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Identification of Outer Membrane Proteins of *Bartonella bacilliformis*

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Purification of the outer membrane of *Bartonella bacilliformis* by sucrose step gradient centrifugation and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) suggest that 14 proteins, ranging from 11.2 to 75.3 kDa, are located in the outer membrane of the pathogen. On the basis of M_rs, eleven of these proteins have counterparts which are labeled by extrinsic radioiodination of intact bartonellae, and two of the proteins are visibly sensitive to extrinsic proteinase K digestion in analysis by SDS-PAGE. While nearly all the extrinsically radioiodinated proteins could be immunoprecipitated with rabbit antibartonella hyperimmune serum, proteins of 31.5, 42, and 45 kDa were prominent immunoprecipitants. Purified lipopolysaccharide from the outer membrane of *B. bacilliformis* produced a diffuse band of approximately 5 kDa on SDS-PAGE and was not detectable on immunoblots developed with rabbit antibartonella hyperimmune antiserum.

Bartonella bacilliformis is the etiologic agent of Oroya fever (Carrión's disease) in humans. The disease is transmitted between humans (10) by nocturnal sand flies in the genera *Phlebotomus* and *Lutzomyia* (11). Oroya fever is endemic to the Andes mountain range of Columbia, Ecuador, and Peru (17), where more than 60% of asymptomatic inhabitants are seropositive for the bacterium (16). Approximately 5 to 10% of the population in these areas are active carriers of the agent (17). In 1987, a rural Peruvian epidemic resulted in the death of a number of residents, with a fatality rate of 88% in untreated individuals (9). The typical mortality rate in the preantibiotic era was approximately 40% (3).

Oroya fever is a biphasic disease characterized by an initial acute hematic phase which occurs within 3 weeks of exposure. The primary stage can present with fever, malaise, and one of the most severe hemolytic anemias known. During the hematic phase, *B. bacilliformis* parasitizes nearly every erythrocyte in the blood (2), and erythrocyte counts decrease by nearly 80% to fewer than 10^6 cells per mm^3 (14). *B. bacilliformis* also invades endothelial cells and can form enlarged membrane-bound vacuoles termed Rocha-Lima inclusions (6). Within 4 to 8 weeks of the primary infection, the secondary or tissue phase of disease presents with hemangiomas (verruga peruana), which erupt in patches on the face and extremities. Hemangioma formation is possibly caused by the hyperproliferation of terminal vascular endothelial cells in response to an angiogenic factor released by the bacterium during the infection (5). The secondary phase also presents with immunosuppression, hepatosplenomegaly, and lymphadenopathy, probably as a result of extensive reticuloendothelial system overload (7). Patients with bartonellosis-induced immunosuppression are susceptible to a variety of opportunistic infections, including shigellosis, salmonellosis, and tuberculosis (3).

Pathogenesis of *B. bacilliformis* is poorly characterized at the molecular and cellular levels. The predilection of bartonellae for erythrocytes is unique among human bacterial pathogens. Viable bacteria attach to a glycolipid moiety on the erythrocyte surface within 30 min of incubation in an energy-dependent reaction (26), with the maximal number of erythrocyte-bacteria complexes formed by 6 h postinoculation (2). Following bacterial attachment to the erythrocyte surface, an endosomal vacuole is formed by deformation and invagination of the cytoplasmic membrane (2). While invasion by bartonellae into epithelial, endothelial, or fibroblast cells is partly facilitated by microfilament-dependent activity of the host cell (12), erythrocyte involvement in the invasion process is necessarily passive, since mature erythrocytes are nonendocytotic. Host cell invasion probably requires the concerted effort of functional flagella (25) and release of deformation factor, a protein which can produce invaginations and trenches in the erythrocyte cytoplasmic membrane independently of the bacterium (20).

Given the importance of surface-exposed determinants to bacterial pathogenesis and immunity, the present study was undertaken to identify the outer membrane proteins (OMPs), to partially characterize the lipopolysaccharide (LPS) of *B. bacilliformis*, and to determine if these components are immunogenic.

Bacterial strains and culture conditions. *B. bacilliformis* KC584 was purchased from the American Type Culture Collection, Rockville, Md. Bartonellae were typically grown at 30°C in a water-saturated atmosphere on heart infusion agar (Difco Laboratories, Detroit, Mich.) containing 5% defibrinated sheep erythrocytes and 5% filter-sterile sheep serum (Colorado Serum Co., Denver, Colo.) by volume. Bacteria were harvested at 5 days postinoculation for all experiments except radioiodination, in which 3-day-old cultures were used.

OMP purification. The outer membrane fraction of *B. bacilliformis* was purified by sucrose step gradient centrifugation, by a modification of the methods of Achtman et al. (1). Bartonellae were harvested into 5 ml of ice-cold 0.2 M Tris–1 mM $MgSO_4$ (pH 8.0), centrifuged at 6,000 × *g* for 5 min at 4°C, and resuspended in 5 ml of 0.2 M Tris (pH 8.0). The suspension was then mixed sequentially with 5 ml of 1 M sucrose in 0.2 M Tris (pH 8.0), 10 μl of 0.5 M EDTA, and 75 μl of freshly prepared egg white lysozyme (25-mg/ml stock) (Sigma Chemical Co., St. Louis, Mo.) and incubated for 3 h on ice. The mixture was treated by forcefully adding 10 ml of ice-cold distilled water by pipette and mixed. After a 30-min incubation on ice, 20 ml of 0.2 M dithiothreitol was added and

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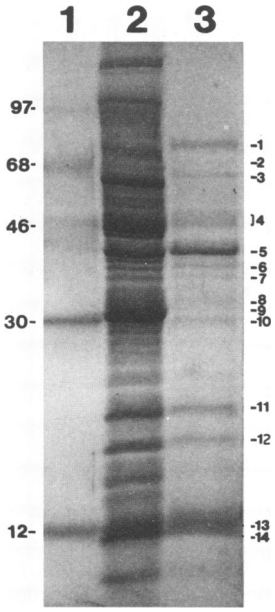

FIG. 1. Coomassie blue-stained SDS-PAGE (12.5% [wt/vol] acrylamide gel) of the outer-membrane fraction of *B. bacilliformis* KC584 following a sucrose step gradient centrifugation of the total membranes. Lanes: 1, protein M_r standards; 2, *B. bacilliformis* KC584 cell lysate; 3, the trichloroacetic acid precipitant from the outer-membrane fraction of bartonellae. The putative OMPs are numbered at the right, and M_rs of the protein standards are given to the left (in thousands).

the mixture was sonicated for 2 min (50% cycle). Cell debris was removed by centrifuging at 10,000 × *g* for 15 min at 4°C. The supernatant was diluted with 1 volume of distilled water and then centrifuged in an 80 Ti rotor (Beckman Instruments, Fullerton, Calif.) at 240,000 × *g* for 2 h at 4°C. The total membrane pellet was resuspended in 0.5 ml of 20% (wt/vol) sucrose–1 mM EDTA–2 mM dithiothreitol and applied to a step gradient containing 0.5-ml steps of 60, 55, 50, 45, 40, 25, and 20% (all wt/vol) sucrose containing 1 mM EDTA–2 mM dithiothreitol. Centrifugation of the gradient was done in an SW 60 Ti rotor (Beckman) at 160,000 × *g* for 36 h at 4°C. The resulting gradient did not produce visible banding. Because of a scarcity of biochemical data on bartonellae, the outer-membrane fraction was then collected on the basis of sucrose density values for *Escherichia coli*, where ρ = 1.25 (1). Proteins were precipitated from the fraction with 4 volumes of 5% (wt/vol) trichloroacetic acid for 16 h at 4°C and then collected by centrifugation at 16,000 × *g* for 15 min in a microcentrifuge. The collected trichloroacetic acid precipitate was resuspended in 0.2 volume of Laemmli sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18) to identify the putative OMPs. SDS-PAGE analysis of the outer-membrane fraction from the sucrose step gradients revealed 14 proteins ranging from 11.2 to 75.3 kDa (Fig. 1). M_rs of the 14 putative OMPs are summarized in Table 1.

A Sarkosyl-insoluble precipitate of bartonella membranes was also prepared, by the methods of Gogolewski et al. (8). However, very poor fractionation of OMPs was achieved on the basis of Sarkosyl insolubility of the outer membrane. The majority of proteins present in the cell lysate of the bacterium were also present in the Sarkosyl-insoluble fraction when analyzed by SDS-PAGE (data not shown). These results

TABLE 1. Correlation of M_rs of the putative OMPs of *B. bacilliformis* with M_rs of protein species of the bacterium

OMP[a]	M_r[b] (10^3)	Correlation with:		
		^{125}I[c]	RIP[d]	Proteinase K[e]
1	75.3			
2	68	+	+	+
3	64.6	+	+	
4	44–48[f]	+	+	
5	42	+	+	
6	38.9			
7	36.3	+		
8	32.9			
9	31.5	+	+	+
10	29.4	+	+	
11	23	+	+	
12	20	+	+	
13	12	+	+	
14	11.2	+	+	

[a] Numbers correspond to the putative OMPs in Fig. 1.
[b] M_rs of the OMPs identified by sucrose step gradient centrifugation–SDS-PAGE analysis (Fig. 1).
[c] A plus sign indicates that an extrinsically iodinated protein with the same M_r exists (Fig. 2).
[d] RIP, radioimmunoprecipitation. A plus sign indicates that a protein species of the same M_r was immunoprecipitated (Fig. 4).
[e] A plus sign indicates that a proteinase K-sensitive protein with the same M_r exists (Fig. 3).
[f] Several protein bands with M_rs ranging from 44,000 to 48,000 were present.

suggest that Sarkosyl insolubility is not a suitable means of obtaining OMPs from total membrane preparations of bartonellae.

Extrinsic radioiodination. To identify proteins which are accessible to extrinsic radioiodination of intact cells of the pathogen and to corroborate the M_rs of the putative OMPs identified by the sucrose step gradient–SDS-PAGE analysis (Fig. 1), radioiodination was done by a modification of the methods of Markwell (19). Briefly, three petri plates of bartonellae were harvested into 100 μl of ice-cold Dulbecco's phosphate-buffered saline (dPBS), pH 7.4 (4). Three Iodobeads (Pierce Chemical, Rockford, Ill.) were added to the suspension and allowed to equilibrate for 5 min. $Na^{125}I$ (New England Nuclear, Boston, Mass.) was then added to the cells at a final concentration of 1 mCi/ml and incubated for 30 min on ice. The reaction was stopped by aspirating the cells away from the Iodobeads to a new microcentrifuge tube. The radiolabeled cells were washed twice with 300 μl of dPBS with 1 min centrifugations at 16,000 × *g*, between washings. The final pellet was suspended in 1 volume of Laemmli sample buffer and analyzed by SDS-PAGE. To ensure that the bartonella cells were intact during the extrinsic radioiodination, cell lysates were prepared by harvesting bartonellae into 100 μl of dPBS containing 1% (wt/vol) SDS and then iodinated as in the whole-cell protocol. Extrinsic radioiodination of bartonellae shows that 15 proteins can be radiolabeled on intact cells of the pathogen (Fig. 2). Eleven of the iodinated proteins have M_rs which are the same as those obtained for OMPs identified by the sucrose step gradient–SDS-PAGE analysis (Fig. 1). A comparison of the results from the two methods is presented in Table 1. Proteins of 16.7, 82.5, 124, and 130.8 kDa were also radioiodinated (Fig. 2, lane 2) but, on the basis of M_r, do not have apparent sucrose step gradient–SDS-PAGE counterparts (Fig. 1 and Table 1). The appearance of numerous additional ^{125}I-labeled protein bands in the protein profiles of bartonella cells which were lysed prior to radioiodination (Fig. 2, lane 1)

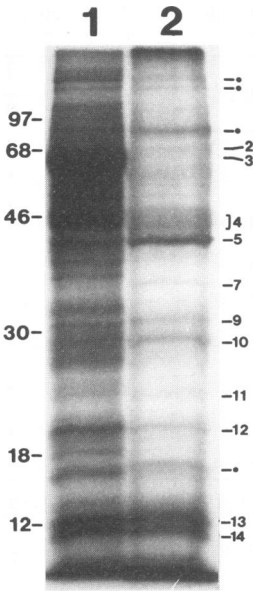

FIG. 2. SDS-PAGE analysis (12.5% [wt/vol] acrylamide) of the extrinsically ^{125}I-labeled proteins of *B. bacilliformis* KC584. An autoradiograph is shown. Lanes: 1, profile of iodinated cell lysates; 2, protein profile of extrinsically radioiodinated intact bartonellae. Numbers to the right correspond to the putative OMPs identified by sucrose step gradient–SDS-PAGE analysis based on M_r (Fig. 1), while closed circles represent extrinsically radioiodinated proteins which have no M_r counterpart in Fig. 1. M_rs of the protein standards are given to the left (in thousands) and were determined by ^{14}C-labeled standards.

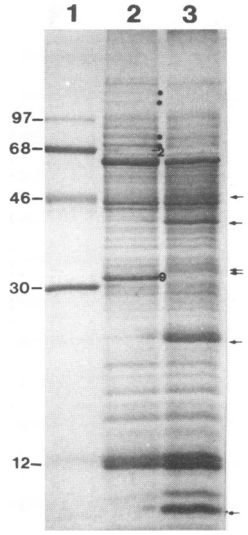

FIG. 3. Coomassie blue-stained SDS-PAGE (12.5% [wt/vol] acrylamide gel) of the proteinase K-sensitive proteins on intact *B. bacilliformis* KC584 cells. Lanes: 1, protein M_r standards; 2, protein profile of untreated (control) bartonella cells; 3, protein profile of intact bartonella cells treated with proteinase K. Numbers to the right of lane 2 correspond to proteins which were proteolytically cleaved during extrinsic proteinase K digestion and have the same M_rs as the putative OMPs identified by sucrose step gradient–SDS-PAGE analysis (Fig. 1), while closed circles represent proteolytically cleaved proteins which have no M_r counterpart in Fig. 1. Novel proteolysis products following the digestion are indicated by arrows (lane 3). M_rs of the protein standards are given to the left (in thousands).

suggests that the bacterial cells used in the extrinsic radioiodination (Fig. 2, lane 2) were indeed intact.

Identification of proteinase K-accessible surface proteins. Extrinsic proteinase K treatment of whole *B. bacilliformis* cells was also used to identify surface-exposed proteins of the bacterium by a modification of the technique of Sadziene et al. (23). Bartonellae were harvested into 0.5 ml of ice-cold dPBS, centrifuged at 6,000 × *g* for 5 min, and then resuspended in dPBS to a final density of 10^9 cells per ml. Two hundred micrograms of proteinase K (Sigma) was added to 490 μl of the cell suspension and allowed to incubate for 40 min at 22°C. The reaction was stopped by adding 25 μg of phenylmethylsulfonyl fluoride (PMSF) in 2.5 μl of 95% ethanol. The suspension was centrifuged for 6,000 × *g* for 5 min, washed in 1 volume of dPBS containing 50 μg of PMSF per ml, and then recentrifuged. The pellet was resuspended in Laemmli sample buffer containing 50 μg of PMSF per ml, boiled for 5 min, and then analyzed by SDS-PAGE (18). The resulting gels were fixed in methanol-acetic acid-water (5:1:5, vol/vol/vol) and then stained with Coomassie brilliant blue-R (24). Extrinsic proteinase K treatment of intact bartonellae resulted in the altered migration of six proteins on SDS-PAGE (Fig. 3). Two proteins, of 31.5 and 68 kDa, have M_rs which correspond to those for two putative OMPs identified by the sucrose step gradient–SDS-PAGE analysis (Fig. 1 and Table 1). Four other proteins, of 71.2, 82.5, 116, and 124 kDa, were also sensitive to proteinase K but have no apparent M_r counterparts in the putative OMPs identified by the sucrose step-gradient–SDS-PAGE analysis (Fig. 1). Treatment of intact bartonella cells with trypsin gave results similar to those from the extrinsic proteinase K treatment and did not reveal additional surface-exposed polypeptides (21). The most prominent surface-exposed protein revealed by the extrinsic proteinase K analysis was a 31.5-kDa polypeptide, termed bb32. The bb32 protein is a major protein band which stains intensely with Coomassie blue in untreated cell lysates of the bacterium (Fig. 3, lane 2) but is not detectable in protein profiles of bartonellae following treatment with proteinase K (Fig. 3, lane 3).

Radioimmunoprecipitation analyses. Lifelong humoral immunity can be obtained following an active infection with *B. bacilliformis* (27). Therefore, identification of immunogenic surface proteins of the bacterium is potentially useful for identifying subunit vaccine candidates. Radioimmunoprecipitation analysis of the extrinsically radioiodinated proteins of bartonellae with rabbit anti-*B. bacilliformis* hyperimmune antiserum was performed to identify OMPs which are immunogenic. Radioimmunoprecipitation was accomplished as previously described (22), with rabbit anti-*B. bacilliformis* hyperimmune antiserum prepared as before (25). Twenty extrinsically radioiodinated proteins were immunoprecipitated with the antiserum (Fig. 4). Ten of these precipitants have the same M_rs as proteins identified as putative OMPs by the sucrose step gradient–SDS-PAGE analysis (Fig. 1 and Table 1), while proteins of 16.7, 18, 41, 61, 71.2, 77.7, 87.3, 92.2, 116, and 124 kDa have no sucrose step gradient–SDS-PAGE counterparts in Fig. 1. The most prominent immunoprecipitants produced by the antiserum included ^{125}I-labeled proteins of 31.5, 41, 42, and 45 kDa (Fig. 4, lane 4). However, on the basis of M_rs only the 31.5-, 42-, and 45-kDa proteins have putative OMP counterparts in the sucrose step gradient–SDS-PAGE analysis (Fig. 1 and Table 1). The immunoprecipitated proteins of 71.2, 116, and 124 kDa also have M_r counterparts which are sensitive to extrinsic proteinase K treatment (Fig. 3).

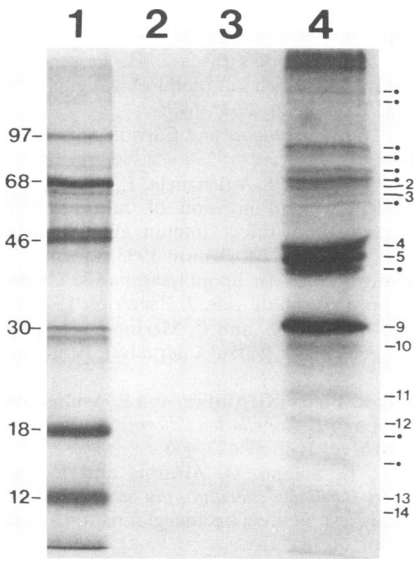

FIG. 4. SDS-PAGE analysis (12.5% [wt/vol] acrylamide) of the ^{125}I-labeled surface proteins immunoprecipitated with rabbit anti-*B. bacilliformis* hyperimmune serum. An autoradiograph is shown. Lanes: 1, ^{14}C-labeled protein M_r standards; 2, precipitate resulting from incubation with dPBS; 3, precipitate resulting from incubation with preimmune (naive) rabbit serum; 4, the immunoprecipitate produced from the antiserum. Numbers to the right correspond to proteins which were precipitated during incubation with antiserum and have the same M_rs as the putative OMPs identified by sucrose step gradient–SDS-PAGE analysis (Fig. 1), while closed circles indicate precipitated proteins which have no M_r counterparts in Fig. 1. M_rs of the protein standards are given to the left (in thousands).

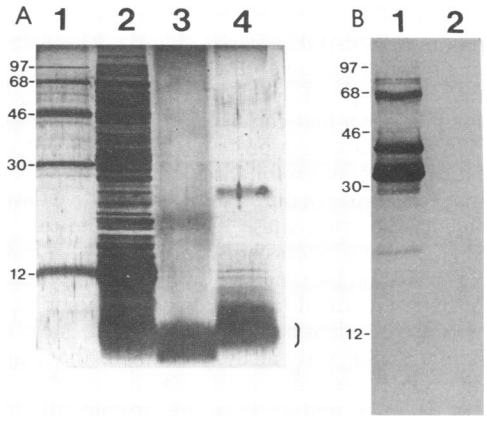

FIG. 5. (A) Silver-stained SDS-PAGE (15% [wt/vol] acrylamide) of the purified LPS from *B. bacilliformis* KC584. Lanes: 1, protein M_r standards; 2, protein profile of the cell lysate of bartonellae; 3, purified LPS (10 μg); 4, proteinase K-resistant fraction of the cell lysate. The position of the LPS and the major band in the proteinase K-resistant fraction is indicated by a bracket. (B) Immunoblot analysis of LPS with rabbit anti-*B. bacilliformis* antiserum. Lanes: 1, cell lysate of bartonellae; 2, purified LPS. M_rs of the protein standards are given to the left of each panel (in thousands).

Immunoprecipitant produced by incubating the extrinsically radioiodinated proteins with dPBS or preimmune (naive) rabbit serum was negligible (Fig. 4, lanes 2 and 3, respectively), suggesting that the antibodies within the antiserum were responsible for the immunoprecipitation results in Fig. 4 (lane 4).

LPS purification. To further characterize the outer membrane of *B. bacilliformis*, LPS was purified from the bacterium by a modification of the procedure of Westphal and Jann (28). Bartonella cells (approximately 300 mg [wet weight]) were harvested into 0.9% (wt/vol) NaCl on ice. The cells were spun for 6,000 × *g* for 10 min at 4°C, and the supernatant was decanted. The pellet was frozen at −20°C and resuspended in 3 ml of sterile distilled water which was preheated to 65°C. To the suspension, 3 ml of hot phenol-water (90% [vol/vol] redistilled phenol in sterile distilled water at 65°C) was added and mixed. The mixture was then incubated for 2 h at 65°C with constant shaking and cooled to 10°C in an ice bath. Aqueous and phenol phases were separated by centrifuging at 10,000 × *g* for 10 min. The aqueous phase was collected and thoroughly dialyzed against distilled water at 4°C (M_r cutoff = 1,000), frozen at −70°C, and then lyophilized and resuspended in sterile distilled water. The LPS extract was then analyzed by SDS-PAGE (18), and the gels were silver stained for LPS by the methods of Hitchcock and Brown (13). The resulting LPS produced a diffuse band of approximately 5 kDa plus a presumed protein contaminant of approximately 21 kDa when analyzed by SDS-PAGE (Fig. 5A, lane 3). The diffuse, low-M_r band of LPS is similar to the only previously reported LPS isolated from strain B13 of *B. bacilliformis*, but unfortunately no M_r of the LPS was given in that study (15). The lack of multiple LPS bands in the silver-stained profile in this and in the previous study (15) suggests that there are only subtle differences in the O side chains within the LPS population of each particular strain of bartonella. The LPS band did not stain with ethidium bromide, nor was it sensitive to proteinase K treatment (21); thus, it is probably not an RNA or protein contaminant. To further investigate whether the band was LPS, bartonella cell lysates were digested with proteinase K by a modification of the procedure of Hitchcock and Brown (13) to determine if the proteinase K-insensitive fraction of the bacterium would comigrate with the LPS band on SDS-PAGE. Approximately 20 mg (wet weight) of bartonellae was harvested into 75 μl of dPBS and lysed with an equal volume of Laemmli sample buffer. The mixture was then solubilized by being boiled for 10 min and was cooled to 50°C by a 15-min equilibration. After being centrifuged for 1 min at 16,000 × *g* in a microcentrifuge, the mixture was digested for 60 min at 50°C with proteinase K, at a final concentration of 0.5 mg of the protease per ml of reaction mixture, and analyzed by SDS-PAGE. The major product within the proteinase K-insensitive fraction of bartonella cell lysates was a broad band of approximately 5 kDa which migrated slightly more slowly on SDS-PAGE than did the purified LPS; there were also minor protease-resistant bands of 11.2, 12, and 25.3 kDa (Fig. 5A, lane 4). Immunoblot analysis of the purified LPS from bartonellae was done as previously described with rabbit anti-*B. bacilliformis* hyperimmune antiserum (25). Data show that recognition of the LPS of bartonellae by antibodies within the antiserum is not detectable (Fig. 5B, lane 2), in contrast to numerous protein bands which are recognized in the cell lysates of the pathogen (Fig. 5B, lane 1). These data imply that the LPS is poorly immunogenic in rabbits. Whether LPS is poorly immunogenic in humans requires further investigation with human convalescent-phase serum samples.

This study is the first attempt to identify the OMPs of *B. bacilliformis* and to determine their immunogenicity in rabbits. Extrinsic radioiodination and extrinsic proteinase K treatment of intact bartonellae were employed to corroborate the M_rs for

the putative OMPs identified by a sucrose step gradient centrifugation–SDS-PAGE analysis (Fig. 1). The data suggest that the majority of putative OMPs identified in the sucrose step gradient–SDS-PAGE procedure have counterpart proteins with similar M_rs that can be extrinsically radioiodinated (Fig. 2). However, extrinsic proteinase K treatment of intact bartonellae was not as useful for identifying OMPs and resulted in the proteolysis of only 2 of the 14 putative OMPs (Fig. 3 and Table 1). The proteinase K data may indicate that the proteinase K has an accessibility problem with respect to majority of OMPs on bartonellae. On the assumption that the M_r counterparts identified in the extrinsic radioiodination are the same proteins identified by the sucrose step gradient centrifugation–SDS-PAGE analysis (Fig. 1), the majority of the putative OMPs are also immunogenic in rabbits. Three proteins, of 31.5, 42, and 45 kDa, are prominently immunoprecipitated and may represent the immunodominant OMPs of the pathogen (Fig. 4). Data from this study also suggest that LPS from *B. bacilliformis* has an apparent M_r of 5,000 on SDS-PAGE and is poorly immunogenic in rabbits (Fig. 5). Studies with monospecific antibody generated against each putative OMP are presently under way to corroborate the results of this study and to help elucidate the function(s) of each particular protein.

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