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The Outer Membranes of *Brucella* spp. Are Not Barriers to Hydrophobic Permeants

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The patterns of susceptibility to hydrophobic and hydrophilic drugs and the uptake of the fluorescent probe *N*-phenyl-naphthylamine in *Brucella* spp., *Haemophilus influenzae*, *Escherichia coli*, and deep rough *Salmonella minnesota* mutants were compared. The results show that the outer membranes of smooth and naturally rough *Brucella* spp. do not represent barriers to hydrophobic permeants and that this absence of a barrier relates at least in part to the properties of *Brucella* lipopolysaccharide.

Gram-negative bacteria have a cell envelope with a cytoplasmic membrane, a periplasmic compartment, and an outer membrane (OM). The OM contains phospholipids, proteins, and a characteristic lipopolysaccharide (LPS) located in the outer leaflet. The distribution and molecular properties of LPS, phospholipids, and porin proteins play a key role in both the protection against some harmful agents and the access of substances to the periplasmic space and transport systems (14). Hydrophilic solutes of appropriate molecular characteristics penetrate the OM through porins, and harmful agents that bind to lipid A of the LPS weaken the OM barrier and penetrate via the self-promoted pathway (6, 14). Moreover, uptake of hydrophobic substances can happen by partition into the lipid phase of the OM, but in most gram-negative bacteria this hydrophobic pathway is not accessible (6, 7, 14). The few exceptions described include the genera with O-chain-lacking LPSs (i.e., *Neisseria* and *Haemophilus*) (6–8) and some deep rough mutants of enteric bacteria (14).

Brucella spp. are facultative intracellular pathogens of both humans and animals. The genus includes species with typical smooth LPS (*Brucella abortus*, *B. melitensis*, *B. suis*, and *B. neotomae*) along with others (*B. ovis* and *B. canis*) that lack the O chain and that are usually designated naturally rough brucellae because of their similarity with the true rough mutants (3). Porins in *Brucella* spp. have been characterized and shown to have sieving properties similar to those of *Escherichia coli* OmpF (4), but evidence for other uptake pathways is only indirect (4, 10). Here, we present evidence for an accessible hydrophobic pathway in the OMs of *B. abortus*, *B. melitensis*, *B. suis*, and *B. ovis*.

The susceptibilities to hydrophilic and hydrophobic drugs of *B. abortus* 2308 (smooth, biotype 1, and virulent), *B. ovis* REO 198 (avirulent and CO_2 independent), *E. coli* O111 K58 H2, *Haemophilus influenzae* 9193 (a clinical isolate of serovar b), and rough *Salmonella minnesota* Rd_1p^+ and Re595 (heptoseless) (1) were examined by standard procedures. The drugs used were those of groups I (hydrophilic) and II (hydrophobic) as defined by Nikaido (13), since increased susceptibility to group II is likely to reflect a functional hydrophobic pathway (13). Although there were differences between naturally rough *B. ovis* and smooth *B. abortus*, both showed a pattern of susceptibility to group II compounds close to that of *H. influenzae* 9193 and *S. minnesota* Re595 but different from that of *E. coli* O111 K58 H2 (Table 1). These results can be taken as indirect evidence for a functional hydrophobic pathway (7).

Direct proof was obtained by using viable cells and *N*-phenyl-naphthylamine (NPN), a fluorescent probe whose quantum yield increases upon transfer from a hydrophilic to a hydrophobic environment (17) and which has been used in OM permeation studies (16). Exponentially growing cells were harvested (centrifuged for 10 min at 5,000 × *g* at 20°C), resuspended in 1 mM KCN–10 mM phosphate-buffered saline (pH 7.2) at an optical density (600 nm) of 0.48 (370 μg [dry weight]/ml), and transferred to 1-cm-diameter fluorimetric cuvettes. NPN (500 μM in acetone) was added after 15 to 20 s to a final concentration of 10 μM. 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; and slit widths, 2.5 nm (16). Whereas only a small increase in fluorescence (up to 25 relative fluorescence units [RFU]) was observed with *E. coli* O111 K58 H2, a progressive and greater increase (up to 125 RFU) was obtained with *B. abortus* 2308 (Fig. 1A), showing that the level of NPN uptake was much higher in smooth *B. abortus* than in bacteria with an efficient barrier to hydrophobic permeants. The *S. minnesota* Re595 heptoseless mutant (1) also took up NPN (Fig. 1B), but the fluorescence increase (75 RFU) was less than that obtained with *B. abortus* 2308. Significantly, the increases in NPN fluorescence obtained with *B. abortus* 2308 and *B. abortus* RB51 (a stable rough mutant of 2308) were similar (Fig. 1), and the same results were also obtained with *B. melitensis* 16M (smooth, biotype 1, and virulent) and *B. melitensis* 115 (rough and avirulent) (data not shown). Other *Brucella* species yielded slightly different results, since with *B. suis* 2 the final fluorescence levels were higher (up to 160 RFU) and both a higher fluorescence level (175 RFU) and a faster NPN uptake were observed with *B. ovis* REO 198 (Fig. 1B). These differences within the genus could relate to quantitative differences in lipid composition and, in the case of *B. ovis*, to an even less effective barrier. Despite these species differences, the data show that NPN uptake by *Brucella* spp. does not relate to the presence or absence of the LPS O chain or to possible changes in the OM that could be indirectly brought about by the rough mutation (14).

NPN uptake in *Brucella* spp. and *H. influenzae*, a species that has been described as showing a hydrophobic pathway,

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TABLE 1. MICs of hydrophilic and hydrophobic agents

Group and agent (partition coefficient)[a]	MIC (μg/ml) for:					
	B. abortus 2308	*B. ovis* REO 198	*H. influenzae* 9193	*S. minnesota*		*E. coli* O111 K58 H2
				Rd_1p^+	Re595	
I (hydrophilic)						
Penicillin (0.02)	2.0	1.0	1.0	2.0	2.0	>8.0
Ampicillin (<0.01)	1.0	0.5	1.0	0.5	0.5	4.0
Tetracycline (<0.07)	<2.0	<2.0	<2.0	<2.0	<2.0	>16.0
Cephalothin (<0.01)	8.0	64.0	8.0	4.0	4.0	16.0
Nafcillin (0.31)	128.0	8.0	32.0	>256.0	>256.0	>256.0
II (hydrophobic)						
Erythromycin	8.0	<0.1	4.0	>16.0	4.0	>16.0
Rifampin (8.8)	2.0	<0.5	<0.5	4.0	<0.5	>4.0
Novobiocin (>20)	16.0	0.5	<0.2	32.0	16.0	128.0
Crystal violet (14.4)	16.0	<0.2	16.0	8.0	2.0	64.0

[a] Partition coefficients in 1-octanol–0.05 M sodium phosphate (pH 7.0) at 24°C (taken from reference 14).

was compared. As judged from the fluorescence increase, the kinetics of NPN uptake in *H. influenzae* 9193 and in the *Brucella* strains tested, with the exception of *B. ovis* REO 198, were similar (Fig. 1). In addition, differences in final fluorescence levels were also observed. When the experiments were repeated with cells with envelopes disrupted by boiling, NPN uptake was instantaneous but the final fluorescence did not change substantially (Fig. 1), proving that the kinetics of uptake by viable cells reflected the integrity of the OM and that the final fluorescence levels represented the saturation of the envelope lipid with the probe. Thus, it seems possible that the differences in final fluorescence between *H. influenzae* and *Brucella* spp. might reflect quantitative and/or qualitative differences in lipid composition. These results show that, as in *S. minnesota* deep rough mutants and in *H. influenzae*, the OM does not act as a barrier to hydrophobic permeants in *Brucella* spp., with the particularity that this property is shared by cells with rough and smooth LPSs.

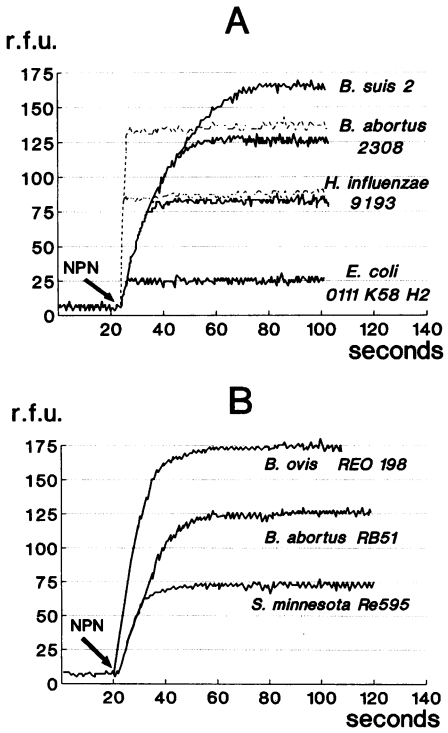

FIG. 1. Uptake of NPN by whole bacterial cells of smooth strains *E. coli* O111 K58 H2, *H. influenzae* 9193, *B. abortus* 2308, and *B. suis* 2 (A) and rough strains *S. minnesota* Re595 (heptoseless mutant), *B. abortus* RB51 (rough mutant of *B. abortus* 2308), and *B. ovis* REO 198 (naturally rough species) (B). Discontinuous lines in panel A represent results with boiled *H. influenzae* 9193 and *B. abortus* 2308.

An enhanced hydrophobic uptake could result from decreased LPS-to-LPS interactions through weaker binding of divalent cations (6). If so, agents that remove divalent cations should have little or no effect on NPN uptake by *Brucella* spp. Figure 2 shows that exposure to 5 mM EDTA increased the NPN uptake by *E. coli* O111 K58 H2. On the other hand, neither the kinetics of NPN uptake by *B. abortus* 2308 nor the final fluorescence values were modified by EDTA, and the same observations were made with *B. melitensis* 16M and *B. suis* 2 (data not shown). This is consistent with the absence of divalent-cation stabilization of LPS in the OM of *Brucella* spp. postulated before (12) and with the comparatively reduced proportions of anionic groups (phosphate and 2-keto-3-deoxyoctonic acid) in *B. abortus* LPS (11).

The results described above confirm the prediction by Martin and Hancock (10) that the susceptibility of *Brucella* spp. to erythromycin and rifampin should reflect an accessible hydrophobic pathway. Moreover, the results link this hydrophobic pathway to the properties of the LPS. Thus, the LPSs of *E. coli* O111 K58 H2 and *B. abortus* 2308 were extracted with phenol-water (9) and purified (by treatment with 1 μg [each] of DNase and RNase per mg of LPS in 10 mM Tris-HCl–5 mM $MgCl_2$ for 4 h at 37°C, treatment with 5 μg of proteinase K per mg of LPS for 24 h at 24°C, and ultracentrifugation). By sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver or periodate-silver stain (18), both LPSs showed no protein and showed the smear characteristic of smooth LPSs (8). A total of 375 μg of each LPS was dispersed by sonication in 1.5 ml of 10 mM phosphate-buffered saline (pH 7.2), and the fluorescence was measured before and after NPN addition as described for whole cells. For *E. coli* LPS, fluorescence increased from 4

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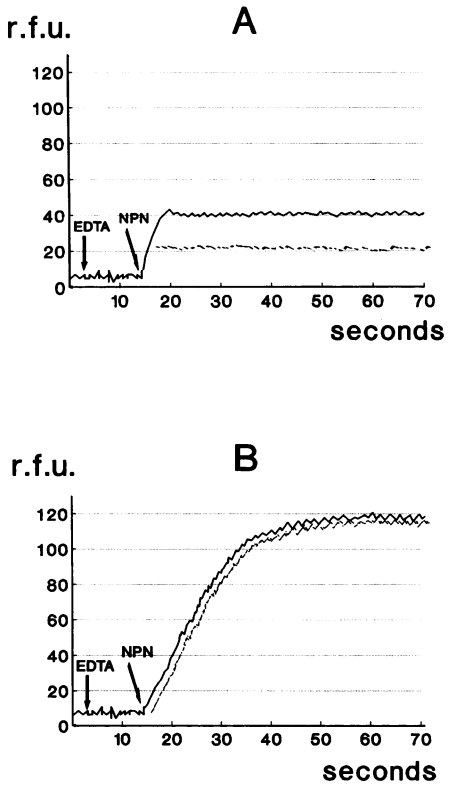

FIG. 2. Effect of 5 mM EDTA on the uptake of NPN by whole bacterial cells. (A) *E. coli* O111 K58 H2; (B) *B. abortus* 2308. Discontinuous lines in both panels represent the results of control experiments performed without addition of EDTA.

to 27 RFU, roughly 30% of the fluorescence obtained with *B. abortus* 2308 LPS (4 to 90 RFU).

In wild-type gram-negative bacteria, the impermeability to hydrophobic drugs is due to the LPS, which excludes the phospholipids from the outer leaflet of the OM, and when phospholipids are present in this leaflet (as in heptoseless mutants) the barrier is no longer effective (14). Thus, although our experiments point to a direct role of *Brucella* LPS in the absence of the barrier to hydrophobic permeants, exposure of phospholipid on the outer surface is also a possible factor. Compared with that in rough *Brucella* spp., the access of antibody to OM proteins is hindered in smooth *Brucella* spp. (15), but this does not mean that the smooth LPS covers all areas where proteins are exposed, since scattered patches of immunogold label are detected on intact smooth cells with antibodies to OM proteins (2, 5). This is compatible with an OM model in which dispersed phospholipid patches alternate with larger areas covered by the smooth LPS. If so, such patches could also play a role in the partition of hydrophobic permeants into the OM of *Brucella* spp.

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