INFECTION AND IMMUNITY, Nov. 1999, p. 6181–6186 Vol. 67, No. 11
0019-9567/99/$04.00+0

The Outer Membrane of *Brucella ovis* Shows Increased Permeability to Hydrophobic Probes and Is More Susceptible to Cationic Peptides than Are the Outer Membranes of Mutant Rough *Brucella abortus* Strains

ENRIQUE FREER,[1] JAVIER PIZARRO-CERDÁ,[2] ANDREJ WEINTRAUB,[3] JOSÉ-ANTONIO BENGOECHEA,[4] IGNACIO MORIYÓN,[4] KJELL HULTENBY,[5] JEAN-PIERRE GORVEL,[2] AND EDGARDO MORENO[6]*

Unidad de Microscopía Electrónica, Universidad de Costa Rica, San José,[1] and Programa de Investigación en Enfermedades Tropicales, Escuela de Medicina Veterinaria, Universidad Nacional, Heredia,[6] Costa Rica; Centre d'Immunologie de Marseille-Luminy, Marseille-Luminy, France[2]; Department of Immunology, Microbiology, Pathology and Infectious Diseases, Division of Clinical Bacteriology, Karolinska Institute,[3] and Clinical Research Center,[5] Huddinge University Hospital, Huddinge, Sweden; and Departamento de Microbiología, Universidad de Navarra, Pamplona, Spain[4]

Received 19 April 1999/Returned for modification 23 June 1999/Accepted 2 September 1999

The permeability of the outer membrane (OM) to hydrophobic probes and its susceptibility to bactericidal cationic peptides were investigated for natural rough *Brucella ovis* and for mutant rough *Brucella abortus* strains. The OM of *B. ovis* displayed an abrupt and faster kinetic profile than rough *B. abortus* during the uptake of the hydrophobic probe *N*-phenyl-naphthylamine. *B. ovis* was more sensitive than rough *B. abortus* to the action of cationic peptides. Bactenecins 5 and 7 induced morphological alterations on the OMs of both rough *Brucella* strains. *B. ovis* lipopolysaccharide (LPS) captured considerably more polymyxin B than LPSs from both rough and smooth *B. abortus* strains. Polymyxin B, poly-L-lysine, and poly-L-ornithine produced a thick coating on the surfaces of both strains, which was more evident in *B. ovis* than in rough *B. abortus*. The distinct functional properties of the OMs of these two rough strains correlate with some structural differences of their OMs and with their different biological behaviors in animals and culture cells.

The genus *Brucella* is a gram-negative, facultative, intracellular pathogen that produces disease in a large number of mammals, including humans (8). The outer membrane (OM) of *Brucella*, which has been implicated in the virulence of the species, is unique in many respects. It has been demonstrated, for instance, that this layer is permeable to hydrophobic permeants and is considerably more resistant to bactericidal cationic peptides than most gram-negative bacteria (9, 15, 16). In smooth pathogenic *Brucella* species (*Brucella abortus*, *Brucella melitensis*, and *Brucella suis*), the polysaccharide O chain of the lipopolysaccharide (LPS) has been implicated as a virulence factor. This proposition is based on the observation that smooth *Brucella* survives and replicates in animals and in cultured macrophages more efficiently than rough mutants, and on the increased susceptibility of rough strains to microbicidal cationic peptides relative to that of their smooth counterparts (14, 16, 31).

Brucella ovis and *Brucella canis*, pathogenic for rams and dogs, respectively, do not possess O-chain polysaccharides; their LPSs react with antibodies against core epitopes specific to the genus and are lysed by the R/C phages specific for rough *Brucella* strains (3, 5, 8). Due to the fact that these properties are shared by mutants derived from smooth strains, these two *Brucella* species have been regarded as natural rough variants (8). Although *B. ovis* has been observed within trophoblasts of placentomes from infected sheep (17), this species attaches in lower numbers to epithelial HeLa cells than the rough mutants *B. abortus* 45/20 and *B. abortus* RB51 (22, 27). *B. abortus*, moreover, is able to proliferate inside nonprofessional phagocytes and induce loss of viability of the infected cells, while *B. ovis* is readily destroyed within lysosomes of these cells and does not induce cellular death (22). Since the differences observed between the natural and mutant rough *Brucella* strains cannot be due to their roughness, we have asked whether this distinct behavior may be correlated with different OM properties displayed by these strains. For this purpose, we have com-

TABLE 1. Minimal lethal concentrations of cationic peptides needed to inhibit the growth of various *Brucella* strains in agar plates

Peptide	Minimum lethal concn (μg/ml)		
	B. abortus S19	*B. abortus* 45/20	*B. ovis* REO 198
Bactenecin 7	4.25 ± 1.08	0.72 ± 0.02	0.45 ± 0.06[a]
Bactenecin 5	24.37 ± 2.57	4.05 ± 0.48	0.07 ± 0.00[a]
Polymyxin B	0.36 ± 0.02	0.13 ± 0.01	0.12 ± 0.01
Lactoferricin B	>95	43.72 ± 2.14	1.63 ± 0.18[a]
Cationic protein 18A	2.85 ± 0.07	1.40 ± 0.06	1.18 ± 0.03[b]
Melittin	1.67 ± 0.12	1.56 ± 0.19	1.05 ± 0.11[b]
Poly-L-lysine	>95	23.67 ± 0.23	12.69 ± 2.45[a]
Poly-L-ornithine	>95	90.03 ± 0.82	66.17 ± 14.83[c]

[a] Significantly different from the results obtained with *B. abortus* 45/20 ($P < 0.001$).
[b] Significantly different from the results obtained with *B. abortus* 45/20 ($P < 0.02$).
[c] Significantly different from the results obtained with *B. abortus* 45/20 ($P < 0.01$).

* Corresponding author. Mailing address: Programa de Investigación en Enfermedades Tropicales (PIET), Escuela de Medicina Veterinaria, Universidad Nacional, 304-3000 Heredia, Costa Rica. Phone: 506-2380761. Fax: 506-2381298. E-mail: emoreno@ns.medvet.una.ac.cr.

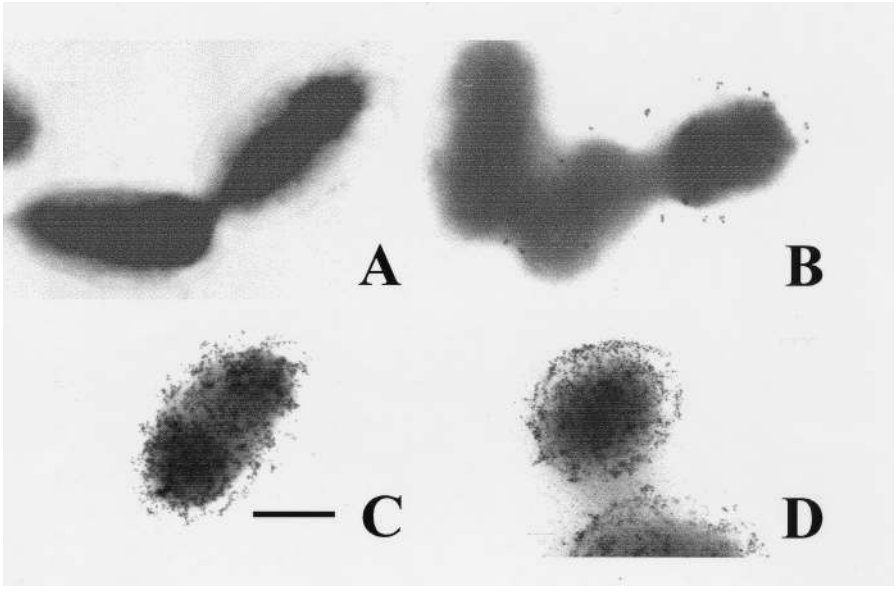


FIG. 1. Immunogold detection of O chain (C/Y) and core polysaccharide epitopes (R) in the surface of rough *Brucella*. (A) *B. ovis* REO 198 stained with anti-O-chain (C/Y epitope) monoclonal antibody and anti-mouse immunoglobulin gold (5 nm) conjugate; (B) *B. abortus* 45/20 stained with anti-O-chain (C/Y epitope) monoclonal antibody and anti-mouse immunoglobulin gold (5 nm) conjugate; (C) *B. ovis* REO 198 stained with anti-R (R1 epitope) monoclonal antibody and anti-mouse immunoglobulin gold (5 nm) conjugate; (D) *B. abortus* 45/20 stained with anti-R (R1 epitope) monoclonal antibody and anti-mouse immunoglobulin gold (5 nm) conjugate. Bar, 0.125 μm.

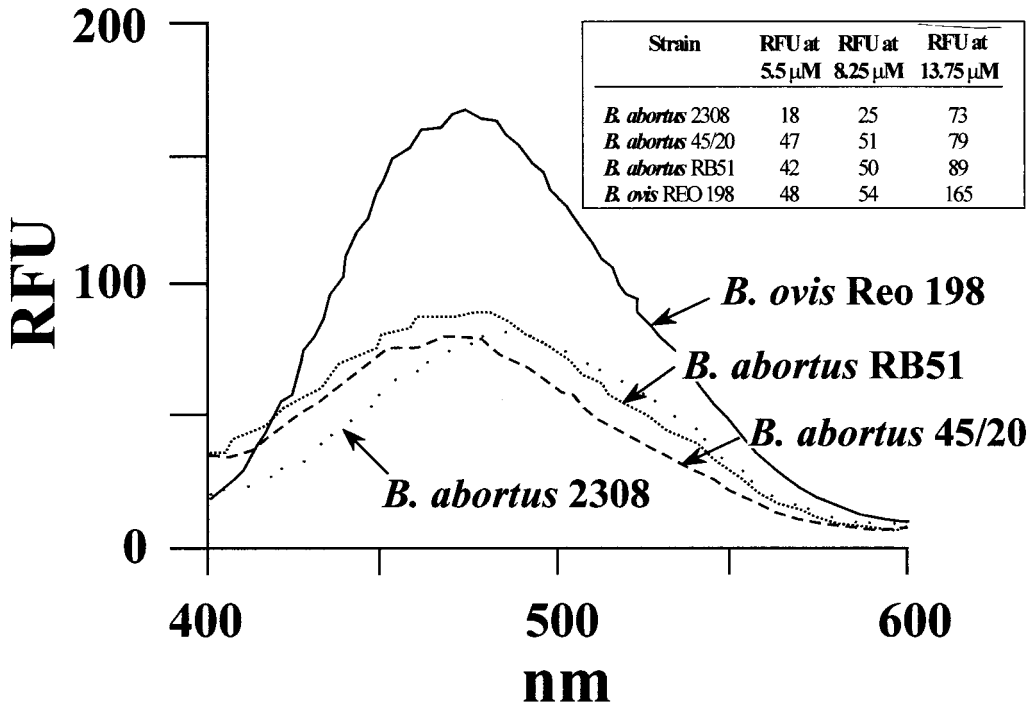
pared the permeabilities of the OM, the susceptibilities to bactericidal cationic peptides, and the levels of binding of the LPS to polymyxin B for mutant rough *B. abortus* strains and natural rough *B. ovis*.

The growth conditions of the bacterial strains used, as well as the purification and characterization of their LPS molecules, have been described elsewhere (6, 10, 15, 16, 18). Briefly, smooth *B. abortus* S19, rough *B. abortus* 45/20, and rough *B. ovis* REO 198 (CO_2 independent) strains were originally obtained from Lois Jones (University of Wisconsin, Madison, Wis.). Attenuated rough *B. abortus* RB51 was obtained from Gerhardt Schurig (Virginia Polytechnic Institute and State University). Smooth *Salmonella montevideo* SH94 serogroup D1 is maintained as part of the collection of the Division of Clinical Bacteriology, Karolinska Institute, Huddinge, Sweden. All strains are maintained in lyophilized stocks at the Veterinary School (National University, Heredia, Costa Rica, and University of Navarra, Pamplona, Spain) and have been demonstrated to be stable strains throughout the years, without detectable phenotypic or biochemical changes (2, 9, 10, 15, 16, 18, 25). *B. abortus* and *S. montevideo* strains were propagated in tryptic soy broth, and *B. ovis* was propagated in the same medium with 0.5% yeast extract (Difco Laboratories, Detroit, Mich.). Bacteria were harvested (5,000 × *g* for 15 min at 4°C) in the exponential phase of growth. Attenuated *B. abortus* S19 is phenotypically a smooth strain with 90% smooth-type LPS, 10% rough-type LPS (10), and a considerable quantity of surface native hapten (NH) polysaccharide (2, 25). The biological, chemical, and physical characteristics of *B. abortus* S19 LPS are indistinguishable from those of preparations isolated from virulent strains (10). Attenuated *B. abortus* 45/20 strain contains more than 99% rough-type LPS and a small number of lipid-bound O-polysaccharide-containing molecules (4, 10, 26), which has been estimated by immunogold electron microscopy with monoclonal antibody against epitope C/Y of the O chain (9) to be from 0 to 12 molecules per cell (Fig. 1). This bacteria does not contain any detectable NH (18). *B. ovis* REO 198 does not possess O chain or NH as demonstrated by immunogold detection (Fig. 1) and by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) analysis (18, 25). The rough-type LPSs of *Brucella* strains demonstrate extensive immunological cross-reactivity and similar chemical and physical properties (18, 25). Cationic protein 18A, bactenecin 5, and bactenecin 7 were provided by R. Gennaro and D. Romeo (Department of Biochemistry, Biophysics and Chemistry of Macromolecules, Trieste University, Trieste, Italy) or synthesized by Chiron Mimotopes Pty. Ltd. (Victoria, Australia). The polymyxin B sulfate, dansyl-polymyxin B, melittin, poly-L-lysine, and poly-L-ornithine were purchased from Sigma Chemical Co. (St. Louis, Mo.). Lactoferricin B was provided by W. Bellamy (Morinaga Dairy Company, Higashihara, Japan). The bactericidal sensitivity assays (results expressed as either the number of viable CFU or the diameters of bactericidal halos in agar

TABLE 2. Reduction of the bactericidal activity against *Escherichia coli* ATCC 29648 after adsorption of the peptides with live *Brucella* or *Salmonella* strains[a]

Peptide	% Reduction in bactericidal activity			
	B. abortus S19	*B. abortus* 45/20	*B. ovis* REO 198	*S. montevideo*
Bactenecin 5	3 ± 0.25	34 ± 4.00	34 ± 1.00	16 ± 1.10
Bactenecin 7	5 ± 0.25	20 ± 2.50	40 ± 5.00	55 ± 2.00
Poly-L-lysine	3 ± 0.25	20 ± 2.50	20 ± 2.50	30 ± 2.50
Poly-L-ornithine	12 ± 1.00	20 ± 2.50	30 ± 2.00	40 ± 3.00
Melittin	17 ± 1.40	41 ± 4.00	53 ± 3.10	41 ± 2.15
Lactoferricin B	5 ± 1.25	25 ± 2.00	50 ± 2.50	60 ± 3.00
Polymyxin B	27 ± 1.5	47 ± 1.50	60 ± 3.50	71 ± 3.50

[a] Ten micrograms of the peptide to be tested was mixed with 10^{10} bacterial cells in a 0.1-ml volume. The mixture was centrifuged and 15 μl of the supernatant was dispensed into wells punched in peptone-glucose-agar plates previously inoculated with *E. coli* ATCC 29648 as target cells, as described previously (9).



Strain	RFU at 5.5 μM	RFU at 8.25 μM	RFU at 13.75 μM
B. abortus 2308	18	25	73
B. abortus 45/20	47	51	79
B. abortus RB51	42	50	89
B. ovis REO 198	48	54	165

FIG. 2. Binding of polymyxin B by LPSs from different *Brucella* strains. LPS suspensions adjusted to 60 to 63 nM concentrations of lipid A were incubated with a 13.75 μM concentration of dansyl-polymyxin B, and the fluorescence was estimated at a range of 400 to 600 nm. The inserted table shows the binding of different concentrations of dansyl-polymyxin B of the different LPSs at 480 nm. Dansyl-polymyxin B alone did not produce detectable fluorescence under these conditions. RFU, relative fluorescence units.

plates) and the method for adsorption of the peptides to different bacteria (reported as the reduction of bactericidal halos in agar plates) were performed as described previously (9). Fluorimetric assays of peptide-treated bacteria with *N*-phenylnaphthylamine (NPN) as the fluorescent probe were performed as reported by Martínez de Tejada and Moriyón (15). Binding of polymyxin B to LPSs isolated from rough and smooth *Brucella* strains was estimated by fluorometric analysis. Briefly, LPS suspensions (yielding concentrations of 60 to 63 nM lipid A in 2.5 mM HEPES, pH 7.2, and prepared by sonic

TABLE 3. Invasiveness and OM properties of rough *B. abortus* and *B. ovis*

Characteristic	*B. abortus* S19[a]	*B. abortus* 45/20	*B. abortus* RB51	*B. ovis* REO 198	Reference
Invasiveness to host and cells[b]					
Ovine infection	Moderate	Moderate	Moderate	Severe	8, 12, 17
Bovine infection	Moderate	Moderate	Moderate	None	1, 8, 21
No. (%) of bacterium-associated cells after 2 h	75	98	100	12	22, 23, 27
No. of adherent bacteria per cell after 2 h	4	40	38	4	22, 23, 27
No. of intracellular bacteria per cell after 2 h	1–2	20	18	0–1	22, 23, 27
No. of CFU within cells at 48 h	5×10^5	6.2×10^6	ND[c]	0	22, 23, 27
No. (%) of infected cells dead at 48 h	0	73	ND	0	22, 23
Bacteria outside lysosomes	+	++	ND	−	22, 23, 27
Skin reaction of *B. melitensis*-infected animals	Positive	Positive	ND	Negative	13, 26
OM properties					
Reactivity with polyclonal anti-*B. canis* R-LPS	Total	Total	Total	Partial	18, unpublished data
Access to surface R epitopes	+	++++	++++	++++	4
Estimated no. of anti-O chain gold conjugate particles per bacterial cell	3,000–5,000	0–12	0	0	4, 10, 26, this work
Heterogeneity of R-LPS in SDS-PAGE (no. of bands)	1	4	3	1	10, 26
OM protein 25	Complete	Complete	Complete	Truncated	4, 7
OM protein 31	Absent	Absent	Absent	Present	4, 29
Sensitivity to hydrophobic antibiotics	+	++	ND	++++	15
Maximal uptake of NPN (s)	200	200	200	10	This work
Binding of polymyxin B by LPS	+	++	++	++++	16, this work
Sensitivity to cationic peptides	+	++	++	++++	16, this work

[a] This is an attenuated vaccine strain. Virulent *B. abortus* 2308 induces severe infections, penetrates cells at an efficiency of close to 100%, evades lysosomes, and replicates within cells (23). LPS preparations from smooth *Brucella* contain close to 10% of rough-type LPS displayed in a single band in SDS-PAGE (10). −, absent; +, present in <25%; ++, present in >25% to <50%; ++++, present in >75%.

[b] Tested in human epithelial HeLa cells.

[c] ND, not done with RB51.

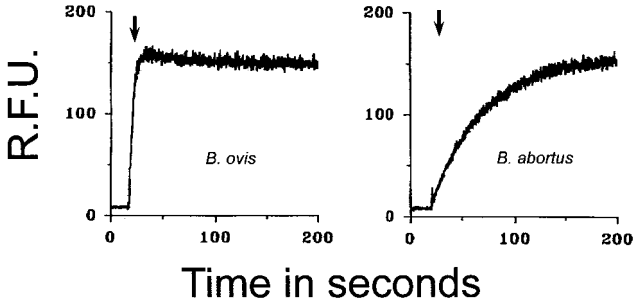


FIG. 3. Fluorimetry assays of bacteria (*B. ovis* and *B. abortus* 45/20 or RB51) treated with bactenecins 5 and 7, lactoferricin B, or polymyxin B. The NPN uptake profiles of *B. abortus* 45/20 and *B. abortus* RB51 are practically the same. The arrow indicates the time of addition of NPN or NPN and the peptide. No effect was observed when NPN or NPN plus peptide was added. RFU, relative fluorescence units.

dispersion) were incubated with different concentrations (5.5, 8.25, and 13.75 μM) of dansyl-polymyxin B. The fluorescence was estimated at room temperature under conditions of excitation at 340 nm, with an LS-50 fluorimeter (Perkin-Elmer Ltd., Beaconsfield, England) with a slit width (for both excitation and emission) of 2.5 nm and a range of 400 to 600 nm. Bacterial cell damage was evaluated by observation of the peptide-treated bacteria on a Hitachi 1100 transmission electron microscope (Hitachi Scientific Instruments, Mountain View, Calif.) operating at 100 kV as previously described (9). Experiments were performed in quadruplicate, and the results were expressed either as the percentage in the reduction of the bactericidal activity inhibition or the lethal concentration of the peptides in micrograms per milliliter (mean ± standard deviation) with respect to the control. Both the Student *t* test and variance analysis were performed for statistical examination.

Just as the reduction of CFU showed the bactericidal action of cationic peptides in various *Brucella* strains (9, 11, 16), the MICs showed that the rough *B. abortus* 45/20 and RB51 strains were more susceptible than their smooth counterparts, although *B. abortus* displayed more resistance than *B. ovis* (Table 1). The results of the adsorption experiments were consistent with those of the bactericidal assays in that *B. ovis* cells adsorbed more peptide than did the rough *B. abortus* cells (Table 2). Moreover, *B. ovis* LPS bound considerably more polymyxin B than did LPSs from both rough and smooth *B. abortus* strains (Fig. 2). As with the binding of cationic peptides by *Brucella* cells, LPS molecules from rough strains captured more polymyxin B than LPS from smooth *B. abortus*, as demonstrated by the increased fluorescence at low concentrations of dansyl-polymyxin B (Fig. 2). At higher concentrations, quenching due to turbidity of the dansyl-polymyxin B-LPS suspensions was observed for *B. abortus* 45/20 LPS (optical density [OD] at 400 nm = 0.723), and to a lesser extent for *B. abortus* RB51 (OD at 400 nm = 0.357). Little quenching was observed with *B. ovis* (OD at 400 nm = 0.288) and smooth *B. abortus* (OD at 400 nm = 0.157) LPSs. These results are consistent with micelle size and solubility of rough *B. abortus* LPS with respect to those of LPS from smooth strains (18).

In previous investigations it was observed that, in contrast to enterobacterial OM, the *Brucella* spp. OMs were not barriers to hydrophobic permeants and that bactericidal cationic peptides did not alter the kinetics during the entrance of the hydrophobic probe NPN (9, 15). Figure 3 shows that the NPN partition kinetics in the OM of *B. ovis* differs considerably from that in the OM of *B. abortus*. None of the peptides tested altered the partition of NPN in the OM of *Brucella* strains, although the abrupt partition of NPN in the *B. ovis* OM makes it difficult to evaluate the action of the peptides in this bacterium.

Transmission electron microscopy demonstrated that the effects induced by bactenecins 5 and 7 were more evident in *S. montevideo* than they were in rough *Brucella* (Fig. 4 and 5). The effects of bactenecins 5 and 7 on rough *B. abortus* and on *B. ovis* appeared to be similar (Fig. 4). The morphological changes observed in peptide-treated *Brucella* were mild and evident only at high concentrations of the bactericidal agents. These changes were characterized by detachment of the internal membrane, vacuolization, and the appearance of electron-dense bodies within the cytoplasm. Most of the time, the OM maintained its appearance. Poly-L amino acids produced a thick coating on rough *B. abortus* and *B. ovis*, being more conspicuous in the latter than in the former bacteria (Fig. 4). This phenomenon hampered the infiltration of the resin, making observation difficult. In spite of this problem, minimal morphological effects were observed in the *Brucella* OM treated with poly-L amino acids. Some spotted precipitation

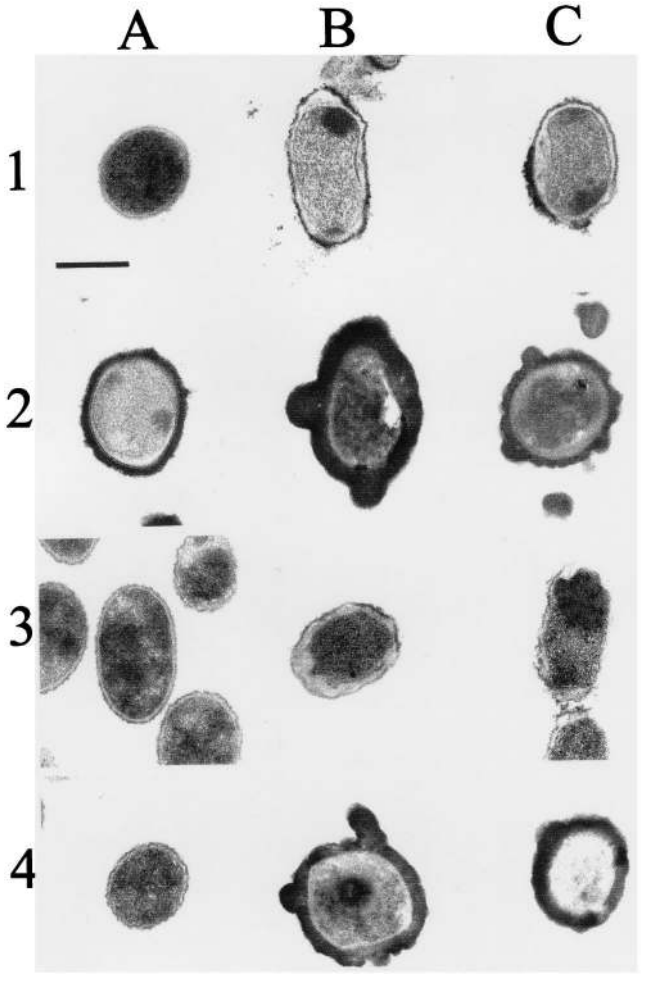


FIG. 4. Transmission electron microscopy of peptide-treated rough *Brucella*. Rows 1 and 2 show *B. ovis* REO 198, and rows 3 and 4 show rough *B. abortus* 45/20. (A) Untreated controls (rows 1 and 3) and bacteria treated with 20 μg of polymyxin B (rows 2 and 4); (B) bacteria treated with 20 μg of bactenecin 5 or 20 μg of poly-L-lysine (rows 2 and 4); (C) bacteria treated with 20 μg of bactenecin 7 (rows 1 and 3) or 20 μg of poly-L-ornithine (rows 2 and 4). Bar, 0.25 μm.

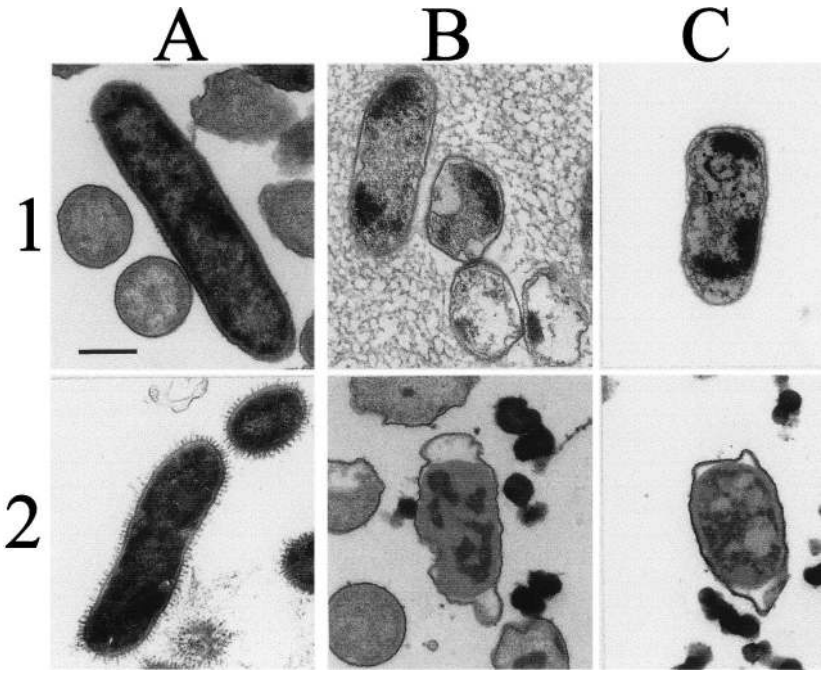


FIG. 5. Transmission electron microscopy of peptide-treated smooth *Salmonella*. Row 1 shows the following: untreated *S. montevideo* SH94 (A) and *Salmonella* treated with 10 μg of bactenecin 5 (B) or 10 μg of bactenecin 7 (C). Row 2 shows results of treatment with 10 μg of polymyxin B (A), 10 μg of poly-L-lysine (B), or 10 μg of poly-L-ornithine (C). Bar, 0.25 μm.

and vacuolization of the cytoplasm was evident; however, the significance of these was unclear. In contrast, poly-L amino acids produced severe damage on *S. montevideo* cells, characterized by severe vacuolization, formation of cytoplasmic electron-dense bodies, and distortion of the cell shape (Fig. 5). These effects were more evident in the bacterial poles. Treatment with a dose of polymyxin B lethal for *S. montevideo* (Fig. 5) did not affect the OM structure of the rough *Brucella* strains. The use of large quantities of polymyxin B resulted in the deposition of an electron-dense layer on the surface of *B. ovis* with little detectable cell damage (Fig. 4), an effect that may be related to the augmented binding of polymyxin B by the LPS of this rough *Brucella* (Fig. 2). Control experiments showed that insoluble polymyxin B in agarose was stained with uranyl and osmium salts in a pattern similar to that of the electron-dense coat observed around polymyxin B-treated *B. ovis* (data not shown). No morphological differences were observed between smooth and rough *B. abortus* control strains.

The OM structure of *Brucella* organisms differ from that of other pathogenic groups (6, 15, 16, 19), including several facultative intracellular bacteria such as *Salmonella* or *Shigella* spp. However, within the genus *Brucella*, which is a close monophyletic bacterial group (28), most of the OM components have been found to be very similar (19). Some of the structural features of the *Brucella* OM correlate well with the observed permeability of the *Brucella* OM to hydrophobic compounds (9, 15), and with the resistance of these bacteria to EDTA, Tris, some detergents (20), and oxygen-dependent and -independent killing mechanisms (13, 16, 24). Features which have been implicated in this resistance are the O chain (linked to the core lipid A) and the NH, both being characteristic of smooth *Brucella* strains (2, 9). Since the resistance of *B. ovis* and rough *B. abortus* strains to cationic peptides has been demonstrated to be lower than that displayed by the smooth *Brucella*, we concluded that the lack of any O chain in the LPS and the absence of NH are factors which contribute to the sensitivity of the OM. It is unlikely that the small amounts of O chains present on the surface of *Brucella* 45/20 account for this difference. Findings supporting this proposition include the almost identical NPN kinetics displayed by the rough *B. abortus* RB51 (which does not possess any O chain) and the *B. abortus* 45/20 cells, and the similar levels of binding of polymixin B of their LPSs (Fig. 2 and 3). Other OM characteristics may also participate in this susceptibility, however, since the pathogenic *B. ovis* is more affected by the peptides than the rough *B. abortus*. In this respect, it has been demonstrated that naturally occurring rough *Brucella* strains expose a uniform set of anionic sites on their surfaces, which are easily visualized with positively charged probes (30). Although the peptides did not have any clear effect on the uptake of NPN in the *Brucella* strains, the partition of this hydrophobic probe on the OM of *B. ovis* was strikingly different in smooth and rough *B. abortus* mutant strains (Fig. 3 and reference 15). The sudden uptake of NPN by the OM of *B. ovis* marked a definitive difference between this species and the other *Brucella* spp. In conclusion, the higher susceptibility of *B. ovis* to cationic peptides, the increased uptake of hydrophobic probes, the augmented polymyxin binding of its LPS, and the distinct structural properties of its OM correlate with the different biological behavior of this rough strain in animal hosts and culture cells (Table 3). We must be cautious, however, since other factors not necessarily related to the structure of the OM may also participate in the host preferences of *B. ovis*, the most differentiated species among the genus *Brucella* (19).

This work was supported in part by grants from the Karolinska International Research Training (KIRT) Program of the Swedish Agency for Research Co-operation with Developing Countries (SAREC/SIDA), the Centre National de la Recherche Scientifique (PICS), and the Institut National de la Santé et de la Recherche

Médicale, Nord-Sud, France; the Dirección General de Investigación Científica y Tecnológica, Madrid, Spain (BIO96-1398-C02-01) and Fundación CR-USA, San José, Costa Rica (R-215-99).

We are grateful to Reynaldo Pereira for his expert assistance in electron microscopy and Daphnne Garita for her unfaltering technical assistance.

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Editor: S. H. E. Kaufmann