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Comparison of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Profiles and Antigenic Relatedness Among Outer Membrane Proteins of 49 *Brucella abortus* Strains

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Outer membrane proteins were solubilized from 49 strains of *Brucella abortus* by sequential extraction of physically disrupted cells with *N*-lauroylsarcosinate and a dipolar ionic detergent (Verstreate et al., Infect. Immun. 35:979–989, 1982). The strains tested included standard agglutination test strain 1119, virulent strain 2308, and eight reference strains representing each of the biotypes; the remainder were isolates from cattle in North America with natural infections and included biotypes 1, 2, and 4. Three principal protein groups with apparent molecular weights of 88,000 to 94,000 (group 1), 35,000 to 40,000 (group 2, now established as porins [Douglas et al., Infect. Immun. 44:16–21, 1984]), and 25,000 to 30,000 (group 3) were observed in every strain. Some variability in banding patterns occurred among strains, but intrastrain variation was sufficient to preclude the use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of outer membrane proteins for differentiating among strains of *B. abortus*. One antigen ([b]) was shared among the porin proteins, and three others ([c], [d], and [e]) were shared among the group 3 proteins of all of the strains tested, indicating that these relationships are probably species wide. These results suggest that it may be possible to use outer membrane proteins from a representative strain of *B. abortus* in a vaccine for species-wide immunization.

We have recently reported on the isolation and characterization of two major outer membrane proteins (OMP) from *Brucella abortus* (49). One of these (group 2; molecular weight, 35,000 to 40,000) has now been identified as a porin by functional assays (16). The other (group 3; molecular weight, 25,000 to 30,000) may be the counterpart of OmpA of *Escherichia coli* (40).

Antibodies directed against the OMP of some gram-negative bacteria have been shown to develop in naturally occurring or experimental infections (20, 24, 32, 34, 42), and OMP have also been used to advantage in identifying subgroups or serotypes of particular gram-negative species (3, 7, 8, 26, 31, 39, 48, 51). In addition, recent studies have reported on the use of OMP in conferring protection actively (1, 9, 12, 17, 27) or passively (23, 27, 46). One of our principal objectives has been to determine whether one or more OMP of *B. abortus* might be effective components in a nonviable vaccine for bovine brucellosis (50). However, the inclusion of OMP in a vaccine requires an understanding of the antigenic diversity of these proteins within a species.

The purposes of this study were to determine (i) whether, in accord with our preliminary data (49), antigenic relatedness among porins and among group 3 proteins is species wide in *B. abortus* and (ii) whether sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of OMP could be used in grouping or distinguishing strains of *B. abortus*.

MATERIALS AND METHODS

Bacterial strains and cultivation. A total of 49 strains of *B. abortus* were used: (i) the standard agglutination test strain (1119) used in the United States; (ii) virulent strain 2308, isolated in 1942 and used commonly in the United States in experimental infection trials (33); (iii) the eight American Type Culture Collection (ATCC) biotype reference strains (biotypes 1 through 7 and 9); and (iv) 39 virulent field strains of diverse geographic origin representing biotypes 1 and 4 with one strain of biotype 2 (Table 1). The field strains from Saskatchewan and Manitoba, Canada, were biotyped at the Animal Pathology Laboratory, Saskatoon, Saskatchewan, Canada. The other field strains were biotyped at the National Animal Disease Center, Ames, Iowa, by standard techniques (2).

Upon receipt each strain was transferred to Schaedler agar plates (BBL Microbiology Systems, Cockeysville, Md.) containing 10% bovine blood and cultivated for 2 days at 37°C in an atmosphere of 10% CO_2. Growth was suspended in Albimi broth (Difco Laboratories, Detroit, Mich.), and stock suspensions were frozen in portions at −70°C. To prepare cells for extraction, stock suspension from one tube was streaked heavily onto Schaedler blood agar plates (diameter, 9 cm) and incubated for 48 h. The confluent growth was suspended in sterile 0.1 M phosphate-buffered saline (pH 7.2) and transferred to several Schaedler blood agar plates (diameter, 15 cm). After incubation for 48 h, the confluent growth was suspended in sterile phosphate-buffered saline and collected by centrifugation. A sample of cells was streaked onto Albimi agar and examined 3 to 4 days later for colony type by crystal violet retention and stability in acriflavine (2). All strains exhibited 100% smooth colony morphology by these criteria. The remainder of the cell suspension was killed by overnight suspension in a solution of 0.25 M trichloroacetic acid at 4°C (15, 49) and then washed thoroughly and frozen as cell pellets at −70°C until use.

Strain 2308 was also grown on two separate occasions in broth cultures (49), and portions of the cells were harvested at 16, 24, and 48 h. These intervals, based on a growth-phase study performed previously on strain 1119 (49), corresponded to mid-log, late-log, and stationary phases, respec-

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TABLE 1. Derivation and properties of *B. abortus* strains used

Strain	Biotype	Origin
Reference strains		
1119	1	NADC[a]
2308	1	NADC
23448	1	ATCC
23449	2	ATCC
17385	3	ATCC
23451	4	ATCC
23452	5	ATCC
23453	6	ATCC
23454	7	ATCC
23455	9	ATCC
Field strains[b]		
CH	1	New York State
CR	1	New York State
FO	1	New York State
FR	1	New York State
GR	1	New York State
Y	1	New York State
2TA(856)	1	Southeastern U.S.
2TA(890)	1	Southeastern U.S.
5TA(866)	1	Southeastern U.S.
6TA(866)	1	Southeastern U.S.
7(874)	1	Southeastern U.S.
J596	4	Saskatchewan
J625	4	Saskatchewan
J864	4	Manitoba
K19	1	Saskatchewan
K68	1	Saskatchewan
K91	4	Saskatchewan
K93	4	Saskatchewan
K164	4	Saskatchewan
K171	4	Saskatchewan
K179	1	Saskatchewan
K188	4	Saskatchewan
K211	4	Saskatchewan
K240	1	Saskatchewan
K247	4	Saskatchewan
K388	1	Saskatchewan
K448	4	Saskatchewan
K450	4	Saskatchewan
M22	4	Manitoba
M33	4	Manitoba
OP6	1	Ontario
5A2	2	Ontario
10	1	Ontario
21	1	Quebec
24	1	Quebec
29	4	Ontario
38	4	Ontario
22W	4	Manitoba
25W	4	Manitoba

[a] NADC, National Animal Disease Center, Ames, Iowa.
[b] Isolated from aborted fetuses, fetal membranes, vaginal swabs, or lymph nodes of cows.

tively. Cells were washed, killed in trichloroacetic acid (15), and frozen as previously described (49).

Extraction of OMP. Disruption of cells and extraction of OMP have been previously described (49). Briefly, the crude membrane fraction was extracted with sodium *N*-lauroylsarcosinate (Pfaltz & Bauer, Stamford, Conn.). After dialysis (4°C for 72 h), the outer membrane-rich fraction was digested overnight at 37°C with 1 mg of egg white lysozyme (Mann Laboratories, New York) per 50 mg of membrane protein. Solubilization was then performed at 37°C for 1 h with a 0.2% solution of Zwittergent 3-14 (Calbiochem-Behring, La Jolla, Calif.) in Tris buffer containing 0.15 M NaCl (1 to 2 mg of membrane protein per ml). After extraction the samples were centrifuged at 100,000 × *g* for 30 min at 4°C, and the supernatants were held at 4°C. For all of the 49 strains, 13 ± 5 (standard deviation) mg (packed cells [wet weight]) of Sarkosyl-insoluble material was required to yield 1 mg of Zwittergent-soluble protein.

In one experiment, group 2 proteins from broth-grown cells of strain 2308 were extracted without lysozyme digestion by the method of Moriyon and Berman (37). Proteins were solubilized in Tris-hydrochloride buffer (pH 7.2) containing 0.2% Zwittergent 3-14 and 50 mM $MgCl_2$ and then purified by sequential ion exchange and gel filtration chromatography (49).

SDS-PAGE. An equal volume of double-strength extraction buffer (43) was added to each sample, and heating was performed for 10 min at 100°C. SDS-PAGE was performed by the method of Laemmli (28) on 10% acrylamide gels (14 by 17.5 cm) (49). Phosphorylase *b* (94,000, molecular weight), bovine serum albumin (68,000, molecular weight), ovalbumin (43,000, molecular weight), carbonic anhydrase (30,000, molecular weight), soybean trypsin inhibitor (21,000, molecular weight), and lysozyme (14,300, molecular weight) (Bio-Rad Laboratories, Richmond, Calif.) were used as reference proteins. Protein staining was performed with Coomassie blue R-250 (Sigma Chemical Co., St. Louis, Mo.).

Chemical analysis. Protein was measured by the method of Lowry et al. as modified by Peterson (41), with bovine serum albumin (Miles Laboratories, Inc., Elkhart, Ind.) as the standard.

Antisera. Antisera had been produced previously in rabbits against group 2 proteins of *B. abortus* strains 2308 (RB 47), Y (RB 35), and 45/20 (RB 44) and group 3 proteins of strain Y (RB 36 and 37) (49). O antibodies were removed by absorption with killed strain 1119 whole cells and trichloroacetic acid extracts (14) derived from strains 1119 or 2308 (49). Absorption was considered to be complete when antisera produced no reaction in immunodiffusion (ID) after 2 days with lipopolysaccharide (LPS) or native hapten in the trichloroacetic acid extracts (35).

Immunological techniques. ID was performed with gels composed of 1.5% agarose (Sea Plaque, Marine Colloids, Inc., Rockland, Maine) in 0.03 M barbital buffer (pH 8.8). ID tests were performed as previously described (49) by using templates (38), and the results were recorded after incubation for 2 days at 23°C in a moist chamber. In some cases gels were dried and stained with 0.25% Coomassie blue R-250.

Counter immunoelectrophoresis (CIE) was done with gels comprised of 1.5% agar (Oxoid; Oxoid Ltd., London, England) and 1% polyethylene glycol 6000 (J. T. Baker Chemical Co., Phillipsburg, N.J.) in 0.03 M barbital buffer (pH 8.8). Distances were 1.5 mm between wells in a row and 2.5 mm between wells in opposite rows. Electrophoresis was performed at 80 V for 20 min in a Gelman chamber (Gelman Instrument Co., Ann Arbor, Mich.). The gels were allowed to develop for 24 h before reactions were recorded. This generally allowed reactions of identity to form between adjacent wells. Relationships between lines produced in ID and CIE were determined by performing ID reactions in wells adjacent to those in which the same reagents had already been subjected to electrophoresis.

RESULTS

SDS-PAGE analyses. All the strains tested, regardless of biotype or geographic origin, displayed three major clusters

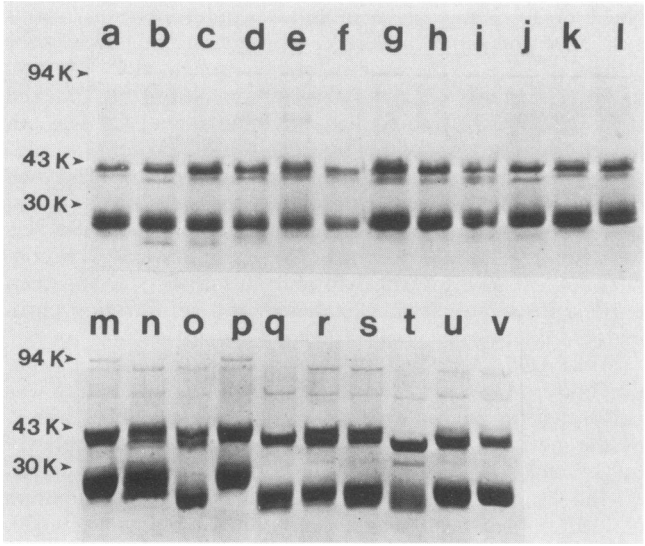

FIG. 1. SDS-PAGE profiles of Zwittergent-solubilized OMP of *B. abortus*. The strains depicted in the lanes are as follows: a, 21; b, 24; c, 29; d, 38; e, 22W; f, 25W; g, 2TA(856); h, 2TA(890); i, 5A2; j, 5TA(866); k, 6TA(866); l, 7(874); m, 23448; n, 23449; o, 17385; p, 23451; q, 23452; r, 23453; s, 23454; t, 23455; u, 1119; v; 2308. Strains in lanes a through l were field isolates; those in lanes m through t were ATCC reference strains (see Table 1). Load per lane was 50 μg of protein. Stains were with Coomassie blue.

of OMP at apparent molecular weights (aMW) of 88,000 to 94,000 (group 1), 34,000 to 40,000 (group 2), and 25,000 to 30,000 (group 3) (Fig. 1). As observed previously (49), groups 2 and 3 were present in higher and generally equivalent concentrations (Fig. 1).

In most strains the principal band in group 1 occurred at an aMW of 94,000 (Fig. 1, lanes a through l), although sometimes the predominant band was noted at 88,000 (Fig. 1, lanes n, o, r, s, u, and v), and in some strains (Fig. 1, lanes a, d, e, and m through v) an additional band was noted between groups 1 and 2.

Group 2 proteins in each of 19 field strains occurred as two bands with aMW of 40,000 and 38,000, of which the 40,000-molecular-weight band was more abundant (Fig. 1, lanes a through l) (49). However, in 6 field strains the two bands were either in approximately equivalent concentrations or there was a predominance of the faster-migrating band, and in 14 others only a single band was resolved in the group 2 region (data not shown). The same kinds of variations were observed in the eight ATCC reference strains, strain 1119, and strain 2308 (Fig. 1, lanes m through v). Small variations in the migration rates of group 2 proteins were noted in some strains. For example, group 2 proteins of ATCC reference strains of biotypes 2, 4, 6, and 7 migrated more slowly than in the majority of strains (Fig. 1, lanes n, p, r, and s), and those of biotype 9 migrated more quickly (Fig. 1, lane t). The number, relative intensities, and migration rates of group 2 proteins were subject to the same variations within a given strain. For example, in repeated extractions of strains 2308 and 1119, two bands of typical appearance (aMW 40,000 and 38,000) were usually noted, but sometimes only one band was resolved (Fig. 1, lanes u and v), and, infrequently, three bands have been observed (D. R. Verstreate, unpublished data). Such variations occurred regardless of whether cells had been grown in solid or liquid medium and could not be ascribed to effects of load, to variations in sample treatment or conditions of electrophoresis, or to growth phase. In two independent experiments, group 2 proteins of strain 2308 from cells extracted at mid-log, late-log, and stationary phases resolved as two bands typical in appearance (e.g., see Fig. 1, lanes a through l) and similar in relative intensities (data not shown). The preparation of purified group 2 proteins of strain 2308 extracted from outer membranes without lysozyme digestion migrated as two discrete bands with aMW of 39,000 and 38,000 (data not shown).

Group 3 proteins also displayed heterogeneity. In 22 field strains and in all of the reference strains, group 3 occurred as a single diffuse band or two very closely spaced bands, with an additional very-minor band sometimes present (Fig. 1). In the remaining 17 field strains, two principal bands were clearly resolved. In some strains the bands were of equivalent intensities, but in others either the faster (eight strains) or slower (three strains) migrating band predominated (data not shown). In a few strains (Fig. 1, lanes n and p), the aMW of a portion of the group 3 band exceeded 30,000. Such variations in group 3 proteins also occurred within strains, and in strain 2308 these variations were not attributable to growth phase (data not shown).

SDS-PAGE analyses from all strains were also performed with lysozyme-digested samples which had not been extracted with Zwittergent. No differences were observed in protein profiles in comparison with the corresponding Zwittergent-soluble fractions (data not shown).

Antigenic comparisons. Group 2 proteins produced two lines in ID; one (antigen [b]) was unique to group 2 (Fig. 2b), and the other (antigen [a]) was shared by group 3 (Fig. 2c). Group 3 proteins developed two lines (antigens [c] and [d]) very close to the antiserum well. The more intense line, closer to the serum well, was designated antigen [d] (Fig. 2a). These antigens were not shared by group 2 (Fig. 2a).

In CIE, extracts of all 49 strains, as well as the purified group 2 preparation derived from outer membranes extracted without lysozyme, developed reactions of identity with antigen [b] (Fig. 3a). Antigen [a] (Fig. 3a, wells 4 and 7) did not always develop on CIE, and when it was present, reactions of identity did not always form. Antigen [a], forming reactions of identity in all instances, was demonstrated by ID in extracts of 22 (45%) of the 49 strains. Strains that contained antigen [a] included strains 1119, 2308, 23452, and 23453 and 18 field strains.

Because of the nature of migration of antigens [c] and [d] in ID, reactions of identity were generally difficult to demonstrate. CIE was therefore used for this purpose. Extracts of all of the strains reacted with antisera specific for group 3 proteins. The major system observed on CIE was antigen [d]. Most extracts produced reactions of identity with antigen [d] (Fig. 3b; wells 1 to 4 and 6 to 8), but reactions of partial identity were occasionally observed (Fig. 3b; well 5). Antigen [c] was more faintly visible (Fig. 3b; wells 3, 4, 7, and 8). Repeated tests demonstrated that antigen [c] was present in extracts of all 49 strains, although these lines frequently failed to join, so relationships of identity of antigen [c] among all of the strains could not be established conclusively. In a few extracts, lines in addition to antigens [c] and [d] were noted. Another weak system (antigen [e]) was demonstrated in CIE by all extracts tested with the group 3 antisera (data not shown). Antigen [e] produced arcs very close to the serum well that frequently failed to join between adjacent wells so that, as with antigen [c], reactions of identity could not be established. Antigen [e] was not identified in ID tests.

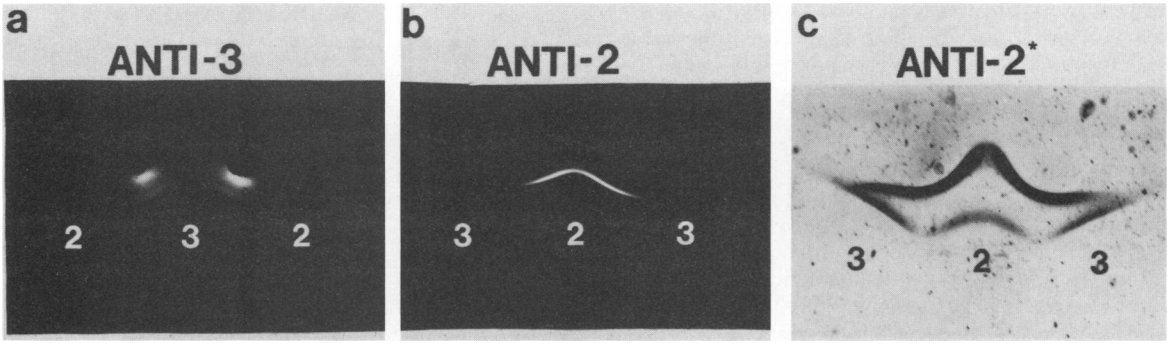

FIG. 2. ID reactions of purified OMP with specific rabbit antisera. All proteins were derived from strain 1119. (a) Bottom wells, group 2 proteins (2 mg/ml) and, in center, group 3 proteins (0.25 mg/ml); top wells, antiserum pool RB 36 and 37 (ANTI-3) specific for group 3 proteins. Antigens [c] and [d] (line closer to serum well) are evident. (b) Bottom wells, group 3 proteins (2 mg/ml) and, in center, group 2 proteins (2 mg/ml); top wells, antiserum RB 35 (ANTI-2) specific for antigen [b]. (c) Bottom wells, group 3 proteins (2 mg/ml) and, in center, group 2 proteins (2 mg/ml); top wells, antiserum pool RB 44 and 47 (ANTI-2*) which produced lines for both antigens [a] (closer to antigen wells) and [b].

DISCUSSION

The occurrence in the *B. abortus* outer membrane of group 2 and group 3 proteins has been confirmed in other laboratories (17, 18, 36, 37), and group 2 proteins have been demonstrated to be porins (16). We have used the methods of Moriyon and Berman (37) in this study and of Dubray and Bezard (17, unpublished data) to verify the identity of proteins derived by these different extraction procedures. The aMW of group 2 and group 3 proteins reported here (Fig. 1) are in general somewhat lower than values that we reported previously (49) and are more in accord with those of others (16–18, 36, 37).

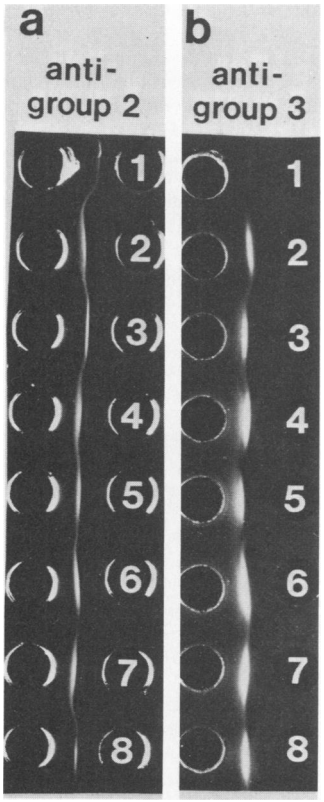

FIG. 3. CIE of Zwittergent-solubilized OMP of *B. abortus*. Numbered wells contain extracts of the following strains: 1, FR; 2, K93; 3, K164; 4, J596; 5, M33; 6, 22W; 7, 25W; 8, 6TA(866). Wells on left (a) contain an antiserum pool (RB 44 and 47) which developed lines for shared antigen [a] and for antigen [b], specific for group 2. Wells on the right (b) contain an antiserum pool (RB 36 and 37) specific for antigens [c] and [d] of group 3.

The numbers of different group 2 and group 3 polypeptides in *B. abortus* are as yet unresolved. Discrete porin bands in *E. coli* and *Salmonella typhimurium* are known to represent distinct gene products (40), but a single band may also contain more than one polypeptide (30). Conversely, we (16, 49) and others (18, 37) have proposed that, in *B. abortus*, multiple bands within each group may also be derived from a single protein, due perhaps to variable amounts of adherent murein fragments or LPS. Results of this study indicate, however, that even in the absence of possible murein fragments, proteins of group 2 can still migrate as discrete bands.

Antigen [b] was specific for group 2 of *B. abortus* (Fig. 1) and was also present in most strains of *B. melitensis*, *B. ovis*, and *B. canis* which have been tested (45). The occurrence of antigen [b] in all 49 strains tested is consistent with, but is not sufficient to prove, the complete antigenic identity of their porins. Shared as well as distinctive determinants have been demonstrated on porin proteins (10, 22) and on heat-modifiable proteins of *Neisseria gonorrhoeae* (14, 47). Antigens [c], [d], and [e] were associated with group 3 proteins (Fig. 1) of all 49 strains, and antigens [c] and [d] also occurred in *B. melitensis*, *B. ovis*, and *B. canis* (45). We hypothesize that these antigens represent separate proteins of group 3, but we have not ruled out that at least one of them may be a group 1 antigen. Preliminary immunoblotting studies with purified group 2 and group 3 proteins have not excluded the latter possibility but are fully consistent with the assignment of antigen [b] as a group 2 protein and assignment of one or more of antigens [c], [d], and [e] as group 3 proteins (J. M. Santos, unpublished data). Absorbtion of antisera has ensured the absence of antibodies specific for the O antigen or the rough LPS core (45) at levels detectable by the serological tests used. In addition, no reactions were observed in ID with any of the absorbed antisera against a *B. abortus* lipid A preparation solubilized by conjugation with bovine serum albumin (V. Y. Perera, unpublished data). We cannot as yet exclude the possibility

that putative murein fragments on some OMP of *B. abortus* consequent to the lysozyme digestion may function as antigenic determinants, although this possibility cannot apply to antigen [b], which was present in proteins extracted without lysozyme. The possession by antigen [a] of a common murein determinant could explain its presence on both group 2 and group 3 proteins of some strains.

It has been proven possible with a number of gram-negative genera (3, 7, 8, 25, 26, 31, 48, 51) to use SDS-PAGE patterns of OMP in typing systems, either by SDS-PAGE profiles alone (3, 25, 31, 39) or in conjunction with serological variations among the proteins (7, 8, 21, 26, 48, 51). If such a classification scheme were applicable to *B. abortus*, it would require a degree of reproducibility in SDS-PAGE profiles within strains that was not achieved in our studies. The reasons for this lack of reproducibility are not certain. Conditions of growth, demonstrated in *Yersinia pestis* to produce quantitative variations in OMP profiles (13), were kept constant. In other species, the phase of growth and liquid versus solid growth medium sometimes did (31) and sometimes did not (39) influence SDS-PAGE profiles of OMP. In the present study, all cultures were grown on solid medium, and the SDS-PAGE profile of strain 2308 produced from such cells was indistinguishable from that of cells grown in broth to late-log phase (M. W. Blab, unpublished data). Furthermore, no effect of growth phase on SDS-PAGE profiles of strain 2308 OMP was observed. It should be noted that, in rough strain 45/20, distinct effects of growth phase on the positions and relative intensities of group 2 and group 3 proteins were observed in two independent trials (45). Other strains must be tested to establish whether this difference is linked to dissociation of strains to the rough phase. It is of interest that, in *Haemophilus influenzae*, growth phase effects were noted in a strain of type B (31) but not in an untypable strain (39).

Another possible explanation for intrastrain variation is that it may be a consequence of lysozyme digestion of the cell wall, required for complete extraction of group 2 and group 3 proteins (49). This requirement is due in part to the extraction of previously killed (49) rather than living (37) cells but also results from the tighter binding of OMP to LPS (37) and to murein (17, 36, 49) in *B. abortus* than in other species, in which extraction of OMP is possible by direct boiling in sodium dodecyl sulfate solution (10, 20, 39, 43, 48). *Brucella* OMP extracted from lysozyme-digested cell walls probably retain some LPS and possibly fragments of murein even after boiling in hot sodium dodecyl sulfate, which could cause anomolous migration in SDS-PAGE (6) and vary sufficiently from one extraction to the next to account for the differences observed. Comparisons of the SDS-PAGE profiles of LPS-free group 2 proteins of one strain derived from successive extraction performed without lysozyme digestion would be required to resolve this question.

The present data emphasize close antigenic relationships of OMP in *B. abortus* that appear to be species wide and uninfluenced by biotype and raise the possibility that incorporation of proteins from a single strain into a vaccine may suffice for species-wide protection. We have as yet no evidence for antigenic variation in exposed determinants of OMP during infection, as has been reported in *N. gonorrheae* (19) and documented with other mural as well as extramural antigens in several bacterial species (4, 5, 11, 19, 29, 44). Additional studies will be required to resolve this question and to verify our hypothesis (50) that immune responses to one or more OMP of *B. abortus* may be relevant to protective immunity.

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