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Porins of *Brucella* Species

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The outer membrane of *Brucella* species contains two major proteins, denoted as group 2 and group 3 proteins (Verstreate et al., Infect. Immun. **35**:979–989, 1982). We reconstituted proteoliposomes from the purified proteins and egg phosphatidylcholine and showed that group 2 proteins, but not a group 3 protein, had the porin activity. The influx rates of sugars of various sizes into the proteoliposomes indicated that the porin channels had apparent diameters in a range comparable to that of *Escherichia coli* OmpF porin and that the channels were predominantly open. Among different *Brucella* species, there were small but detectable differences in the channel diameter, and it was possible to explain the differential sensitivity of several *Brucella* species to diagnostic dyes on the basis of these observed differences.

Cells of all gram-negative bacteria so far investigated are known to be covered by the outer membrane(20), which contains proteins producing largely nonspecific, water-filled diffusion channels, i.e., porins (14, 15), and at the same time serves as a diffusion barrier to those molecules that cannot pass through the porin channel (2, 5, 17–19). Although porins of *Escherichia coli* (21–23, 27–29) and to some extent those of *Salmonella typhimurium* (14) have been studied in depth, only a few other organisms (for example, see references 3, 6 and 34) have been investigated as to the identity and properties of the porin channel. The outer membrane of *Brucella* strains was found to contain two major protein species, one called group 2, with an apparent molecular weight of ca. 35,000 to 40,000, and the other called group 3, with an apparent molecular weight of ca. 30,000 (32). In this study we show that the group 2 proteins are indeed porins, as predicted on the basis of their amino acid composition and oligomeric structure (32), and we report on the properties of these porin channels. These organisms were of special interest, partly because dye sensitivity tests, which could be measuring the outer membrane permeability to various dyes, have been used extensively in the identification of species and biotypes (1, 33) and partly because they are capable of surviving within phagocytic cells of the host (33).

MATERIALS AND METHODS

Bacterial strains and the preparation of proteins. *Brucella abortus* 19, 2308, 45/20, and 1119.3 were provided by B. L. Deyoe, National Animal Disease Center, Ames, Iowa. *Brucella melitensis* B115 was obtained from G. G. Schurig, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg. *Brucella canis* RM666 was obtained from L. E. Carmichael, New York State College of Veterinary Medicine, Cornell University, Ithaca.

All of the strains except *B. abortus* 19 were grown in Albimi broth in an incubator-shaker as previously described and were harvested in the late exponential phase of growth (32). The isolation of group 2 and group 3 proteins was carried out as described previously (32).

Strain 19 was grown in liquid culture as specified previously (1) and was supplied as acetone-killed powder by B. L. Deyoe. The group 2 protein was isolated as follows. Dried cells (0.75 g) were suspended in 30 ml of 0.01 M Tris-hydrochloride buffer (pH 7.3), washed three times by centrifugation in the same buffer, and resuspended in 15 ml of the same buffer containing 0.3% sodium *N*-lauroylsarcosinate (Sigma Chemical Co.). The suspension was kept at room temperature for 20 min with intermittent mixing, and the insoluble fraction was recovered by centrifugation and washed three times in the 0.01 M Tris-hydrochloride buffer as described above. The pellet was resuspended in 15 ml of 0.01 M Tris buffer (pH 7.3) containing 200 μg each of lysozyme, pancreatic DNase, and pancreatic RNase, and the mixture was incubated at 37°C for 3 h with intermittent mixing. The insoluble fraction was recovered by centrifugation, washed with 0.01 M Tris buffer as described above, and extracted again with 0.3% sodium *N*-lauroylsarcosinate at room temperature. After centrifugation at 200,000 × *g* for 20 min, the translucent pellet was collected and washed three times with the 0.01 M Tris-hydrochloride buffer, pH 7.3, by centrifugation under the same conditions. The pellet was then extracted with 0.2% Zwittergent 3.14 (Calbiochem-Behring) in 0.01 M Tris-hydrochloride buffer. After centrifugation at 17,000 × *g* for 10 min to remove the undigested debris, the supernatant was centrifuged at 200,000 × *g* for 40 min. The extracted group 3 protein and lipopolysaccharide (LPS) remain in the supernatant, and the group 2 protein is recovered in the pellet.

Swelling assay of porin channel permeability. The swelling assay of porin channel permeability has been described previously (11, 21, 22). In short, egg phosphatidylcholine (2.4 μmol) and dicetylphosphate (0.1 μmol) were dried as a film at the bottom of a tube, and the aqueous solution of porin (or, in one case, group 3 protein) was added. The suspension was homogenized by sonication and dried again under reduced pressure. Finally, the porin-phospholipid mixture was gently suspended in 15% (wt/vol) dextran T-40 (Pharmacia Fine Chemicals, Inc.) containing 5 mM Tris-hydrochloride buffer (pH 7.4), the liposomes were diluted into isotonic solutions of various sugars, and the initial rate of swelling of the liposomes was measured turbidimetrically.

Radioisotope efflux assay of porin channel permeability. The radioisotope efflux assay of porin channel permeability was carried out essentially as described previously (14), except that LPS was omitted from the reconstitution mix-

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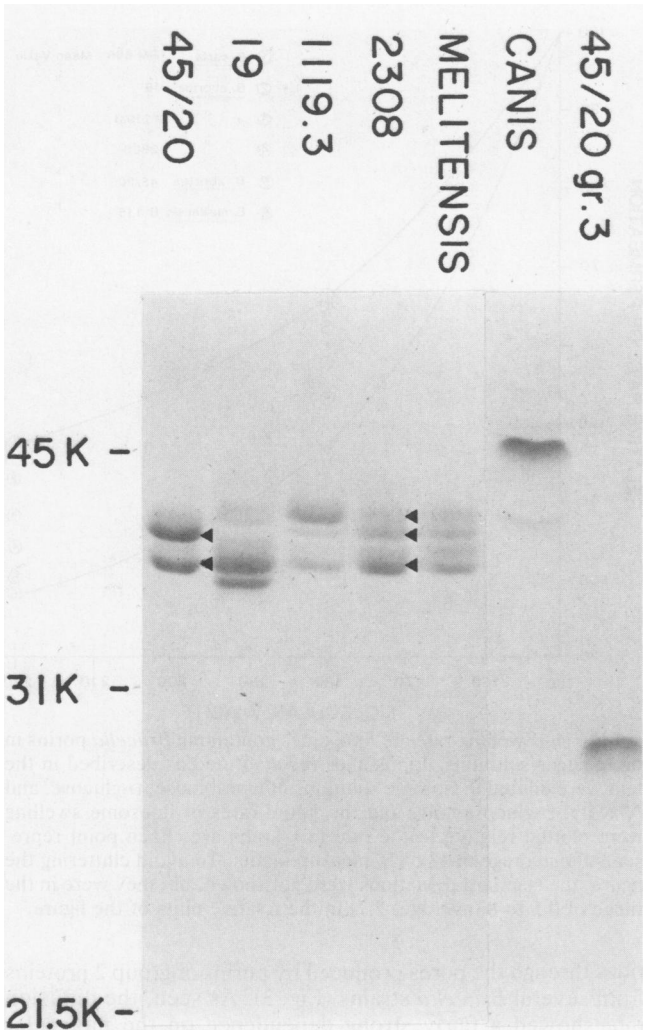

FIG. 1. SDS-polyacrylamide gel electrophoresis of group 2 and 3 proteins. Approximately 7 μg of the purified proteins was applied to a slab gel 25 cm long, and after electrophoresis the protein bands were stained with Coomassie brilliant blue. Except for the lane at the extreme right, to which the group 3 protein of *B. abortus* 45/20 was applied, all lanes contained group 2 proteins. *B. abortus* strains are denoted by strain numbers only. For the strain numbers of other species, see the text. Only the central part of the gel is shown. The molecular weight standards used were ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500). The arrowheads show the bands cut out and used for the peptide mapping experiment shown in Fig. 2.

ture. The phospholipid used was the mixture of egg phosphatidylcholine (1 μmol) and dicetylphosphate (0.1 μmol). The porin-containing liposomes prepared in the presence of ^{14}C-dextran and ^{3}H-oligosaccharide or ^{3}H-dextran and ^{14}C-oligosaccharide were applied to a column of Sepharose 6B, and the radioisotope content of the liposomes eluted at the void volume was determined.

Chemicals. Chemicals used were generally of the highest grade commercially available. Egg phosphatidylcholine (Sigma type IX-E) was purified as described previously (8, 22). ^{3}H-dextran and ^{14}C-dextran (both from New England Nuclear Corp.) were purified by gel filtration on a column of Sephacryl S200 to remove low-molecular-weight components. [^{3}H]raffinose and [^{3}H]stachyose were prepared as described before (5). Thionine blue (British Drug Houses) was the kind gift of Margaret E. Meyer, University of California, Davis.

Other methods. Protein was determined by the method of Lowry et al. (10). Sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis was carried out as described by Laemmli (9). The apparent partition coefficient in 1-octanol–0.05 M sodium phosphate buffer, pH 7.0, was measured as described earlier (17).

RESULTS

Purity of the proteins. The proteins purified from the *Brucella* strains were examined by SDS-polyacrylamide gel electrophoresis (Fig. 1). The group 2 proteins migrated with apparent molecular weights of ca. 37,000 to 42,000, whereas one group 3 protein showed an apparent molecular weight of 26,000. The group 2 proteins usually produced multiple bands. However, peptide patterns after partial digestion with staphylococcal protease V8 (4) were very similar in bands from the same strain (Fig. 2), and the multiple bands appear to represent modified forms of a single protein. Since the protein was obtained after the lysozyme digestion of peptidoglycan, it seems possible that different bands contain either peptidoglycan fragments of different sizes, different amounts of adherent LPS molecules, or both. Despite the presence of multiple bands from each strain, the migration patterns of group 2 proteins seemed to be different in different strains.

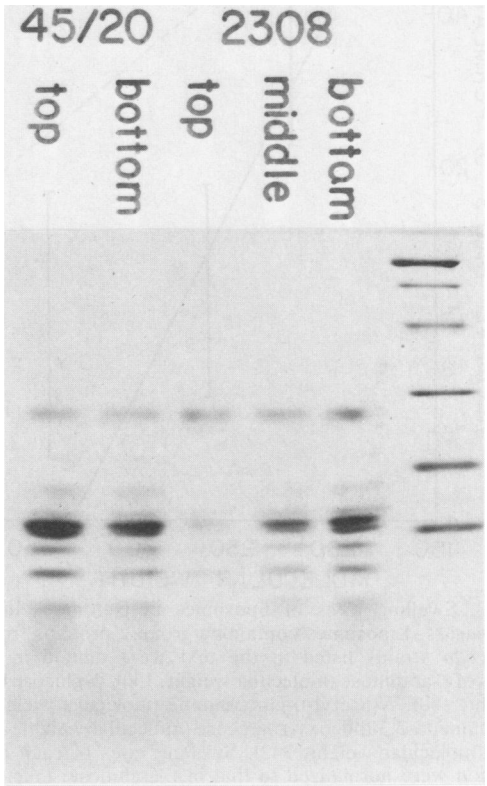

FIG. 2. Peptide mapping of group 2 protein bands. The bands indicated by the arrowheads in Fig. 1 were cut out, digested with staphylococcal protease V8, and reelectrophoresed in the SDS-polyacrylamide slab gel system, following the method of Cleveland et al. (4). The gel was stained with Coomassie brilliant blue. The lane on the extreme right shows molecular weight markers, which included phosphorylase *b* (92,500), bovine serum albumin (66,000), and lysozyme (14,400), in addition to those listed in the legend to Fig. 1.

Permeability of porin channels studied with swelling assay. In the swelling assay (see above), all group 2 protein preparations showed strong pore-forming activity. Reconstitution of 0.1 μg of protein with 2.4 μmol of phosphatidylcholine was sufficient to produce a very rapid swelling of liposomes upon dilution into isotonic L-arabinose, corresponding to the initial rate of decrease in optical density at 400 nm of 0.2 to 0.35/min. This was 50 to 90% of the rate of swelling of liposomes containing the same amount of *E. coli* OmpF porin (22). In contrast, the group 3 protein from *B. abortus* 45/20 was totally inactive in increasing the permeability of liposomes, even when added at levels of up to 10 μg/2.4 μmol of phospholipids.

The rate of penetration of solutes through porin channels is affected strongly by the size of the solute molecule, even when the size is within the "exclusion limit" of the channel (21, 22). Thus, it is possible to calculate the equivalent diameter of the channel from this rate of penetration (25). We therefore determined the rates of diffusion of various saccharides through the pores produced by porins of group 2 proteins from several *Brucella* strains (Fig. 3). As seen, the diffusion rate showed a fairly strong dependence on the molecular size, and the slope of the line was similar to that seen with *E. coli* OmpF porin (22), a result suggesting that the size of pore in porins of group 2 proteins is similar to that of the OmpF porin, i.e., ca. 1.2 nm in diameter.

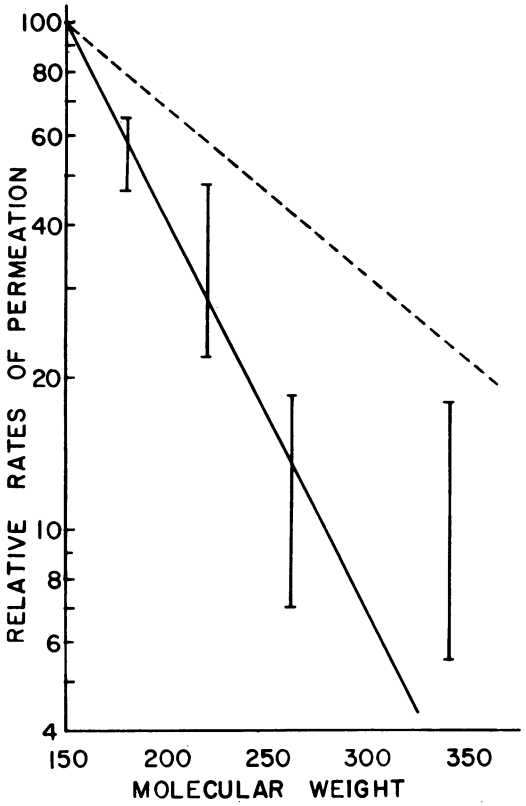

FIG. 3. Swelling rates of liposomes in isotonic solutions of various sugars. Liposomes containing group 2 proteins from all of the *Brucella* strains listed in the text were diluted in isotonic solutions of L-arabinose (molecular weight, 150), D-glucose (molecular weight, 180), *N*-acetyl-D-glucosamine (molecular weight, 221), 2,3-diacetamido-2,3-dideoxy-D-glucose (molecular weight, 262), and sucrose (molecular weight, 342). Swelling rates of each liposome preparation were normalized to that in L-arabinose, taken as 100, and the range of values found was plotted. Only one or two experiments were performed for each protein preparation, as the object of the experiment was to find the approximate range of permeability in a wide range of sugars. The solid and broken lines show the behavior of *E. coli* OmpF porin (22) and *P. aeruginosa* porin, respectively (34), for comparison. The wide deviation of the experimental values for sucrose from the solid line is probably an artifact, as the swelling rates measured with this sugar were too low to be measured accurately.

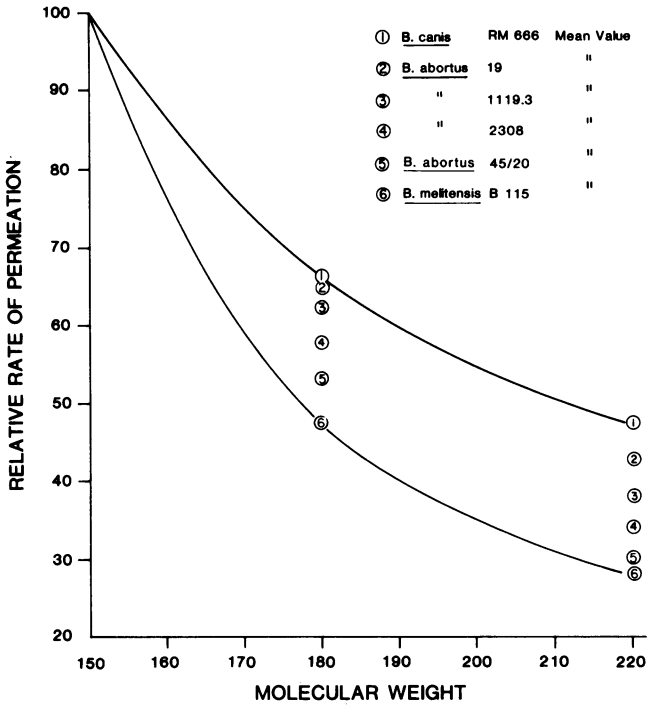

FIG. 4. Swelling rates of liposomes containing *Brucella* porins in three sugar solutions. Liposomes reconstituted as described in the text were diluted in isotonic solutions of L-arabinose, D-glucose, and *N*-acetyl-D-glucosamine, and the initial rates of liposome swelling were plotted relative to the rate in L-arabinose. Each point represents the average of 12 to 15 measurements. To avoid cluttering the figure, the standard deviations were not shown, but they were in the range of 0.5 to 8 (average, 2.7) in the relative units of the figure.

Comparison among different *Brucella* porins was performed more carefully by using three solutes, L-arabinose, D-glucose, and *N*-acetyl-D-glucosamine (Fig. 4). The results showed small but significant differences in the apparent pore sizes of different organisms. Thus, *B. canis*, *B. abortus* 19, and *B. abortus* 1119.3 appeared to produce relatively large pores, in contrast to *B. melitensis* B115 and *B. abortus* 45/20, which produced the smallest pores. The size of the *B. abortus* 2308 channel seemed to be in between these classes.

Radioisotope efflux assay of porin function. Because the isotope efflux assay is often more sensitive to small differences in pore sizes, this assay was carried out with most of the porin preparations. The porins of *B. abortus* 19 and 1119.3 allowed the efflux of more than 85% of [^{3}H]raffinose (molecular weight, 505) during the gel filtration step, in contrast to the efflux of less than 35% found in vesicles containing *B. abortus* 45/20 porin (Table 1). Similar differences were found in the efflux of a larger sugar, [^{3}H]stachyose (molecular weight, 666; Table 1). These results confirm that a significant difference in pore size exists between *B. abortus* 19 and 1119.3 on the one hand and *B. abortus* 45/20 on the other. Since stachyose essentially will not pass through *E. coli* porin channels, we conclude that *B. abortus* 19 and 1119.3 pores are slightly larger than the *E.*

TABLE 1. Radioisotope efflux assay of *Brucella* porins

Porin added		Oligosaccharide diffused out of vesicles during gel filtration (%)[a]	
Source	Amt (μg)	Raffinose	Stachyose
B. melitensis B115	10	34 ± 1.8	ND[b]
B. abortus 45/20	10	29 ± 1.8 (4)	4 ± 1.3 (4)
	20	66 (1)	ND
B. abortus 1119.3	10	87 ± 0.1 (3)	24 ± 1.0 (3)
B. abortus 19	10	90 ± 0.1 (6)	35 ± 1.8 (6)

[a] Figures show the percentage of the initially present intravesicular oligosaccharide that diffused out, plus or minus the standard deviation. The numbers in parentheses are the numbers of experiments repeated.

[b] ND, Not determined.

coli OmpF channel. *E. coli* channels, on the other hand, allowed partial efflux of raffinose under similar conditions (15, 22), and the narrower *Brucella* channels, such as that of *B. abortus* 45/20, behaved very similarly, again confirming the results of the liposome swelling assay.

Hydrophobicity of the dyes used in the classification. Basic fuchsin, thionine, and thionine blue (Fig. 5) have been widely used in the identification of *Brucella* species and biotypes (1). Because the passage of solute molecules through the porin channel as well as through the lipid bilayer region of the outer membrane is strongly influenced by the hydrophobicity of the solute (17, 22), we assessed the hydrophobicity of these dyes by measuring the partition coefficient in the system containing 1-octanol and 0.05 M sodium phosphate buffer, pH 7.0. The apparent partition coefficients at room temperature (25°C) were 8.2, 0.56, and 1.67 for basic fuchsin, thionine, and thionine blue, respectively, showing that thionine had an exceptionally low hydrophobicity among dyes (see also data listed in reference 17).

DISCUSSION

The cell envelope fraction of *Brucella* species contains two major proteins. One, called group 2 proteins (32), was predicted to be the porin of these organisms on the basis of the following observations. (i) The proteins migrated as though their molecular weight was about 115,000 when treated with SDS at 23 or 37°C, but heating in SDS at 100°C

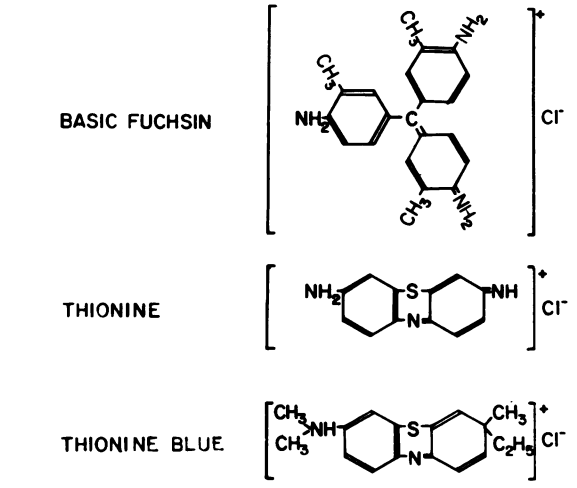

FIG. 5. Structure of the dyes often used in the identification of *Brucella* species.

dissociated these SDS-resistant oligomers into a monomeric form with an apparent molecular weight of ca. 40,000. Existence as SDS-stable trimers is a characteristic property of porins from *E. coli* and *S. typhimurium* (16, 24, 31, 35). (ii) The group 2 proteins resembled *E. coli* porins in their amino acid composition, containing relatively large amounts of tyrosine, valine, alanine, aspartic acid or asparagine, and glycine (32). (iii) Like *E. coli* porin (26), the *Brucella* group 2 proteins appeared to be strongly associated with the peptidoglycan layer, and in fact efficient extraction with detergents could be achieved only after digestion of the peptidoglycan layer with lysozyme.

In this work we found that group 2 proteins, but not a group 3 protein, showed strong porin activity in liposome reconstitution assays. The magnitude of permeability produced in these assays suggested that at least a large fraction of the *Brucella* porin channels were "open," in contrast to the *Pseudomonas aeruginosa* porin in which most channels appeared to be "closed" (3, 34). Predominantly open but narrow channels produce a strongly selective permeability that tends to exclude moderately large, hydrophobic, or negatively charged solutes, such as many antibiotic molecules (22). Indeed, the *Brucella* strains are known to have only moderate sensitivity to a number of antibiotics, including β-lactams, tetracyclines, and aminoglycosides (7, 26), generally comparable to the sensitivity levels of *E. coli* and other enteric bacteria. This finding is obviously consistent with the similarity between the *E. coli* and *Brucella* porins in terms of their size and the proportion of open channels. The reported high sensitivity of *Brucella* to erythromycin and rifampin (7), however, forms a striking contrast to the high resistance of *E. coli* strains to these large, hydrophobic agents (17) and is difficult to explain by the properties of the porin channel. Possibly these agents penetrate the outer membrane via lipid bilayer regions. In this connection, it should be noted that the remarkable lack of permeability of the lipid bilayer regions of the *E. coli* outer membrane is dependent on the structure and properties of LPSs (30) and that *Brucella* LPSs are very different from those of enteric bacteria in the structure of the hydrophobic, lipid A portion (12). It has also been reported that *Brucella* LPSs contain tightly bound protein, which cannot be removed by the treatment that removes proteins from enterobacterial LPSs (12).

Sensitivity to various dyes and antibiotics has been used in the classification of *Brucella* species (1, 13, 33; Table 2). Although more extensive study is needed to reach a definitive interpretation, it seems profitable to formulate a tentative hypothesis that will explain these sensitivity data on the basis of the permeability properties of the pore (Table 2). (i) Except for *B. suis* most *Brucella* species can grow in the presence of 10 μg of basic fuchsin per ml. Since basic fuchsin is a triphenylmethane dye (Fig. 5) with a minimum diameter of ca. 1.5 nm (measured with the use of CPK atomic models) and because it is so hydrophobic (see above), it is very unlikely to penetrate any of the *Brucella* porin channels. Thus, if it penetrates the outer membrane, it must do so through the lipid bilayer region. The higher sensitivity of *B. suis* to this dye suggests that the lipid bilayer region of the outer membrane may have a different structure in this species. In *S. typhimurium*, mutants producing different and much more permeable lipid bilayer regions are known, and these "deep rough" mutants are inhibited much more strongly by triphenylmethane dyes such as crystal violet and malachite green (17). (ii) Another dye used extensively in the classification of *Brucella* species, thionine, has a minimum

TABLE 2. Dye sensitivity and pore diameter in various *Brucella* species

Species	Growth in the presence of[a]:			Pore diam
	Basic fuchsin (20 μg/ml)	Thionine (10 μg/ml)	Thionine blue (2 μg/ml)	
B. melitensis, all biotypes	+	+		Narrow
B. abortus biotype 1 (except strain 19)	+	−	+	Medium
B. abortus biotype 1, strain 19	+	−	−	Wide
B. suis biotypes 1 and 2	−	+		ND[b]
B. canis	−	+		Wider

[a] The data are from reference 1.
[b] ND, Not determined.

diameter (0.7 nm) much smaller than that of basic fuchsin and is also very hydrophilic among commonly used dyes (see above). Thus, it is likely that porin channels represent the major pathway of penetration for this dye. *B. melitensis* strains are usually resistant to this dye, and indeed the porin from one *B. melitensis* strain examined was found to have a narrower pore than do other *Brucella* species (Fig. 4). In contrast, *B. abortus* strains, which are sensitive to this dye, did produce wider pores that would favor the penetration of the dye (Fig. 4). It must be admitted, however, that the large pore diameter of *B. canis* porin, which is resistant to thionine, is difficult to explain on this basis. However, all *B. canis* strains are rough, and possibly this fact has an influence on the diffusion of the dye across the outer membrane. (iii) Thionine blue, which has additional, bulky, hydrophobic substituents (Fig. 5) in comparison with thionine, fails to inhibit all strains of *B. abortus* biotype 1, and this finding is compatible with the known properties of these narrow porin channels that tend to exclude more hydrophobic compounds (22). However, thionine blue does inhibit strain 19, which was found to have porin channels larger than those found in other *B. abortus* strains (Fig. 4 and Table 1). In studies with *E. coli*, a small (<10%) difference in pore diameter was shown to produce a very large (10-fold or even more) difference in the diffusion rates of large or hydrophobic solutes, and it is most likely that the difference in pore sizes of strain 19 and other strains produces a marked difference in the diffusion rates of thionine blue. (iv) Finally, the observation that *B. abortus* 1119.3 and 19 had pores larger than those of *B. abortus* 45/20 and 2308 is interesting in view of the fact that strain 1119.3 was isolated from a source that could have been contaminated by strain 19 (B. L. Deyoe, U.S. Department of Agriculture, personal communication). Although the group 2 protein or porin from strain 19 seemed to have mobility different from that of strain 1119.3 in SDS-polyacrylamide gel electrophoresis (Fig. 1), this difference could have been due to differences in the structure of adherent LPS, since strain 19 has always been cultivated to produce less smooth types of LPS (Deyoe, personal communication).

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