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Membrane Protein Variations Associated with In Vitro Passage of *Borrelia burgdorferi*

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***Borrelia burgdorferi*, the causative agent of Lyme disease, undergoes a loss in virulence with repeated passage in vitro. Defining the changes which occur after conversion to avirulence may assist in identifying virulence factors and mechanisms of pathogenesis. We have used a cross-adsorption technique and two-dimensional nonequilibrium pH gradient electrophoresis to compare virulent (low-passage) and avirulent (high-passage) variants of *B. burgdorferi* B31. Using cross-adsorbed rabbit sera to probe immunoblots, we identified 10 low-passage-associated proteins (relative molecular masses of 78, 58, 49, 34, 33, 28, 24, 20, and 16 kDa) unique to the virulent strain B31. Cross-adsorbed human serum detected five proteins of similar sizes (78, 58, 34, 28, and 20 kDa), suggesting that several of these proteins were expressed during human infection. By probing inner and outer membranes, two proteins (58 and 33 kDa) that localized specifically to the outer membrane were observed. An additional low-passage-associated protein (28 kDa) was identified when outer membranes from low- and high-passage variants of strain B31 were compared by two-dimensional nonequilibrium pH gradient electrophoresis.**

One approach that has been employed to identify virulence factors from *Borrelia burgdorferi* involves comparisons of virulent, low-passage *B. burgdorferi* strains with their high-passage avirulent variants, a tactic that has been applied to the study of numerous pathogenic bacteria (9, 16, 20, 29). *B. burgdorferi*, when passaged in Barbour-Stoenner-Kelly (BSKII) medium in vitro, undergoes changes that result in irreversibly avirulent variants (27). Norris et al. (22) investigated this conversion in order to identify potential virulence factors. By comparing cell extracts from low-passage (virulent) and high-passage (avirulent) variants of strain B31 by two-dimensional nonequilibrium pH gradient electrophoresis (2D-NEPHGE), they were able to identify and clone the gene encoding OspD. *ospD* resides on a 38-kb linear plasmid that was lost during prolonged in vitro culture, suggesting that plasmid loss was at least partially responsible for loss of virulence. However, it is possible that events other than the loss of the 38-kb plasmid may be responsible for the virulence-to-avirulence conversions.

We have adopted alternative strategies to compare virulent strains with their avirulent variants. First, we made use of a procedure that involved the cross-adsorption of sera that were raised against a low-passage, virulent strain with cell extract isolated from the high-passage, avirulent variant of the same strain. This yields an antibody probe that can be used for identifying proteins that are expressed only in the low-passage strain. This immune cross-adsorption technique identified several proteins that appeared to be expressed only in low-passage *B. burgdorferi* B31. Second, since most virulence factors of bacterial pathogens localize to the cell surface, where they can directly interact with the host, we isolated outer and inner membranes using a separation technique we have developed (5) and probed these cell fractions with cross-adsorbed sera. Two of the low-passage-associated proteins localized specifically to the outer membranes. Finally, to identify proteins that were not detected with adsorbed sera, purified inner and outer membranes from low- and high-passage variants of strain B31 were compared by 2D-NEPHGE. An additional low-passage outer membrane protein was observed by this method. These techniques have revealed previously uncharacterized low-passage-specific outer membrane proteins from *B. burgdorferi.*

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MATERIALS AND METHODS

Bacterial strains. *B. burgdorferi* B31 (8) and HB19 (30) were grown in modified BSKII medium (1) under an atmosphere of 3 to 5% O_2–5% CO_2–90% N_2. The cells were examined and enumerated by dark-field microscopy until the desired cell density of 5×10^7 cells per ml was reached. Avirulent, high-passage variants of *B. burgdorferi* B31 were obtained by transferring cells into fresh BSKII medium at a dilution of 1:100. High-passage isolates were tested for virulence in mice as described by Barthold et al. (4).

Isolation of subcellular fractions. Five-hundred-milliliter cultures of *B. burgdorferi* were grown to a density of 5×10^7 cells per ml and harvested by centrifugation (10,000 × *g*, 15 min, 4°C). Cell pellets were gently washed once in 50 ml of 25 mM NaCl in 20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, pH 7.6 (HEPES buffer), and cells were harvested by centrifugation. The cells were suspended in 5 ml of HEPES buffer containing 2 mM Pefabloc (Boehringer Mannheim, Indianapolis, Ind.) and lysed by being passed twice through a French pressure cell (12,000 lb/in^2; SLM-Aminco, Urbana, Ill.), and cell debris was removed by centrifugation (10,000 × *g*, 15 min, 4°C). Aliquots of the cell lysate were stored at −80°C. Soluble and membrane components were separated from the cell lysate by centrifugation (150,000 × *g*, 1 h, 4°C). The supernatant (soluble protein) was collected, the membrane pellet (total membranes) was resuspended in 15 ml of HEPES buffer, and the washed membranes were harvested by centrifugation. Total membranes were resuspended in 0.5 ml of HEPES buffer by using a tissue homogenizer (Kontes Glass Co., Vineland, N.J.), and aliquots of soluble and membrane fractions were stored at −80°C. Inner and outer membranes from *Borrelia* strains were isolated by the method of Bledsoe et al. (5). The protein concentrations of the various subcellular fractions were determined by a modified Lowry protein assay (19) with bovine serum albumin as a standard.

Sera used for cross-adsorption experiments. Antisera to low-passage, virulent *B. burgdorferi* B31 were raised by the following method. Two rabbits were bled for control sera, and a total of 10^6 virulent, passage 3 (p3) *B. burgdorferi* cells were injected at four intradermal sites on the backs of the rabbits. At 14-day intervals, blood samples were collected from the marginal ear vein and processed to sera (7, 17), which were tested for reactivity with *B. burgdorferi* proteins by Western blot (immunoblot) (31). A human serum sample (no. 91-2111; provided by Roy Campbell, Centers for Disease Control and Prevention, Fort Collins, Co.) was collected from a patient with late-stage Lyme disease. Titers of all serum samples were determined by enzyme-linked immunosorbent assay (ELISA) (18).

Serum cross-adsorption. Antiserum to virulent strain B31 (collected on day 28) was extensively cross-adsorbed with cell lysate isolated from the avirulent variant of B31 (p100+) by a modification of the method of Gherardini and Salyers (12). Cell lysate was treated as follows. One milliliter of cell lysate (5 mg/ml) was heated for 5 min at 100°C, cooled on ice, and then added to an equal volume of unheated cell lysate. By adding heated, denatured cell lysate, antibodies directed against primary protein structures as well as those directed against secondary epitopes are effectively removed by cross-adsorption. Two milliliters of this mixed cell lysate was added to 5 ml of phosphate-buffered saline (PBS) containing 25 μl of sera. The reaction mixture was continuously inverted for 1 h at 37°C, and antibody-antigen complexes were removed by centrifugation (150,000 × *g*, 2 h, 4°C). Two milliliters of mixed cell lysate was added to the supernatant, and the adsorption was repeated four times. The supernatant from the final centrifugation was collected and diluted to a final volume of 25 ml with PBS containing 5% (wt/vol) dry milk (final dilution = 1:1,000), and the cross-adsorbed serum was stored at −80°C. Human convalescent-phase serum (no. 91-2111) was cross-adsorbed by the same method.

Electrophoresis and immunoblotting. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (5) by using a Hoefer SE600 gel apparatus (Hoefer Scientific, San Francisco, Calif.). One hundred micrograms of protein was applied to each lane. 2D-NEPHGE was performed as described by O'Farrell (23) and modified as described by Bledsoe et al. (5). Proteins were visualized by staining with Coomassie blue R250 or by silver staining (21). Molecular weight standards were purchased from Bio-Rad Laboratories, Hercules, Calif.

For immunoblotting, proteins were electrophoretically transferred to nitrocellulose (0.45-μm-pore-size Protran membrane; Schleicher & Schuell, Keene, N.H.) as described by Towbin et al. (31) by using a Bio-Rad Trans Blot Cell (100 mA, 12 h, 4°C). After transfer, proteins were visualized by using Ponceau Red (0.1% Ponceau Red dye in 1.0% acetic acid) and the standards were marked. Nitrocellulose membranes were blocked with 5% nonfat dry milk in PBS solution (12 h, 4°C), and cross-adsorbed antiserum (primary antibody) was applied to the blot (2 h, 4°C). The blot was washed twice in 100 ml of PBS for 10 min to remove residual primary antibody. Secondary antibody (alkaline phosphatase [AP]-conjugated goat anti-rabbit or anti-human antibody) was diluted 1:5,000 in 2% Tween 20 in PBS and applied to the blot for 45 min, and then three washes with 200 ml of PBS were done. Reactive bands were detected by using 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (X-phosphate) as described in the Boehringer Mannheim technical manual.

RESULTS

Comparison of protein profiles from low- and high-passage variants of strain B31 by using cross-adsorbed sera. To identify protein differences between low- and high-passage isolates of *B. burgdorferi* B31, we first compared protein profiles by SDS-PAGE. When cell lysate, soluble protein, and membrane fractions were compared by Coomassie blue staining, few differences were detected (Fig. 1A), as reported previously by Norris et al. (22). We decided to use cross-adsorbed rabbit antisera to increase sensitivity for detection of differences between low- and high-passage strains. Anti-*Borrelia* rabbit sera were generated by infecting rabbits with low-passage, virulent *B. burgdorferi* B31. Using this method, we were able to generate sera that routinely gave titers of 1:1,500 by ELISA. In addition, analysis of the sera by using immunoblots demonstrated reactivity with a significant number of *B. burgdorferi* proteins. Preinfection control sera did not react with *B. burgdorferi* proteins (data not shown).

Sera that showed extensive reactivity with *B. burgdorferi* proteins were exhaustively cross-adsorbed with protein isolated from high-passage, avirulent strain B31 as described in Materials and Methods. When cell fractions were probed with cross-adsorbed sera, 10 proteins (relative molecular masses of 78, 58, 49, 34, 33, 28, 24, 20, and 16 kDa) that appeared to be produced only in low-passage *B. burgdorferi* B31 were identified (Fig. 1B). The 78-kDa protein appeared to localize specifically to the soluble fraction, while the nine remaining proteins localized to the total membrane fraction. Reaction of the cross-adsorbed sera with subcellular fractions from high-passage B31 showed that the cross-adsorption had almost completely removed antibody to high-passage proteins. In addition, no bands were visible at 31 or 34 kDa in lanes containing high-passage proteins, even though there were significant amounts

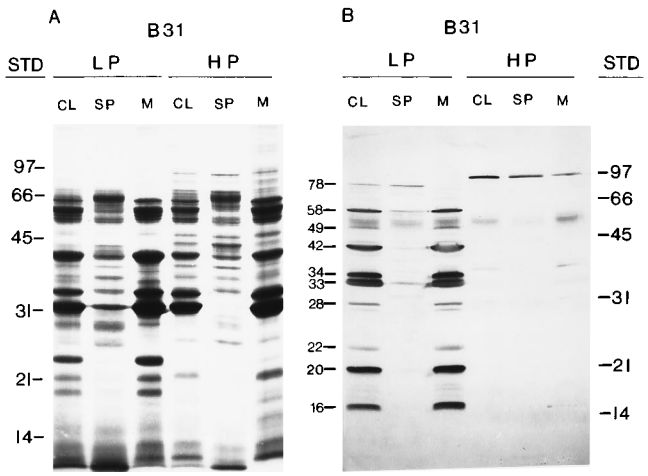

FIG. 1. Coomassie blue-stained SDS-polyacrylamide gel (12.5% polyacrylamide) (A) and immunoblot (B) of proteins from low-passage (LP) and high-passage (HP) variants of *B. burgdorferi* B31. Lanes marked CL, SP, and M contained cell lysate, soluble protein, and membranes, respectively. The immunoblot was probed with cross-adsorbed rabbit serum (collected on day 28), and proteins were detected with AP-conjugated anti-rabbit antibody. Numbers to the left of panel B represent the estimated sizes of low-passage-associated proteins (in kilodaltons). Numbers on the left and right of panels A and B, respectively, indicate protein standards (STD) (in kilodaltons).

of OspA and OspB in these samples. This suggested that all antibodies to these proteins had been effectively removed by the cross-adsorption process. The cross-adsorbed sera detected many more differences between high- and low-passage isolates than were previously reported by Norris et al. (22) using SDS-PAGE, 2D-NEPHGE, and immunoblotting techniques.

Effect of passage on protein changes. The original cross-adsorption experiments compared p5 with p100 proteins. It has been reported by several groups that *B. burgdorferi* may become avirulent as early as p10 to p15 or as late as p20 to p25 (13, 27, 28). Therefore, we investigated the protein changes at three different passages: (i) p5 (virulent strains), (ii) p22 (avirulent strains), and (iii) p100 (avirulent strains). Proteins from total membranes of these passages were blotted and probed with cross-adsorbed sera (Fig. 2). Eight proteins that were not present in p22 membranes (78, 42, 34, 33, 28, 22, 20, and 16 kDa) were detected in p5 membranes (Fig. 2, lane p5, asterisks). Two additional proteins that were not present in p100 membranes (58 and 49 kDa) were detected in p22 membranes. These data suggest that most of the observed changes occurred before p22 and during the virulence-avirulence conversion.

Comparison of proteins of low-passage *B. burgdorferi* B31 and HB19. From the previous experiments, we identified 10 proteins, 8 of which were lost by p22, that appeared to be produced only in low-passage B31. Because it has been demonstrated that various *Borrelia* species undergo antigenic variation, we probed an additional strain (e.g., HB19) in order to identify proteins common to both. Such proteins would be of interest as vaccine candidates and could possibly play a direct role in the pathogenesis of Lyme disease.

Cross-adsorbed sera were used to probe immunoblots containing total membranes isolated from *B. burgdorferi* HB19 (p4) (Fig. 3). We were able to identify at least five proteins produced in HB19 that had similar-molecular-weight counterparts in B31 (78, 49, 34, 33, and 28 kDa) (Fig. 3, arrows). Five proteins identified in strain B31 had no homolog in strain HB19 (58-, 42-, 22-, 20-, and 16-kDa proteins). Thus, some of the low-passage proteins could be conserved between *B. burg-*

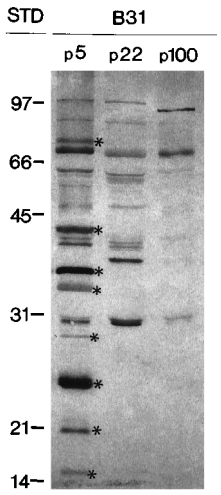

FIG. 2. Immunoblot of *B. burgdorferi* B31 total membranes. Lanes contained membrane proteins from the indicated passages. The immunoblot was probed with cross-adsorbed rabbit serum, and proteins were detected with AP-conjugated anti-rabbit antibody. Asterisks to the right of lane p5 indicate bands that were lost between passages 5 and 22. Numbers to the left indicate sizes of the protein standards (STD) (in kilodaltons).

dorferi strains. Also, it was interesting that four high-molecular-weight proteins detected in strain HB19 had no homolog in strain B31. Since this immunoblot was probed with serum raised against strain B31, the presence of these bands suggests that some event(s) that has generated hybrid proteins has occurred. This could be the result of rearrangements in the genes encoding some of the low-passage-associated proteins. The fact that both strains B31 and HB19 used in these experiments were low-passage, virulent strains suggested that these observed changes did not result in conversion to avirulence but were the result of antigenic variation between divergent strains. This would be expected to occur in bacteria that make use of antigenic variation to evade a host immune system.

Cross-adsorption of serum from a Lyme disease patient. In the initial cross-adsorption experiments, we used anti-*Borrelia* sera that had been raised in rabbits. In order to determine if the same proteins were expressed during Lyme disease, we cross-adsorbed serum from a Lyme disease patient with cell extract from high-passage *B. burgdorferi* B31 (Fig. 4). This cross-adsorbed serum was used to probe total membranes from low- and high-passage variants of strain B31. Five proteins were similar in size to proteins detected by cross-adsorbed rabbit sera (78-, 58-, 34-, 28-, and 20-kDa proteins), suggesting that some of the proteins detected with cross-adsorbed rabbit sera were expressed and elicited an immune response during human infection. It is interesting that the human serum sample used in this blot (patient 90-2111) was collected from a patient who acquired Lyme disease in a geographic area of the United States where strain B31 was originally isolated. In fact, when the same human serum was cross-adsorbed with protein from high-passage HB19 and used to probe low-passage HB19 total membranes, only one very weak reactive band (34 kDa) was observed (data not shown), demonstrating the importance of using the same strain as, or one closely related to, the strain to which the person was exposed for cross-adsorption of human serum.

Identification of immunoreactive outer membrane proteins by using cross-adsorbed sera. We intend to concentrate our efforts on the characterization of low-passage-associated proteins that localize to the outer membranes of *B. burgdorferi*. Cell lysate, soluble protein, total membranes, inner membranes, and outer membranes were blotted and probed with cross-adsorbed rabbit sera (Fig. 5). The adsorbed sera detected eight proteins that localized to the inner membranes of low-passage strain B31 (Fig. 5, lane IM, asterisks; 58-, 49-, 43-, 42-, 34-, 23-, 22-, and 16-kDa proteins). Additional bands (Fig. 5, lane IM) that were not observed in previous blots were detected.

Two proteins (58 and 33 kDa) localized to the outer membrane fraction of low-passage *B. burgdorferi* B31 (Fig. 5, lane OM, arrows). Despite the similarity in size, the 33-kDa protein does not appear to be related to OspA (31 kDa) or OspB (34 kDa). The cross-adsorbed sera did not react with any proteins at 34 or 31 kDa in high-passage cell extracts or membrane fractions (Fig. 1B), in spite of the fact that these samples contain large amounts of OspA and OspB (Fig. 1A). The 58-kDa protein appears to localize to both the inner and outer membrane fractions (Fig. 5, lanes IM and OM, arrow). At this time, we do not know if the 58-kDa outer membrane protein that we observed represents the same polypeptide as that observed in the inner membrane. It is possible, as we have previously shown, that proteins (e.g. OspA) localize to both membrane fractions in *B. burgdorferi* (5). We feel that the 33- and 58-kDa low-passage-associated outer membrane proteins represent previously uncharacterized proteins from *B. burgdorferi* B31.

Comparison of low- and high-passage inner and outer membranes by 2D-NEPHGE. Cross-adsorbed serum is extremely

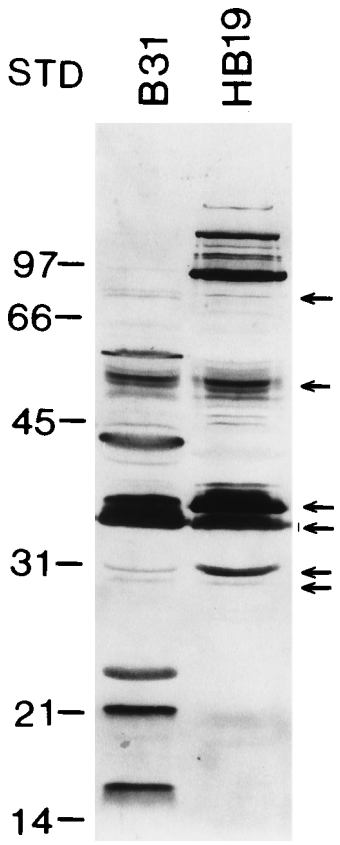

FIG. 3. Immunoblot of total membranes from low-passage *B. burgdorferi* B31 and HB19. The blot was probed with cross-adsorbed rabbit serum and detected with AP-conjugated anti-rabbit antibody. Arrows on the right indicate bands of similar size in the two strains. Numbers to the left indicate sizes of the protein standards (STD) (in kilodaltons).

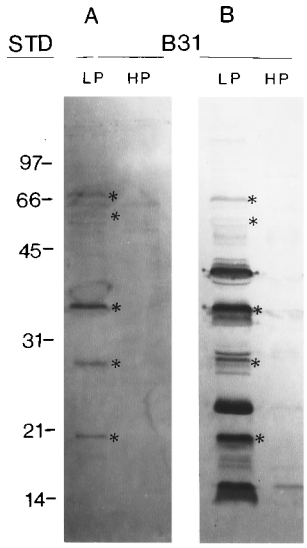

FIG. 4. Immunoblot comparing total membranes of low-passage (LP) and high-passage (HP) variants of *B. burgdorferi* B31 probed with convalescent-phase serum from a human Lyme disease patient (no. 90-2111) which was cross-adsorbed with cell lysate from high-passage strain B31 and detected with AP-conjugated anti-human antibody (A) or cross-adsorbed low-passage rabbit serum raised against strain B31 and detected with AP-conjugated anti-rabbit antibody (B). Asterisks denote bands with similar mobility which were detected by both the human and the rabbit cross-adsorbed sera. Numbers to the left indicate sizes of the protein standards (STD) (in kilodaltons).

sensitive at detecting differences between low- and high-passage isolates but will not detect low-passage proteins that are nonimmunogenic or that are synthesized at reduced levels in high-passage strains. Therefore, we also compared protein profiles of low- and high-passage inner (Fig. 6) and outer (Fig. 7) membranes using 2D-NEPHGE. The low-passage inner membranes contain >50 distinct spots (Fig. 6A). Several of these were not present in high-passage inner membranes (Fig. 7B; 58-, 49-, 43-, 24-, 23-, and 16-kDa proteins) and corresponded to low-passage-specific proteins identified with cross-adsorbed sera (Fig. 5). Three proteins that were detected in low-passage inner membranes with cross-adsorbed sera, including the strongly immunoreactive 34-kDa protein, were not observed by 2D-NEPHGE. This is most likely due to poor solubility of some of these proteins under conditions used for NEPHGE (data not shown). More importantly, no novel low-passage-associated proteins were discovered in inner membranes by 2D-NEPHGE.

The outer membranes from low- and high-passage variants of strain B31 were also compared by 2D-NEPHGE (Fig. 7). Low-passage outer membranes contained 18 to 20 distinct polypeptides, four of which were not observed in high-passage outer membranes (Fig. 7A; 28-, 26-, 18-, and 16-kDa polypeptides). No low-passage-associated proteins that corresponded to the 33- and 58-kDa proteins seen in immunoblots were detected by silver staining. It is possible that these proteins do not stain effectively (as observed with some proteins of other bacteria [32]) or that they are not effectively solubilized before 2D-NEPHGE. A prominent low-passage-associated outer membrane protein observed on 2D-NEPHGE was 28 kDa in size (Fig. 7A) and exhibited a very basic pI. Cross-adsorbed sera had not detected a protein of this size in low-passage outer membranes. The very basic nature of this protein suggests that it is not related to the 28-kDa protein (OspD) previously identified by Norris et al. (22) and represents a previously unidentified low-passage outer membrane protein. Three additional proteins (26, 18, and 16 kDa) were detected only in low-passage-outer-membrane 2D-NEPHGE patterns and were not detected on immunoblots. Like the 28-kDa protein, it is possible that these proteins also represent previously unidentified low-passage-associated outer membrane proteins.

DISCUSSION

One approach that has been used to identify virulence factors from *B. burgdorferi* has been to compare protein profiles of virulent (low-passage) and avirulent (high-passage) variants of *B. burgdorferi*. Norris et al. (22) used 2D-NEPHGE and immunoblotting to compare cell extracts from low-passage (p5) and high-passage (p200) variants of *B. burgdorferi* B31. They identified four low-passage-associated proteins (35, 28, 24, and 20 kDa) that are not expressed or weakly expressed in high-passage, avirulent *B. burgdorferi* B31. However, experiments based on comparisons of cell lysates may be relatively insensitive to minor changes in protein composition and major proteins, such as the Osps, may interfere with the identification of proteins of similar size.

In order to increase the sensitivity of detection, we made use of a cross-adsorption technique to compare virulent and avirulent strains. This yields an antibody probe that can be used for the identification of proteins that are expressed only in low-passage, virulent strains. Using this immune adsorption technique, we identified 10 proteins that appear to be expressed only in low-passage *B. burgdorferi* B31. Preliminary localization experiments indicate that nine of these proteins localize to the total membrane fraction, showing that almost all of the observed changes had affected membrane proteins. Five proteins similar in size to those identified with adsorbed rabbit sera were detected from adsorbed human serum. Thus, it appears that many of the low-passage-associated proteins detected by this technique could elicit a humoral response during human infection. Previous studies comparing virulent and avirulent variants had not detected so many differences. The number of

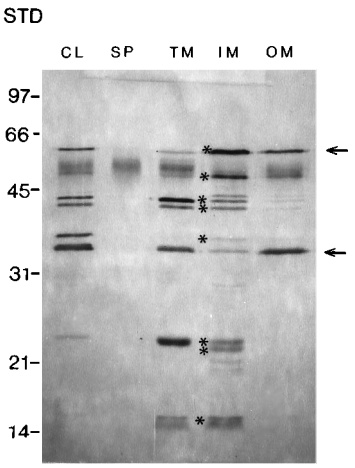

FIG. 5. Immunoblot of subcellular fractions from low-passage *B. burgdorferi* B31. The blot was probed with cross-adsorbed rabbit serum raised against strain B31 and detected with AP-conjugated anti-rabbit antibody. Lanes: CL, cell lysate; SP, soluble protein; TM, total membranes; IM, inner membrane; OM, outer membranes. The asterisks indicate low-passage-associated bands which localize to the inner membrane. The arrows indicate low-passage-associated bands which localize to the outer membrane. The numbers to the left of the figure indicate sizes of protein standards (STD) (in kilodaltons).

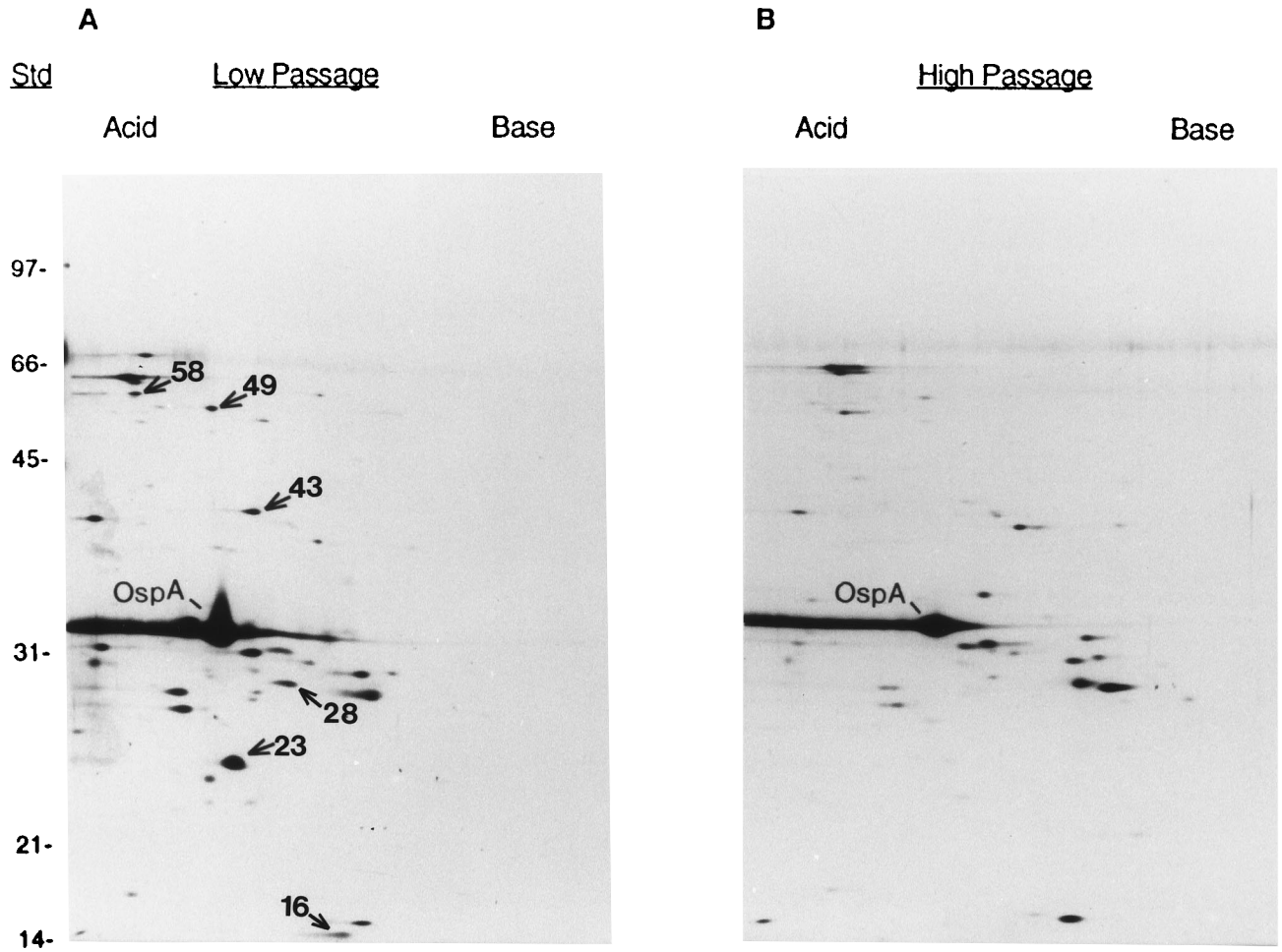

FIG. 6. 2D-NEPHGE analysis of purified inner membranes from low-passage (A) and high-passage (B) variants of *B. burgdorferi* B31. Proteins were detected by silver staining. The arrows indicate proteins which are unique to the low-passage purified inner membranes. The gel is oriented so that the acidic end of the first dimension is to the left and the basic end is to the right. Numbers to the left indicate sizes of protein standards (Std) (in kilodaltons).

proteins observed illustrates the sensitivity of the technique and, more importantly, the extent of the changes taking place.

There are several possible reasons for these changes. (i) Protein differences could result from metabolic changes as *B. burgdorferi* adapts to in vitro growth (e.g., altered transport systems). (ii) Changes could be due to antigenic variation as low-passage strains are passaged in vitro, and this type of change has been extensively reported (2, 6, 26, 27, 33–35). (iii) Changes could also result from altered gene expression due to some unidentified regulatory factor. Certainly, some or all of these mechanisms could be responsible for the observed alterations, thus identifying a subset of proteins that are not related to virulence or to in vivo survival of *B. burgdorferi*. However, some of these changes could be directly related to the observed loss of virulence.

Of the proteins detected with cross-adsorbed sera, three (34-, 28-, and 20-kDa proteins) are similar in size to the proteins identified by Norris et al. (22). We did not detect a 24-kDa protein which was reported to be synthesized at reduced levels in high-passage strains. This points out one problem with the cross-adsorption technique. It is relatively insensitive to differences in levels of synthesis. If a protein is produced in sufficient quantities in the avirulent variant, it has the capability to bind and remove antibodies from the polyclonal sera during cross-adsorption. Thus, proteins synthesized at reduced levels may go undetected when protein extracts from the low-passage, virulent isolates are probed. It should be noted that a 23-kDa low-passage-associated protein was observed by using silver-stained 2D-NEPHGE (Fig. 6), and this protein may be related to the 24-kDa protein (22).

Until recently, reliable methods for the localization of outer membrane proteins from spirochetes were lacking. The methods most commonly used to localize proteins of *B. burgdorferi* (2, 3, 10, 11, 15) are subject to artifacts due to the labile nature of the outer membrane of *B. burgdorferi*. Most recently, we have developed a technique using isopycnic centrifugation (5) that allows the isolation of outer as well as inner membranes, and this has permitted more accurate localization of low-passage-associated proteins from *B. burgdorferi* (5). Cross-adsorbed sera were used to probe purified inner and outer membranes isolated from low-passage strain B31. Eight low-passage proteins were identified in strain B31 inner membranes. This was not unexpected, since the inner membrane has been shown to be much more complex than the outer membrane by freeze fracture (5, 14, 24, 25) and 2D-NEPHGE (Fig. 6). In addition, it is also logical that the inner membrane, which harbors numerous enzyme systems, would undergo changes as the bacterial cells adapt physiologically to in vitro growth. Finally, we

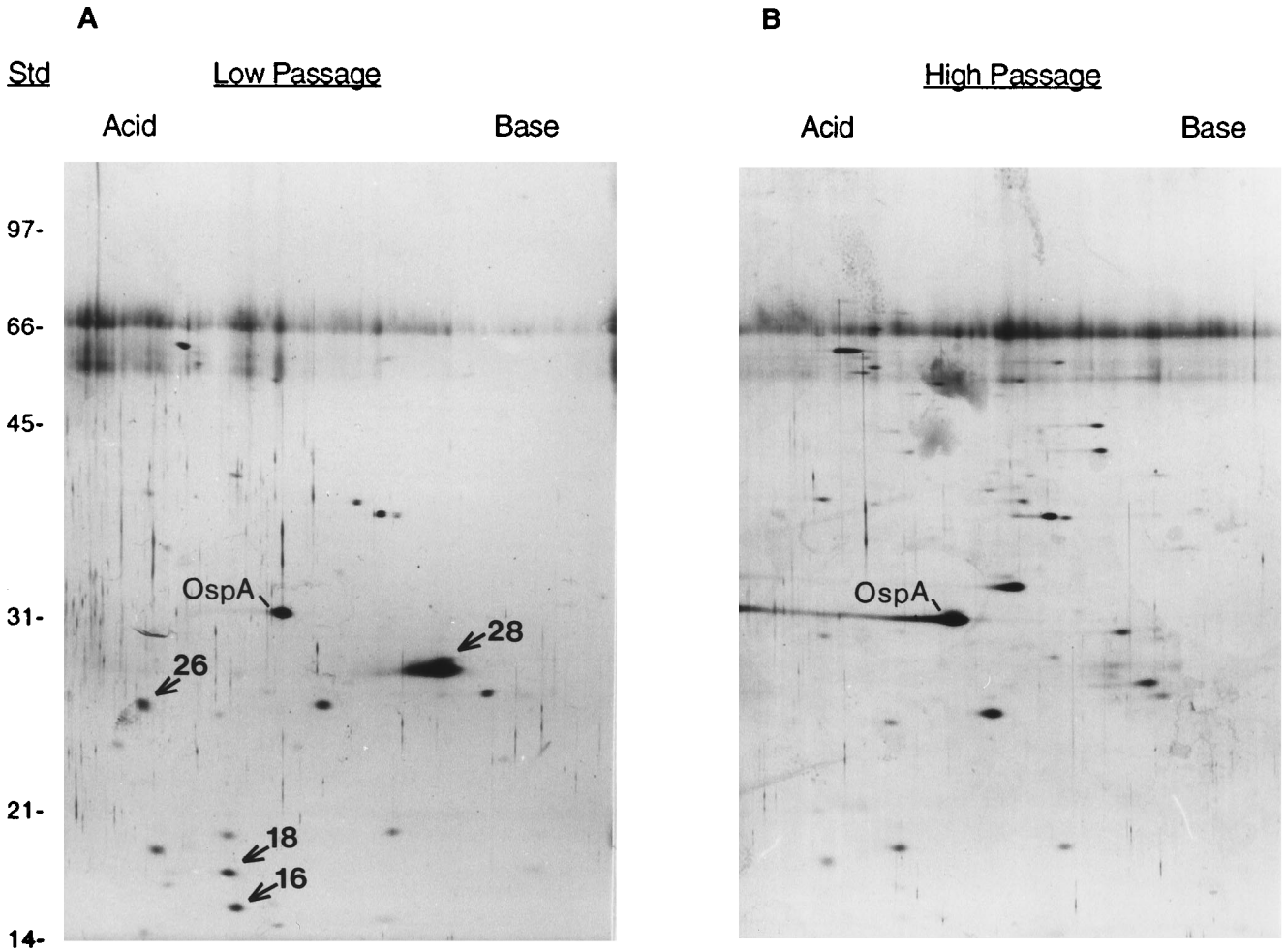

FIG. 7. 2D-NEPHGE analysis of purified outer membranes from low-passage (A) and high-passage (B) variants of *B. burgdorferi* B31. Proteins were detected by silver staining. The arrows indicate proteins which are unique to the low-passage purified outer membranes. The gel is oriented so that the acidic end of the first dimension is to the left and the basic end is to the right. Numbers to the left indicate the relative mobilities of the molecular mass markers (Std) (in kilodaltons).

have identified two low-passage-associated proteins (58 and 33 kDa) from the outer membrane of *B. burgdorferi* B31. Preliminary analysis of the 58- and 33-kDa proteins suggests that they are clearly unique, different from previously characterized *B. burgdorferi* proteins.

In addition to being insensitive to detecting proteins which are synthesized at reduced levels or virulence factors which are produced in avirulent as well as virulent strains, cross-adsorption will not detect proteins that have not stimulated an immune response. Therefore, we compared low- and high-passage inner and outer membranes using 2D-NEPHGE. By using purified membrane fractions, we hoped that we would simplify the protein patterns in order to facilitate the detection of minor membrane components. Low-passage inner membranes had six proteins that were not detected in high-passage inner membranes. These all were similar in size to proteins detected with cross-adsorbed sera. However, low-passage outer membranes had four proteins that were low passage associated that were not detected by cross-adsorbed sera. Three of these (26, 18, and 16 kDa) are minor components of the outer membrane, while one very basic protein (28 kDa) appears to be synthesized at significant levels. The amount of the 28-kDa protein observed on 2D-NEPHGE hints that this protein may be worth further investigation.

The approaches we have taken to identify the 58-, 33-, and 28-kDa low-passage outer membrane proteins are intended to provide valuable information about the pathogenesis of *B. burgdorferi*. Ultimately, our goal is to identify surface proteins of *B. burgdorferi* that are essential for virulence. Such proteins are excellent candidates for vaccine development. By targeting these proteins, one could block the initial ability of *B. burgdorferi* to establish an infection. Because of the potential importance of these low-passage-associated outer membrane proteins, we intend to target the 58-, 33-, and 28-kDa proteins for further characterization.

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REFERENCES

1. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57:**521–525.
2. **Barbour, A. G., S. L. Tessier, and S. F. Hayes.** 1984. Variation in a major surface protein of Lyme disease spirochetes. Infect. Immun. **45:**94–100.
3. **Barbour, A. G., S. L. Tessier, and W. J. Todd.** 1983. Lyme disease spirochetes

and ixodid tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. Infect. Immun. **41:**795–804.
4. **Barthold, S. W., M. S. de Souza, J. L. Janotka, A. L. Smith, and D. H. Persing.** 1993. Chronic Lyme borreliosis in the laboratory mouse. Am. J. Pathol. **143:**959–971.
5. **Bledsoe, H. A., J. A. Carroll, T. R. Whelchel, M. A. Farmer, D. W. Dorward, and F. C. Gherardini.** 1994. Isolation and partial characterization of *Borrelia burgdorferi* inner and outer membranes by using isopycnic centrifugation. Infect. Immun. **176:**7447–7455.
6. **Bundoc, V. G., and A. G. Barbour.** 1989. Clonal polymorphism of outer membrane protein OspB of *Borrelia burgdorferi*. Infect. Immun. **57:**2733–2741.
7. **Burgdorfer, W.** 1984. Discovery of the Lyme disease spirochete and its relation to tick vectors. Yale J. Biol. Med. **57:**609–612.
8. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease—a tick borne spirochetosis? Science **216:** 1317–1319.
9. **Catrenich, C. E., and W. Johnson.** 1988. Virulence conversion of *Legionella pneumophila*: a one-way phenomenon. Infect. Immun. **56:**3121–3125.
10. **Coleman, J. L., J. L. Benach, G. Beck, and G. S. Habicht.** 1986. Isolation of the outer envelope from *Borrelia burgdorferi*. Zentralbl. Bakteriol. Hyg. **263:** 123–126.
11. **Cunningham, T. M., D. D. Thomas, S. D. Thompson, J. N. Miller, and M. A. Lovett.** 1988. Identification of *Borrelia burgdorferi* surface components by Triton X-114 phase partitioning. Ann. N. Y. Acad. Sci. **539:**376–378.
12. **Gherardini, F. C., and A. A. Salyers.** 1987. Characterization of an outer membrane mannanase from *Bacteroides ovatus*. J. Bacteriol. **169:**2031–2037.
13. **Johnson, R. C., F. W. Hyde, and C. M. Rumpel.** 1984. Taxonomy of the Lyme disease spirochetes. Yale J. Biol. Med. **57:**529–537.
14. **Jones, J. D., K. W. Boerell, M. V. Norgard, and J. D. Radolf.** 1995. Membrane topology of *Borrelia burgdorferi* and *Treponema pallidum* lipoproteins. Infect. Immun. **63:**2424–2434.
15. **Luft, B. J., W. Jiang, P. Munoz, R. J. Dattwyler, and P. D. Gorevic.** 1989. Biochemical and immunological characterization of the surface proteins of *Borrelia burgdorferi*. Infect. Immun. **57:**3637–3645.
16. **Luzar, M. A., and T. C. Montie.** 1985. Avirulence and altered physiological properties of cystic fibrosis strains of *Pseudomonas aeruginosa*. Infect. Immun. **50:**572–576.
17. **Magnarelli, L. A., J. F. Anderson, and J. B. McAninch.** 1990. Serologic analyses of cottontail rabbits for antibodies to *Borrelia burgdorferi*. J. Clin. Microbiol. **28:**890–893.
18. **Magnarelli, L. A., J. M. Meegan, J. F. Anderson, and W. A. Chappell.** 1984. Comparison of an indirect fluorescent-antibody test with an enzyme-linked immunosorbent assay for serological studies of Lyme disease. J. Clin. Microbiol. **20:**181–184.
19. **Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87:**206–210.
20. **Mehigh, R. J., A. K. Sample, and R. R. Brubaker.** 1989. Expression of the low calcium response in *Yersinia pestis*. Microb. Pathog. **6:**203–217.
21. **Morrissey, J. H.** 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. **117:** 307–310.
22. **Norris, S. J., C. J. Carter, J. K. Howell, and A. G. Barbour.** 1992. Low-passage-associated proteins of *Borrelia burgdorferi* B31: characterization and molecular cloning of OspD, a surface-exposed, plasmid-encoded lipoprotein. Infect. Immun. **60:**4662–4672.
23. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.
24. **Radolf, J. D., K. W. Bourell, D. R. Akins, J. S. Brusca, and M. V. Norgard.** 1994. Analysis of *Borrelia burgdorferi* membrane architecture by freeze-fracture electron microscopy. J. Bacteriol. **176:**21–31.
25. **Radolf, J. D., M. S. Goldberg, K. Bourell, S. I. Baker, J. D. Jones, and M. V. Norgard.** 1995. Characterization of outer membranes isolated from *Borrelia burgdorferi*, the Lyme disease spirochete. Infect. Immun. **63:**2154–2163.
26. **Rosa, P. A., T. Schwan, and D. Hogan.** 1992. Recombination between genes encoding major outer surface proteins A and B of *Borrelia burgdorferi*. Mol. Microbiol. **6:**3031–3040.
27. **Schwan, T. G., and W. Burgdorfer.** 1987. Antigenic changes of *Borrelia burgdorferi* as a result of in vitro cultivation. J. Infect. Dis. **156:**852–853.
28. **Schwan, T. G., W. Burgdorfer, and C. F. Garon.** 1988. Changes in infectivity and plasmid profile of the Lyme disease spirochete, *Borrelia burgdorferi*, as a result of in vitro cultivation. Infect. Immun. **56:**1831–1836.
29. **Spanier, J. G., S. J. Jones, and P. Cleary.** 1984. Small DNA deletions creating avirulence in *Streptococcus pyogenes*. Science **225:**935–938.
30. **Szczepanski, A., and J. L. Benach.** 1991. Lyme borreliosis: host responses to *Borrelia burgdorferi*. Microbiol. Rev. **52:**21–24.
31. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
32. **Wedege, E., and L. O. Froholm.** 1986. Human antibody response to a group B serotype 2a meningococcal vaccine determined by immunoblotting. Infect. Immun. **51:**571–578.
33. **Wilske, B., A. G. Barbour, S. Bergstrom, N. Burman, B. I. Restrepo, P. A. Rosa, T. Schwan, E. Soutschek, and R. Wallich.** 1992. Antigenic variation and strain heterogeneity in *Borrelia* spp. Res. Microbiol. **143:**583–596.
34. **Wilske, B., V. Preac-Mursic, S. Jauris, A. Hofmann, I. Pradel, E. Soutschek, E. Schwab, G. Will, and G. Wanner.** 1993. Immunological and molecular polymorphisms of OspC, an immunodominant major outer surface protein of *Borrelia burgdorferi*. Infect. Immun. **61:**2182–2191.
35. **Wilske, B., V. Preac-Mursic, G. Schierz, R. Kuhbeck, A. G. Barbour, and M. Kramer.** 1988. Antigenic variability of *Borrelia burgdorferi*. Ann. N. Y. Acad. Sci. **539:**126–143.

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