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Transposon-Derived *Brucella abortus* Rough Mutants Are Attenuated and Exhibit Reduced Intracellular Survival

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The O antigen of *Brucella abortus* has been described as a major virulence determinant based on the attenuated survival of fortuitously isolated rough variants. However, the lack of genetic definition of these mutants and the virulence of naturally occurring rough species, *Brucella ovis* and *Brucella canis*, has confused interpretation. To better characterize the role of O antigen in virulence and survival, transposon mutagenesis was used to generate *B. abortus* rough mutants defective in O-antigen presentation. Sequence analysis of DNA flanking the site of Tn*5* insertion was used to verify insertion in genes encoding lipopolysaccharide (LPS) biosynthetic functions. Not surprisingly, each of the rough mutants was attenuated for survival in mice, but unexpected differences among the mutants were observed. In an effort to define the basis for the observed differences, the structure of the rough LPS and the sensitivity of these mutants to individual killing mechanisms were examined in vitro. All of the *B. abortus* rough mutants exhibited a 4- to 5-log-unit increase, compared to the smooth parental strain, in sensitivity to complement-mediated lysis. Little change was evident in the sensitivity of these organisms to hydrogen peroxide, consistent with an inability of O antigen to exclude relatively small molecules. Sensitivity to polymyxin B, which was employed as a model cationic, amphipathic peptide similar to defensins found in phagocytic cells, revealed survival differences among the rough mutants similar to those observed in the mouse. One mutant in particular exhibited hypersensitivity to polymyxin B and reduced survival in mice. This mutant was characterized by a truncated rough LPS. DNA sequence analysis of this mutant revealed a transposon interruption in the gene encoding phosphomannomutase (*pmm*), suggesting that this activity may be required for the synthesis of a full-length core polysaccharide in addition to O antigen. *B. abortus* O antigen appears to be essential for extra- and intracellular survival in mice.

Rough *Brucella* organisms are characterized by a defect in O-antigen presentation and arise spontaneously in cultures in a process classically referred to as dissociation or, perhaps more appropriately, smooth-to-rough phase variation (6, 25). Several environmental factors, including nutrient availability, temperature, and aeration, have been shown to influence the rate of appearance of rough *Brucella* organisms in culture (5). Several studies have shown that phase variation is the result of changes occurring at random and is not the selection of variants within the original inoculum (7, 25). Similar systems have been described for the biosynthesis of lipooligosaccharides in *Haemophilus influenzae* and *Neisseria meningitidis* (45). However, the underlying molecular mechanism for phase variation in *Brucella* has not been defined.

Phase variation in *Brucella* is a reversible phenomenon, and the accumulation of rough organisms in culture suggests that O-antigen expression is not essential under these conditions and is metabolically demanding. Experimental evidence suggests that rough *Brucella* variants would be rapidly destroyed by complement-mediated lysis or by the phagocytic cells of the infected host. Since the organism is not known to occupy an environmental niche outside the infected host, the need for variable expression of O antigen is not immediately apparent. One possible explanation for phase variation has been a putative selective advantage under as yet unidentified conditions. For example, L forms lacking lipopolysaccharide (LPS) have been frequently observed in the tissues of animals chronically infected with *Brucella* (39). However, the low frequency at which such organisms are observed during primary isolation has otherwise hampered interpretation. Smooth organisms have been shown to arise spontaneously from rough isolates during growth in culture or in the infected host. In the latter situation, smooth organisms will have a selective advantage (37, 38).

Understanding of the role of O antigen in the resistance of *Brucella* to killing activities is based on experiments using fortuitously isolated rough variants and, as a result, is characterized by a lack of genetic definition. In many cases, the parental strain for the rough variant is not available, and under the best conditions, it is several generations removed. Thus, a relationship between the observed rough phenotype and loss of O antigen has not been directly confirmed. For these reasons, we have generated rough mutants via transposon mutagenesis and have characterized the locus encoding the LPS biosynthetic genes. The results presented here focus on four genes which encode proteins required for the stable production of LPS. Transposon insertion in any of these genes prevents O-antigen expression and results in sensitivity to complement and cationic peptides and reduced survival in mice. However, unexpected differences in sensitivity suggested that the genetic defects were not equivalent. We describe one example in which the inactivated gene may also function in the synthesis of core oligosaccharide, and inactivation has a dramatically increased effect on survival.

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MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. *Brucella abortus* S2308, S19, and RB51 were kindly provided by B. L. Deyoe, National Animal Disease Center,

Ames, Iowa, and RB51 was provided by G. G. Schurig, Virginia/Maryland Regional College of Veterinary Medicine, Blacksburg. *Escherichia coli* DH10B was purchased from Life Technologies. *E. coli* XL1-Blue and XL1-Blue MRF′(p-Bluescript KSII+) were purchased from Stratagene. *E. coli* SM10(pSUP2021) was generously provided by R. Simon through S. Boyle, Virginia/Maryland Regional College of Veterinary Medicine (54). *Brucella* strains were cultured in tryptic soy broth (TSB) (Difco Laboratories), potato infusion agar (PIA) (Difco Laboratories), or SOC (0.5% [wt/vol] yeast extract, 2% [wt/vol] tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSo_4$, 20 mM $MgSO_4$) medium at 37°C in an atmosphere containing 5% CO_2 (4). Kanamycin was added at a concentration of 50 μg/ml unless otherwise indicated. *E. coli* strains were cultured at 37°C in Luria-Bertani medium containing kanamycin at 50 μg/ml and/or ampicillin at 100 μg/ml as needed (4).

Transposon mutagenesis. For the preparation of electrocompetent cells, *B. abortus* S2308 was incubated as described above for 48 h on PIA. Cells were harvested and resuspended to a concentration of 1×10^{10} to 5×10^{10} CFU/ml for electroporation based on a procedure by Reschke et al. (47). Five micrograms of CsCl-purified pSUP2021 carrying Tn*5* was added to 75 μl of S2308 cells and incubated on ice for 30 min (4, 33, 54). Electroporation was performed at 15 kV/cm with a BTX flat-pack cuvette (Genetronics, Inc.) by a procedure based on that described by Lai et al. (33). The cell suspension was washed from the cuvette with 1 ml of SOC medium into a microcentrifuge tube and incubated for 24 h with agitation. The bacteria were pelleted at 2,000 × *g* for 5 min at room temperature and then resuspended in 200 μl of SOC medium and plated onto TSA (TSB with agar) plates containing kanamycin at 10 μg/ml, which were incubated for 4 to 7 days.

Selection of rough mutants. Individual *Brucella* mutants were inoculated into the wells of a microtiter dish containing 200 μl of TSB-kanamycin (10 μg/ml) and replica plated onto TSA plates containing kanamycin. The plates were incubated for 48 to 72 h, and individual colonies were tested for the uptake of crystal violet (2% [wt/vol] crystal violet dye, 20% [vol/vol] ethanol, 0.8% [wt/vol] ammonium oxalate) as described by Alton et al. as an initial screening method for the identification of rough mutants (2).

Acriflavine and serum agglutination. The phenotype of the selected rough mutants was further determined by acriflavine agglutination and serum agglutination tests (2, 7). Frozen bacterial stock or an isolated colony was inoculated onto TSA-kanamycin plates and grown to saturation after 48 to 72 h of incubation. Harvested bacteria were resuspended at a concentration of 10^{11} to 10^{12} CFU per 3 ml of phenol saline (0.85% [wt/vol] NaCl, 0.1% [vol/vol] phenol) and heat killed at 65°C for 2 h. For the acriflavine agglutination test, 10 μl of a 0.1% (wt/vol) aqueous solution of acriflavine was mixed with 10 μl of heat-killed cell suspension. For the agglutination test, 10 μl of heat-killed cell suspension was mixed separately with an equal volume of 1:50 anti-A, anti-M, and anti-R monospecific sera (obtained from the National Animal Disease Center) based on the procedure described by Alton et al. (2). Biotype analysis was performed as described by Alton et al. (2).

Electrophoresis transfer and immunoblotting. Whole-cell lysates of *Brucella* strains were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis by the protocols of Tsai and Frasch (60) and Hitchcock and Brown (26). Western immunoblotting procedures were performed as described elsewhere (4). Nylon membranes were probed with a primary mouse monoclonal antibody (MAb) specific for *B. abortus* O antigen (MAb 39) or lipid A (MAb 177) (4, 15, 27). For exposure to MAb 177, the membrane was hydrolyzed according to procedures described by de Kievit and Lam (15). Membrane-bound antigens were visualized via an alkaline phosphate-based chromogenic visualization system (4).

Southern blot analysis. Genomic DNA was isolated from *Brucella* by using the cetyltrimethylammonium bromide method as described previously (4). Isolated DNA was digested with the restriction enzyme *Eco*RI or *Bam*HI, the latter cutting once within Tn*5* (30). Genomic DNA fragments were separated by electrophoresis, and the fragments containing Tn*5* were identified by Southern blot analysis with a Tn*5* probe labeled with [α-^{32}P]ATP (Du Pont, NEN Research Products) (19, 56). Based on Southern blot analysis, *Eco*RI genomic DNA fragments containing Tn*5* flanked by *Brucella* DNA were excised from a 0.8% (wt/vol) preparative agarose gel following electrophoresis and subcloned into the *Eco*RI site of pBluescript KSII+ dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim). The size of the *Eco*RI fragments containing Tn*5* (5.8 kb) ranged from 7.8 to 10.8 kb. The recombinant plasmids, designated pCA180, pCA353, pCA533, and pCA613, were transformed into competent *E. coli* DH10B (24). Digestion of these plasmids with *Bam*HI and *Eco*RI, followed by ligation, was performed to generate the following subclones for the purpose of sequence analysis: pCA1801, pCA1802, pCA3531, pCA3532, pCA5331, pCA5332, pCA6131, and pCA6132. Restriction endonucleases were purchased from Boehringer Mannheim and New England Biolabs.

Nucleotide sequence analysis. *Brucella* genomic DNA, flanking the site of Tn*5* insertion, was sequenced by using the Tn*5* primer IR.1 (5′-CTGGAAAACGG GAAAGGTTCCG-3′) on the ABI Prism 377 DNA sequencer (Perkin-Elmer) (42, 53). Sequence was analyzed by using MacVector Sequence Analysis software (International Biotechnologies, Inc.). Characterization of recombinant DNA and putative gene products was done with the National Center for Biotechnology Information Blast server with the SWISS-PROT database (3).

Survival in the mouse model. To test in vivo survival, 6- to 8-week-old female BALB/c mice (The Jackson Laboratory), in groups of 15, were inoculated intraperitoneally (i.p.) with 10^4 CFU of bacteria harvested from a 48-h PIA plate with phosphate-buffered saline (145 mM NaCl, 49 mM KH_2PO_4, 21 mM Na_2HPO_4, pH 6.4) per ml. At 1-, 2-, and 6-week intervals postinoculation, five mice from each treatment group were sacrificed for blood and spleen collection. The presence of O-antigen antibody in the mouse sera was determined via Western blotting with strips of S2308 whole-cell lysate (as described above) (4, 27). Bacterial survival was determined following homogenization of the mouse spleens in phosphate-buffered saline at a weight-to-volume ratio of 1:10 with an Omni International 2000 tissue homogenizer. Serial dilutions of the spleen homogenates were plated in triplicate on TSA to determine bacterial counts.

Bactericidal assays. *Brucella* strains were tested for sensitivity to bovine serum by a procedure based on that described by Corbeil et al. (12). Sera were collected from the blood of five naive Angus bovine calves and pooled for storage at −80°C. The in vitro sensitivity of *Brucella* to the killing activity of macrophages was determined with bovine peripheral blood monocyte-derived macrophages. Bovine macrophages, isolated from a cow with a known genetic susceptibility to *B. abortus* S2308, were infected at a ratio of five bacteria per macrophage as previously described with the following changes: mononuclear cells, collected from blood on Percoll, were allowed to adhere overnight and were incubated for 72 h prior to infection (8). Bacterial counts after 12 h of intracellular growth were determined in triplicate and expressed as a percentage of the survival observed for the parental strain, S2308. For the polymyxin B (PmB) MIC assay, brucellae were grown to log phase in TSB, pelleted at 4,200 × *g*, resuspended in 10 mM phosphate buffer (pH 7.2) at approximately 4×10^4 CFU/ml, and incubated for 1 h over a range of PmB concentrations (36, 51). Following the 1-h incubation period, the cell suspensions were diluted 1:10 in 10 mM phosphate buffer (pH 7.2), and 100 μl was plated on triplicate TSA plates. Average results of three assays were expressed as a percentage of the brucellae surviving in wells incubated in the absence of bactericidal agents. For the hydrogen peroxide susceptibility assay, bacteria were exposed to 30% hydrogen peroxide in a disk sensitivity assay described by Elzer et al. (17).

Statistical analyses. Data was analyzed by using the statistics software GraphPad Prism 2.0. Analysis of variance was used to calculate the levels of significance of the complement sensitivity and bactericidal assays. To determine the significance of differences in the mouse infection experiment, the Kruskal-Wallis test and the Dunn procedure were used.

Nucleotide sequence accession numbers. DNA sequences were deposited in GenBank under the following accession numbers: CA180, AF022366; CA353, AF021920; CA533, AF021921; and CA613, AF021922.

RESULTS

Identification of rough mutants. In order to identify *B. abortus* rough mutants, a primary screening was performed with crystal violet, which is excluded from the surface of smooth colonies by O antigen (2, 32, 35). Phase transition has been shown to occur in *B. abortus* cultures at rates that vary depending on external conditions and the bacterial strain (5). In order to account for this potential background, electroporation without exogenous DNA was performed in parallel, and the rate of appearance of naturally occurring rough mutants of S2308 was shown to be less than 1 in 1,000 CFU. Rough organisms were identified following electroporation at a frequency of 1 in 200 kanamycin-resistant CFU selected from a bank of *B. abortus* mutants.

Crystal violet adsorbed to four colonies, designated CA180, CA353, CA533, and CA613, which were verified as rough organisms via agglutination tests with antisera specific for O antigens from *B. abortus* (A serovar), *Brucella melitensis* (M serovar), *Brucella canis* (R serovar), and acriflavine (2). Consistent with their preliminary identification, the mutants failed to agglutinate with antismooth antibody (A and M) but did agglutinate with antirough sera and acriflavine. Selected mutants also demonstrated resistance to the lytic action of Tblisi phage, which requires *B. abortus* O antigen as the receptor for infection (13). Biotype analysis, which employs oxidative metabolic tests designed to distinguish species and biovars of the *Brucella* genus, confirmed that the mutants were *B. abortus* biovar 1 isolates, consistent with the phenotype of the parental strain (2). These results confirm that the isolated organisms exhibit the rough phenotype and are derived from the parental strain *B. abortus* biovar 1 S2308 (35).

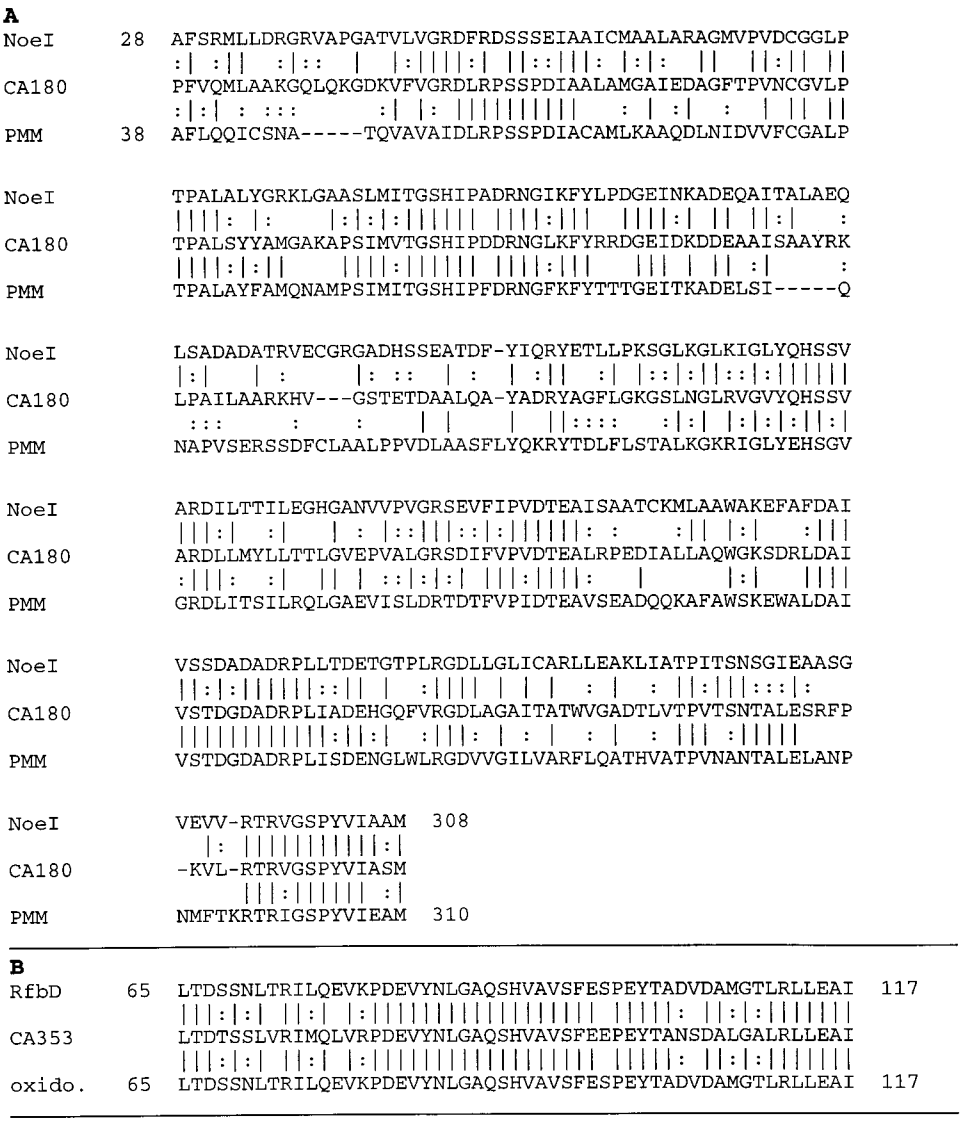

FIG. 1. Deduced amino acid sequence of Tn5-flanking DNA from the *B. abortus* rough mutants aligned with amino acid sequences from GenBank. (A) Alignment of CA180 ORF product with *Rhizobium* sp. strain NoeI and *Vibrio cholerae* PMM; (B) alignment of CA353 ORF product with *V. cholerae* RfbD-like gene product and *V. cholerae* oxidoreductase (oxido.); (C) alignment of CA533 ORF product with *V. cholerae* RfbV and an outer membrane protein (OMP) precursor of *V. cholerae*; (D) alignment of CA613 ORF product with RfbA of *P. aeruginosa* and TrsF of *Y. enterocolitica*. Conserved amino acids are indicated by vertical lines, and similar amino acids are indicated by colons. Numbers at the beginnings and ends of sequences indicate the positions of published amino acids. Dashes indicate spaces inserted to improve alignment. Alignments were performed with CLUSTAL V. See the text for complete descriptions of the genes.

Genetic characterization of the rough mutants. Southern blot analysis and DNA sequence analysis were used to identify and isolate genomic *Eco*RI fragments containing the Tn*5* insertion in each of the isolated mutants. Sequence analysis of each subclone reveals that in each of the rough mutants, Tn*5* is inserted into a gene whose putative protein product has homology to proteins involved in LPS biosynthesis (Fig. 1). The open reading frame (ORF) identified in pCA180 encoded a protein having homology with an undefined nodulation protein of *Rhizobium* sp. strain NoeI and with phosphomannomutase (PMM) of *Vibrio cholerae* (11, 21). The interrupted ORF in pCA353 encoded a protein having homology with an RfbD-like protein in *V. cholerae*, a putative GDP–D-mannose dehydratase (accession number 1002384), and *V. cholerae* oxidoreductase (59). The interrupted ORF in pCA533 encoded a protein having homology with *V. cholerae* RfbV, an undefined protein involved in LPS biosynthesis, and a probable outer membrane protein precursor of *V. cholerae* (accession number 280156) (18). RfbA of *Pseudomonas aeruginosa*, and TrsF of *Yersinia enterocolitica*, both LPS biosynthesis proteins, had homology with the protein encoded by the CA613 ORF (14, 55).

Characterization of O-antigen synthesis. The absence of O antigen on the cell surface of the rough mutants (now designated *lps*), suggested by hydrophobic dye exclusion and agglutination, was verified by using MAbs specific for the *N*-formylperosamine monomers of A-type O antigen (MAb 39) or lipid A (MAb 177) (15, 40). Smooth strains expressing O antigen, the distal portion of LPS, produce a nondiscrete ladder-like profile with preferred clusters, representing a wide range of O-antigen lengths, as visualized by immunoblotting with MAb 39 (Fig. 2) (20, 48). Similar profiles have been described for *E. coli* O8 and O9 and *Y. enterocolitica* O:3 and O:9, which ex-

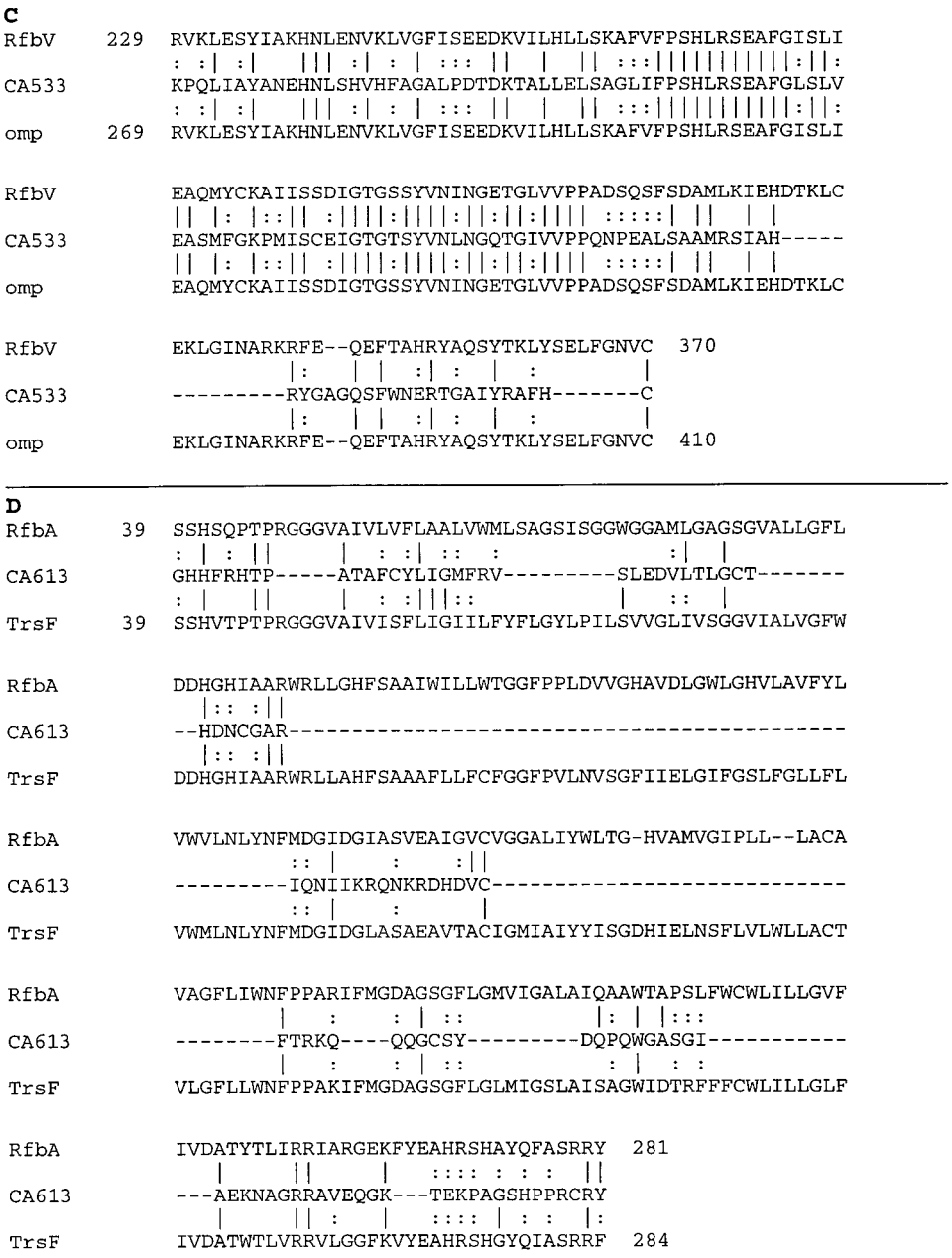

FIG. 1—*Continued.*

press O-antigen homopolymers (1, 28, 49, 63). O-antigen expression was undetectable in CA180, CA353, CA533, and CA613 by using MAb 39 (Fig. 2).

The inability to detect O antigen on the cell surface of the *lps* mutants by using MAb 39 suggests two possibilities. First, O-antigen expression was blocked, resulting in its absence, or second, the structure of O antigen was altered, rendering it nonactive with MAb 39. In order to distinguish between these possibilities, a broad-range antibody (MAb 177) directed against lipid A, the hydrophobic anchor of the LPS molecule, was used to characterize O-antigen expression via detection of lipid A-containing cell components (48). The profile of lipid A-containing molecules detectable in smooth strains matches the distribution of O-antigen-containing molecules, indicating that O antigen and lipid A comigrate as part of the same LPS molecule (Fig. 3). Mutant strains CA180, CA353, CA533, and CA613 produced very few bands, which migrated near the bottom of the gel. Mutant CA180 produced a different pattern which suggests a possible defect in the synthesis of core, the structural link between lipid A and O antigen (Fig. 3, lane 8) (48). These data confirm that changes in the migration of LPS in the *lps* mutants are consistent with defects in O-antigen or core biosynthesis (15).

Survival of *lps* mutants in the mouse model. The ability of *B. abortus* to proliferate and persist within the BALB/c mouse has been shown to correlate with virulence in the natural bovine host (16). Although the ultimate fates of the organism in these species are different, virulence in mice is an excellent predictor

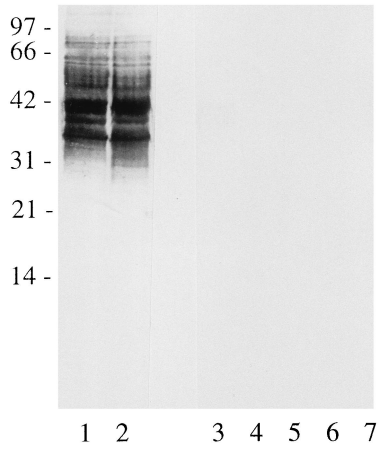

FIG. 2. Immunoblot of whole-cell lysates from *B. abortus* smooth and rough strains electrophoresed on a 12% (wt/vol) gel, transferred to a polyvinylidene difluoride membrane, and reacted with anti-O-antigen antibody (MAb 39). Lane 1, S2308; lane 2, S19; lane 3, RB51; lane 4, CA613; lane 5, CA533; lane 6, CA353; lane 7, CA180. Positions of molecular mass markers (in kilodaltons) are shown on the left.

of virulence in cattle (44). Groups of 15 mice were inoculated i.p. with each rough strain or parental smooth strain S2308, and five mice per strain were sacrificed at weeks 1, 2, and 6 postinoculation, at which time their spleens were examined for *Brucella* proliferation. Serology was used to confirm the absence of antibody against O antigen in the mouse sera for each *lps* mutant and is consistent with the rough phenotype (data not shown). Although mouse-to-mouse variation was apparent, especially at the earlier time points, the general trend revealed attenuated survival of the *lps* mutants in the mouse model. However, among the organisms tested, only CA180 was significantly different from the parental smooth strain at each week examined ($P < 0.05$) (Table 1). CA353 survived for an extended period in the spleens of mice but its survival was significantly different ($P < 0.05$) from that of the parental strain by 6 weeks postinoculation. CA533 and CA613 survived for extended periods in the spleens of mice, albeit at much reduced levels, and their lengths of survival were never significantly different from that of the parental smooth strain. Overall, survival in mice revealed greater differences among the *lps* mutants than were expected based on previous descriptions of rough organisms. In only one case can we suggest a potential cause for the variability, i.e., the truncated rough LPS observed via Western blotting in CA180 (Fig. 3) and the putative core defect. The experiments described in the following sections were designed to determine which of several potential killing mechanisms was responsible for the decreased survival and variation observed among the *lps* mutants.

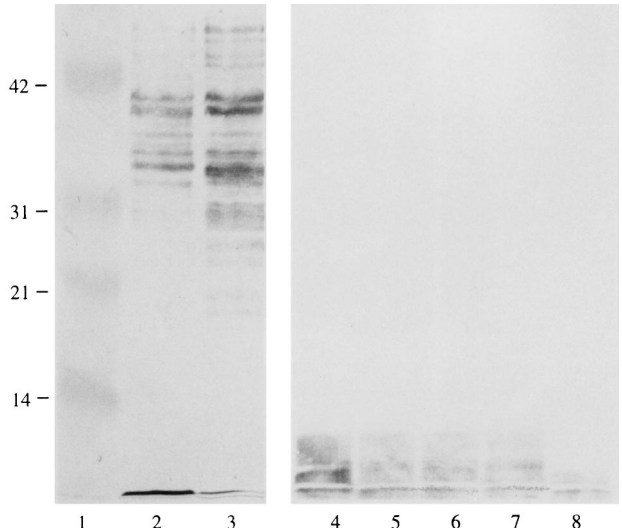

FIG. 3. Immunoblot of whole-cell lysates from *B. abortus* smooth and rough strains electrophoresed on a 12% (wt/vol) gel, transferred to a polyvinylidene difluoride membrane, hydrolyzed in 10% acetic acid, and reacted with anti-lipid A antibody (MAb 177). Lane 1, molecular mass markers (sizes in kilodaltons are shown on the left); lane 2, S2308; lane 3, S19; lane 4, RB51; lane 5, CA613; lane 6, CA533; lane 7, CA353; lane 8, CA180.

Sensitivity of *lps* mutants to complement-mediated lysis. Brucellae are sensitive to complement-mediated lysis via the classical pathway (12). Using *B. abortus* 45/20 and RB51, Corbeil et al. concluded that increased sensitivity to the killing activity of complement was attributable to the lack of O antigen (12). Sensitivity to complement was used as a predictor of sensitivity to extracellular killing within the host (Fig. 4). Each of the *lps* mutants exhibited a greater-than-4-log-unit increased sensitivity to bovine serum complement compared to that of the parental smooth strain ($P < 0.0001$). The additional defect noted in the rough LPS produced in CA180 has no apparent effect on the resistance to complement-mediated lysis. The increased sensitivity of the *lps* mutants to complement-mediated lysis is consistent with the role of O antigen in preventing the membrane attack complex from reaching the surface of the bacterium. The differences in survival observed in mice are not consistent with the observed differences in complement-mediated lysis, indicating that survival in the mouse is not controlled by extracellular killing activities. Differences in survival observed in mice must be attributable to differences in intracellular survival.

Sensitivity of *lps* mutants to phagocytic killing. The ability to control infection by *B. abortus* has been attributed to phagocytic cells of the host (8). Previous studies using fortuitously isolated *B. abortus* rough strains has revealed attenuated intracellular survival in both macrophages and polymorphonuclear leukocytes (50). Peripheral blood macrophages obtained from a cow susceptible to *B. abortus* infection were used to study intracellular survival (Fig. 5). The use of peripheral blood macrophages from susceptible animals was originally chosen to maximize differences in survival among the *lps* mutants, at a sacrifice to overall killing. Survival of each *lps* mutant was reduced relative to that of the parental smooth strain; however, only the survival of CA180, CA353, and CA613 was significantly different from that of the parental strain ($P < 0.05$). When the mouse macrophage cell line J774A.1 was employed, the *lps* mutants exhibited increased sensitivity relative to that of the parental smooth organism (data not shown). However, distinction among the *lps* mutants based on macrophage sensitivity was not possible in these experiments. The contribution of polymorphonuclear leukocytes to the overall killing of *B. abortus* has not been clearly described. However, it is clear that rough strains exhibit increased sensitivity to intraleukocytic killing mechanisms (51). The contribution of these activities to survival or elimination of *B. abortus* will be discussed further below following an examination of the sensitivity or resistance to individual killing activities.

Sensitivity of rough mutants to intracellular activities. Intracellular survival and replication within host phagocytes have been described as constituting the primary mechanism by which brucellae persist in the host and ultimately cause disease (9, 29). The uptake of rough organisms by phagocytic cells has

TABLE 1. Bacterial counts in mouse spleens examined 1, 2, and 6 weeks after i.p. infection

Strain	CFU/spleen[a] at wk:		
	1	2	6
Wild type (S2308)	4.88×10^6 (2.83×10^5–3.06×10^7)	2.34×10^6 (6.45×10^5–4.45×10^6)	1.34×10^6 (1.12×10^6–3.69×10^6)
Mutants			
CA180	0 (0–1.06×10^2)	0 (0–4.93×10^2)	0 (0)
CA353	1.94×10^3 (1.23×10^1–3.51×10^4)	8.4×10^1 (0–5.59×10^3)	0 (0)
CA533	1.19×10^4 (2.20×10^2–7.28×10^4)	5.51×10^2 (2.50×10^2–4.27×10^4)	0 (0–1.62×10^4)
CA613	7.52×10^2 (2.17×10^2–3.20×10^3)	5.51×10^2 (0–7.38×10^3)	0 (0–1.12×10^2)

[a] Bacterial counts in the mouse spleens are represented as medians, with the lower (25%) and upper (75%) quartiles presented in parentheses.

been shown to be unaffected by the absence of O antigen (51). Thus, any differences in survival observed in the mouse model must be attributable to differences in sensitivity to intracellular killing mechanisms.

Defensins are low-molecular-weight, cationic, amphipathic peptides which are thought to function within phagocytic cells by permeabilizing the cytoplasmic cell membranes of gram-negative organisms (34, 52, 61). Although defensins have not been generally described for macrophages, it is possible that molecules present in these cells share this general structural feature or mechanism of action. PmB, an amphipathic peptide shown to form ionic interactions with oligosaccharide components of LPS, including 2-keto-3-deoxyoctulosonic acid and phosphate, has the greatest bactericidal affect against *B. abortus* (36, 57, 61). When PmB was used as a model defensin, the survival of the *lps* mutants was shown to be reduced relative to that of S2308 over a PmB concentration range of 1 to 40 μg/ml (Fig. 6). CA180 and CA353 exhibited the greatest sensitivity to PmB at all concentrations tested and were significantly different from the parental strain at 5 to 10 μg of PmB per ml ($P < 0.05$). At PmB concentrations exceeding 20 μg/ml, all of the *lps* mutants tested exhibited significant differences ($P < 0.001$) in sensitivity to PmB compared to S2308. These results confirm the function of O antigen in the inhibition of intracellular host defense mechanisms, possibly by providing a physical barrier which prevents bactericidal components released within the phagosome from reaching the cell surface (31). The increased sensitivity of CA180 to PmB is probably the result of the truncated rough LPS.

Hydrogen peroxide, a reactive oxidative agent produced during the respiratory burst within phagocytes, is toxic to *Brucella* (51). In vitro exposure revealed no appreciable increase in the sensitivity of the *lps* mutants to H_2O_2 (data not shown). O antigen is not expected to exclude this compound from the surface of the cell. The resistance of the *lps* mutants to H_2O_2 is presumably due to the production of superoxide dismutase and catalase, which inactivate O_2^- and H_2O_2 (29). Taken together, the results confirm the important role of phagocytic cells in controlling infection with *B. abortus*. However, these results also predict that the killing observed is controlled by activities associated with phago-lysosome fusion. Unless these activities enter the phagosome by alternative methods, these results suggest that the maturation of the phagosomal compartment in cells infected with the *lps* mutants may proceed normally, resulting in attenuated survival. Alternatively, undefined mechanisms may be responsible for the elimination of the *lps* mutants.

DISCUSSION

Although there has been much written about the phenotype of rough mutants, those reports have relied upon the characterization of fortuitous rough isolates. Since nothing was known about the genetic defects in those organisms, any biological properties attributed to the lack of O antigen influenced the interpretation of subsequent experimental observations. For example, *Brucella ovis* and *B. canis* are naturally occurring

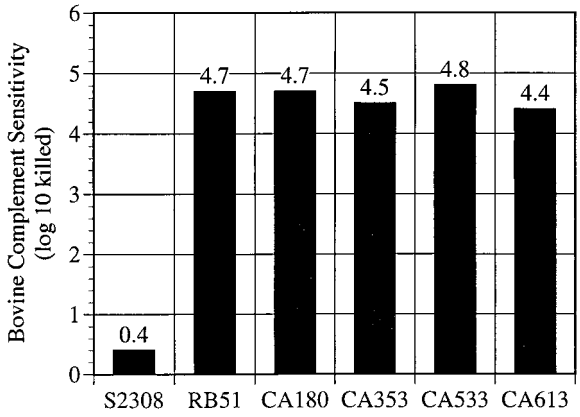

FIG. 4. Complement-mediated killing of *B. abortus* strains in bovine sera. Complement-mediated killing was performed as described in Materials and Methods. Bacterial counts are reported as log CFU/ml (fresh sera) − log CFU/ml (heat-inactivated sera). The results represent means from four independent assays ($P < 0.0001$).

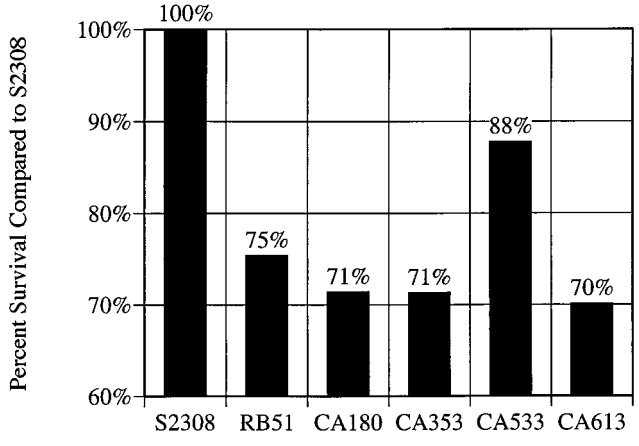

FIG. 5. Percent survival of *B. abortus* strains in bovine peripheral blood macrophages. Macrophage-mediated killing was performed as described in Materials and Methods. Results are expressed as percent survival in comparison to that of S2308 and represent the averages from three independent trials ($P < 0.05$).

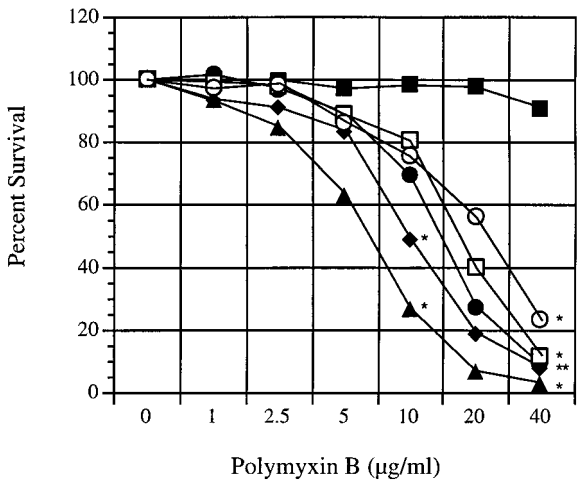

FIG. 6. Bactericidal effect of PmB on *B. abortus* strains. PmB-mediated killing was performed as described in Materials and Methods. Average results of three assays are expressed as percentages of the brucellae surviving in wells incubated in the absence of PmB. Asterisks indicate statistically significant results as noted in the text. Symbols: ■, S2308; ●, RB51; ▲, CA180; ◆, CA353; □, CA533; ○, CA613.

rough species which are pathogenic in their preferred hosts and resistant to complement-mediated lysis (10, 46). As a result, most researchers have maintained that these organisms must express a shortened (somehow undetectable) O antigen (43). However, recent work in our laboratory with the broadly reactive anti-lipid A antibody (MAb 177) described in this paper indicates the presence of LPS in *B. ovis* and *B. canis* which is indistinguishable from that observed in the *lps* mutants (CA353, CA533, and CA613) of *B. abortus* (data not shown). The differences in phenotype (virulent versus avirulent) may be most easily explained by changes (perhaps several) which have occurred since the divergence of these organisms, and simple comparisons are not possible. Careful genetic characterization of the *lps* mutants as performed here indicates that the lack of O antigen in *B. abortus* alone is responsible for the attenuated phenotype observed.

The most significant findings of this work are (i) the preliminary identification of the *Brucella lps* locus and (ii) that insertion into the *lps* locus within the *pmm* gene results in an organism exhibiting a faster-migrating LPS (i.e., deep rough) with decreased survival in the mouse model. The differences observed in the mouse model support the long-held assumption that the virulence of *Brucella* organisms reflects the ability to survive intracellularly in phagocytes. All of the *lps* mutants examined exhibited elevated and indistinguishable sensitivity to complement-mediated lysis. If this was the most important factor controlling virulence in the mouse model, then little difference in the number of surviving organisms colonizing the spleen would be expected. Differences in sensitivity to phagocyte-mediated killing mechanisms were also observed in vitro and reflected the differences observed in the macrophage assay. The selection of permissive rather than restrictive macrophages was made to enhance differences in survival among the rough organisms (8). Of course, it is not possible to completely imitate the in vivo host-agent interaction either in vitro or in tissue culture, and a combination of factors acting on the organism may cause the observed differences.

The absence of O antigen in the *lps* mutants is predicted to increase sensitivity to cationic peptides as a result of increased membrane penetration of the cell (61). CA180 exhibited the highest sensitivity to PmB and macrophages and was quickly eliminated from the mouse. In contrast, CA533 exhibited both resistance to in vitro activities and macrophage killing and was eliminated more slowly from the mouse. The dissimilarity observed among the *lps* mutants in the mouse model and in vitro presumably reflects differences in membrane permeability resulting from defects in LPS biosynthesis. The best example of these differences is the rough LPS of CA180, which produces a truncated core polysaccharide, thus increasing the organism's sensitivity to several agents (Fig. 3). Apparently, these defects have no effect on the uptake of *Brucella*.

The correlation between PmB sensitivity and survival in the mouse suggests that neutrophils may play a predominant role in the clearing. It is clear from other studies that neutrophils are potent killers of *Brucella*. For example, *B. abortus* is extremely sensitive to the myeloperoxidase-halide-peroxide system found only in neutrophils (9). Although the role of defensins in macrophage killing is not well established, we cannot rule out the possibility that PmB sensitivity reveals a general membrane defect which sensitizes these organisms to cellular products present in all phagocytic cells. For this reason, we cannot rule out increased clearance of the *lps* mutants as a result of either macrophages or neutrophil killing.

The distinct LPS structure of CA180 may be explained by the nature of the gene interrupted by transposon insertion. Sequence analysis of CA180 revealed protein homology to PMM, which is responsible for the interconversion of mannose-6-phosphate and mannose-1-phosphate and is a key biosynthetic enzyme in O-antigen production (Table 1) (11, 22). In *Brucella*, mannose is both an important precursor in the O-antigen biosynthetic pathway and in the production of the inner core moiety of LPS (64). A defective PMM would result in incomplete LPS core production as observed in *P. aeruginosa* (22).

Like *B. abortus*, *V. cholerae* O1 produces an O-antigen homopolymer constructed of perosamine units (58). A single *rfb* operon of *V. cholerae* (16 to 19 kb) was originally thought to contain all of the genes necessary for O-antigen biosynthesis. More recent work suggests that three additional genes required for O-antigen biosynthesis are located outside the previously described *rfb* operon. Mutations in these three genes, *rfbV*, *rfbU*, and *rfbW*, result in the loss of O-antigen biosynthesis (18). Having identified the *Brucella lps* locus, we may now be able to answer questions regarding the expression of LPS. For example, what is the nature of the mechanism controlling *Brucella* phase variation (referred to as dissociation or smooth-to-rough transition)? Is it related to the mechanisms used to vary surface-exposed structures in *H. influenzae* and *Neisseria gonorrhoeae*, in which frameshifting, or single-strand mispairing, results in the loss or gain of nucleotides within repetitive sequences, causing the translation initiation codon to be out of phase with the remaining ORF (41, 62)? Consistent with this possibility, the *Brucella* genome is known to harbor repeated palindromic DNA sequences, Bru-RS1 and Bru-RS2. Bru-RS1 contains a tetramer sequence, 5′-GAAA-3′, in multiple complete or partial copies (23). Future experiments are designed to characterize the genetic organization of the *Brucella lps* locus.

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