INFECTION AND IMMUNITY, Dec. 2009, p. 5262–5271 Vol. 77, No. 12
0019-9567/09/$12.00 doi:10.1128/IAI.00911-09

Proteomic Analysis of *Rickettsia parkeri* Strain Portsmouth▽

Walairat Pornwiroon,[1] Apichai Bourchookarn,[1,2] Christopher D. Paddock,[3] and Kevin R. Macaluso[1]*

Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University, Skip Bertman Drive, SVM-3213, Baton Rouge, Louisiana 70803[1]; Department of Technology and Industries, Faculty of Science and Technology, Prince of Songkla University, Pattani 94000, Thailand[2]; and Infectious Disease Pathology Branch, Mailstop G-32, Centers for Disease Control and Prevention, 1600 Clifton Rd., Atlanta, Georgia 30333[3]

Received 11 August 2009/Returned for modification 28 August 2009/Accepted 15 September 2009

***Rickettsia parkeri*, a recently recognized pathogen of human, is one of several *Rickettsia* spp. in the United States that causes a spotted fever rickettsiosis. To gain insights into its biology and pathogenesis, we applied the proteomics approach to establish a two-dimensional gel proteome reference map and combined this technique with cell surface biotinylation to identify surface-exposed proteins of a low-passage isolate of *R. parkeri* obtained from a patient. We identified 91 proteins by matrix-assisted laser desorption ionization–tandem time of flight mass spectrometry. Of these, 28 were characterized as surface proteins, including virulence-related proteins (e.g., outer membrane protein A [OmpA], OmpB, β-peptide, and RickA). Two-dimensional immunoblotting with serum from the *R. parkeri*-infected index patient was utilized to identify the immunoreactive proteins as potential targets for diagnosis and vaccine development. In addition to the known rickettsial antigens, OmpA and OmpB, we identified translation initiation factor 2, cell division protein FtsZ, and cysteinyl-tRNA synthetase as immunoreactive proteins. The proteome map with corresponding cell surface protein analysis and antigen detection will facilitate a better understanding of the mechanisms of rickettsial pathogenesis.**

Rickettsia parkeri, a member of the spotted fever group *Rickettsia* (SFGR), was first isolated from the Gulf Coast tick, *Amblyomma maculatum*, in 1937 (29). In 2004, the first confirmed human infection with *R. parkeri* was reported in a 40-year-old man from the Tidewater area of coastal Virginia. The agent was isolated in cell culture from an eschar biopsy specimen and designated the Portsmouth strain (28). Recently, the first recognized case of tick bite-associated human infection was described (43); however, the epidemiology of *R. parkeri* is not well defined. In the United States, *R. parkeri* has been detected in *A. maculatum* and *A. americanum*; the geographical overlap between *R. parkeri* and these ticks with that of the vectors of *R. rickettsii* (the etiological agent of Rocky Mountain spotted fever [RMSF]) suggests that many cases of *R. parkeri* infection have been misidentified as RMSF (27, 35). For example, Western blot analysis of serum specimens from 15 U.S. patients previously diagnosed with RMSF identified four serum specimens reactive with a 120-kDa protein of *R. parkeri*, suggesting infection with *R. parkeri* rather than *R. rickettsii* (30). However, a serologic test specific for this pathogen is not available (43), and little is known about its biology.

Due to their obligate intracellular nature, genetic manipulation of *Rickettsia* has proven difficult. Alternatively, protein expression profiles (proteomes) are utilized to identify the mechanisms of pathogenesis and differentiate rickettsial species recognizing host immune response specificity to cell surface molecules, referred to as outer membrane proteins (Omps). The presence or absence of some Omps allows for differentiation between the typhus group and the SFGR, and the response to some species within the SFGR is specific (2). Proteomes have been developed for *R. prowazekii* (7), *R. conorii* (31), and *R. felis* (26) by using two-dimensional liquid chromatography-tandem mass spectrometry (2D LC-MS/MS), two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and MS, or sodium dodecyl sulfate (SDS)-PAGE and nanoLC-MS/MS, respectively. More recently, an emphasis has been placed on better understanding surface protein expression profiles for obligate intracellular bacteria in the family *Anaplasmataceae* since it is well recognized that Omps for these bacteria are critical for host cell invasion (12, 13). Likewise, the rickettsial Omps are critical for bacterial attachment and invasion of host cells (21, 23, 40).

To better understand the molecular basis of virulence of *R. parkeri*, we utilized 2D PAGE with a pH 3-10 immobilized pH gradient (IPG) coupled with matrix-assisted laser desorption ionization–tandem time of flight (MALDI-TOF/TOF) MS in order to establish the protein expression profile of a low-passage strain of *R. parkeri* isolated from an infected patient. This reference map will be useful for comparative analyses of protein profiling of *R. parkeri* as it is maintained under differing microenvironments (e.g., in the arthropod vector and vertebrate host). Biotinylation of cell surface proteins and 2D immunoblotting analysis were also used to identify the surface-exposed proteins and immunoreactive proteins as potential targets for diagnosis and vaccine development.

MATERIALS AND METHODS

Rickettsial culture and purification. *R. parkeri* strain Portsmouth, isolated from a skin biopsy specimen obtained from the index case of *R. parkeri* rickettsiosis (28), were grown in African green monkey kidney cell line (Vero E6) in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (HyClone, Logan, UT) in a humidified 5% CO_2 incubator at 34°C. All rickettsiae used in subsequent purifications were obtained

* Corresponding author. Mailing address: Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University, Skip Bertman Drive, SVM-3213, Baton Rouge, LA 70803. Phone: (225) 578-9677. Fax: (225) 578-9701. E-mail: kmacaluso@vetmed.lsu.edu.

▽ Published ahead of print on 21 September 2009.

from 3 to 11 passes of the initial isolate in Vero E6 cells. When more than 90% of the cells were infected, as determined by Diff-Quik (Dade Behring, Deerfield, IL) staining according to the manufacturer's protocol, rickettsiae were purified from cell cultures as previously described by Weiss et al. (42), with modifications. Cells were collected and centrifuged at 500 × *g* for 15 min at 4°C. The resulting pellet was resuspended in K36 buffer (0.1 M potassium chloride, 0.015 M sodium chloride, 0.05 M potassium phosphate buffer [pH 7.0]), and the cells were lysed by passing the suspension several times through a 25- and a 27-gauge needle attached to a 10-ml syringe. Large cell debris was removed by centrifugation at 100 × *g* for 5 min at 4°C, and the supernatant was filtered through a 5-μm-pore size syringe filter (Millex-SV; Millipore, Billerica, MA). The rickettsiae in the filtrate were harvested by centrifugation at 9,500 × *g* for 30 min at 4°C. The obtained pellet was washed and resuspended in K36 buffer. The rickettsial suspension was layered over the discontinuous Renografin (Merry X-Ray Corp., Lake Charles, LA) gradient (15 to 37.5%) and centrifuged in an OptimaXL-100K ultracentrifuge at 90,000 × *g* using an SW41-Ti rotor (Beckman Coulter, Fullerton, CA) for 1.5 h at 4°C. The rickettsial band was drawn into a syringe through a 27-gauge needle and washed twice with K36 buffer by centrifugation at 13,000 × *g* for 10 min at 4°C. The homogeneity of *Rickettsia* was examined by Diff-Quik staining, and the *Rickettsia* pellet was either immediately used for biotinylation of rickettsial surface proteins or stored in protease inhibitor cocktail (Roche, Indianapolis, IN) at −80°C until being subjected to protein preparation.

Biotinylation of surface proteins. Freshly purified *R. parkeri* were enumerated by using a *Bac*Light viability stain kit (Molecular Probes, Carlsbad, CA) as described previously (37). Viable rickettsiae corresponding to 5×10^{10} cells were washed three times with phosphate-buffered saline (PBS; pH 8.0) by centrifugation at 13,000 × *g* for 10 min at 4°C. The bacteria were surface labeled by incubation with 417 μM sulfosuccinimidyl-6-[biotin-amido]hexanoate (Sulfo-NHS-LC-Biotin; Pierce, Rockford, IL) in PBS (pH 8.0) or in PBS alone (negative control) at 4°C for 5 min. Excess Sulfo-NHS-LC-Biotin was quenched and removed by three washes in 100 mM glycine-PBS with incubation for 15 min at 4°C for the first wash. The bacterial pellet was washed with PBS (pH 8.0) and stored in protease inhibitor cocktail at −80°C until used for protein extraction.

Protein preparation. The rickettsial pellet was resuspended in lysis buffer (8 M urea, 2 M thiourea, 60 mM dithiothreitol [DTT], 0.2% Triton X-100, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS]) (5) and incubated for 15 min at room temperature. The suspension was subjected to cell lysis by continuous sonication for 5 min at 4 to 5°C in an ice-bath sonicator (Tru-Sweep model 275TA; Crest Ultrasonics, Trenton, NJ), followed by centrifugation at 13,000 × *g* for 10 min at 4°C. The procedure was repeated at least two more times to ensure complete cell lysis. Proteins in the supernatant were precipitated overnight with an equal volume of methanol and 4 volumes of acetone at −20°C. The protein pellet was collected by centrifugation at 13,000 × *g* for 15 min at 4°C, air dried, and dissolved in lysis buffer. The protein concentration was determined by a Bio-Rad protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard. Samples were divided into aliquots and stored at −80°C.

2D PAGE. An IPG strip (7 cm, linear pH 3 to 10; Bio-Rad) was passively rehydrated for 12 h with either 40 μg (for construction of proteome and cell surface proteome map), 20 μg (for detection of surface proteins), or 30 μg (for detection of immunoreactive proteins) of total protein in lysis buffer with an addition of 0.25% (vol/vol) Bio-Lyte 3/10 ampholyte (Bio-Rad) and 0.001% bromophenol blue. The first-dimension separation by isoelectric focusing (IEF) was then carried out in a Protean IEF cell apparatus (Bio-Rad) at 20°C with the following parameters: for 40 μg of total protein, 100 V for 1 h, 250 V for 1 h, 1,000 V for 30 min, 6,000 V for 2.5 h, and 6,000 V for 50,000 V · h; for 20 and 30 μg of total protein, 100 V for 30 min, 250 V for 30 min, 1,000 V for 30 min, 6,000 V for 6,000 V · h; and a final focusing step (20 μg of protein, 6,000 V for 30,000 V · h; 30 μg of protein, 6,000 V for 40,000 V · h). The focused IPG strip was incubated in 1× NuPAGE LDS sample buffer (Invitrogen) containing 1% (wt/vol) DTT for 15 min, followed by incubation for 15 min in 1× NuPAGE LDS sample buffer containing 2.5% (wt/vol) iodoacetamide. The second-dimension SDS-PAGE was conducted on the XCell SureLock Mini-Cell System (Invitrogen) using NuPAGE Novex 4 to 12% Bis-Tris Zoom gels (Invitrogen) at 100 V until the tracking dye font reached the bottom of the gel. After the electrophoretic run, gels were fixed in 10% methanol–7% acetic acid for 1 h and overnight stained with SYPRO Ruby protein gel stain (Bio-Rad). Gels were digitized by using Molecular Imager Gel Doc XR System (Bio-Rad) and kept at 4°C for spot excision and protein identification.

Western blot analysis. Unlabeled or biotin-labeled *R. parkeri* proteins separated by 2D PAGE were electroblotted onto Immun-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad) by using an XCell II blot module (Invitrogen) according to the manufacturer's protocol. After protein transfer, membranes were blocked for 2 h with 3% skim milk in Tris-buffered saline–0.1% Tween 20 (TBST; 20 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.1% Tween 20). For identification of immunoreactive proteins, the membranes were probed with a convalescent-phase serum sample obtained from the index patient with *R. parkeri* rickettsiosis (28) at a dilution of 1:200 for 2 h at room temperature, followed by a secondary antibody (horseradish peroxidase [HRP]-conjugated rabbit anti-human immunoglobulin G [IgG]; Sigma, St. Louis, MO) at a dilution of 1:80,000 for 1 h at room temperature. The 2D blot probed with secondary antibody alone served as the negative control. Rickettsial surface proteins were detected by incubation of the membrane with streptavidin-HRP conjugate (Invitrogen) at a dilution of 1:6,000 for 1 h at room temperature. Before and after addition of the secondary antibody, the membranes were rinsed and washed twice for 10 min each time with TBST. The membranes were developed by using a SuperSignal West Pico chemiluminescent substrate kit (Pierce). The developed membranes were stained with an MemCode reversible protein stain kit (Pierce), according to the manufacturer's protocol, to match the location of proteins on the membrane with the Western blot signals. Protein spots on a SYPRO Ruby-stained gel were aligned with the positive signals on a 2D immunoblot by using ImageMaster 2D Platinum software (version 5.0; Amersham Biosciences, Piscataway, NJ). The immunoreactive spots were excised by using the EXQuest spot cutter (Bio-Rad) and identified by MALDI-TOF/TOF MS.

Protein digestion, MALDI-TOF/TOF MS, and data analysis. Protein digestion and MS were performed by the Nevada Proteomics Center, University of Nevada (Reno, NV) as follows: excised protein spots were digested on an Investigator Proprep (Genomic Solutions, Ann Arbor, MI) using a previously described protocol (33) with some modifications. Samples were washed twice with 25 mM ammonium bicarbonate and 100% acetonitrile, reduced and alkylated using 10 mM DTT and 100 mM iodoacetamide, and incubated with 75 ng of trypsin in 25 mM ammonium bicarbonate for 6 h at 37°C. Samples were prepared and spotted onto a MALDI target with ZipTipu-C18 (Millipore). Samples were aspirated, dispensed three times, eluted with 70% acetonitrile and 0.2% formic acid, and then overlaid with 0.5 μl of a 5-mg/ml MALDI matrix (α-cyano-4-hydroxycinnamic acid) and 10 mM ammonium phosphate. All MS data were collected by using an ABI 4700 MALDI TOF/TOF apparatus (Applied Biosystems, Foster City, CA). The data were acquired in reflector mode from a mass range of 700 to 4,000 Da, and 1,250 laser shots were averaged for each mass spectrum. Each sample was internally calibrated on trypsin's autolysis peaks. The eight most intense ions from the MS analysis, which were not on the exclusion list, were subjected to MS/MS. For MS/MS analysis, the mass range was 70 to precursor ion with a precursor window of −1 to 3 Da, with an average 5,000 laser shots for each spectrum. The data were stored in an Oracle database. The data were extracted from the Oracle database and a peak list was created by using GPS Explorer software (Applied Biosystems) from the raw data generated from the ABI 4700. This peak list was based on signal-to-noise filtering and an exclusion list and included deisotoping. The resulting file was then searched by Mascot (Matrix Science, Boston, MA) with database search parameters including a mass tolerance of 20 or 50 ppm, one missed cleavage, oxidation of methionines, and carbamidomethylation of cysteines. Only matched proteins with significant scores ($P < 0.05$) were reported.

In silico analyses. The identified proteins were grouped according to the clusters of orthologous groups (COGs) functional classification (http://www.ncbi.nlm.nih.gov/COG/) (38). The signal peptide at the N terminus of the protein was predicted by the programs SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) (3) and LipoP 1.0 (http://www.cbs.dtu.dk/services/LipoP/) (20).

RESULTS

Proteome reference map of *R. parkeri* strain Portsmouth. To establish the proteome map of *R. parkeri*, a 2D PAGE of rickettsial protein extract was performed by using a 7-cm pH 3 to 10 IPG strip, followed by a 4 to 12% Bis-Tris gel. Approximately 270 protein spots with isoelectric points (pIs) ranging from 4.5 to 9.5 and molecular masses ranging from 10 to 240 kDa could be visualized by SYPRO Ruby protein gel stain. The majority of proteins were located in the pI range of 5 to 8 and molecular-mass range of 20 to 100 kDa (Fig. 1). The intense protein spots of various pIs and molecular masses were excised and identified by MALDI-TOF/TOF MS. The 110 identified spots represent 91 unique proteins (Table 1). Of

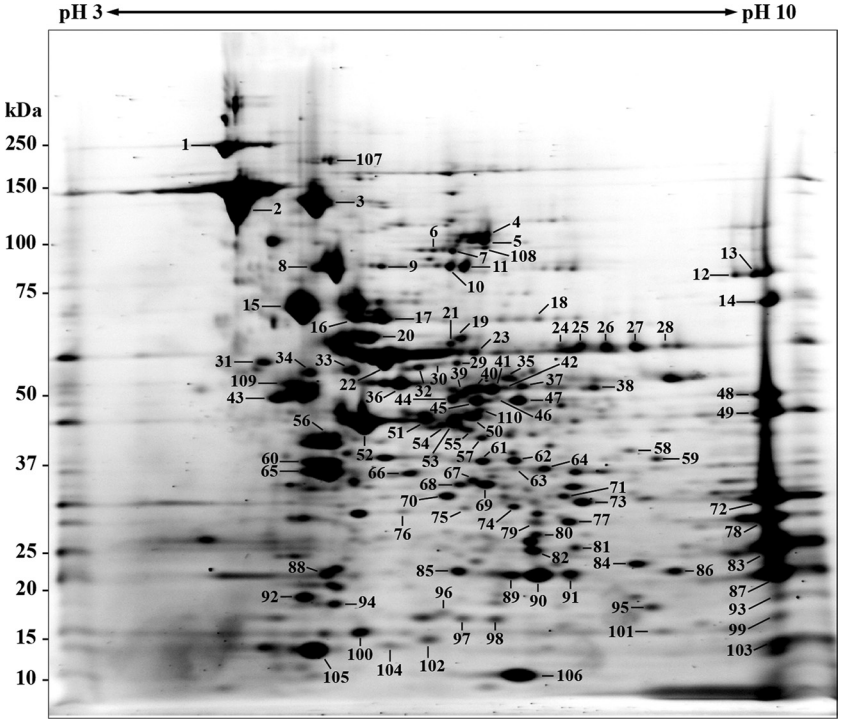

FIG. 1. 2D gel proteome reference map of *R. parkeri* strain Portsmouth. IEF was performed with total protein extract of *R. parkeri* using a 7-cm pH 3 to 10 immobilized gradient strip, followed by SDS-PAGE on a 4 to 12% Bis-Tris gel and stained with SYPRO Ruby protein gel stain. The numbers refer to the protein identities shown in Table 1. The molecular masses of the Precision Plus Protein Kaleidoscope standards (Bio-Rad) are indicated on the left.

these, 12 proteins with predicted molecular masses of >70 kDa, 54 proteins with predicted molecular masses of 30 to 70 kDa, and 25 proteins with predicted molecular masses of <30 kDa were identified. Fourteen of the gene products, including OmpB (spots 2 and 107), preprotein translocase subunit SecA (spots 4 and 5), polyribonucleotide nucleotidyltransferase (spots 10 and 11), Omp1 (spots 12 and 13), chaperonin GroEL (spots 21 and 22), methyltransferase (spots 24 to 28), trigger factor (spots 40 to 42), heat shock protease (spots 46 and 47), hypothetical protein (spots 48 and 49), CapD protein (spots 62 and 63), 30S ribosomal protein S2 (spots 68 and 69), 3-deoxy-manno-octulosonate cytidylyltransferase (spots 79 and 80), thioredoxin peroxidase 1 (spots 89 and 90), and single-stranded DNA-binding protein (spots 97 and 98) were found in multiple spots indicating isoforms (Table 1). These isoforms are apparent as either a vertical or horizontal pattern of spots on a 2D gel. The sequence coverage of the identified proteins ranged from 4% (spot 1, cell surface antigen rOmpA) to 98% (spot 106, 10-kDa chaperonin). OmpB, protein PS 120, elongation factor G, DnaK protein, and elongation factor Tu were the highly abundant proteins on the *R. parkeri* proteome map. In most cases, observed molecular mass values were in good agreement with that of predicted, except for spot 72, which was identified as OmpB. This protein spot had a molecular mass of 32 kDa, much lower than its predicted molecular mass (164 kDa), suggesting protein cleavage. It has been demonstrated that the whole molecule of OmpB is processed into the mature 120-kDa protein and the 32-kDa fragment, known as β-peptide (16). Because the Mascot search result of this spot showed that all six matched peptides were located in the carboxy-terminal region of the full-length OmpB, a finding consistent with the β-peptide, we identified spot 72 as the OmpB β-peptide. Among the identified protein spots, 12 spots were identified as 11 different unknown or hypothetical proteins in which seven of them had pIs ranging from 5 to 8, and the remaining exhibited basic pIs (pH > 9).

The identified proteins were classified into different functional categories according to COGs and found to be distributed in 17 different orthologous groups (Fig. 2). The majority of these proteins are involved in translation, ribosomal structure, and biogenesis (COG:J, 23.1%); posttranslational modification, protein turnover, and chaperones (COG:O, 17.6%); energy production and conversion (COG:C, 13.2%); and cell wall/membrane biogenesis (COG:M, 8.8%). There were 4.4% of proteins identified that were not in COGs, and 2.2% belong to the unknown function orthologous group (COG:S).

Identification of *R. parkeri* surface-exposed proteins by cell surface biotinylation. We performed biotinylation of viable *R. parkeri* using water-soluble and membrane-impermeable reagent, Sulfo-NHS-LC-Biotin, to identify surface-exposed proteins. The protein products resolved by 2D PAGE were visualized by SYPRO Ruby protein gel stain or transferred to a PVDF membrane. The biotinylated proteins were detected by using HRP-linked streptavidin and enhanced chemiluminescence. The 2D blot of unlabeled rickettsial proteins incubated with streptavidin-HRP, which served as the negative control to check for endogenous biotin-containing polypeptides, showed

TABLE 1. *R. parkeri* (Portsmouth) proteins identified by MALDI-TOF/TOF MS

Category and spot no.[a]	GenInfo identifier no.	Protein description[b]	Score	No. of peptides matched	Predicted molecular mass (kDa)	Predicted pI	Sequence coverage (%)
Translation, ribosomal structure, and biogenesis (J)							
8	15892097	Elongation factor G	1160	60	78.00	5.22	76
10	15619751	Polyribonucleotide nucleotidyltransferase*	120	28	82.07	6.15	35
11	34580431	Polyribonucleotide nucleotidyltransferase*	1040	50	82.17	6.31	47
16	20139895	Aspartyl-tRNA synthetase	274	40	67.97	5.35	51
20	15619842	30S ribosomal protein S1	226	28	63.59	5.49	28
23	34580946	Arginyl-tRNA synthetase	91	14	65.22	6.24	28
30	34581409	Glutamyl-tRNA synthetase	471	25	58.43	5.81	32
33	157828067	Aspartyl/glutamyl-tRNA amidotransferase subunit B	555	28	54.47	5.29	33
51	15893139	Seryl-tRNA synthetase	884	33	48.66	5.62	54
52	22087329	Elongation factor Tu	306	8	42.97	5.50	22
59	15892504	Phenylalanyl-tRNA synthetase subunit alpha	582	31	40.48	7.77	56
65	15619155	Elongation factor EF-Ts	810	35	33.72	5.21	81
67	157828584	Tryptophanyl-tRNA synthetase	519	22	37.54	6.42	32
68	34580930	30S ribosomal protein S2*	472	21	32.94	6.34	58
69	15619153	30S ribosomal protein S2*	231	20	32.84	6.34	46
84	34580453	Probable sigma(54) modulation protein	101	12	21.88	7.71	37
86	157828071	Ribosome recycling factor	481	16	20.91	7.88	50
93	15892102	50S ribosomal protein L10	263	15	18.16	9.55	51
99	167471261	50S ribosomal protein L19	251	16	15.86	10.15	58
104	161723851	30S ribosomal protein S6	364	17	13.92	5.82	84
105	42453334	Ribosomal protein L7/L12	257	15	15.01	6.41	78
108	15892739	Translation initiation factor IF-2†	438	35	91.07	6.52	40
110	15892034	Cysteinyl-tRNA synthetase†	179	18	53.25	6.23	24
Transcription (K)							
34	34581537	N utilization substance protein A	900	41	56.57	4.99	48
60	34581393	DNA-directed RNA polymerase alpha chain	387	29	38.23	5.10	60
92	34581077	Transcription elongation factor EF	319	23	18.13	4.99	67
103	15619757	Unknown (RC0668)	126	7	14.39	9.48	29
Replication, recombination, and repair (L)							
18	34581050	DNA mismatch repair protein MutL	382	25	69.19	7.04	24
56	15619665	DNA polymerase III beta chain	214	31	42.21	5.27	65
61	15893105	Recombinase A	802	35	39.65	6.93	63
97	157829131	Single-strand DNA-binding protein*	236	11	17.44	6.07	57
98	157829131	Single-strand DNA-binding protein*	157	11	17.44	6.07	48
Signal transduction mechanisms (T)							
82	15619141	Transcriptional activator protein czcR	88	12	26.59	6.85	39
101	13235447	Hypothetical protein	82	10	17.17	7.83	47
Cell wall/membrane biogenesis (M)							
12	34580844	Outer membrane protein Omp1*	195	41	86.90	8.61	41
13	34580844	Outer membrane protein Omp1*	144	47	86.90	8.61	49
37	67004914	Carboxyl-terminal protease	91	21	50.46	6.18	38
57	15619530	Putative UDP-*N*-acetylglucosamine 2-epimerase	92	22	43.33	6.25	62
62	15619529	CapD protein*	218	27	38.39	6.68	52
63	15619529	CapD protein*	66	10	38.39	6.68	18
77	157828329	Putative dTDP-4-dehydrorhamnose reductase	760	30	32.27	7.64	52
79	34580558	3-Deoxy-manno-octulosonate cytidylyltransferase*	173	18	27.45	6.45	58
80	34580558	3-Deoxy-manno-octulosonate cytidylyltransferase*	280	23	27.45	6.45	50
83	34581125	Hypothetical protein	464	24	26.36	9.54	53
87	34581124	Hypothetical protein	471	20	24.02	9.38	60
Intracellular trafficking, secretion, and vesicular transport (U)							
4	167472009	Preprotein translocase subunit SecA*	110	21	103.51	6.28	28
5	53732210	Preprotein translocase subunit SecA*	87	31	103.60	6.17	31
Posttranslational modification, protein turnover, chaperones (O)							
6	34580983	ClpB protein	117	24	95.98	5.93	32

Continued on following page

TABLE 1—*Continued*

Category and spot no.[a]	GenInfo identifier no.	Protein description[b]	Score	No. of peptides matched	Predicted molecular mass (kDa)	Predicted pI	Sequence coverage (%)
15	34580814	DnaK protein	493	52	67.94	5.00	62
17	34581106	Heat shock protein HtpG	289	35	70.76	5.61	44
21	53732249	Chaperonin GroEL*	163	20	58.59	5.62	32
22	53732249	Chaperonin GroEL*	294	36	58.59	5.62	60
31	34580880	Periplasmic serine protease	688	25	55.58	4.71	34
40	34581103	Trigger factor*	505	32	50.98	6.06	48
41	34581103	Trigger factor*	178	24	50.98	6.06	33
42	34581103	Trigger factor*	135	29	50.98	6.06	58
44	42453569	ATP-dependent protease HslVU (ClpYQ), ATPase subunit	115	27	49.62	6.05	49
46	15892157	Heat shock protease*	87	16	55.54	7.68	33
47	34580813	Heat shock protease*	175	34	55.53	7.68	42
64	15892560	Thioredoxin reductase	517	23	37.52	7.15	40
78	34581487	Protein export protein prsA precursor	94	19	31.40	9.04	50
88	15620090	GrpE protein	159	13	20.16	5.18	47
89	157828322	Thioredoxin peroxidase 1*	444	21	22.49	6.62	62
90	15892374	Thioredoxin peroxidase 1*	493	24	22.74	6.62	67
91	157828616	ATP-dependent Clp protease proteolytic subunit	393	19	22.86	6.60	42
95	34581294	Bacterioferritin comigratory protein	336	15	17.90	7.74	51
100	34580701	Heat shock protein	211	18	18.95	6.34	76
106	34581406	10-kDa chaperonin	429	21	10.53	6.74	98
Cell division and chromosome partitioning (D)							
109	157828869	Cell division protein FtsZ†	525	26	48.45	4.89	56
Energy production and conversion (C)							
7	157829065	Aconitate hydratase	78	16	97.35	5.88	15
9	15892430	Malic enzyme	88	20	84.42	5.61	21
19	157964195	Succinate dehydrogenase flavoprotein subunit	237	17	65.95	6.23	23
32	161723840	F0F1 ATP synthase subunit alpha	1150	45	56.13	5.85	51
43	53732375	F0F1-type ATP synthase, beta subunit	296	31	51.07	5.00	62
45	167471897	Dihydrolipoamide dehydrogenase	761	40	49.32	6.17	57
53	15619278	Dihydrolipoamide acetyltransferase component	301	25	42.77	6.89	60
55	157829141	Type II citrate synthase	413	28	49.40	6.20	39
58	53732427	NAD/NADP transhydrogenase alpha subunit	311	26	40.60	7.66	54
66	34580715	Pyruvate dehydrogenase e1 component alpha subunit precursor	100	23	36.67	5.76	49
70	34580561	Malate dehydrogenase	623	25	33.64	6.01	62
73	34580487	Succinyl-CoA synthetase alpha chain	143	18	30.24	6.66	48
Carbohydrate transport and metabolism (G)							
76	34581482	Hypothetical protein	77	14	34.45	5.44	40
Amino acid transport and metabolism (E)							
36	15619233	Aminopeptidase A	342	28	53.99	5.69	39
38	167471346	Thermostable carboxypeptidase	462	27	57.24	6.73	33
39	34580709	Isocitrate dehydrogenase	115	22	53.89	6.10	30
71	157828466	Dihydrodipicolinate synthase	221	12	32.81	7.08	30
81	157828064	Dihydrodipicolinate reductase	275	15	26.58	7.05	44
Nucleotide transport and metabolism (F)							
75	15892328	FAD-dependent thymidylate synthase	483	23	34.58	6.23	48
102	34580958	Nucleoside diphosphate kinase	134	12	14.71	5.77	59
Coenzyme transport and metabolism (H)							
50	34581632	Hypothetical protein	523	26	51.09	6.24	44
Lipid transport and metabolism (I)							
35	34581415	Propionyl-CoA carboxylase beta chain precursor	136	17	57.05	6.58	26
54	167472306	3-Oxoacyl-(acyl carrier protein) synthase II	365	21	45.58	5.81	38
Inorganic ion transport and metabolism (P)							
85	34580412	Superoxide dismutase	310	17	24.83	6.25	52

Continued on following page

TABLE 1—*Continued*

Category and spot no.[a]	GenInfo identifier no.	Protein description[b]	Score	No. of peptides matched	Predicted molecular mass (kDa)	Predicted pI	Sequence coverage (%)
96	157828723	Hypothetical protein A1G_04785	207	15	19.51	5.50	41
General function prediction only (R)							
24	165933779	Methyltransferase*	332	23	63.38	7.68	22
25	165933779	Methyltransferase*	748	34	63.38	7.68	37
26	165933779	Methyltransferase*	95	16	63.38	7.68	21
27	165933779	Methyltransferase*	764	40	63.38	7.68	44
28	165933779	Methyltransferase*	641	38	63.38	7.68	42
29	34581008	Hypothetical protein	261	26	61.54	6.18	36
74	34580794	Hypothetical protein	623	29	35.61	6.37	56
Function unknown (S)							
1	62861417	Cell surface antigen rOmpA†	94	9	210.38	5.27	4
2	6969950	OmpB*†	287	14	164.16	5.20	12
72	6969950	OmpB β-peptide	322	6	164.16	5.20	4
107	6969950	OmpB*†	289	26	164.16	5.20	12
Not in COGs							
3	13568657	Protein PS 120	460	67	110.63	5.18	60
14	34581459	Hypothetical WASP N-WASP MENA proteins	286	33	59.45	9.27	44
48	15892021	Hypothetical protein RC0098*	173	6	48.05	9.24	12
49	34580943	Hypothetical protein*	551	39	48.07	9.24	60
94	15619114	Unknown (RC0076)	225	20	21.14	5.56	78

[a] The COG category abbreviations are given in parentheses.
[b] *, Protein isoforms; †, immunoreactive proteins recognized by serum from the *R. parkeri*-infected index patient. CoA, coenzyme A.

only one positive spot with an apparent molecular mass of ~68 kDa and a pI of 5.6 (data not shown). This spot was not included in the analysis of rickettsial surface proteins. A total of 59 intense protein spots in a SYPRO Ruby-stained gel corresponding to Western blot signals were subjected to MALDI-TOF/TOF analysis. Among the 59 selected spots, 40 of which represent 28 proteins including known immunodominant surface-exposed proteins, Omps A and B, were successfully identified in the present study (Table 2). Some of the labeled proteins appeared as chains of spots with slightly different pIs (Fig. 3) that were not observed in 2D gel of unlabeled sample (Fig. 1). SignalP and LipoP analyses revealed a putative N-terminal signal peptide sequence with the cleavage site for signal peptidase I (SpI) in seven identified proteins including OmpA, OmpB, Omp1, protein export protein prsA precursor, and three hypothetical proteins (spots 48, 83, and 87).

***R. parkeri* immunoreactive proteins recognized by patient serum.** To identify immunoreactive antigens of *R. parkeri*, proteins separated by 2D PAGE were electroblotted onto a PVDF

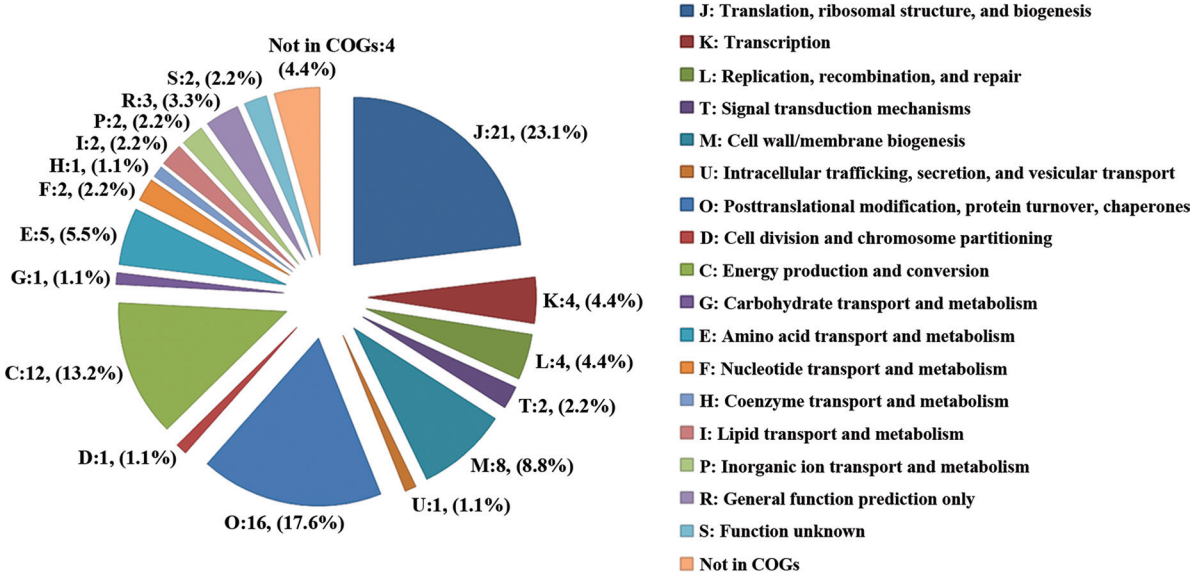

FIG. 2. Functional distribution of identified *R. parkeri* proteins. The pie chart displays the proportion of identified proteins assigned to different functional categories according to the COGs functional classification (http://www.ncbi.nlm.nih.gov/COG). The number and percentage of identified proteins associated with each COG functional category are shown.

TABLE 2. Surface-exposed proteins of *R. parkeri* (Portsmouth) identified by MALDI-TOF/TOF MS

Spot no.	GenInfo identifier no.	Protein description	Score	No. of peptides matched	Predicted molecular mass (kDa)	Predicted pI	Sequence coverage (%)
1	1778893	rOmpA	50	1	110.11	5.30	1
2	6969950	OmpB	298	14	164.21	5.20	10
8a-b	15892097	Elongation factor G	163	30	78.00	5.22	35
10	34580431	Polyribonucleotide nucleotidyltransferase	254	28	82.17	6.31	27
11	34580431	Polyribonucleotide nucleotidyltransferase	293	32	82.17	6.31	36
12	34580844	Outer membrane protein Omp1	161	22	86.90	8.61	15
14	34581459	Hypothetical WASP N-WASP MENA proteins	191	16	59.45	9.27	19
15	34580814	DnaK protein	618	46	68.05	5.00	48
22a-b	15892891	Chaperonin GroEL	475	39	58.68	5.62	60
23	15892018	Arginyl-tRNA synthetase	48	2	65.25	6.24	5
32	34581170	ATP synthase alpha chain	253	22	56.11	5.74	30
33	34580853	Glutamyl-tRNA amidotransferase subunit B	221	24	54.37	5.35	23
36a-b	34580862	Aminopeptidase A	95	14	54.25	5.62	19
38	15892151	Thermostable carboxypeptidase	129	15	57.23	6.70	17
40	34581103	Trigger factor	192	27	50.98	6.06	48
41	34581103	Trigger factor	634	42	50.98	6.06	66
43	34581172	ATP synthase beta chain	442	28	51.21	4.87	46
45	34581573	Dihydrolipoamide dehydrogenase precursor	220	17	49.31	6.38	29
48	15892021	hypothetical protein RC0098	252	25	48.05	9.24	45
49	15892021	hypothetical protein RC0098	186	21	48.05	9.24	41
52a-d	61223562	Elongation factor Tu (EF-Tu)	429	28	43.02	5.50	53
61	15893105	Recombinase A	212	22	39.65	6.93	45
65	15892036	Elongation factor Ts	226	21	33.78	5.21	60
72	6969950	OmpB β-peptide	293	23	164.21	5.20	9
73	34580487	Succinyl-CoA synthetase alpha chain	255	14	30.58	6.66	35
78a-c	34581487	Protein export protein prsA precursor	148	11	31.40	9.04	28
83	15893204	Hypothetical protein RC1281	486	23	26.92	9.58	51
87	34581124	Hypothetical protein	549	21	24.02	9.38	51
90	15892374	Thioredoxin peroxidase 1	478	23	22.74	6.62	64
92	34581077	Transcription elongation factor EF	96	12	18.13	4.99	46
93	15892102	50S ribosomal protein L10	61	1	18.16	9.55	4
103	15892591	Hypothetical protein RC0668	54	1	14.50	9.48	8

membrane and probed with a serum specimen obtained from the patient from whom *R. parkeri* strain Portsmouth was also isolated. The 2D immunoblots were then incubated with anti-IgG antibody, which was tested to have no reactivity with *R. parkeri* proteins (data not shown). Protein spots on a SYPRO Ruby-stained gel, which aligned to antigenic spots on a 2D immunoblot, were excised and analyzed by MALDI-TOF/TOF MS. Serum from the index patient reacted with seven protein spots with the observed molecular masses ranging from 50 to 240 kDa (Fig. 4). Six immunoreactive spots corresponding to five proteins—OmpA (spot 1), OmpB (spots 2 and 107), translation initiation factor IF-2 (spot 108), cell division protein FtsZ (spot 109), and cysteinyl-tRNA synthetase (spot 110)—were successfully identified (see Table 1). Moreover, the typical ladder pattern of lipopolysaccharide was recognized by this serum.

DISCUSSION

Although *R. parkeri* was first identified more than 70 years ago, there are relatively few data that describe its biology, and none that identify molecular constituents involved in its pathogenic behavior in human hosts. In the present study, a 2D gel proteome reference map of *R. parkeri* strain Portsmouth was constructed. A total of 110 spots representing 91 proteins were identified by MALDI-TOF/TOF. A variety of analytical methods have been used for rickettsial proteome analyses. The number of identified proteins in the present study was less than that of *R. prowazekii* analyzed by a 2D LC-MS/MS technique (7) and *R. felis* characterized by using two proteomic approaches: 2D PAGE coupled with MALDI-TOF and SDS-PAGE with nanoLC-MS/MS (26). However, our identification rate was comparable to that of *R. felis* analyzed only by 2D PAGE and MS. Among the 91 *R. parkeri* proteins that we identified, 60 were orthologs not reported in the *R. felis* 2D proteome map by this technique (26). The theoretical and experimentally observed molecular mass and pI values of the identified proteins were in general agreement, except for spot 72, which was identified as OmpB β-peptide. A good correlation between the predicted and observed molecular mass and pI of *R. parkeri* β-peptide was found when the molecular mass and pI values were calculated based on the amino acid sequence reported in the GenBank database (accession number FJ644549) using the pI/molecular-mass tool in the Expasy proteomic server (http://www.expasy.org/tools/pi_tool.html). The presence of several protein isoforms as either a vertical or

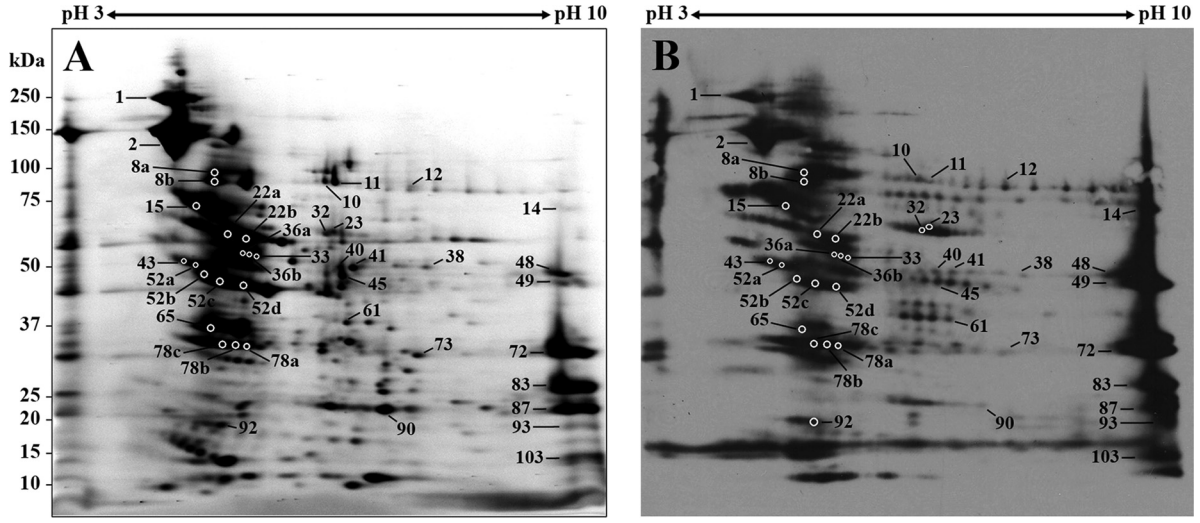

FIG. 3. 2D gel and blot of biotin-labeled *R. parkeri* surface proteins. The biotinylated proteins resolved by 2D PAGE were stained with SYPRO Ruby protein gel stain (A) or transferred to a PVDF membrane and detected using streptavidin-HRP conjugate (B). The numbers refer to the protein identities shown in Table 2. The molecular masses of the Precision Plus Protein Kaleidoscope standards (Bio-Rad) are indicated on the left.

horizontal pattern of spots on the 2D map of *R. parkeri* was likely due to posttranslational modifications (PTMs). Similar observations were made in other rickettsial proteomic studies (26, 31). In bacteria, PTMs play important roles in protein stability, signaling process, and host-pathogen interaction and in determining antigenicity (44). Identifying proteins that undergo PTMs, as reported here, facilitates future studies designed to decipher the biological significance of PTMs in *Rickettsia* spp.

Several of the *R. parkeri* proteins that we identified have been implicated in the virulence of other *Rickettsia* spp. The WASP N-WASP MENA proteins or RickA are involved in actin-based motility through activation of the Arp2/3 complex

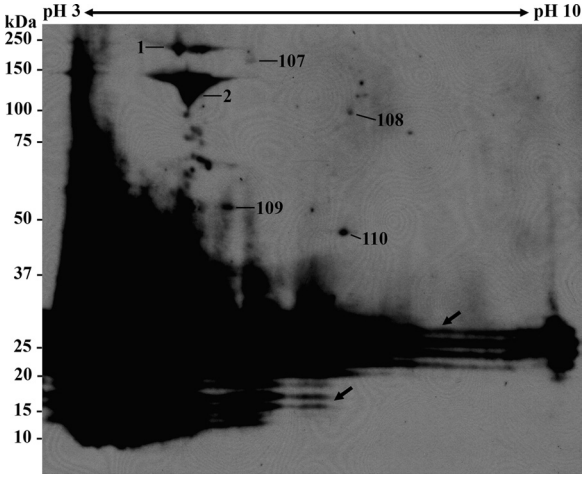

FIG. 4. 2D immunoblot of *R. parkeri* protein extract. The proteins separated by 2D PAGE were transferred to a PVDF membrane and probed with *R. parkeri* index patient serum. The numbers refer to the protein identities shown in Table 1. Arrows indicate the typical ladder pattern of lipopolysaccharide. The molecular masses of the Precision Plus Protein Kaleidoscope standards (Bio-Rad) are indicated on the left.

utilized by SFGR to exit from the host cell (15, 18, 19). The ability of *R. parkeri* to form actin tails was reported by Heinzen et al. (17). The role of methyltransferase in the pathogenesis of *R. prowazekii* has been suggested. This protein encoded by open reading frames RP028 and RP027 was expressed in the virulent Breinl strain but not in the avirulent Madrid E strain (8). The finding was supported by a frameshift mutation of the gene only in the avirulent Madrid E strain (46). BLASTP analysis showed that a hypothetical protein of *R. parkeri* (spot 29) shared 86 and 87% amino acid sequence identity to *R. prowazekii* proteins encoded by RP027 and RP028, respectively, suggesting the expression of methyltransferase in *R. parkeri*. We also identified three surface cell antigen proteins, including OmpA, OmpB, and protein PS 120. OmpA and OmpB are involved in rickettsial adhesion to and invasion of host cells (21, 23, 40). In contrast to other rickettsial proteome analyses (7, 26), only one protein involved in the secretion system, preprotein translocase subunit SecA, was successfully identified in the current study. In addition, we were unable to detect any type IV secretion system (T4SS) proteins. T4SS genes have been identified in all *Rickettsia* genomes analyzed to date, including the earliest diverging species, *R. bellii* (25), and the closely related *R. africae* (10). In this context, it is likely that one or more functional T4SS genes also exist in *R. parkeri*. Although we identified >90 *R. parkeri*-associated proteins, it is possible that some others elaborated by this pathogen were not detected because of the amount of bacterial protein used in the analyses or low-level expression during the particular growth conditions. However, when the complete genome sequence of *R. parkeri* becomes available, these data will allow for prediction of these and other genes and better assessment of the complete guild of proteins associated with this SFGR.

Two additional hypothetical proteins detected in our proteome analysis, as well as the β-peptide of *R. parkeri*, are orthologs of putative rickettsial adhesins. The β-peptide and *R. conorii* protein encoded by RC1281, which has sequence similarities to *R. parkeri* proteins of unknown function (spots 83

and 87), act as adhesin molecules that bind to surface proteins of Vero cells (32). The confirmed expression of these virulence determinants is consistent with other pathogenic rickettsial species and the ability of *R. parkeri* to cause disease in humans.

All identified proteins were analyzed for their COGs functional classifications. We observed a similar expression profile to previously reported rickettsial proteomes in which a large portion of identified *R. parkeri* proteins belongs to the functional category of translation, ribosomal structure and biogenesis (7, 26). Moreover, the most common genes identified in the genomes of *Rickettsia*, *Orientia*, and *Wolbachia* are involved in translation (11, 14). Further analysis of the unique requirements for protein synthesis associated with arthropod versus vertebrate host should illuminate novel mechanisms of pathogenesis.

We further applied cell surface biotinylation and a proteomics approach to identify 28 distinct surface proteins of *R. parkeri*. Of these, seven proteins, including OmpA, OmpB, Omp1, protein export protein prsA precursor, and three hypothetical proteins, were predicted to have the signal peptide sequences with the cleavage site for SpI. The findings of the present study corroborate the results reported by Ammerman et al. (1) in which an *Escherichia coli*-based alkaline phosphatase assay identified OmpB, Omp1, protein export protein prsA, and proteins of unknown function encoded by open reading frames RT0064, RT0815, and RT0816, which are the orthologs of three *R. parkeri* hypothetical proteins identified in the present study, to be Sec-dependent extracytoplasmic proteins. The surface expression of two *R. parkeri* hypothetical proteins (spots 83 and 87) and β-peptide is supported by previous work showing that orthologs of these proteins function as putative adhesins in *R. conorii* (32).

The rest of the surface proteins identified in the present study lack a putative N-terminal signal sequence and are generally considered cytosolic proteins. These proteins could be secreted by an unknown mechanism or released from bacteria with damaged cell membranes, an artifact of the purification step, and subsequently bound to the surface of intact cells. However, homologs of these proteins, including elongation factor G, polyribonucleotide nucleotidyltransferase, DnaK protein, chaperonin GroEL, two tRNA synthetases, aminopeptidase A, trigger factor, ATP synthase beta chain, dihydrolipoamide dehydrogenase, elongation factor Tu, recombinase A, and elongation factor Ts, were detected in the membrane fraction of *R. conorii* and other gram-negative bacteria (4, 12, 13, 22, 31, 34). The localization of the WASP N-WASP MENA proteins or RickA on the cell surface of *R. conorii* has been demonstrated. This protein activates Arp2/3 and stimulates actin polymerization (15). Because surface proteins are known to play crucial roles in host cell adhesion and invasion, further studies should be conducted to examine the functions of identified surface proteins in the virulence of *R. parkeri*.

In addition to the proteome map and surface-associated protein identification, the immunoreactive proteins of *R. parkeri* were identified by 2D immunoblotting analysis. Five proteins reacted with a convalescent-phase serum sample from the index patient. As expected, OmpA and OmpB were identified as major antigens and as surface-exposed proteins in the present study. It has been shown that both proteins are able to stimulate protective immunity against rickettsiosis in laboratory animals (6, 9, 36, 41). To the best of our knowledge, the immunogenicity of the remaining three antigenic proteins—translation initiation factor IF-2, cell division protein FtsZ, and cysteinyl-tRNA synthetase—has not been described for other *Rickettsia* spp.; it is unknown whether these represent immunologically reactive proteins unique to *R. parkeri* or whether antigenic homologs exist among other SFGR. Further studies will require screening with serum specimens from additional patients. However, the antigenicity of translation initiation factor IF-2 and cell division protein FtsZ homologs has been reported in previous studies of several bacteria (4, 24, 45). Moreover, the FtsZ-like protein has been suggested to be involved in pathogenesis of *Bacillus anthracis* (39).

In summary, we established a 2D reference map of proteins expressed in *R. parkeri* and identified 91 distinct proteins by MALDI-TOF/TOF. Of these, 28 were characterized as surface-exposed proteins by using cell surface biotinylation technique, including virulence-related proteins. Our data provide a basis for understanding the pathogenesis of *R. parkeri*. The proteome reference map will facilitate comparative analyses of differential protein expression under various environmental conditions or during the infection process. Finally, we identified novel immunoreactive proteins recognized by serum from the index patient which may serve as potential targets for diagnosis and disease prevention.

ACKNOWLEDGMENTS

We thank J. A. Macaluso for helpful comments.

Protein identification at the Nevada Proteomics Center, University of Nevada, Reno, was supported by National Institutes of Health grant P20 RR-016464 from the INBRE Program of the National Center for Research Resources. This research was supported by the National Institute of Allergy and Infectious Diseases (AI070705).

The findings and conclusions presented in the present study are those of the authors and do not necessarily represent the views of the U.S. Department of Health and Human Services.

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Editor: R. P. Morrison