Identification and Characterization of a *Brucella abortus* ATP-Binding Cassette Transporter Homolog to *Rhizobium meliloti* ExsA and Its Role in Virulence and Protection in Mice

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***Brucella abortus* is a facultative intracellular bacterial pathogen that causes abortion in domestic animals and undulant fever in humans. The mechanism of virulence of *Brucella* spp. is not fully understood yet. Furthermore, genes that allow *Brucella* to reach the intracellular niche and to interact with host cells need to be identified. Using the genomic survey sequence (GSS) approach, we identified the gene encoding an ATP-binding cassette (ABC) transporter of *B. abortus* strain S2308. The deduced amino acid sequence encoded by this gene exhibited 69 and 67% identity with the sequences of the ABC transporters encoded by the *exsA* genes of *Rhizobium meliloti* and *Mesorhizobium loti*, respectively. Additionally, *B. abortus* ExsA, like *R. meliloti* and *M. loti* ExsA, possesses ATP-binding motifs and the ABC signature domain features of a typical ABC transporter. Furthermore, ortholog group analysis placed *B. abortus* ExsA in ortholog group 6 of ABC transporters more likely to be involved in bacterial pathogenesis. In *R. meliloti*, ExsA is an exopolysaccharide transporter essential for alfalfa root nodule invasion and establishment of infection. To test the role of ExsA in *Brucella* pathogenesis, an *exsA* deletion mutant was constructed. Replacement of the wild-type *exsA* by recombination was demonstrated by Southern blot analysis of *Brucella* genomic DNA. Decreased survival in mice of the *Brucella* Δ *exsA* mutant compared to the survival of parental strain S2308 demonstrated that ExsA is critical for full bacterial virulence. Additionally, the *B. abortus* *exsA* deletion mutant was used as a live vaccine. Challenge experiments revealed that the *exsA* mutant strain induced superior protective immunity in BALB/c mice compared to the protective immunity induced by strain S19 or RB51.**

Brucellosis is a major zoonotic disease that causes abortion in domestic animals and undulant fever in humans. *Brucella* proliferates within macrophages of the host and thereby successfully bypasses the bactericidal effects of phagocytes (26, 34). Thus, *Brucella* virulence is associated with the capacity of the organisms to multiply inside the host cells. Therefore, cell-mediated immunity and subsequent activation of macrophages are essential for host clearance of infection (16). Once inside cells, *Brucella* prevents fusion of the phagosome with the lysosome by altering the intracellular traffic of the early phagosome vesicle (24). It has recently been demonstrated that brucellae replicate in a vesicle compartment containing reticuloendoplasmic markers reached after fusion between phagosomes and lysosomes is prevented (25). However, the genes that allow *Brucella* to invade and reach the appropriate intracellular replication niche remain to be identified.

To be successful in infection, a pathogenic intracellular bacterium requires four steps: adherence, invasion, establishment, and dissemination within the host (15). The interaction between host and pathogen also includes uptake and secretion of

substances, which are facilitated by a family of proteins termed transporters. ATP-binding cassette (ABC) transporters are some of the active transport systems that are common in bacteria and eukaryotic cells (14). ABC transporters use the free energy of ATP hydrolysis to pump substances across the membrane against a concentration gradient into or out of cells (27). These transporters can use a variety of substrates, such as amino acids, sugars, inorganic ions, polysaccharides, peptides, and proteins like toxins. In *Rhizobium meliloti*, a member of the alpha subgroup of the *Proteobacteria* closely related to *Brucella*, the *exsA* gene was identified as a gene that encodes an ABC transporter of the exopolysaccharide succinoglycan (EPS I) (4). EPS I is essential for the invasion of alfalfa root nodules by *R. meliloti*. Mutants of *R. meliloti* devoid of EPS I are unable to establish an effective symbiosis with alfalfa (22). Since EPS I from *R. meliloti* is a surface polysaccharide, like *Brucella* lipopolysaccharide (LPS), which is considered an important virulence factor involved in many host-pathogen interactions (11) and therefore pathogenesis, we decided to isolate and further characterize the *B. abortus* *exsA* gene and the product that it encodes.

In this study, we identified the gene encoding *B. abortus* ExsA and performed a nucleotide and deduced amino acid sequence analysis. The amino acid sequence analysis revealed a high degree of identity among the *B. abortus* ABC trans-

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TABLE 1. Bacterial strains and vectors used in this study

Strain or plasmid	Characteristics	Source
<i>E. coli</i>	<i>rfbD endA1 hsdR17 supE44 thi-1 F'</i>	Gibco BRL
<i>Brucella</i> strains		
<i>B. abortus</i> S2308	Wild-type, smooth, virulent	Laboratory stock
<i>B. abortus</i> S19	Vaccine strain, smooth	Laboratory stock
<i>B. abortus</i> RB51	Rif ^r , rough mutant of S2308	Laboratory stock
<i>B. abortus</i> Δ exsA	Kan ^r , Δ exsA mutant of S2308	This study
Plasmids		
pUC4K	ColE1, Amp ^r Kan ^r	Amersham Pharmacia
pUC12	ColE1, Amp ^r	Gibco BRL
pUC64	pUC12 plus 2.1 kb containing <i>B. abortus</i> <i>exsA</i> gene	This study
pGR64	pUC64 containing kanamycin resistance gene	This study

porter and *R. meliloti* and *Mesorhizobium loti* ExsA proteins. The sequence similarity and the presence of ABC transporter motifs in the *Brucella* ExsA sequence suggest a role for this molecule in the polysaccharide transport function critical in bacterial pathogenesis (11, 33). Furthermore, a mutant with a defined mutation in the *exsA* gene of *B. abortus* was obtained by gene replacement. The survival of the mutant was analyzed in the mouse model, and it was determined that ExsA is required for full virulence during *Brucella* infection. Additionally, the *B. abortus* Δ exsA mutant induced greater protective immunity in BALB/c mice than the commercially available strain S19 vaccine induced.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *B. abortus* virulent strain S2308 and *B. abortus* vaccine strains RB51 and S19 were obtained from G. Splitter (University of Wisconsin-Madison, Madison, Wis.). They were grown in brucella broth medium (Becton Dickinson, Sparks, Md.) for 3 days at 37°C. If necessary, the medium was supplemented with ampicillin or kanamycin at a concentration of 25 µg/ml and with 0.1% erythritol. *Escherichia coli* DH5α was cultured at 37°C in Luria-Bertani medium containing kanamycin (50 µg/ml) or ampicillin (100 µg/ml) as needed (28).

Isolation of *B. abortus* *exsA* and DNA and amino acid sequence analysis. The *exsA* gene was isolated in a gene identification program by using the genomic survey sequence strategy. This approach was based on end sequencing of random clones of a *B. abortus* S2308 genomic library constructed in our laboratory by using the plasmid vector pUC12. Five hundred clones were sequenced, and a clone designated clone 64 (pUC64) with high homology to the *R. meliloti* *exsA* gene was identified. This clone, which was approximately 2.1 kb long, was sequenced by primer walking. Double-stranded DNA sequencing was performed by the dideoxy chain termination method (29) by using an automated laser fluorescence DNA sequencer from Amersham Pharmacia Biotech (Piscataway, N.J.). The clone was sequenced with a Thermo Sequenase kit, and the primers used were M13 reverse sequence, M13 universal sequence from Amersham Pharmacia Biotech, and specific primers purchased commercially. The sequences of the specific primers used were as follows: 1F, 5'-CTG ATG GGC CTG ATG GTG-3'; 2F, 5'-CGC AAC CTG CAA CGC TCG-3'; 3F, 5'-TCA CAG GCC GAG ATC GAG-3'; 1R, 5'-GTT TTC CGC CAG CGT CCG-3'; 2R, 5'-TGT CCC CTG GAT GAA ACG-3'; and 3R, 5'-GTC GGT CGA AAA GCG TTG-3'. The sequence data were compiled and analyzed by using the sequence analysis program DNASIS V5.00 (Hitachi Software). Subsequent homology searches were performed by using BLAST programs (2). Amino acid sequences were aligned by using the ClustalW service of the European Bioinformatics Institute available on the internet (<http://www2.ebi.ac.uk/clustalw>). The potential transmembrane domains of the ExsA protein were predicted by the Kyte-Doolittle method by using a computer program available at the Tokyo University of Agriculture & Technology website (<http://sosui.proteome.bio.tuat.ac.jp/sosui/frame0E.html>).

Determination of the *exsA* gene copy number in the *B. abortus* genome. Genomic DNA from *B. abortus* strain S2308 was isolated as described previously (17). For DNA hybridization, 10 µg of genomic DNA was digested with *Bam*HI,

*Eco*RI, and *Eco*RV. *Bam*HI and *Eco*RI did not cut within the open reading frame (ORF), whereas *Eco*RV did cut in the middle of the *exsA* gene. The fragments obtained were separated by 1% agarose gel electrophoresis, passively transferred onto a nylon membrane (Hybond-N; Amersham Pharmacia Biotech), and UV cross-linked. The *exsA* fragment was used as a DNA probe. This fragment was labeled with [α -³²P]dCTP by random priming (Gibco BRL). Hybridization was performed under stringent conditions (38°C, 50% formamide) as described previously (28). The nylon membrane was subjected to autoradiography for 18 h at -80°C.

Construction of *B. abortus* Δ exsA deletion mutant. The deletion plasmid used for *exsA* gene replacement in *B. abortus* was constructed as follows. Plasmid pUC4K (Amersham Pharmacia Biotech) was digested with *Eco*RI, which produced a 1.2-kb fragment containing the kanamycin resistance cassette. This DNA fragment was blunt ended with T4 DNA polymerase (Amersham Pharmacia Biotech) and ligated into the unique *Eco*RV site of the *exsA* gene present in pUC64 to generate plasmid pGR64 (Fig. 1). To prepare *B. abortus* S2308 competent cells, bacteria were grown in 200 ml of brucella broth for 6 h at 37°C to the log phase (optical density at 600 nm, 0.4 to 0.6). The bacterial cells were harvested by centrifugation at 5,000 × g for 10 min, and they were washed three times with cold double-distilled water plus 10% glycerol and resuspended in 0.65 ml of chilled 10% glycerol. After that, 0.05-ml aliquots were stored immediately at -80°C. Five micrograms of pGR64 plasmid DNA was added to 50 µl of *B. abortus* competent cells in sterile electroporation cuvettes with 0.2-cm electrode gaps (Bio-Rad Laboratories, Richmond, Calif.), and then electroporation was performed with a Gene Pulser II transfection apparatus (Bio-Rad Laboratories) at 25 µF, 2.5 kV, and 400 Ω. One milliliter of SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose; Gibco BRL Life Technologies, Inc., Gaithersburg, Md.) was added, and the cells were grown with agitation at 37°C for 16 h and plated on brucella agar containing 25 µg of kanamycin per ml. After 3 to 5 days of incubation at 37°C, transformants were examined by replica plating on brucella agar containing 25 µg of kanamycin per ml and 25 µg of ampicillin per ml. Then colonies that were Kan^r Amp^s and Kan^r Amp^r were selected as colonies in which double and single crossover events had occurred, respectively.

Southern blot analysis of the *B. abortus* Δ exsA deletion mutant. To provide genetic evidence that the wild-type *exsA* gene was replaced by an *exsA* gene interrupted by the Kan^r cassette, 10 µg of genomic DNA isolated from both the mutant strain and the wild-type strain (S2308) was digested with *Eco*RI and then loaded onto a 0.8% agarose gel to perform Southern blotting. The genomic DNA was transferred to a nylon membrane by capillary transfer overnight in 10× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). DNA bound to the membrane was exposed to UV cross-linking and then prehybridized at 42°C for 4 h in 25 ml of a prehybridization solution (6× SSC, 0.2% sodium dodecyl sulfate, 10× Denhardt's solution, 100 µg of salmon sperm per ml, 50% formamide). The *exsA*, Kan^r, and Amp^r genes were used as probes and were labeled with [α -³²P]dCTP by using a random-primed labeling kit (Gibco BRL), and unincorporated ³²P was removed by Sephadex G-50 column chromatography. Hybridization was conducted at 42°C in the same prehybridization solution for 16 h. The blots were washed three times with 2× SSC-0.1% sodium dodecyl sulfate at 60°C for 30 min. After the last wash, the membrane was subjected to autoradiography for 6 h at -80°C.

Survival in the mouse model. Virulence was determined by measuring the numbers of CFU of wild-type and mutant strains in mouse spleens. Female BALB/c mice that were 6 to 8 weeks old were injected intraperitoneally (i.p.) with 1 × 10⁵ CFU of brucellae in 0.1 ml of phosphate-buffered saline (PBS) (145

FIG. 1. Schematic map of clone 64 encoding the *exsA* gene of *B. abortus* and construction of the pGR64 target vector for generation of the Δ *exsA* mutant by gene replacement. The arrow indicates the translation direction, and the cross-hatched bar indicates the *exsA* gene. Kan^r, kanamycin resistance gene.

mM NaCl, 49 mM KH₂PO₄, 21 mM Na₂HPO₄; pH 7.2). Groups of eight mice were injected with either *B. abortus* S2308 or the *B. abortus* Δ *exsA* mutant strain. At 1, 2, and 6 weeks postinoculation, all mice in each group were killed, and bacterial survival was determined following homogenization of the mouse spleens in 10 ml of PBS. Tenfold serial dilutions of the homogenized spleens were plated on brucella agar containing kanamycin to determine the number of Δ *exsA* CFU per spleen compared to the number of wild-type CFU.

Immunization of mice with the *B. abortus* Δ *exsA* mutant. Female BALB/c mice that were 6 to 8 weeks old were immunized i.p. with 1×10^5 CFU of brucellae in 0.2 ml of PBS. Groups containing eight mice each were immunized with either *B. abortus* S19, *B. abortus* RB51, or *B. abortus* Δ *exsA*. Nonimmunized, control mice were injected i.p. with 0.2 ml of PBS. Six weeks after immunization, all mice in each group were challenged by i.p. injection of 1×10^5 CFU of *B. abortus* S2308. Experimentally infected mice were killed 2 weeks later by cervical dislocation, and the spleens were collected and disrupted in 10 ml of PBS. Tenfold serial dilutions were plated on brucella agar containing kanamycin or 0.1% erythritol for differentiation of *B. abortus* Δ *exsA*, *B. abortus* strain S19, and *B. abortus* strain S2308. After 3 days of incubation at 37°C, colonies were visualized, and the number of CFU of *B. abortus* S2308 per spleen was determined after the number of *B. abortus* S19 or *B. abortus* Δ *exsA* found by replica plating was subtracted. The degrees of protection in immunized animals and controls were expressed as the mean CFU of *B. abortus* S2308 for each mouse group obtained after challenge and log₁₀ conversion. Log₁₀ units of protection were obtained by subtracting the mean log₁₀ CFU for the experimental group from the mean log₁₀ CFU for the control group, as previously described (19).

Statistical analyses. Statistical analyses were performed with Student's *t* test by using the MINITAB computer software package (Minitab Inc., State College, Pa.).

Nucleotide sequence accession number. The nucleotide sequence of the *exsA* gene of *B. abortus* has been deposited in the GenBank database under accession number AF218367.

RESULTS

***B. abortus* ABC transporter is a homolog of *R. meliloti* ExsA.** A 1,791-bp ORF was identified in a 2.1-kb clone (Fig. 1). The nucleotide sequence of the *B. abortus* *exsA* gene and the amino acid sequence encoded by this gene are shown in Fig. 2. The coding region was translated by using the computer program DNASIS V5.00. This ORF and this protein sequence were subjected to nucleic acid and protein homology searches by using the Basic Local Alignment Search Tool (BLAST) service of the National Center for Biotechnology Information. These searches revealed that the ORF encoding the *Brucella* ABC

transporter amino acid sequence has 69 and 67% identity with the ABC transporter-encoding *exsA* genes of *R. meliloti* and *M. loti*, respectively. On the basis of the alignment of the *Brucella* ExsA amino acid sequence with *R. meliloti* and *M. loti* ExsA sequences, we determined that *Brucella* ExsA is probably missing 5 to 15 amino acids, including the start codon, in our clone (Fig. 3). The *Brucella* *exsA* gene encodes a polypeptide consisting of at least 596 amino acids with a predicted molecular mass of approximately 66 kDa.

***B. abortus* ExsA contains all ABC transporter motifs.** Analysis of the *B. abortus* ExsA sequence revealed the presence of Walker A and B motifs and an ABC signature that are conserved ATP-binding domains in the ABC transporter protein family (Fig. 3). *B. abortus* ExsA has conserved features of a typical ABC transporter. First, ABC transporters possess typical ATP-binding motifs, such as Walker A (GXSGXGKST), Walker B (hhhhDXT), and the ABC signature, where X and h indicate any amino acid and hydrophobic amino acid residues, respectively (Fig. 3). Furthermore, the Kyte-Doolittle analysis of hydrophobicity revealed that *Brucella* ExsA is a typical transmembrane protein containing five membrane-spanning segments at the amino acid terminal region, like *R. meliloti* and *M. loti* ExsA (Fig. 4). Additionally, an ABC transporter ortholog group analysis performed by Junko Yabuzaki and Minoru Kanehisa (Kyoto University, Kyoto, Japan) revealed that *B. abortus* ExsA belongs to ortholog group 6 (data not shown), which includes toxin and glucan transporters which are important bacterial virulence factors (18, 35). Therefore, according to Tomii and Kanehisa (32), ABC transporters classified in ortholog group 6 are more likely to be involved in bacterial pathogenesis than transporters which belong to other ABC transporter ortholog groups.

Characterization of *B. abortus* Δ *exsA* deletion mutant. Before constructing a *B. abortus* Δ *exsA* deletion mutant, we determined the copy number of the *exsA* gene in *B. abortus*. Southern blot analysis revealed that a single copy of the *exsA* gene is present in the *B. abortus* genome (data not shown). Furthermore, a defined Kan^r Amp^s Δ *exsA* deletion mutant of

1	ATC	GAT	TTC	CGG	GGG	CCT	GCG	TTC	CGC	AGC	GTT	CTC	GGC	TAC	ACG	TTC	AGC	CAC	TGG	CGA
1	I	D	F	R	G	P	A	F	R	S	V	L	G	Y	T	F	S	H	W	R
61	AGT	CAG	CCC	TTG	CGG	CTG	TCG	GCG	ATG	ATT	ATC	GGC	ATG	TTG	CTG	GCG	ACA	CCG	CCC	GAC
21	S	Q	P	L	R	L	S	A	M	I	I	G	M	L	L	A	T	P	P	D
121	GTT	CTC	ACC	CCG	CTC	TAT	TCC	GGG	CGG	CTG	GTG	GAT	TCG	CTG	TCG	CTG	GGC	AGC	AAT	GCA
41	V	L	T	P	L	Y	S	G	R	L	V	D	S	L	S	L	G	S	N	A
181	TGG	GAT	GCG	GCG	ATC	CAC	GCC	CTC	ATC	GCC	CTG	CTC	TCG	CTG	GGT	GCG	CTG	GCG	CTC	GTT
61	W	D	A	A	I	H	A	L	I	A	L	L	S	L	G	A	L	A	L	V
241	TCG	CGG	GCA	GGT	CAC	GTT	TTT	ATG	TCC	TG	ACC	GAC	TTC	ACC	TTG	AAA	ATC	ATG	AGC	GAC
81	S	R	A	G	H	V	F	M	S	L	T	D	F	T	L	K	I	M	S	D
301	ATG	GCG	GCA	AGC	GCC	TTT	CGC	AAG	CTG	CAA	CGC	TTT	TCG	ACC	GAC	TGG	CAC	GCG	AAT	GCC
101	M	A	A	S	A	F	R	K	L	Q	R	F	S	T	D	W	H	A	N	A
361	TTT	GCC	GGC	TCG	ACG	GTG	CGC	AAG	GTG	ACA	CGC	GGC	ATG	TGG	GCG	CTC	GAC	CTG	CTG	AAC
121	F	A	G	S	T	V	R	K	V	T	R	G	M	W	A	L	D	L	L	N
421	GAC	ACG	CTC	ATC	ATC	GCG	CTT	CTG	CCC	TCG	CTC	CTG	ATG	CTC	GTG	GGT	TCG	GTG	GTG	CTG
141	D	T	L	I	I	A	L	L	P	S	L	L	M	L	V	G	S	V	V	L
481	CTG	GCA	TGG	TTC	TGG	CCA	CTG	ATG	GGC	CTG	ATG	GTG	GCA	ATC	GGC	TCG	GTG	CTT	TAT	ATC
161	L	A	W	F	P	L	M	G	L	M	V	A	I	G	S	V	L	Y	I	
541	GCC	TGC	ACC	ATC	GTC	ATC	TCG	CTT	GGT	TTC	GTG	GCG	CCT	GCG	GCC	ACG	CTC	GCA	AAT	AGC
181	A	C	T	I	V	I	S	L	G	F	V	A	P	A	A	T	L	A	N	S
601	TGG	GAT	ACG	CGC	ATG	GGC	GGT	GCA	CTG	GCC	GAT	TCC	ATG	TCG	TGC	AAC	GCC	GTG	GTC	AAG
201	W	D	T	R	M	G	G	A	L	A	D	S	M	S	C	N	A	V	V	K
661	GCT	TTC	GGC	GCA	GAA	GCG	CGC	GAG	GAT	GAA	CGG	CTC	GCC	ATG	GTC	GTG	AAC	AAG	TGG	CGC
221	A	F	G	A	E	A	R	E	D	E	R	L	A	M	V	V	N	K	W	R
721	ACG	CAC	ACG	GCG	CAC	ATG	GCT	GCA	GGT	CTG	CAC	GCA	GGA	TCC	CGT	TTC	ATC	CAG	GGG	ACA
241	T	H	T	A	H	M	A	A	G	L	H	A	G	S	R	F	I	Q	G	T
781	AAC	CTT	CTG	GTG	ATG	CGC	GGC	GTC	ATC	ATC	GCT	TTT	GCC	CTT	TAT	CTG	TGG	ACC	AGG	GGG
261	N	L	L	V	M	R	G	V	I	I	A	F	A	L	Y	L	W	T	R	G
841	CAA	GCG	ACG	GCT	GGC	GAT	ATA	ACC	TTT	CGT	TCT	CAC	CGC	CTT	TTC	TTC	ATG	CTG	CAA	GGC
281	Q	A	T	A	G	D	I	T	F	R	S	H	R	L	F	F	M	L	Q	G
901	TAT	CTG	CGT	GAT	GTG	GGC	ATG	CAT	ATC	GCC	AAC	CTG	CAA	CGC	TCG	GTC	AAT	CAC	ATG	GAG
301	Y	L	R	D	V	G	M	H	I	R	N	L	Q	R	S	V	N	H	M	E
961	GAA	CTG	GTC	GAT	ATC	GAA	GCC	CAG	CTA	CGC	ATT	GCC	GAC	AAG	CCC	GGC	GCG	CCC	GCG	ATC
321	E	L	V	D	I	E	A	Q	L	R	I	A	D	K	P	G	A	P	A	I
1021	AGG	ATC	GGC	AAT	GGC	GAG	ATC	ACC	TTC	AAT	CAC	GTC	ACC	TTC	CAT	TAT	GGC	AGG	CAC	GAA
341	R	I	G	N	G	E	I	T	F	N	H	V	T	F	H	Y	G	R	H	E
1081	GCC	GCG	CTC	TAC	GCG	GAC	TTC	TCG	GTG	CGG	ATC	GAA	GCT	GGC	GAG	CGG	GTC	GGT	CTG	GCG
361	A	A	L	Y	R	D	F	S	V	R	I	E	A	G	E	R	V	G	L	A
1141	GGC	TCC	GGC	TCC	GAG	AAG	ACG	ACC	TTC	GTG	AAG	CTC	ATC	CAA	CGG	CTT	TAT	GAC	GTG	AAT
381	G	S	G	S	G	K	T	T	F	V	K	L	I	Q	R	L	Y	D	V	N
1201	GGC	GGC	GCA	ATC	CTG	ATC	GAC	GGG	CAA	AAC	ATC	GCC	GAT	GTC	ACA	CAG	GCG	TCC	CTG	CGT
401	G	G	A	I	L	I	D	G	Q	N	I	A	D	V	T	Q	A	S	L	R
1261	TCG	CAG	ATC	GCC	ATC	GTG	CAG	CAG	GAA	CCA	ATC	CTG	TTT	CAC	CGG	ACG	CTG	GCG	GAA	AAC
421	S	Q	I	A	I	V	Q	Q	E	P	I	L	F	H	R	T	L	A	E	N
1321	ATC	GCC	TAT	GCC	CGC	CCC	GGC	GCA	TCA	CAG	GCC	GAG	ATC	GAG	GAA	GCC	GCT	GCG	CTT	GCC
441	I	A	Y	A	R	P	G	A	S	Q	A	E	I	E	E	A	A	R	L	A
1381	AGC	GCG	CAT	GAC	TTT	ATC	AGC	CGG	CTG	CCC	AAG	GGC	TAC	CGG	ACG	CTG	GTG	GGC	GAG	CGC
461	S	A	H	D	F	I	S	R	L	P	K	G	Y	R	T	L	V	G	E	R
1441	GGC	GTG	AAG	CTT	ACC	GGC	GGT	GAG	CGT	CAG	CGC	GTG	GCA	ATT	GCC	CGC	GCC	TTC	CTC	GCG
481	G	V	K	L	T	G	G	E	R	Q	R	V	A	I	A	R	A	F	L	A
1501	GAT	GCG	CCG	ATC	CTC	ATT	CTG	GAC	GAG	ACC	ACA	TCG	AGC	CTC	GAT	TCG	GAA	TCG	GAA	GTG
501	D	A	P	I	L	I	L	D	E	A	T	S	S	L	D	S	E	S	E	V
1561	CTG	ATC	CAG	CAC	GCC	ATG	GAA	CGG	CTG	ATG	GTG	GGG	GCG	ACC	ACG	CTT	GTC	ATC	GCC	CAC
521	L	I	Q	H	A	M	E	R	L	M	V	G	R	T	T	L	V	I	A	H
1621	CGT	CTT	TCC	ACA	GTG	CGC	GCG	CTC	GAC	CGG	CTT	CTG	GTG	TTC	GAC	AAG	GCG	CGC	ATT	GTG
541	R	L	S	T	V	R	A	L	D	R	L	L	V	F	D	K	G	R	I	V
1681	GAA	GAA	GGC	AAT	CAT	GAC	GCG	CTG	ATC	CGC	AAG	TCA	GAC	GGC	ATT	TAT	CGT	CGC	CTG	TTC
561	E	E	G	N	H	D	A	L	I	R	K	S	D	G	I	Y	R	R	L	F
1741	GAG	CGC	CAG	GCG	CTG	GAA	CTG	ACC	AAG	GGA	TTT	GGA	AGA	CAT	ATC	TGT	TAA			
581	E	R	Q	A	L	E	L	T	K	G	F	G	R	H	C	*				

FIG. 2. Nucleotide sequence of the *B. abortus* *exsA* gene and deduced amino acid sequence. The asterisk indicates the termination code.

B. abortus S2308 was constructed by chromosomal gene replacement. After electroporation, transformants were examined by replica plating on brucella agar containing kanamycin and ampicillin or kanamycin alone. Ten clones of the two phenotypes, Kan^r Amp^r and Kan^r Amp^s, were selected to confirm that the *exsA*-deficient (Kan^r Amp^s) clones were the result

of an allelic exchange. Chromosomal DNA was isolated from these clones and from the parental strain for Southern blot analysis. The same hybridization profile was observed for all transformants selected from each different phenotype group, as shown in Fig. 5. DNA hybridization of *Eco*RI-digested chromosomal DNA by using the *exsA* probe produced one frag-

FIG. 4. Hydrophobic profiles of ExsA proteins of *B. abortus* (A), *R. melioidi* (B), and *M. loti* (C). The analysis was performed by using the Kyte-Doolittle method. The vertical axis indicates the relative hydrophobicity (above the horizontal line) or hydrophilicity (below the horizontal line). The boxes indicate the potential transmembrane regions, and the numbers at the bottom are the amino acid positions.

FIG. 5. Characterization of the *B. abortus* $\Delta exsA$ mutant by Southern blot analysis. *Eco*RI-digested genomic DNA was probed with the *exsA* gene (A), *Amp*^r (B), or *Kan*^r (C) DNA fragments. Lanes 1, *B. abortus* $\Delta exsA$ mutant; lanes 2 and 3, clones in which a single recombination event took place; lanes 4, *B. abortus* S2308. Lanes MW contained molecular weight markers.

to genomic DNA of wild-type *B. abortus* S2308, which was used as a negative control (Fig. 5C). The size difference in the fragments observed in Fig. 5C between the *Kan*^r *Amp*^s and *Kan*^r *Amp*^r clones again was due to integration of the deletion plasmid in the ampicillin-resistant bacterial cells.

Attenuation of *B. abortus* $\Delta exsA$ mutant in BALB/c mice. The ability of *B. abortus* to persist within BALB/c mice has been shown to correlate with virulence in the natural host (12). Groups of mice were inoculated i.p. with the $\Delta exsA$ mutant or *B. abortus* parental strain S2308 to determine differences in persistence in the mouse model. The *Brucella* CFU were evaluated 1, 2, and 6 weeks postinfection in the spleen of each animal. The *B. abortus* $\Delta exsA$ mutant strain displayed reduced virulence at all times tested compared to the virulence of the wild type (Fig. 6). The smallest difference in CFU between the $\Delta exsA$ mutant and S2308 was observed at week 2 (log 0.23), and the greatest difference was observed at week 6 (log 0.92). Nevertheless, the log CFU difference between these two strains was statistically significant at all times studied.

Immunoprotection conferred by vaccination with the *B. abortus* $\Delta exsA$ mutant strain. To determine if the *B. abortus* $\Delta exsA$ mutant strain is able to induce protective immunity against infection, mice immunized with the $\Delta exsA$ mutant or with strain S19 or RB51 were challenged with the *B. abortus* virulent strain (S2308). The numbers of bacterial CFU in the spleens were determined 6 weeks after challenge, since Araya et al. (3) showed that nonspecific resistance to infection with unrelated bacteria is very low 6 weeks after immunization with *Brucella*. At this time, mice immunized with the *B. abortus* $\Delta exsA$ mutant strain had significantly ($P < 0.05$) fewer splenic brucellae than nonimmunized animals (Table 2). Additionally, we observed higher log units of protection in mice immunized with the $\Delta exsA$ mutant strain (2.74 log units) than in mice immunized with commercial vaccine strain S19 (1.44 log units) or RB51 (0.84 log unit) following challenge. Thus, the *B. abortus* $\Delta exsA$ mutant strain induced significantly enhanced resistance to experimental infection.

DISCUSSION

Bacterial pathogens modulate their gene expression to adapt to the various environments to which they are exposed during the course of infection. The mechanism of *Brucella* virulence is not yet fully understood. *Brucella* sensing unfavorable conditions produces diverse gene products that contribute to the survival and multiplication of this bacterium inside host cells (13). Understanding the molecular events that allow brucellae to reach an intracellular niche where the bacteria freely multiply should help determine the mechanism of pathogenesis. Little is known regarding the nature of bacterial factors responsible for *Brucella* entry and multiplication in different cell types. An increasing number of pathogenic bacteria are being

FIG. 6. Persistence of *B. abortus* S2308 and the *B. abortus* $\Delta exsA$ mutant in BALB/c mice. Eight mice were infected i.p. with a dose of 10^5 CFU of bacteria. Spleens were harvested at different times, and the number of CFU in disrupted tissue was determined by 10-fold serial dilution and plating. The values are means $\pm$ standard deviations. The asterisks indicate statistically significant differences between the results obtained for the group that received the mutant and the results obtained for the group that received *B. abortus* parental strain S2308 ($P < 0.05$).

TABLE 2. Protection of mice against challenge with *B. abortus* S2308 after immunization with the *B. abortus* Δ exsA mutant or with the *B. abortus* S19 or RB51 vaccine strain

Vaccine	Log ₁₀ CFU of <i>B. abortus</i> S2308 in spleen (mean $\pm$ SD) ^a	Log ₁₀ units of protection
PBS control	7.38 $\pm$ 0.16	
<i>B. abortus</i> RB51	6.54 $\pm$ 0.30	0.84 ^b
<i>B. abortus</i> S19	5.94 $\pm$ 0.78	1.44 ^b
<i>B. abortus</i> Δ exsA	4.64 $\pm$ 0.77	2.74 ^{b,c}

^a Mice were immunized with 10⁵ CFU and 6 weeks later were challenged i.p. with 10⁵ CFU of strain S2308.

^b Significantly different from value for the PBS control group ($P < 0.05$).

^c Significantly different from value for the group immunized with S19 and from value for the group immunized with RB51 ($P < 0.05$).

found to secrete effector molecules that affect host cell functions (8, 10). In *Brucella* infection, the type IV secretion system with which this bacterium transports macromolecular complexes across its envelope has been described as an important virulence factor (5, 23). Therefore, the cell secretion machinery that uses bacterial transporters is critical for the maintenance of *Brucella* pathogenesis.

In this study, we identified, sequenced, and disrupted the *exsA* gene of *B. abortus*. The deduced amino acid sequence for the ORF from clone 64 exhibited homology to ABC transporters of different bacterial species, mainly ExsA of *R. meliloti* and *M. loti*, with which the levels of sequence identity were 69 and 67%, respectively. The ABC transporters are a major class of cellular translocation machinery in all bacterial species (32). Based on computer analysis of the predicted amino acid sequence, Walker and ABC signature motifs that could form an ATP-binding site were identified in the C-terminal domain of *Brucella* ExsA. Additionally, the ABC transporter transmembrane region in the highly hydrophobic segment was predicted. In *R. meliloti*, the *exsA* gene encodes an ABC transporter involved in EPS I transport (4). EPS I is essential for the invasion of alfalfa root nodules by *R. meliloti*. Mutants of *R. meliloti* devoid of EPS I are unable to establish an effective symbiosis with alfalfa (22). Since like *Rhizobium* and *Mesorhizobium*, *Brucella* belongs to the alpha-2 subgroup of the *Proteobacteria* (21), whose members are characterized by their ability to interact pericellularly or intracellularly with eukaryotic cells, we suggest that *Brucella* ExsA functions as a polysaccharide transporter and may be crucial for the maintenance of *Brucella* pathogenesis.

To address the role of *Brucella* ExsA in bacterial virulence, a mutant was constructed. In mice the Δ exsA mutant exhibited a different level of spleen colonization than wild-type strain S2308, indicating that virulence in vivo was altered by the absence of ExsA (Fig. 6). *Brucella* ExsA also showed a high degree of homology to the *Sinorhizobium meliloti* MsbA-like saccharide-exporting ABC transporter (data not shown). An *msbA* mutant strain of *Francisella novicida*, a facultatively intracellular bacterium like *Brucella* that survives and grows in macrophages by preventing phagolysosomal fusion, was unable to grow in macrophages and mice, and it was found to be sensitive to serum (20). Therefore, we speculate that the *Brucella* Δ exsA mutant has an altered polysaccharide architecture, as demonstrated previously for *E. coli* (9, 11), and therefore is sensitive to complement-mediated lysis and/or phagocyte-me-

diated killing mechanisms. Macrophages and neutrophils are potent killers of *Brucella*, and this bacterium is extremely sensitive to the myeloperoxidase-halide-peroxide system found in the host phagocyte system (7). This phenomenon has been demonstrated in *B. abortus* LPS mutants (1). Even though changes in polysaccharide structure enhanced bacterial clearance, the *B. abortus* Δ exsA mutant has a smooth phenotype, like parental strain S2308 (data not shown).

Besides its importance in helping understand *Brucella* pathogenesis, our study also could have an impact on vaccine development. Bacterial vaccines are usually based on either live or inactivated whole-cell or subunit preparations (6). Live vaccines for intracellular organisms are generally more efficacious and consist of attenuated variants of particular pathogens that have lost the ability to cause clinical disease but are still able to establish self-limiting infections and hence immune responses in the hosts (36). As genes required for intracellular growth and survival of brucellae are identified, attenuated variants harboring defined genetic defects can be evaluated. Rational genetic attenuation of a pathogen could lead to a new generation of live bacterial vaccines that are safer and do not revert to full virulence. As demonstrated here, the *B. abortus* Δ exsA mutant induced superior protective immunity compared to the protective immunity induced by the *Brucella* vaccine strains currently available, S19 and RB51. RB51 induced lower resistance to challenge with the pathogenic strain S2308, probably due to rapid clearance of this strain (30) and to the absence of antibodies against LPS (31). As for strain S19, we suggest that a lower level of protection was induced in mice because of more rapid bacterial clearance with this strain than with the Δ exsA mutant. At 6 weeks postinfection, *B. abortus* S19 was cleared more rapidly than the *B. abortus* Δ exsA mutant (data not shown). Ideally, a live-vaccine strain conferring solid immunity without host restrictions would be a significant improvement over available vaccines.

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