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The Outer Membranes of *Brucella* spp. Are Resistant to Bactericidal Cationic Peptides

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The actions of polymyxin B, rabbit polymorphonuclear lysosome extracts, 14 polycationic peptides (including defensin NP-2, cecropin P1, lactoferricin B, and active peptides from cationic protein 18 and bactenecin), EDTA, and Tris on *Brucella* spp. were studied, with other gram-negative bacteria as controls. *Brucella* spp. were comparatively resistant to all of the agents listed above and bound less polymyxin B, and their outer membranes (OMs) were neither morphologically altered nor permeabilized to lysozyme by polymyxin B concentrations, although both effects were observed for controls. EDTA and peptides increased or accelerated the partition of the hydrophobic probe *N*-phenyl-naphthylamine into *Escherichia coli* and *Haemophilus influenzae* OMs but had no effect on *Brucella* OMs. Since *Brucella* and *H. influenzae* OMs are permeable to hydrophobic compounds (G. Martínez de Tejada and I. Moriyón, J. Bacteriol. 175:5273–5275, 1993), the results show that such unusual permeability is not necessarily related to resistance to polycations. Although rough (R) *B. abortus* and *B. ovis* were more resistant than the controls were, there were qualitative and quantitative differences with smooth (S) brucellae; this may explain known host range and virulence differences. *Brucella* S-lipopolysaccharides (LPSs) had reduced affinities for polycations, and insertion of *Brucella* and *Salmonella montevideo* S-LPSs into the OM of a *Brucella* R-LPS mutant increased and decreased, respectively, its resistance to cationic peptides. The results show that the core lipid A of *Brucella* LPS plays a major role in polycation resistance and that O-chain density also contributes significantly. It is proposed that the features described above contribute to *Brucella* resistance to the oxygen-independent systems of phagocytes.

Brucella spp. are gram-negative pathogens that infect both animals and humans (5). This genus includes species whose outer membranes (OMs) contain smooth lipopolysaccharides (S-LPSs) (*B. abortus*, *B. melitensis*, *B. suis*, and *B. neotomae*) as well as others (*B. ovis* and *B. canis*) that lack true O chains and are designated naturally rough (R) brucellae because of their similarities with R mutants of the S *Brucella* species (5). The survival of *Brucella* spp. within mononuclear cells has been well documented (3, 5, 32–34), and it has been shown that *B. abortus* cells elicit little respiratory burst and only reduced (34) but significant (25) levels of degranulation in polymorphonuclear (PMN) leukocytes. Moreover, Riley and Robertson (33, 34) found that compared with R-LPS *Salmonella typhimurium* mutants, both S-LPS and R-LPS *B. abortus* cells are resistant to the oxygen-independent mechanisms of PMN cells. More recently, Rasool et al. (32) found that highly purified preparations of *B. abortus* S-LPS are at least 200 times less active in stimulating PMN cells than *S. typhimurium* S-LPS preparations are, an observation that explains the reduced respiratory burst and degranulation induced by *B. abortus* (33).

The resistance of *Brucella* spp. to oxygen-independent mechanisms has not been explained on a structural basis. Oxygen-independent mechanisms include the synergistic actions of several cationic proteins and peptides which, in a first step, bind to OM anionic targets and render the cell envelope permeable and susceptible to lytic enzymes, thereby blocking cell functions that depend on membrane integrity (11, 18, 40, 45). Accordingly, the virulence of some intracellular gram-negative bacteria, such as *Salmonella* spp., is linked in part to resistance to cationic peptides (11, 41), including not only peptides from phagocytes but also bactericidal cationic peptides from other animal and bacterial sources that act first on the OM by a closely related mechanism (11, 45). In this work, we probed the OMs of S and R *Brucella* spp. with various cationic peptides and found reduced affinities for all of them, thereby providing experimental evidence for the hypothesis (20, 32) that in *Brucella* spp., resistance to oxygen-independent systems is linked to the OM and S-LPS properties. In addition, since ionic groups are the target of polycations and part of the OM barrier to hydrophobic agents, we also studied whether the unusual permeability of *Brucella* OMs to such agents (21) was necessarily related to resistance to polycations. Appropriate controls of other gram-negative bacteria were used throughout these studies.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used are listed in Table 1. *B. abortus*, *B. melitensis*, *Yersinia enterocolitica*, *Escherichia coli*, and *Salmonella montevideo* were grown in tryptic soy broth (Oxoid Ltd., London, England), and *B. ovis* and *Haemophilus influenzae* were grown in the same medium with 0.5% yeast extract or 3% nutrient supplement (Fildes; Difco Laboratories, Detroit, Mich.), respectively. Bacteria were harvested (5,000 × *g*, 15 min, 4°C) in the exponential phase of growth and for sensitivity, polymyxin B binding, and OM stability studies (see below) were resuspended immediately in the appropriate buffer. Alternatively, when sensitivity was tested according to protocol B (see below), bacteria were grown on blood (*Brucella* spp.) or MacConkey (*E. coli*) agar plates. For S-LPS extraction, bacteria were propagated in

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TABLE 1. Bacterial strains used in this study

Strain	Characteristics	Reference
B. melitensis 16M	S-LPS, virulent, biotype 1, serotype M	1
B. melitensis Ether	S-LPS, virulent, biotype 3, serotype AM	1
B. melitensis 115	R-LPS, avirulent	1
B. abortus 2308	S-LPS, virulent, biotype 1, serotype A	19
B. abortus S19	S-LPS, vaccine strain, biotype 1, serotype A	1
B. abortus B3196	S-LPS, virulent, biotype 5, serotype M	1
B. abortus 45/20	R-LPS, attenuated	33
B. abortus RB51	R-LPS, attenuated, derived from *B. abortus* 2308	37
B. ovis Reo 198	Natural R-LPS species, CO_2-independent strain	1
E. coli O111 K58H2	S-LPS, enteropathogenic, used in OM permeability studies	21
E. coli ATCC 29648	S-LPS, used in studies of sensitivity to defensins	38
E. coli K-12 ATCC W1485	R-LPS of Ra chemotype	
S. montevideo SH94	S-LPS, serogroup D1	16
H. influenzae 9193	Serovar b, clinical isolate	21
Y. enterocolitica MY79	Serotype 9, LPS O chain of *Brucella* serotype A	26

2-liter flasks (500 ml per flask) on an orbital shaker (200 rpm) and, when appropriate, inactivated with 0.5% phenol at 37°C for 24 h.

LPS preparations. The S-LPSs of *B. abortus* 2308, S19, *E. coli* serotype O111, and *S. montevideo* were used as representatives of *Brucella*- and enterobacterial-type S-LPSs. *B. abortus* S-LPS (fraction 5) was obtained from the phenol phase of a water-phenol extract and purified by treatment with chaotropic agents and detergents according to published procedures (22, 23). Alternatively, fraction 5 was dispersed (20 mg/ml) by sonication in 5 mM $MgCl_2$–10 mM Tris-HCl (pH 7.5) and digested with nucleases (20 μg each of DNase I and RNase A [Sigma Chemical Co., St. Louis, Mo.] per ml for 4 h at 37°C) and proteinase K (65 μg/ml [E. Merck, Darmstadt, Germany] for 24 h at room temperature), and S-LPS was recovered by ultracentrifugation (100,000 × *g*, 6 h, 4°C) and freeze dried. By standard analytical methods (6), these S-LPS preparations had 0.8% 2-keto-3-deoxyoctonic acid (KDO), 2.9% protein, and up to 6% free lipids distributed among ornithine lipids and the major classes of *Brucella* phospholipids (43). In some experiments, free lipids were removed by threefold extraction with water-chloroform-methanol (1:1:2). *E. coli* O111 and *S. montevideo* S-LPSs were obtained by the standard phenol-water method (6) and purified with nucleases and proteinase K as described above. These preparations had 1.5% KDO, less than 1.0% protein, and trace amounts of free lipids. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining for LPS (44), all of these preparations showed the characteristic pattern of S-LPSs.

Cationic peptides. The characteristics and reported actions of the peptides used are summarized in Table 2. Polymyxin B (sulfate), egg lysozyme, poly-L-lysine, poly-L-ornithine, cecropins, melittin, magainins, and lactoferrin were purchased from Sigma. Lactoferricin B was kindly provided by W. Bellamy (Morinaga Dairy Company, Higashihara, Japan), the cationic protein 18 (CAP18) peptide (amino acids 106 through 125) and bactenecin peptides 5 (amino acids 0 through 31) and 7 (amino acids 1 through 35) were provided by R. Gennaro and D. Romeo (Department of Biochemistry, Biophysics, and Chemistry of Macromolecules, Trieste University, Trieste, Italy), and defensin NP-2 was provided by R. I. Lehrer (Department of Medicine, University of California, Los Angeles).

Lysosomal extract from rabbit PMN leukocytes. White New Zealand rabbits weighing 2.5 kg each were sensitized by three intraperitoneal injections of 150 ml of 0.1% oyster glycogen in sterile saline at 37°C administered at weekly intervals. Four hours after the last injection, each rabbit was anesthetized, the peritoneal cavity was cannulated, and the exudate was collected in saline with 50 mg of heparin per liter. Over 90% of cells were PMN leukocytes, as judged by Giemsa staining of smears. After assessment of cell viability (trypan blue exclusion test), PMN leukocytes were suspended (10^7 to 10^8 cells per ml) in 0.34% sucrose and disrupted in a Potter-type homogenizer. The homogenate was clarified (125 × *g*, 15 min, 4°C), cytoplasmic granules were sedimented (20,000 × *g*, 30 min, 4°C), and cationic components were extracted with HCl as described previously (14).

Sensitivity assays. Depending on the amount of peptide available, bacterial sensitivity was measured as the effect of increasing concentrations of an agent on cell viability (protocol A) and/or as the percentage of cell viability after exposure to a single concentration of each agent (protocol B).

(i) Protocol A. The assay described by Riley and Robertson (34) was used with some modifications. Stock solutions of agents were prepared in sterile 0.133 M NaCl–0.1 M NaH_2PO_4 (pH 4.6) or 10 mM phosphate buffer (pH 7.0), and serial dilutions were made directly in sterile 96-well tissue culture clusters (Costar, Cambridge, Mass.) by using the same buffer as a diluent (100 μl per well). Bacteria were resuspended in the same buffer solution at approximately 4×10^4 CFU/ml, 100 μl of this suspension was dispensed in duplicated series of wells (4×10^3 CFU per well in 200 μl), and plates were incubated for 1 h at 37°C. Viable counts were performed by spreading 100 μl from each well on tryptic soy agar (Oxoid Ltd.) plates, and the results were expressed as the percentage of survival with respect to that of controls incubated without the appropriate agent (no bacterial multiplication occurred under those conditions).

TABLE 2. Origins and relevant characteristics of cationic peptides

Peptide(s)	Origin(s)	Relevant features	Reference(s)
Bactenecin	Bovine neutrophils	Amphiphilic; bactericidal for gram-negative bacteria; permeabilizes OMs and cytoplasmic membranes	10
CAP18	Rabbit PMN cells	Bactericidal; binds to LPS; increases cell permeability	13
Cecropin P1	Pig intestine	Bactericidal; forms channels in lipid bilayers	17, 39
Cecropin A	Cecropia moth hemolymph	Amphiphilic (two alpha-helices); bactericidal; forms voltage-dependent channels in lipid bilayers	2
Defensin NP-2	PMN cells	Binds to LPS; forms voltage-dependent channels in lipid bilayers	15, 38
Lactoferrin	Milk and mucosal secretions	Complex structure (alpha-helix and beta-sheet); Fe and divalent cation chelator; bactericidal; binds to OMs	9, 28, 48
Lactoferricin B		OM-binding peptide derived from lactoferrin	48
Lysozyme	Egg white	Muramidase; binds to LPS	42
Magainins 1 and 2	Frog skin and stomach	Amphiphilic alpha-helix; bactericidal; binds to LPS; forms channels in membranes	31, 45
Melittin	Bee venom	Amphiphilic (two alpha-helices); bactericidal; binds to LPS; forms voltage-dependent channels in membranes	7, 12, 45
Poly-L-lysine	Synthetic	Alpha-helix; bactericidal for gram-negative bacteria; binds to LPS	45
Poly-L-ornithine	Synthetic	Bactericidal for gram-negative bacteria	45
Polymyxin B	Bacteria	Amphiphilic lipopeptide; bactericidal; binds to LPS; produces OM blebbing	45

(ii) Protocol B. Bacteria were suspended in 10 mM phosphate buffer (pH 7.0) at approximately 4×10^6 CFU/ml, and 100-μl aliquots were mixed with 10 to 20 μg of the appropriate agent (suspended in 3 μl of deionized distilled water), and the mixture was incubated at 37°C for 20 or 180 min, depending on the agent (see footnotes to Table 3). For CFU counting, the suspension was diluted with 900 μl of buffer and 100 μl was plated out. All experiments were run in quadruplicate, and the results were expressed as the percentage of inhibition (mean ± standard deviation) of controls without the agent.

Binding of polymyxin by viable cells. Cell suspensions (4.5×10^{10} CFU/ml for *Brucella* spp. and 10^{10} CFU/ml for *E. coli* and *Y. enterocolitica* serotype O:9) were prepared in 0.133 M NaCl–0.1 M NaH_2PO_4 (pH 4.6), and serial dilutions were made in 2-ml Eppendorf-type tubes by using the same buffer solution. An equal amount of polymyxin B was added to each tube (350 U/ml of cell suspension), and after incubation for 30 min at 37°C, cells were removed by centrifugation (12,000 × *g*, 15 min). To measure unbound polymyxin B, 3-mm-diameter wells were punched on petri dishes with peptone-glucose-agar containing 10^7 CFU of *E. coli* K-12 per ml, 50-μl aliquots of supernatants were dispensed into wells, and plates were incubated overnight. The amount of polymyxin B was estimated from the diameter of the inhibition halo by comparison with polymyxin B standards on the same plate.

Assessment of OM damage. (i) Electron microscopy. Bacteria were resuspended in 10 mM phosphate-buffered saline (PBS; pH 7.2) and incubated with polymyxin B (500 U/ml) for 20 min at 37°C. Cells were sedimented (12,000 × *g*, 5 min), fixed first in 4% glutaraldehyde–0.1 M cacodylate buffer (pH 7.2) for 1 h at 4°C, washed with 2 M sucrose—50 mM cacodylate (pH 7.2), and fixed again in 1% OsO_4–100 mM cacodylate (pH 7.2) for 2 h at 4°C. The fixing solution was removed with two washes of 340 mM Veronal sodium (pH 7.4), and bacteria were stained with 0.5% uranyl acetate in the same buffer (30 min, 4°C). After two additional Veronal washes, bacteria were resuspended in molten Noble agar (Difco Labortories), the gel was included in Epon-812, and thin sections were contrasted with uranyl acetate. Observations were carried out with a Zeiss EM10CR electron microscope (Carl Zeiss Germany, Oberkochen, Germany).

(ii) Permeability to lysozyme. Exponentially growing cells were resuspended in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.2) at an optical density (at 500 nm) of 0.8, and lysozyme (50 μg/ml) and polymyxin B (100 U/ml) were added. Incubation was performed at room temperature, and cell lysis was monitored by measuring the A_{500}. Since sensitivity to lysozyme has been shown for *Brucella* spp. (8) and *E. coli*, cell lysis was taken as evidence of lysozyme uptake (46). Controls were bacterial suspensions with only lysozyme or polymyxin B or without either agent.

(iii) Fluorimetry. Exponentially growing cells were resuspended in 1 mM KCN–10 mM PBS (pH 7.2) (*Brucella* spp. and *H. influenzae*) or 1 mM KCN–10 mM HEPES (pH 7.2) (*E. coli*) at an optical density (at 600 nm) of 0.48 and transferred immediately to 1-cm-diameter fluorimetric cuvettes. After 15 to 20 s, *N*-phenyl-naphthylamine (NPN; 500 μM in acetone) was added to a final concentration of 10 μM. Peptides and EDTA were added either before or after NPN at the following final concentrations to cuvettes: EDTA, 5 mM; polymyxin B, 100 U/ml; melittin, 10 μg/ml; cecropin P1, 6.5 μg/ml; magainins 1 and 2, 30 μg/ml (each); and lysosomal extract, 20 μg of protein per ml. In preliminary experiments, the concentrations of peptides listed above were selected because they were bactericidal (see Results) and did not generate quenching. Fluorescence was monitored at 20°C with an LS-50 fluorimeter (Perkin-Elmer Ltd., Beaconsfield, England) set as follows: excitation, 350 nm; emission, 420 nm; slit width, 2.5 nm. The results were expressed in relative fluorescence units (RFU).

Binding of polymyxin B and cationic peptides by S-LPSs. Appropriate amounts of S-LPS were dispersed by ultrasonic treatment (10 W, 20 s) in deionized distilled water or 0.133 M NaCl–0.1 M NaH_2PO_4 (pH 4.6), and the solutions were clarified by brief centrifugation (12,000 × *g*, 15 s). By using those suspensions, 5 μg of the agent to be tested was mixed with 1.25 to 25.0 μg of S-LPS in a volume of 50 μl, aliquots of this mixture were dispensed into wells punched in peptone-glucose-agar plates previously inoculated with *E. coli* as described above, and plates were incubated overnight. For each agent, the effect of S-LPS was estimated as the percentage of reduction in the halo of inhibition with respect to that obtained with the corresponding agent and no LPS.

The amount of polymyxin B bound by S-LPS was measured by high-performance liquid chromatography (HPLC). Aliquots of S-LPS suspensions were incubated (10 min, 37°C) with increasing amounts of polymyxin B, and the unbound lipopeptide was separated on a TSK3000SW column (30.0 by 0.8 cm) (TosoHaas GmbH, Stuttgart, Germany) fitted to a 625 LC unit (Waters Associates, Inc., Milford, Mass.) with a Waters 486 detector set at 220 nm (previously calibrated by using polymyxin B as the standard). HPLC was performed at 0.5 ml/min in 13 mM NaCl–10 mM NaH_2PO_4 (pH 4.6).

Coating of R *B. abortus* with S-LPSs. One hundred microliters of a *B. abortus* 45/20 suspension (8×10^7 CFU/ml in 10 mM phosphate buffer [pH 7.0]) was mixed with 100 μl of a suspension (20 mg/ml) of S-LPS from either *B. abortus* S19 or *S. montevideo*, and the mixture was sonicated briefly (three pulses of 1 s). After incubation for 18 h at 40°C, cells were washed three times and resuspended in 200 μl of 10 mM phosphate buffer (pH 7.0), and 100 μl of this suspension was incubated for 20 min with 10 μg of the agent to be tested and then plated out for CFU counting. Experiments were run in quadruplicate, and the results were expressed as the percentage of inhibition (mean ± standard deviation) of controls without the agent. Insertion of S-LPS into the OM of *B. abortus* 45/20 was shown by immunofluorescence (16) with either rat monoclonal antibody to the *S. montevideo* O chain (D1 serogroup) (kindly provided by A. Weintraub, Department of Clinical Bacteriology, Karolinska Institute, Huddinge Hospital, Huddinge, Sweden) or mouse monoclonal antibody to the *B. abortus* O-chain common C/Y epitope (35) and rabbit anti-rat immunoglobulin G or anti-mouse immunoglobulin-fluorescein isothiocyanate conjugates (Sigma). Compared with that of uncoated controls, coating had no effect on the viability of *B. abortus* 45/20.

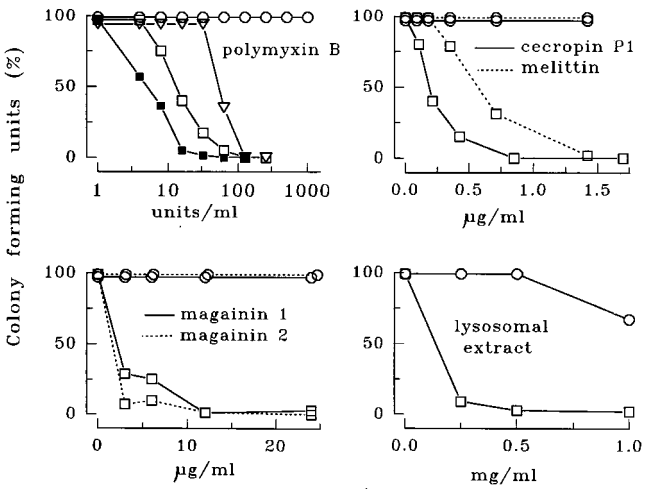

FIG. 1. Effects of polymyxin B (upper left panel), melittin and cecropin P1 (upper right panel), magainins 1 and 2 (lower left panel), and lysosomal extract (lower right panel) on the viabilities of *B. abortus* 2308 (○), *Y. enterocolitica* O:9 (▽), *E. coli* K-12 (■), and *E. coli* O111 K58H2 (□).

RESULTS

Effects of EDTA, polymyxin B, and cationic peptides on cell viability. The effects of increasing concentrations of polymyxin B on the viabilities of *B. abortus* 2308, S and R *E. coli*, and *Y. enterocolitica* O:9 are illustrated in Fig. 1. In this assay, *B. abortus* 2308 was remarkably more resistant than the controls were, and identical results were obtained with the remaining S and R *Brucella* strains within the range of concentrations tested (data not shown). It can also be seen that the *E. coli* R strain was more sensitive than the S strain was and that *B. abortus* O-chain-bearing *Y. enterocolitica* O:9 was less affected by polymyxin B than *E. coli* was at concentrations of less than 50 U/ml. Similar experiments were performed with melittin, cecropin P1, magainins, and lysosomal extract (Fig. 1), and the results resembled those obtained with polymyxin B in that *B. abortus* was resistant to peptide and lysosomal extract concentrations that were lethal for *E. coli*.

Table 3 shows the effects of a single concentration of each cationic peptide, Tris, and EDTA on the viabilities of S *B. abortus*, an R *B. abortus* mutant, and *B. ovis*. It can be seen that the three *Brucella* strains were more resistant to all of the agents tested than the *E. coli* control was, with the conspicuous exception of bactenecin 7 and *B. ovis*. This peptide reduced the growth of *E. coli* by 97%, and although it had a limited effect on S and R *B. abortus*, it showed maximal activity on *B. ovis* (100% growth reduction). The peptides that were more active on S *B. abortus* (strain S19) were polymyxin B and lactoferricin B, and although the results of both protocols were in good agreement (Fig. 1 and Table 3), protocol B also showed that the S *B. abortus* strain was more resistant than the homologous R strain was (Table 3). *B. ovis* showed a sensitivity spectrum somewhat different from that of *B. abortus*, since in addition to bactenecin 7, it was comparatively sensitive to poly-L-ornithine and poly-L-lysine. No pattern of activity that related to the

TABLE 3. Activities of cationic peptides, EDTA, and Tris against *E. coli* and S and R brucellae[a]

Agent	Activity against:			
	E. coli	*B. abortus* S19	*B. abortus* 45/20	*B. ovis* Reo 198
Bactenecin 7	97.3 ± 0.7	1.4 ± 0.8	8.6 ± 2.2	100 ± 0.0
Bactenecin 5	76.8 ± 5.2	0.6 ± 0.7	5.9 ± 1.1	11.9 ± 1.4
CAP18	91.1 ± 3.0	1.1 ± 0.8	9.9 ± 1.7	14.3 ± 2.5
Cecropin A[b]	100	0.4	0.5	0.6
Cecropin P1[b]	97.8	4.9	22.5	24.0
Defensin NP-2[c]	93.9 ± 3.7	0.6 ± 0.4	1.5 ± 0.3	ND[d]
Lactoferricin B[e]	93.9 ± 1.2	10.9 ± 1.5	23.1 ± 1.0	25.6 ± 0.5
Lactoferrin[f]	85.8 ± 1.9	0.6 ± 0.3	9.5 ± 0.7	15.6 ± 2.3
Lysozyme[g]	81.1 ± 2.5	7.0 ± 2.2	27.6 ± 1.5	30.3 ± 1.8
Magainin 1[b]	84.9	0.0	0.5	0.9
Magainin 2[b,e]	93.7	1.0	1.3	2.4
Melittin	100.0 ± 0.0	0.6 ± 0.3	23.9 ± 1.0	32.8 ± 1.5
Poly-L-lysine	99.6 ± 0.3	0.5 ± 0.3	0.95 ± 1.3	55.9 ± 2.3
Polymyxin B	100.0 ± 0.0	19.0 ± 2.1	31.6 ± 2.5	39.9 ± 2.6
Poly-L-ornithine	99.5 ± 0.4	0.9 ± 0.7	14.7 ± 3.4	57.2 ± 4.1
EDTA[g]	97.3 ± 0.8	0.9 ± 0.7	2.6 ± 1.4	6.7 ± 4.6
Tris[g]	76.6 ± 2.0	1.4 ± 1.1	2.2 ± 2.1	3.4 ± 1.5

[a] Unless indicated otherwise, activity is expressed as the percentage of inhibition (mean ± standard deviation) after exposure for 20 min to 10 μg of the indicated agent per ml.
[b] Only one assay was performed.
[c] Incubation was carried out for 90 min.
[d] ND, not done.
[e] Incubation was carried out for 180 min.
[f] Incubation was carried out for 180 min with 10 μg of the agent per ml and 10 μg of lysozyme per ml.
[g] Incubation was carried out for 180 min with 200 μg of the agent per ml.

known structural features (Table 2) of these peptides was observed.

Binding of polymyxin B by live cells. The results of polymyxin B binding experiments are presented in Fig. 2. Whereas 10^{10} CFU of *E. coli* and *Y. enterocolitica* O:9 bound over 300 U of polymyxin B, the same number of *Brucella* CFU bound less than 50 U of polymyxin B. No differences between S *B. abortus* and *B. melitensis* and their respective R mutants were observed within the range of polymyxin B concentrations tested (Fig. 2).

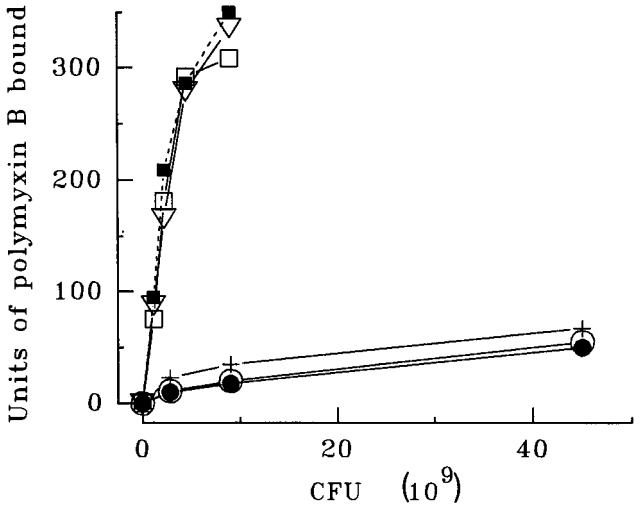

FIG. 2. Polymyxin B binding by increasing amounts of viable cells of *B. abortus* 2308 (○), *B. abortus* RB51 (●), *B. melitensis* 115 (✚), *Y. enterocolitica* O:9 (▽), *E. coli* K-12 (■), and *E. coli* O111 (□).

In these experiments, HPLC and SDS-PAGE analyses of the supernatants of cells treated with polymyxin B showed the release of large amounts of medium- to low-molecular-weight materials by *E. coli*, not *B. abortus* (data not shown).

Assessment of OM damage by EDTA, polymyxin B, and cationic peptides. Figures 3A and B are electron microscope pictures of *E. coli* O111 and *B. abortus* 2308 cells exposed to polymyxin B. Whereas this treatment induced extensive OM blebbing in the control, it had no effect on *B. abortus* 2308. Figure 3C shows that polymyxin B increased the permeability of *E. coli* O111 to lysozyme, as demonstrated by the turbidity drop (cell lysis) of the cell suspension. In contrast, the turbidity of the *B. abortus* 2308 suspension remained constant (Fig. 3C). *E. coli* K-12 and *B. abortus* RB51 also gave equivalent results; viability was 100% for *B. abortus* and less than 5% for *E. coli* at the end of this experiment.

The possibility of more subtle changes in cell surfaces was studied by examining the partition of NPN into OMs (Fig. 4), with bacteria with OM permeability properties different from (*E. coli*) and similar to (*H. influenzae*) those of *Brucella* spp. used as controls. In the absence of EDTA, polymyxin B, and cationic peptides, only a limited amount of NPN was partitioned into the OM of *E. coli* O111, as shown by the small (20 RFU) increase in fluorescence measured after NPN addition (Fig. 4, upper left panel, discontinuous line). In *H. influenzae* (Fig. 4, middle panel, discontinuous line), a progressive increase in fluorescence was observed after NPN addition, a result which is due to the accessibility of the hydrophobic pathway in this OM (21). Although the OMs of controls had almost opposite barrier properties, both were stabilized by divalent cations since EDTA caused a small but significant increase in fluorescence in *E. coli* (up to 45 RFU) (Fig. 4, upper left panel, continuous line) and an acceleration in the kinetics of NPN entry in *H. influenzae* (Fig. 4, middle left panel, continuous line). In agreement with these results, the peptides tested (polymyxin B, melittin, cecropin P1, and magainins 1 and 2) brought about a sharp increase (up to 120 RFU) in the entry of NPN into the OM of *E. coli* O111 (Fig. 4, upper right panel) and accelerated NPN uptake in *H. influenzae* (Fig. 4, middle right panel). In contrast, although NPN partitioned spontaneously into *B. abortus* OMs (Fig. 4, lower left panel, discontinuous line), the kinetics of NPN uptake into *B. abortus* 2308 OMs was not altered by either EDTA or the peptides tested (Fig. 4, lower panels, continuous lines). Additional controls performed with heat-shocked *B. abortus* 2308 showed a quick increase in fluorescence (Fig. 4, lower right panel, discontinuous line), confirming that OM damage accelerates the partition of NPN into the OM. Similar results were obtained with the remaining *B. abortus* and *B. melitensis* strains (data not shown). Finally, NPN uptake by *E. coli* O111 was promoted by lysosomal extract, particularly in combination with EDTA, but lysosomal extract had no effect on *B. abortus* 2308 under the same conditions (data not shown).

Binding of polymyxin B and cationic peptides by S-LPSs. As a first approach, the binding of peptides by S-LPSs was assessed as the reduction in peptide antibiotic activity after incubation with the S-LPS tested. At the maximal amount tested (25 μg), *S. montevideo* S-LPS caused 70 to 80% reductions in the activities of magainins, melittin, CAP18 peptide, cecropins, and polymyxin B and a 47% reduction in the activity of lactoferricin B. With *B. abortus* S-LPS, there were reductions of 66% for polymyxin B; of about 50% for cecropin P1, magainins, and melittin; and of only 15% for CAP18 peptide, cecropin A, and lactoferricin B.

Since polymyxin B was the agent with the highest affinity for *B. abortus* S-LPS, direct measurements of the amounts of poly-

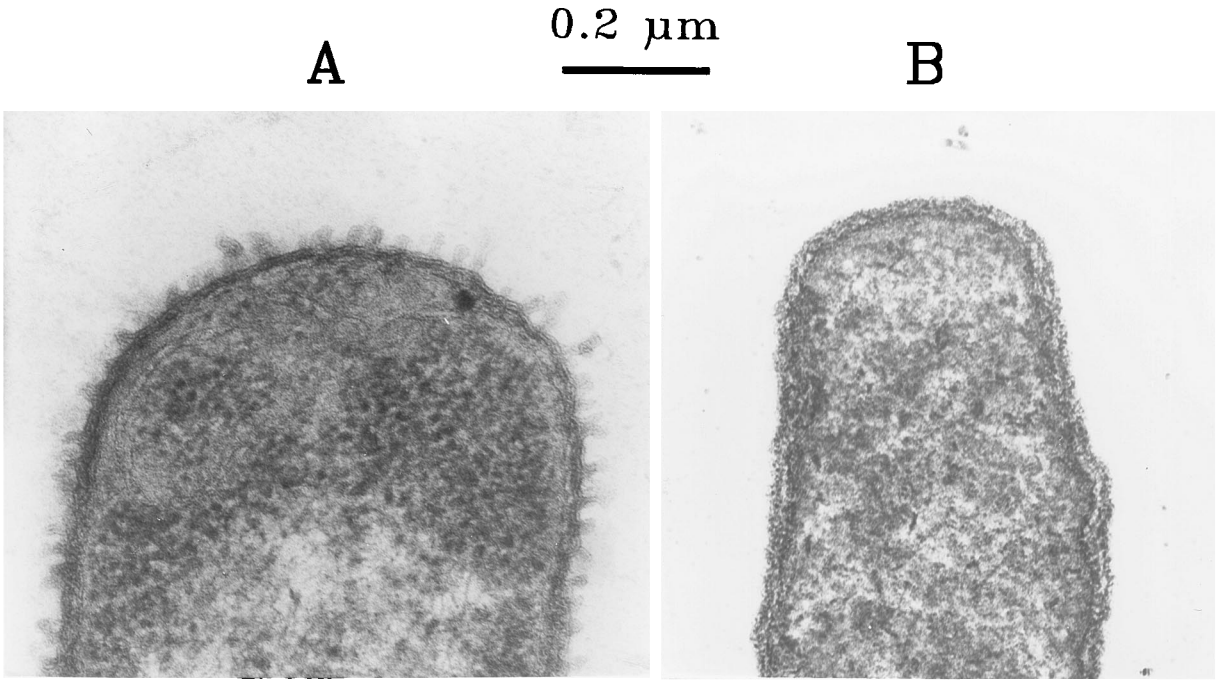

FIG. 3. Effects of polymyxin B on the OMs of *B. abortus* and *E. coli*. Electron microscope pictures of *E. coli* O111 after exposure to polymyxin B at 500 U/ml for 20 min at 37°C (A) and *B. abortus* 2308 after exposure to polymyxin B at 500 U/ml for 20 min at 37°C (B). (C) Cell lysis induced by exposure to polymyxin B (100 U/ml) and lysozyme (50 μg/ml).

myxin B bound by the S-LPSs of *B. abortus* 2308 and *E. coli* O111 were carried out by HPLC analysis of S-LPS–polymyxin B mixtures. The results showed that on the basis of either dry weight (Fig. 5A) or polymyxin B bound by nanomoles of KDO (Fig. 5B), the S-LPS of *B. abortus* 2308 bound less polymyxin B than did the S-LPS of *E. coli* O111. The results did not change substantially when the S-LPSs of *B. abortus* S19 and *S. montevideo* were used or when *B. abortus* S-LPSs were purified by different protocols. No differences were observed between *B. abortus* S-LPS preparations which contained phospholipids and ornithine lipids and those from which free lipids had been removed by solvent extraction (data not shown).

Effects of polymyxin B and cationic peptides on R *B. abortus* coated with S-LPSs. Compared with the sensitivities of S *B. abortus* and *E. coli*, R *B. abortus* 45/20 had an intermediate sensitivity to polycationic molecules (Table 3). By taking advantage of this observation, an experimental protocol was designed to test the activities (percentages of growth inhibition) of polycations on R *B. abortus* 45/20 cells coated with S-LPSs from resistant (S *B. abortus*) and sensitive (enterobacteria) bacteria (Table 4). Coating with *B. abortus* S-LPS brought about significant increases in resistance to the CAP18 peptide, lactoferricin B, melittin, polymyxin B, and poly-L-ornithine which paralleled the higher resistance observed for the S *B. abortus* strain in the same sensitivity assay (Table 3). In contrast, coating with *S. montevideo* S-LPS rendered *B. abortus* 45/20 sensitive to the agents tested (Table 4) at levels close to those observed for S-LPS enterobacteria (Table 3).

DISCUSSION

Previous studies have shown that the *Brucella* cell envelope has permeability properties (21) and sensitivities to detergents and EDTA (21, 24) that are different from those common to gram-negative bacteria, and on this basis, a relationship between *Brucella* OM properties and pathogenicity has been suggested (20, 32). In this work, *B. abortus*, *B. melitensis*, and *B. ovis* were studied for their resistance to Tris, polymyxin B, lysosomal extract, and bactericidal cationic peptides, including several (bactenecins 5 and 7, CAP18 peptide, cecropin P1, defensin NP-2, lactoferrin, and lysozyme) that are physiological in mammals. In two complementary protocols, those agents manifested their lethal actions on controls (*E. coli* and *Y. enterocolitica* O:9), but with one exception (bactenecin 7 and *B. ovis*), they showed only limited actions on *Brucella* spp. The fact that *Brucella* spp. were equally resistant to peptides with various structures and some differences in reported actions (Table 2) suggests that resistance occurs at an early step common to all of these peptides, and insensitivity to EDTA hinted at the lack of OM ionic groups for electrostatic binding. This hypothesis was tested first with polymyxin B. The results showed that both R and S *Brucella* cells bound comparatively reduced amounts of polymyxin B and that this potent lipopeptide did not cause OM morphological alterations or permeability to lysozyme under conditions in which both effects were observed for controls. Moreover, the identical kinetics of NPN

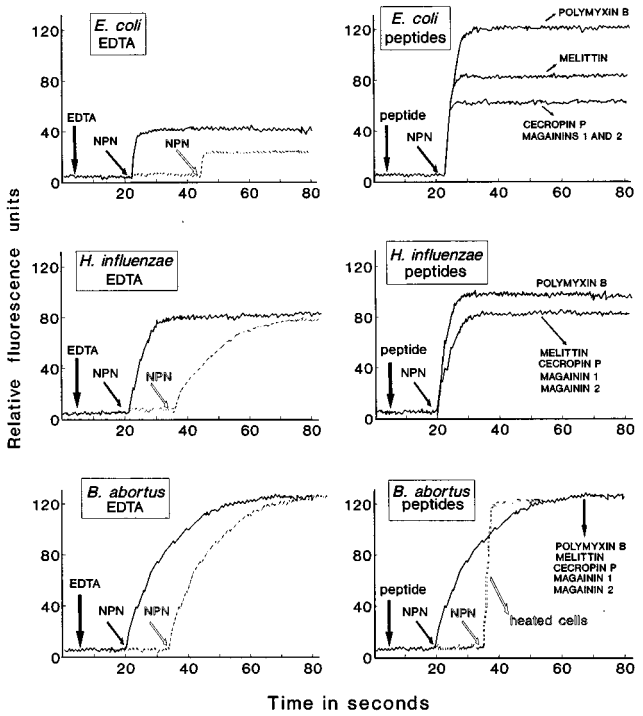

FIG. 4. Effects of EDTA, polymyxin B, and cationic peptides on the partition of NPN into the OMs of live cells of *E. coli* O111 K58H2 (upper panels), *H. influenzae* 9193 (middle panels), and *B. abortus* 2308 (lower panels). Arrows indicate the times of addition of the agent(s) listed and NPN, and fluorescence (in RFU) corresponding to polymyxin B and peptides has been plotted. The discontinuous line in the lower right panel corresponds to a control in which NPN was added to *B. abortus* 2308 cells previously damaged by heating. Discontinuous lines in all other panels are control experiments with viable cells and NPN only.

partition into the OMs of *Brucella* spp. in the absence and presence of EDTA and cationic peptides contrasted with the effects observed for the two complementary controls used (*E. coli* and *H. influenzae*). Since it is known that little changes in the OMs of gram-negative bacteria caused by cationic peptides instantaneously alter the partition of NPN into the OM (29, 30, 36, 45), the results of fluorimetric experiments extend the results obtained in binding experiments with whole cells. The fact that *H. influenzae* and *Brucella* OMs presented similar levels of permeability to hydrophobic probes but different sensitivities to EDTA and cationic peptides also means that, in general, there is no tight connection between the ionic groups which allow the electrostatic binding of cationic peptides and the impermeability of OMs to hydrophobic agents.

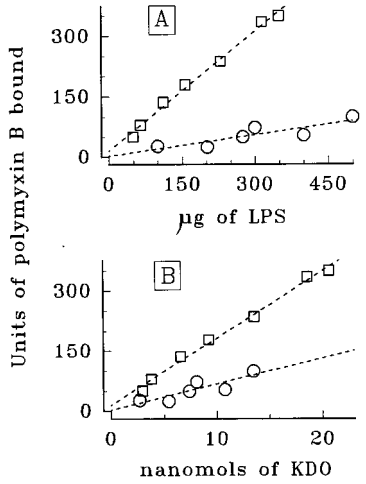

FIG. 5. Polymyxin B binding by enterobacterial-type S-LPS (*E. coli*) (□) and *Brucella*-type S-LPS (*B. abortus*) (○) according to LPS dry weight (A) and nanomoles of KDO (B).

TABLE 4. Activities of cationic peptides against R *B. abortus* 45/20 coated with S-LPSs from S *B. abortus* and *S. montevideo*[a]

Agent	Coating		
	None	*B. abortus* S19 S-LPS	*S. montevideo* S-LPS
Bactenecin 7	8.6 ± 2.2	7.4 ± 0.9	98.7 ± 1.1
CAP18	9.9 ± 1.7	7.2 ± 0.5[b]	97.8 ± 0.5
Lactoferricin B	23.1 ± 1.0	17.2 ± 0.8[c]	97.4 ± 2.3
Melittin	31.6 ± 2.5	16.4 ± 1.1[c]	99.1 ± 0.4
Polymyxin B	23.9 ± 1.0	8.9 ± 0.7[c]	99.1 ± 0.9
Poly-L-ornithine	14.7 ± 3.4	11.1 ± 2.0[d]	98.1 ± 1.4

[a] See Table 3, footnote *a*.
[b] Significantly different from the results for uncoated controls ($P < 0.01$).
[c] Significantly different from the results for uncoated controls ($P < 0.001$).
[d] Significantly different from the results for uncoated controls ($P < 0.02$).

In other gram-negative bacteria, LPS is the major target of several cationic peptides (Table 2); therefore, it is not surprising that LPS plays a role in the resistance of *Brucella* spp. to polycationic compounds at both the O-chain and core lipid A levels. A role for the O chain can be postulated since S *B. abortus* was more resistant than R *B. abortus* was and since coating the latter with homologous S-LPS increased resistance. Obviously, this could account in part for the well-known, yet unexplained loss of virulence in R *Brucella* mutants (5). An overall anionic charge on the surface of R *B. abortus* has previously been shown (47); as in other gram-negative bacteria (4, 27, 41), the O polysaccharide could simply mask inner anionic sites. In addition, the composition of *Brucella* O polysaccharide (a homopolymer of *N*-formyl-perosamine [26]) could enhance the effect of polysaccharide length, since *B. abortus* O-chain-bearing *Y. enterocolitica* O:9 (26) was less affected by subinhibitory polymyxin B concentrations than *E. coli* O111 was. A major role for the core lipid A of *Brucella* LPS is demonstrated by the resistance of R *Brucella* mutants and *B. ovis* and by binding experiments with purified LPSs. Since phosphate and KDO are the LPS sites with affinities for polymyxin B and cations (41, 45) and the core lipid A of *Brucella* LPS contains reduced amounts of KDO (23), this could account in part for comparatively reduced affinity. Moreover, the fact that the ratio of bound polymyxin B to nanomoles of KDO for *E. coli* and *S. montevideo* was higher than that for *B. abortus* suggests the unavailability on *Brucella* LPS of anionic binding sites other than KDO, perhaps in relation to the absence of phosphate in lipid A (23). The shielding of LPS anionic groups by cationic ornithine lipids has also been suggested as a mechanism of resistance (20), and although we found that S-LPS purified of lipids had the same reduced affinity for cationic peptides, such shielding could be an additional resistance factor in an intact OM.

The results of this research demonstrate that the OMs of *Brucella* spp. have properties that are helpful in withstanding the actions of relevant components of the oxygen-independent systems of phagocytes. Moreover, they suggest subtle differ-

ences among *Brucella* species. In a previous study, we found differences in the kinetics of NPN partition into the OM between *B. ovis* on one hand and *B. melitensis* and *B. abortus* on the other (21) that could be related to their respective LPS structures, and in this work, we observed that *B. ovis* was extremely sensitive to bovine bactenecin 7. Unfortunately, little is known of the structure of either bovine bactenecin 7 or *B. ovis* LPS. However, since *B. ovis* infections are restricted to sheep (5), it is tempting to speculate that differences in OM and host defense peptides account in part for known differences in virulence and host range among *Brucella* species (5).

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