot, 45% aqueous phenol as described previously (5). The phenol phase was filtered through a no. 40 Whatman filter, the LPS was precipitated from the phenol phase with cold methanol reagent, and the precipitate was extracted with distilled water and concentrated (5). The crude LPS was reprecipitated with cold methanol, suspended in distilled water, and lyophilized. The smooth LPS was purified by enzyme digestion and hot phenol extraction as described previously (20), except that the aqueous phase was removed and the phenol phase was filtered as described above. The LPS was precipitated with cold methanol reagent and suspended in distilled water twice and lyophilized. The final methanol supernatant was measured at A_{260} and A_{280} to confirm the purity of the LPS.

LPS analysis. Following separation of extracted LPS in sodium dodecyl sulfate-polyacrylamide gel with a 15% separating gel containing 4 M urea, LPS

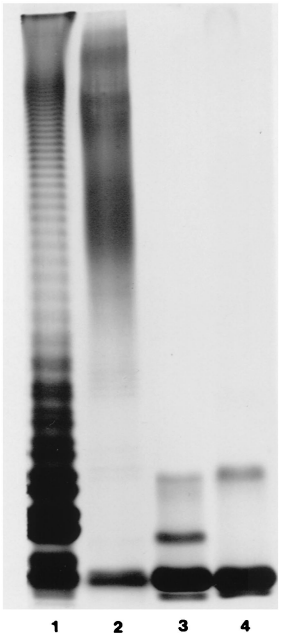

FIG. 1. Silver-stained LPS profiles of *B. abortus* and *Salmonella* strains. LPSs were extracted and separated by denaturing gel electrophoresis as described in Materials and Methods. Lane 1, *S. typhimurium*; lane 2, *B. abortus* 2308; lane 3, *B. abortus* RA1; lane 4, *B. abortus* RB51.

heterogeneity was visualized by silver staining (44). Fatty acids and alditol acetate or trimethylsilyl derivatives of the glycoses were determined by gas chromatography (GC) (21) and GC-mass spectrometry at the Complex Carbohydrate Research Center, The University of Georgia, Athens, Ga. The absence of the O side chain was also assessed by immunoblot analysis with MAb Bru-38, specific for the O side chain (39).

Mouse spleen clearance. Groups of five BALB/c mice each were inoculated intraperitoneally with 2×10^8 CFU of either strain RA1 or strain RB51. Mice were killed, and their spleens were cultured for the presence of *Brucella* by previously described methods (40). Sera were obtained by cardiac puncture and used in immunoblotting to analyze *B. abortus* strains and *Y. enterocolytica* O:9 LPS as described previously (40).

Nucleotide sequence accession number. The nucleotide sequence of the putative *wboA* gene of *B. abortus* has been submitted to GenBank; the accession number is AF107768.

RESULTS

Screening of *B. abortus* Tn*5* insertion mutants for the rough phenotype. A previously created Tn*5* insertion library of *B. abortus* (25) was screened by colony immunoblotting with MAb Bru-38. Over 2,200 clones of *B. abortus* Tn*5* mutants that did not react with MAb Bru-38 were initially isolated. The absence of the O side chain was further verified by demonstrating the uptake of crystal violet by the colonies (46) (data not shown). One of these rough Tn*5*-induced mutants, strain RA1, was chosen for further study.

LPS analysis. In order to assess the degree of the roughness of strain RA1, the LPS electrophoretic profile was examined (Fig. 1). The silver-stained gel showed no O side chain associated with strain RA1 or vaccine strain RB51 when each strain was compared with *B. abortus* 2308 (smooth) and *S. typhimurium* (smooth). A major low-molecular-mass band of the same size (~4.2 kDa) was present in the LPSs of strains 2308, RB51, and RA1, indicating that strains RA1 and RB51 have a complete core. An additional higher-molecular-mass band was present in strain RA1 and RB51 LPSs, and a band of intermediate size was present in strain RA1 LPS only. Immunoblot analysis with MAb Bru-38 did not reveal any reactivity (data not shown), suggesting that these bands may represent aggregates of core LPS without the O side chain. Compositional analysis indicated that LPSs of strains RB51 and RA1 contained similar amounts of KDO and glucose but that strain RA1 LPS contained substantially more galactose and mannose (Table 2). Whether the differences in these glycoses were responsible for the additional band in strain RA1 LPS could not be determined. *N*-Acetyl glucosamine was apparently present only in the lipid A moiety because it was not found in lipid-free oligosaccharide. Heptose was also not detected in the LPS (data not shown). The fatty acids identified in *Brucella* LPS included β-hydroxymyristic acid, palmitic acid, β-hydroxysteric acid, and a large proportion of 27-OH C_{28}, which has not been previously reported. The proportions of these fatty acids were similar in these strains, except there was approximately twice as much palmitic and β-hydroxysteric acids in the LPS of strain 2308 as in the LPS of strain RB51 or RA1.

TABLE 2. Glycose compositions of LPSs from *B. abortus* RB51, RA1, and 2308

Strain	Component[a] (μg/mg)				
	Glucose	Galactose	Mannose	KDO	OV[b]
RB51	32.6	0.9	4.9	114.6	
RA1	44.8	7.4	12.5	122.5	1.1
2308	22.2	2.9	22.3	56.2	31.2

[a] Heptose was not identified in the LPS, and amino sugars were not identified in the lipid-free oligosaccharide by colorimetric analysis.
[b] 2-Amino-2,6-dideoxy-D-glucose (quinovosamine).

DNA analysis. In order to identify the mutated gene(s) affecting O-side-chain synthesis, the Tn*5* element in strain RA1 was located by physical mapping of the chromosome by pulsed-field gel electrophoresis. Upon Southern blot analysis, the Tn*5* element was located within a 30-kb *Xba*I fragment (data not shown). Since *Xba*I does not cut the Tn*5* element (23), this restriction enzyme was used to digest the chromosome of *B. abortus* RA1 and to produce a fragment containing Tn*5* and the flanking chromosomal regions. This fragment was ligated into the *Xba*I site of pGEM3-Z to create pJM6. The actual size of this fragment was estimated to be 25.2 kb. Southern blot hybridization with both the Tn*5* element and *Brucella* chromosomal DNA as probes further confirmed that pJM6 contained both the Tn*5* element and the flanking genomic DNA from *B. abortus* RA1 (data not shown). Further analysis revealed that a 11.6-kb *Eco*RI fragment from the insert of pJM6 contained the Tn*5* element; cloning of this fragment in pGEM-3Z created pJM63 (Table 1).

Nucleotide sequence analysis. To facilitate sequencing, two *Eco*RI-*Pst*I fragments from pJM63 were cloned into pUC18 to create pRW4424 and pRW4425. Plasmids pRW4424 and pRW4425 contained a 2.1- and a 5.9-kb insert, respectively; these inserts represented the DNA sequences flanking the Tn*5* insertion and 0.7 kb of Tn*5* DNA. Nucleotide sequencing of these inserts was performed to obtain the DNA sequence flanking the Tn*5* insertion in strain RA1. Computer analysis of the nucleotide sequence revealed an open reading frame (ORF) capable of coding for a protein of 410 amino acids in length. Based on the deduced amino acid sequence, the encoded protein was 46.5 kDa in size and had an isoelectric point of 6.19. The ORF was preceded by a purine-rich ribosomal binding site. Two putative promoter sequences were identified upstream of the ribosomal binding site. The G+C content of the ORF as well as of the flanking regions was calculated to be 46.5%, which is lower than that typical of *Brucella* spp. (55 to

TABLE 3. Homologies of the putative *B. abortus* glycosyltransferase with other bacterial glycotransferases

Gene	Organism	Protein size (amino acids)	Putative enzyme	Homology[a]	Accession no. (GenBank)
rfbU	*Salmonella typhimurium*	353	Mannosyltransferase	25/43/104	141359
rfbW	*Synechocystis* sp.	365	Mannosyltransferase	19/39/212	1001656
wbpX	*Pseudomonas aeruginosa*	460	Glycosyltransferase[b]	24/37/186	3249545
mtfB	*Aquifex aeolicus*	374	Mannosyltransferase B	20/38/210	2983150
wbdP	*Escherichia coli*	404	Glycosyltransferase	18/37/249	3435176
wbpY	*Pseudomonas aeruginosa*	371	Glycosyltransferase[b]	22/38/169	3249551

[a] Homologies were determined with Gapped BLAST (3). Numbers shown are percentages of identity/percentages of similarity/numbers of amino acids in the region of homology.

[b] The functions of these enzymes have been demonstrated to transfer D-rhamnose by means of an α-1,2 linkage or an α-1,3 linkage (37).

58%) (19). A BLAST search (3) with the deduced amino acid sequence of the ORF revealed limited homology with various bacterial glycosyltransferases (Table 3). According to the newly suggested nomenclature, this *Brucella* gene has been named *wboA* (32a, 33).

Conversion of *B. abortus* 2308 from a smooth to a rough phenotype. *B. abortus* 2308 transformed with either pJM6 or pJM63 generated 25 kanamycin-resistant and ampicillin-sensitive clones which retained crystal violet and did not react with MAb Bru-38, indicating the loss of the O side chain. DNA hybridization with a Tn*5* probe confirmed that the rough phenotype was due to the replacement of the wild-type DNA with the DNA bearing the Tn*5* insertion (data not shown).

Complementation of strain RA1. The *wboA* gene along with the putative promoter sequences was amplified from *B. abortus* 2308 genomic DNA and cloned into pCR2.1 (Invitrogen). This fragment was subcloned into the *Sal*I and *Bam*HI sites of pBBR1MCS, a broad-host-range plasmid that can replicate in *Brucella* (24). The ensuing plasmid was designated pAB-3 and electroporated into *B. abortus* RA1. The resultant chloramphenicol-resistant colonies excluded crystal violet and reacted with MAb Bru-38 in an immunoblot analysis (Fig. 2). The plasmids from these colonies were isolated and shown to be pAB-3 based on restriction mapping and Southern blot analysis (data not shown). These results demonstrate the complementation of the interrupted *wboA* genomic allele with the intact gene on plasmid pAB-3.

Immune response and splenic clearance. BALB/c mice were injected with 1×10^8 to 2×10^8 CFU of either strain RA1 or strain RB51. The mice were killed at various intervals, and the bacterial CFU in their spleens were determined (Table 4). All mice inoculated with strain RA1 had over 10^3 CFU/spleen at 5 weeks postinfection, and three of five mice were still infected at 7 weeks postinoculation. In contrast, all mice inoculated with strain RB51 were *Brucella* free at 4 weeks postinfection. All *Brucella* isolates recovered from spleens were rough and did not contain O side chains as determined by immunoblot analysis (38). All sera from infected mice were negative in the tube agglutination test with smooth *Brucella* as the antigen and did not react with *B. abortus* 2308 or *Y. enterocolytica* O:9 in Western blot analysis (data not shown).

DISCUSSION

In this study, we isolated and characterized *B. abortus* RA1, a transposon-derived rough mutant of standard strain 2308. We further identified the gene disrupted by Tn*5* in strain RA1 as *wboA*, which encodes a putative glycosyltransferase, an enzyme which appears to be essential for the biosynthesis of the O side chain in *B. abortus*. LPS analysis of strains RA1 and RB51 indicated that both strains lack the O side chain. However, the reason for the presence of the intermediate LPS band in strain RA1 that is lacking in strain RB51 is not known (Fig. 1). One possibility is that the mutation in strain RA1 results in exposure of a glycose that can be glycosylated by glycosyltransferases that are not able to attach specific sugars in the parent or in strain RB51. The result may be a side branch in the core, generating a new glycoform. Evidence for this is the relatively high amount of galactose present in RA1, whereas only a trace amount of this glycose is present in RB51. Unfortunately, the nature of the mutation causing the RB51 phenotype is unknown. The neutral sugar content of strain 2308 LPS has been reported by Phillips et al. (30) and includes 50% glucose, 20% mannose, 0.5% KDO, 0.63% rhamnose, 0.9% galactose, and 20% unidentified peaks. The detailed structure of strain 2308 core oligosaccharide is not yet known. Although the contents of LPSs from rough mutants of strain 2308 have not been previously reported, our results were most similar to those of

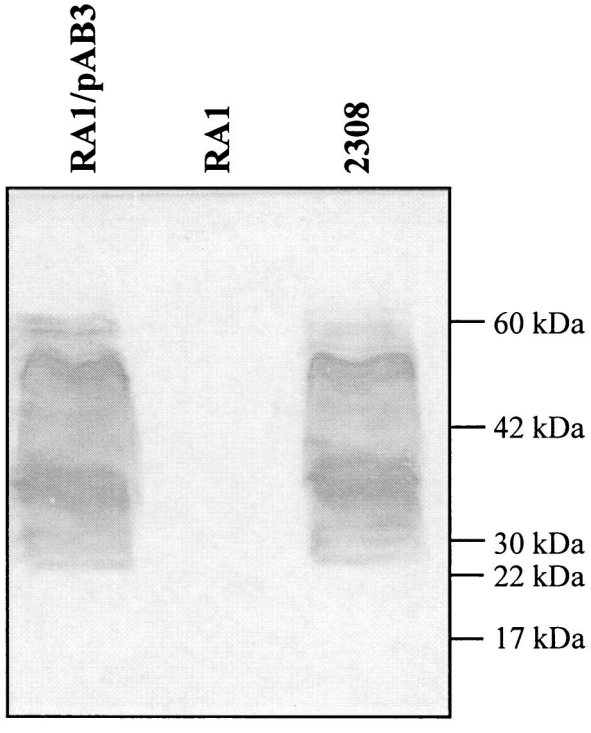

FIG. 2. Western blot reactions of the indicated *B. abortus* strains (RA1, RA1/pAB3 [RA1 complemented with a functional *B. abortus rfbU* gene], and 2308) with the O-side-chain-specific MAb Bru-38. Numbers at the right indicate approximate protein molecular masses.

TABLE 4. Clearance of *B. abortus* RA1 and RB51 from mice spleens[a]

No. of days postinfection	Mean $\log_{10}$ CFU/spleen (±SD) in strain:	
	RB51	RA1
7	4.08 (3.03)	4.58 (0.73)
14	3.19 (2.13)	4.94 (0.41)
21	2.25 (1.39)	4.31 (0.32)
28	—[b]	3.57 (0.41)
35	—	3.12 (0.28)
42	—	2.10 (0.98)
49	—	0.95 (0.31)

[a] Infection dose, 2×10^8 CFU (log = 8.3), given intraperitoneally to each of five mice per group.
[b] —, the infection had cleared.

Moreno et al. (29). The LPS from strain RB51 was greatly reduced in mannose content and void of quinovosamine, indicating the lack of the O side chain. The intermediate amount of mannose and small amount of quinovosamine in strain RA1 LPS suggest that the mutation may be leaky and that some of the O side chain may be present. However, the epitope reactive with MAb Bru-38 was lacking or below detection in RA1. In contrast, KDO was the predominant glycose in the rough LPSs of strains RA1 and RB51. The deep, rough nature of strain RB51 LPS was also indicated when this LPS was subjected to mild acid hydrolysis. More than 90% of strain RB51 LPS was lipid A, and less than 10% was oligosaccharide. In contrast, substantially more oligosaccharide was isolated from strain RA1 LPS after acid hydrolysis (data not shown). Since MAb Bru-38 did not react with either of these bands (data not shown), it is reasonable to conclude that the bands are most likely part of the core LPS.

DNA sequence analysis demonstrated that the Tn*5* element in strain RA1 disrupted the *wboA* gene, which encodes a protein with homology to several bacterial glycosyltransferases. However, until the substrate utilization by the putative *wboA* gene product and the actual reaction it catalyzes are confirmed, it is difficult to establish a functional relationship between this *B. abortus* protein and the other bacterial polysaccharide synthesis gene product(s). Based on the composition of the *B. abortus* O side chain (9, 12), it can be proposed that the putative WboA is a mannosyltransferase which forms α-1,2 linkages. Several other genes that code for proteins essential for the biosynthesis of the *Brucella* O side chain have recently been identified (1, 15, 16). In *B. melitensis*, a gene encoding mannosyltransferase which can form α-1,3 linkages has been reported (16); in the O side chain of *B. melitensis*, every fifth residue is linked by an α-1,3 linkage (9). As in most other bacterial species, all the *Brucella* LPS synthesis genes identified so far, including our *wboA*, also contain low G+C contents (44 to 49%) compared to that of whole genomic DNA (56 to 58%).

B. abortus rough vaccine strain RB51 is highly attenuated and was cleared from mice within 28 days of an intraperitoneal injection of 10^8 CFU. It is well documented in the literature that inoculation of mice with as few as 10^4 CFU of strain 2308 results in chronic infections and that 10^6 CFU of bacteria can be recovered from their spleens up to 12 weeks postinoculation (31). In contrast, only low numbers of strain RA1 were isolated at 6 weeks from the spleens of mice infected with 10^8 CFU, suggesting that strain RA1 is more virulent than strain RB51 but less virulent than strain 2308. Furthermore, sera from mice infected with strains RA1 and RB51 did not contain antibodies to the O side chain, as was indicated by the lack of reactivity by their sera with the smooth *Brucella* and *Y. enterocolytica* O:9 LPSs. These data indicate that the loss of the O side chain by *B. abortus* results in significant attenuation, as has been demonstrated by others (1, 40). A direct comparison of the degree of in vivo attenuation of strain RA1 with those of Tn*5*-induced mutants of the other putative polysaccharide synthesis genes (*rfbA*, *rfbD*, and *rfbV*) cannot be made, since our study was carried out with an infectious dose of 10^8 CFU and that by Allen et al. (1) was carried out with an infectious dose of 10^4 CFU. Nevertheless, intraperitoneal injection of a few mice with 10^4 CFU of strain RA1 did not lead to infection beyond 5 days postinoculation (data not shown).

With the *wboA* gene identified in this study, deletion mutants of rough phenotype have been constructed from *B. melitensis* and *B. suis* and have been designated VTRM1 and VTRS1, respectively (48). This indicates that the *wboA* gene product is essential for O-antigen synthesis in other *Brucella* spp. of smooth phenotype. It was also demonstrated that, like strain RA1, strains VTRM1 and VTRS1 were less virulent than their parent strains, indicating the essential role of smooth LPS in the virulence of these *Brucella* species (48).

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AUTHOR'S CORRECTION

Genetic Characterization of a Tn*5*-Disrupted Glycosyltransferase Gene Homolog in *Brucella abortus* and Its Effect on Lipopolysaccharide Composition and Virulence

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