Infection and Immunity

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Surface-Exposed Proteins of *Ehrlichia chaffeensis* \(*\dagger\)

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Received 5 February 2007/Returned for modification 12 March 2007/Accepted 14 May 2007

The surface proteins of *Ehrlichia chaffeensis* provide an important interface for pathogen-host interactions. To investigate the surface proteins of *E. chaffeensis*, membrane-impermeable, cleavable Sulfo-NHS-SS-Biotin was used to label intact bacteria. The biotinylated bacterial surface proteins were isolated by streptavidin-agarose affinity purification. The affinity-captured proteins were separated by electrophoresis, and five relatively abundant protein bands containing immunoreactive proteins were subjected to capillary-liquid chromatography-nanospray tandem mass spectrometry analysis. Nineteen out of 22 OMP-1/P28 family proteins, including P28 (which previously was shown to be surface exposed), were detected in *E. chaffeensis* cultured in human monocytic leukemia THP-1 cells. For the first time, with the exception of P28 and P28-1, 17 OMP-1/P28 family proteins were demonstrated to be expressed at the protein level. The surface exposure of OMP-1A and OMP-IN was verified by immunofluorescence microscopy. OMP-1B was undetectable either by surface biotinylation or by Western blotting of the whole bacterial lysate, suggesting that it is not expressed by *E. chaffeensis* cultured in THP-1 cells. Additional *E. chaffeensis* surface proteins detected were OMP58, hypothetical protein ECH_0525 (here named Esp73), immunodominant surface protein gp47, and 11 other proteins. The identification of *E. chaffeensis* surface-exposed proteins provides novel insights into the *E. chaffeensis* surface and lays the foundation for rational studies on pathogen-host interactions and vaccine development.

Human monocytic ehrlichiosis (HME) is an emerging tick-borne zoonosis in the United States (12). It is an acute febrile systemic disease that can cause severe and potentially fatal disease, especially in immunocompromised and elderly people (12, 34). The etiologic agent of HME is *Ehrlichia chaffeensis*, which belongs to the family *Anaplasmataceae* (11). In North America, the major vector of *E. chaffeensis* is the Lone Star tick, *Amblyomma americanum*, and the white-tailed deer is considered to be the major reservoir of *E. chaffeensis* (14, 26).

*E. chaffeensis* is a gram-negative, obligatory intracellular bacterium which has tropism for monocytes/macrophages. The entry and proliferation of *E. chaffeensis* involve host caveolae, glycosylphosphatidylinositol-anchored proteins, and incorporation of cholesterol into the bacterial membrane (25). After internalization by host monocytes, *E. chaffeensis* has the ability to subvert the hostile environment by residing in an early endosome-like compartment, which does not fuse with lysosomes (3). As an obligate intracellular bacterium, *E. chaffeensis* needs to exchange nutrients and metabolites with the host cell cytoplasm. These events are ehrlichial surface related. However, the corresponding bacterial surface components have not been characterized.

Studies on bacteria from the family *Anaplasmataceae* have revealed an important role for the bacterial outer membrane proteins (OMPs) in the stimulation of the host immune response and protection of the host from infection. Immunization with recombinant P28 (one of the major OMP-1/P28 family members) protected mice from *E. chaffeensis* challenge (33). Immunization of calves with *Anaplasma marginale* OMPs induced stronger protection against challenge compared to individual major surface proteins, e.g., MSP-1 and MSP-2 (1, 6, 35, 36, 48). Along this line, efforts have been made to identify the global composition of *A. marginale* outer membrane immunogens (28).

Polyclonal antibodies against *E. chaffeensis* or monoclonal antibody against OMP-1g (P28) mediated protection of SCID mice from *E. chaffeensis* fatal infection (24, 53). While many *E. chaffeensis* antigenic protein bands have been revealed by Western blotting with whole organisms (7, 8, 42), bacterial surface exposure of *E. chaffeensis* antigens or proteins other than P28 (33), gp47 (13), and gp120 (39) has not been determined.

Despite the importance of *E. chaffeensis* surface proteins as a critical interface for pathogen-host interactions, as mentioned above, there has been no systematic investigation of the surface proteins of *E. chaffeensis*. Therefore, this study focused on the characterization of *E. chaffeensis* major surface proteins via surface biotinylation with cleavable sulfosuccinimidyl-2-[biotin-amiido]ethyl-1,3-dithiopropionate (Sulfo-NHS-SS-Biotin) labeling (17, 45), streptavidin affinity purification of biotinylated proteins, and identification of the purified proteins by proteomic analysis with the recently published *E. chaffeensis* genome sequence database (20). Bacterial surface exposure of two proteins was confirmed by immunofluorescence microscopy with surface-exposed-epitope-specific antibodies.

**MATERIALS AND METHODS**

*E. chaffeensis* and cell culture. The *E. chaffeensis* Arkansas strain (11) was propagated in THP-1 cells, a human monocytic leukemia cell line, in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (US Bio-Technologies, Parkerford, PA) and 2 mM L-glutamine (Invitrogen) in a humidified 5% CO₂–95% air atmosphere at 37°C. No antibiotic was used throughout the study. The degree of bacterial infection in host cells was assessed...
bonds in Sulfo-NHS-SS-Biotin (45). The biotinylated bacterial lysates were preparing the Sulfo-NHS-SS-Biotin-labeled bacterial lysates, additional oxidized Lysates were incubated on ice for 30 min with occasional gentle vortexing. For 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS] containing a 1:100 quenching by washing in 500 mM glycine–PBS three times. Bacterial lysates were The biotinylation reaction was performed at 4°C for 30 min. Free biotin was within one of the extracellular loops predicted by PRED-TMBB, were chosen to verify their surface localization by immunofluorescence microscopy. To design peptides for developing antibodies recognizing extracellular epitopes, transmembrane β strands of OMP-1A and OMP-1N and their topology with respect to the outer membrane lipid bilayer were predicted by the posterior decoding method at the PRED-TMBB web server (http://bioinformatics.biol.uoa.gr/PRED-TMBB) (2). PRED-TMBB is a web server capable of predicting the transmembrane strands and topology of β-barrel OMPs of gram-negative bacteria, which is based on a hidden Markov model. It is trained with nonhomologous OMPs with structures known at atomic resolution according to the conditional-maximum-likelihood criterion (2).

Two sequentially highly antigenic and hydrophilic peptide fragments, located within one of the extracellular loops predicted by PRED-TMBB, were chosen from the OMP-1A and OMP-1N amino acid sequences on the basis of Protein was sequenced in the DNAStar software (http://www.dnastar.com) and assigned the 15-mer peptide CDELKGEFEPKAEDLT where the underlined sequence corresponds to 14 amino acids (aa) of OMP-1A (aa 154 to 167), and the 15-mer peptide CEISISGNPANNKEY, where the underlined sequence corresponds to OMP-1A aa 154 to 167, was synthesized and conjugated to keyhole limpet hemocyanin, and rabbit antibodies were developed by Sigma Genosys (St. Louis, MO). According to BLAST search results for short, nearly exactly matching sequences in the NCBI nonredundant database, each of these two peptide sequences is unique and has little to no homology to any other known proteins (E > 10).

For immunofluorescence microscopic analysis of the surface localization of E. chaffeensis OMP-1A and OMP-1N, organisms were fixed by the paraformaldehyde method (18, 52). Briefly, host cell-free bacteria were pelleted and washed in PBS. All subsequent steps were performed at room temperature. Bacteria were fixed in 2% paraformaldehyde for 45 min. After quenching in PBS (137 mM NaCl, 2.68 mM KCl, 10 mM Na2HPO4, 1.76 mM KH2PO4 [pH 7.4]) containing 0.1 M glycerol and subsequent washing in PBS, bacteria were incubated with a 1:10 dilution of rabbit antiserum against OMP-1A, rabbit antiserum against OMP-1N, rabbit preimmune serum, or rabbit antiserum against an irrelevant peptide in PBG buffer (0.2% gelatin in PBS) for 1 h. After washing in PBG buffer, the bacteria were labeled with Alexa Fluor 488 goat anti-rabbit immunoglobulin G (IgG; Invitrogen) at a dilution of 1:100 in PG buffer for 1 h. The bacteria were washed in PG buffer, resuspended in PBS, and observed with a Nikon Eclipse E400 fluorescence microscope with a x10-nylon light source (Nikon Instruments, Melville, NY). For an uninfected host cell control, THP-1 cells were fixed in paraformaldehyde as mentioned above or in cold methanol for 5 min.

Western blotting analysis. Protein samples were subjected to 10% SDS-PAGE and transferred to PVDF membranes (Westron S; Schleicher & Schuell BioScience, Keene, NH). Recombinant E. chaffeensis OMP-1B (full length with no signal peptide cloned into pET29a between the [5’] NdeI and [3’] Xhol sites) was used. The membrane was incubated with rabbit antiserum against OMP-1B peptide CEAPINGNTSITKKV (OMP-1B sequence underlined), human choriocarcinoma patient serum (42), or sera from experimentally E. chaffeensis-infected, specific-pathogen-free dogs at a dilution of 1:200 at 4°C overnight. The membranes were washed in TBST (15 mM NaCl, 5 mM Tris-HCl [pH 7.4], 0.02% Tween 20) four times for 10 min each time, which was followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (heavy plus light chains); human IgA, IgM, and IgG; or anti-dog IgG(γ) (KPL, Gaithersburg, MD) at a 1:1,000 dilution at room temperature for 1 h. For the biotin blot, the membrane was incubated with 1:1,000-diluted, HRP-conjugated streptavidin (Invitrogen) at room temperature for 1 h. The blots were then washed in TBST four times for 10 min each time and developed by the enhanced-chemiluminescence technique (Pierce).
RESULTS

Detection of biotinylated proteins. Since the Sulfo-NHS-LC-Biotin or Sulfo-NHS-SS-Biotin labeling reagent is charged by the sodium sulfoxide group on the succinimidyl ring, it is water soluble and membrane impermeable, which makes the reagent suitable for labeling of cell surface proteins via reaction with primary amines (10, 17). Because of the extremely strong affinity between biotin and avidin (dissociation constant of roughly $10^{-15}$ M, i.e., ~1 fM), it is not possible to efficiently separate the complex and isolate Sulfo-NHS-SS-Biotin-labeled proteins. Considering this, Sulfo-NHS-SS-Biotin has the advantage of enabling isolation of biotin-labeled cell surface proteins by streptavidin affinity chromatography via cleavage with a reducing agent (45). Since the biotin residue is removed after cleavage with a reducing agent, biotin would no longer interfere with detection of the protein mass, which makes Sulfo-NHS-SS-Biotin suitable for proteomics. This feature nonetheless prohibits the use of reducing agents in the SDS-PAGE sample buffer when observing Sulfo-NHS-SS-Biotin-labeled proteins via biotin blotting, which makes molecular size pattern discrimination inaccurate. Therefore, we first labeled cell-free E. chaffeensis with Sulfo-NHS-LC-Biotin with a spacer arm length similar to that of Sulfo-NHS-SS-Biotin (22.4 and 24.3 Å, respectively) to determine the approximate molecular sizes of biotinylated proteins in the presence of a reducing agent. The biotinylated proteins were blotted onto the membrane and probed with HRP-conjugated streptavidin. As shown in Fig. 1, Sulfo-NHS-LC-Biotin labeling revealed E. chaffeensis surface proteins with approximate molecular masses of 22, 28, 29, 36, 38, 40, 47, 55, 60, 75, 80, 90, 120, and 200 kDa (lane 1). As described above, to preserve the disulfide bonds in Sulfo-NHS-SS-Biotin, there was no reducing agent added to the sample buffer of Sulfo-NHS-SS-Biotin-labeled proteins (Fig. 1, lane 3). The biotinylation of lysed E. chaffeensis (Fig. 1, lane 2) showed much more proteins labeled compare to the selective surface labeling (Fig. 1, lane 1).

Streptavidin affinity purification of biotinylated E. chaffeensis surface proteins. To identify biotinylated E. chaffeensis surface proteins, Sulfo-NHS-SS-Biotin-labeled intact host cell-free bacteria were solubilized in RIPA buffer. The biotinylated proteins were purified by streptavidin affinity gel chromatography. The disulfide bond in Sulfo-NHS-SS-Biotin was cleaved with the reducing agent to elute the streptavidin affinity-captured proteins. The eluted proteins were separated by SDS-PAGE. As shown in Fig. 2A, with GelCode blue protein staining in the SDS-PAGE (with the reducing agent in the sample buffer), there were five bands of relatively abundant proteins corresponding to molecular masses of approximately 28, 40, 55, 60, and 80 kDa. The proteins with similar molecular masses were detected by Sulfo-NHS-LC-Biotin surface labeling (Fig. 1, lane 1).

Immunogenicity of E. chaffeensis surface proteins. While there was some banding pattern variation across human and dog sera, the human ehrlichiosis patient serum (lane 1) provided by the Centers for Disease Control and Prevention (CDC, Atlanta, GA) (42), dog 3918815 anti-E. chaffeensis Arkansas serum (lane 3) all recognized E. chaffeensis surface proteins with molecular masses of approximately 28 kDa (Fig. 2B). In addition, the human ehrlichiosis patient serum
exposed. Esp73 is an ortholog of E. chaffeensis (lane 1) weakly reacted with 60-kDa proteins (Fig. 2B). Dog 3836/GE AND RIKIHISA INFECT. IMMUN. shown in Table 1, some host cell data). Band 2, corresponding to approximately 40 kDa, was face-exposed proteins (Y. Ge and Y. Rikihisa, unpublished material). Among the OMP-1/A and OMP-1N paralogs, and their topology with respect to the outer membrane lipid bilayer have not been reported. We examined the 2-D structures of OMP-1A and OMP-1N, which were phylogenetically distinct and have acidic and alkaline isoelectric points, respectively (32). Both proteins have a 2-D structure of amphipathic and antiparallel multipass transmembrane β strands (Fig. 4A and B) predicted by the posterior decoding method with the dynamic programming algorithm in PRED-TMBB (2).

To verify the localization of OMP-1A and OMP-1N on the surface of E. chaffeensis, peptides of OMP-1A (aa 154 to 167) (Fig. 4A) and OMP-1N (aa 142 to 155) (Fig. 4B) located within one of the extracellular loops on the basis of 2-D structure prediction were synthesized and rabbit antibodies were developed. Host cell-free E. chaffeensis was prefixed with paraformaldehyde to prevent antibody permeabilization, incubated with either anti-OMP-1A or -OMP-1N peptide serum, and examined by immunofluorescence microscopy. As shown in Fig. 4C, the OMP-1A peptide antiserum labeled the surface of individual bacteria in the typical ring-like surface staining pattern, as observed with A. phagocytophilum surface protein P44 (52); so did the OMP-1N peptide antiserum. In contrast, antiserum from a rabbit immunized with an irrelevant peptide (Fig. 4C) and preimmune rabbit serum (data not shown) did not recognize E. chaffeensis. As a negative control, uninfected THP-1 cells fixed with paraformaldehyde were incubated with anti-OMP-1A and -OMP-1N peptide sera. The result showed no significant fluorescence labeling (data not shown). Uninfected THP-1 cells were also fixed with methanol to permeabilize the cells. These THP-1 cells had diffuse and insignificant fluorescence labeling with OMP-1A (Fig. 4C) and OMP-1N (data not shown) peptide antisera compared to cell-free E. chaffeensis-specific surface labeling. The data indicated that the antibody labeling of the E. chaffeensis surface OMP-1A and OMP-1N proteins were specific and thus confirmed their surface localization, as identified by the biotinylation method in this study (Table 1). Hence, immunofluorescence labeling results for OMP-1A and OMP-1N with rabbit antiserum against each peptide experimentally validated the 2-D prediction of bacterial surface exposure of the particular peptide region by PRED-TMBB.

Expression of OMP-1B by E. chaffeensis cultured in THP-1 cells. Among the E. chaffeensis OMP-1/P28 family proteins, OMP-1B, OMP-1T, and P28-2 (Ohashi’s designation) were not detected by surface biotinylation (Fig. 3). Since OMP-1B is the only OMP-1/P28 paralog detected at the translational level in ISB6 tick cell culture (46) or at the transcriptional level in ticks (49), to determine whether the OMP-1B protein was expressed by E. chaffeensis cultured in THP-1 cells, Western blot analysis of OMP-1B with rabbit anti-OMP-1B peptide serum was performed. In lane 1 of Fig. 5, there was no detectable OMP-1B band around 28 kDa in E. chaffeensis-infected THP-1 cells. To verify the reactivity of anti-OMP-1B peptide serum, BL21(DE3) competent cells were transformed with recombinant PET29a-omp-1B or the pET29a vector with no insert and expression of proteins was induced with isopropyl-β-D-thiogalactopyranoside.

Nano-LC/MS/MS. The five bands of streptavidin affinity-captured proteins separated by SDS-PAGE were subjected to proteomic analysis. Band 1 consisted mainly of the OMP-1/P28 protein family. With the exception of three E. chaffeensis OMP-1/P28 paralogs, i.e., OMP-1T (designated P28-4 by Yu et al. [55]), OMP-1B (designated P28-14 by Yu et al.) and P28-2 (designation of Ohashi et al. [32]; designated P28-21 by Yu et al.), 19 out of 22 E. chaffeensis OMP-1/P28 family proteins were surface exposed (Table 1 and Fig. 3). Every OMP-1/P28 paralog was identified by the detection of paralog-specific sequences. This was verified by a BLAST search of each peptide against the entire E. chaffeensis annotated genome sequencing data (20), as indicated by percent paralog-specific amino acid sequence coverage in Table 1. For these OMP-1/P28 paralog-specific peptide sequences, see Table S1 in the supplemental material. Except for P28 (YP_507927) and P28-1 (Ohashi’s designation; YP_507928) (33, 46), 17 of these 19 OMP-1/P28 family proteins have not been directly proven to be expressed at the protein level. Except for P28, surface exposure of other OMP-1/P28 family proteins has not been shown previously.

The conjugal transfer protein VirB9-1 (YP_506989), a type IV secretion system subunit, was detected from band 1. The VirB9-1 ortholog (YP_505897) has been shown to be surface exposed on Anaplasma phagocytophilum (31) but previously was not observed on the surface of E. chaffeensis.

A protein from the serine protease DO/DeqQ family (YP_507837) was detected from band 3. This is the first time the surface exposure of a member of this protein family was shown on E. chaffeensis. The identification of the immunodominant surface protein gp47 (AAZ40202) is in agreement with the recent proposal of this protein as surface exposed on the basis of immunoelectron microscopy (13). This protein migrated as band 3, with a molecular mass of approximately 50 kDa (Fig. 2), which was much different from its predicted molecular mass of 33,040 Da. The major protein in band 4 was the 60-kDa chaperonin GroEL (YP_507185). Band 5 contained OMP85 (YP_507856), which is conserved in gram-negative bacteria (51) but has not been shown to be expressed or surface exposed on E. chaffeensis. For the first time, the hypothetical protein ECH_0525 (YP_507340), here named Esp73 (Ehrlichia surface protein, 73 kDa), was revealed to be surface exposed. Esp73 is an ortholog of A. phagocytophilum hypothetical proteins APH_0404 and APH_0405, newly uncovered surface-exposed proteins (Y. Ge and Y. Rikihisa, unpublished data). Band 2, corresponding to approximately 40 kDa, was host cell β-actin (NP_001092) (not shown in Table 1). As shown in Table 1, some E. chaffeensis proteins of relatively low abundance also were identified in this study, such as translation elongation factor G (YP_507748) (band 5), the ATP synthesis F1 β subunit (YP_507384) (band 4), dihydrolipicolate reductase (YP_507114) (band 1), disulfide oxidoreductase (YP_5077114) (band 1), and the antioxidant ApC/Tsa family (YP_507536) (band 1).
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<td>29,614</td>
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<sup>a</sup> Identified by using the MASCOT MS/MS search engine and Turbo SEQUEST algorithm in the BioWorks 3.1 Software.

<sup>b</sup> Identified by blasting each peptide sequence against the entire *E. chaffeensis* annotated genome sequencing data.

<sup>c</sup> The program SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP) was used to predict the presence of N-terminal signal peptides.
lactopyranoside. As shown in Fig. 5, the OMP-1B peptide antiserum recognized the recombinant OMP-1B protein (lane 3) but not pET29a-transformed E. coli proteins (lane 2). This result suggests that OMP-1B is not expressed by E. chaffeensis when cultured in THP-1 cells at 37°C. Expression of the OMP-1T or P28-2 protein was not determined.

### DISCUSSION

By a surface biotinylation method, the present study uncovered many E. chaffeensis proteins that had not been shown previously to be surface exposed (Table 1). Detection of two proteins known to be surface exposed, P28 and gp47, by surface biotinylation attests to the utility of this method. OMP-1/P28 family proteins are the most abundantly expressed E. chaffeensis surface-exposed integral membrane proteins. For the first time, 18 out of 21 E. chaffeensis OMP-1/P28 family proteins have been shown to be surface exposed and 17 have been detected directly at the protein level.

Since surface proteins of bacteria in the family Anaplasmataceae are exposed to the host immune system, they compose immunodominant antigens (23, 29, 37). P28 (33) and gp47 (13) have been shown to be immunoreactive with E. chaffeensis-infected dog serum. By Western blotting analysis with whole bacterial lysates, a series of E. chaffeensis immunoreactive proteins have been reported as approximately 74-, 70-, 64-, 47-, 31-, and 29-kDa proteins (42) and those with molecular masses of approximately 200, 120, 66, 58, 55, 29, 28, and 22 kDa (7, 8). However, surface exposure of these proteins or the identities of these proteins have been mostly unknown. In the present study, the major immunoreactive E. chaffeensis proteins with molecular masses of approximately 75, 60, and 55 kDa for the first time have been shown to be surface exposed. Therefore, the newly identified proteins have advanced the knowledge of E. chaffeensis surface immunogens, which extends the range of candidates for diagnostic antigens and vaccine development.

OMP-1/P28 family members are the most-studied E. chaffeensis OMPs with multiple transmembrane β strands. They are encoded by a pleomorphic multigene family composed of 22 paralogous genes clustered in a 27-kb gene locus of the E. chaffeensis genome (32). The gene organization and the genomic locus of the E. chaffeensis omp-1/p28 gene cluster are conserved among Ehrlichia species such as Ehrlichia canis (32) and Ehrlichia ruminantium (4). The mRNA expression of various numbers of the omp-1/p28 gene family has been reported in E. chaffeensis growing in DH82 cells, e.g., in 1 of 5 (40), 6 of 10 (55), and 16 of 22 (27) paralogs tested. All 22 E. canis omp/p30 paralogs are transcribed in DH82 cells, and more than two-thirds of them are cotranscribed (32). Unver et al. detected the transcripts of 16 of 22 E. chaffeensis omp-1/p28 paralogs in the blood monocytes of infected dogs (49).

In contrast, few studies have been undertaken concerning the protein expression of OMP-1/P28 family members. By enzyme-linked immunosorbent assay with synthetic peptides of P28 OMPs (OMP-1/P28 paralogs) as antigens, Zhang et al. have reported that all 22 P28 OMPs are expressed in infected dogs (57). By N-terminal amino acid sequencing, Ohashi et al. directly detected P28 expression by E. chaffeensis in DH82 cells (33). Recently, by proteomic analysis, two OMP-1/P28 family members, P28-Omp19 (Ohashi’s P28) and P28-Omp20 (Ohashi’s P28-1), were identified in E. chaffeensis cultured in DH82 cells (46). In the present study, 19 E. chaffeensis OMP-1/P28 proteins (cultured in THP-1 cells), including P28 and P28-1 (Ohashi’s designation), were directly identified by proteomics. These 19 OMP-1/P28 family proteins coexist in E. chaffeensis cultured in THP-1 cells. Considering the cotranscription of most of the omp/p30 paralogs in DH82 cells (32) and the fact that most of the E. chaffeensis organisms in a given bacterial population express the P28, OMP-1A, or OMP-1N protein, it is possible that each organism expresses more than one of these proteins. Furthermore, it is also possible that each individual organism expresses different combinations of them but that in a given bacterial population, all 19 OMP-1/P28 family proteins are expressed. Western blotting data on OMP-1B suggest that it is not expressed by E. chaffeensis cultured in THP-1 cells at 37°C. Of note, OMP-1B is the only omp-1/p28 paralog transcript detected in three developmental stages of A. americanum ticks before or after E. chaffeensis transmission to naive dogs (49). The P28-Omp14 (Ohashi’s OMP-1B) protein was also the only OMP-1/P28 paralog detected by proteomics in E. chaffeensis cultured in a tick cell line, ISE6 (46). In the present study, besides OMP-1B, OMP-1T and P28-2 (Ohashi’s designation) were the other two OMP-1/P28 family members not detected by surface biotinylation, suggesting that these two proteins are either not expressed at a detectable level or not surface exposed by E. chaffeensis cultured in THP-1 cells at 37°C. Therefore, the present data favor the notion that the E. chaffeensis OMP-1/P28 multigene family is differentially expressed in mammalian and tick hosts, which may be important for ehrlichial adaptation to different host environments (46, 49). Recently, Zhang et al. proposed that the expression of P28-19 (Ohashi’s P28) was up-regulated in E. chaffeensis reticulate cells and down-regulated in dense-cored cells (58). In future studies, it would be of interest to determine the global pattern of surface-exposed proteins during the de-
Development cycles of *E. chaffeensis* and the mechanism and functions of the differential expression by *E. chaffeensis* cultured in different host cells or in different ehrlichial developmental stages.

Esp73 is an ortholog of the newly identified *A. phagocytophilum* integral OMPs APH_0404 and APH_0405, which have predicted secondary structures of 22 transmembrane β strands and are very likely to function as transporters (Ge and Rikihisa, unpublished). Except for the genus *Neorickettsia*, the orthologs have been found in the genera *Anaplasma*, *Ehrlichia*, and *Wolbachia* in the family *Anaplasmataceae* by BLAST search of the GenBank database with an E value below e\(^{-15}\), suggesting evolutionary pressure for conservation within the family (Ge and Rikihisa, unpublished).

The theoretical molecular mass of the immunodominant surface protein gp47 is different from the migration distance in a 10% SDS-PAGE gel (Table 1 and Fig. 2). It has been proposed that this is caused by protein glycosylation (13). Recombinant gp47 has been shown to be immunoreactive with serum from an *E. chaffeensis*-infected dog (13). gp47 was differentially expressed at different developmental stages in DH82 cell culture (13). gp47 does not have a signal peptide on the basis of prediction by the SignalP 3.0 program, suggesting that it is not secreted by the conventional Sec-dependent pathway. Therefore, how this protein is secreted to the bacterial surface remains to be investigated. The *E. chaffeensis* serine protease (YP_507837), with a definitive N-terminal signal peptide sequence predicted by SignalP 3.0, was identified as a surface-exposed protein in the present study. There are reports of serine proteases existing on the surfaces of other bacteria. For example, serine protease HtrA (high-temperature requirement) has been described as a surface-exposed protease in the family *Staphylococcaceae* (41). This protein homolog is involved in the virulence of many pathogens via its roles in thermal stability, resistance to oxidative stress, and bacterial survival (9). It would be interesting to characterize the function of this protein.

Some proteins which were considered bacterial cytoplasmic, periplasmic, or inner membrane proteins were identified in the present study, such as chaperone protein GroEL, DnaK, translation elongation factor G, cytosol aminopeptidase, disulfide oxidoreductase, and ATP synthase F1. These proteins may have been released from spontaneously lysed bacteria and bound to the intact bacteria within the host inclusions. Another possibility is that some bacterial outer membrane are damaged during the isolation of cell-free bacteria before biotin labeling.
However, the surface localization and some surface-related function of these proteins have been reported in other bacteria. For example, by immunofluorescence or immunoelectron microscopy, GroEL has been shown on the surfaces of Helicobacter pylori (22), Legionella pneumophila (16), Haemophilus ducreyi (15), and Clostridium difficile (19). DnaK was detected on the surface of H. pylori (22). Evidence for specific secretion rather than autolysis has been reported in the release of some H. pylori proteins, including GroEL (50). H. ducreyi GroEL has been reported to mediate the binding of this bacterium to host carbohydrate receptors (38). Cytosol aminopeptidase has been found in the outer membrane fraction of A. marginale (28). Disulfide oxidoreductase of E. chaffeensis (eDb) has been occasionally observed on the bacterial surface by immunoelectron microscopy (30). Recently, elongation factor G and the ATP synthase F1 alpha and beta subunits have been detected on the cell envelope of S. aureus (17). Therefore, we cannot deny the possibility that these proteins with well-known functions in the cytoplasmic, periplasmic, or inner membrane are present on the surface of E. chaffeensis and play unexpected roles in E. chaffeensis-host interaction.

One obvious host protein band captured by streptavidin affinity purification was β-actin, which is one of the most abundant cytoskeleton proteins of eukaryotic cells. This may be due to the binding of host cell actin to bacterial surface proteins during the isolation of host cell-free bacteria or via a functional association. Actin association with various strains of A. marginale was previously reported (47).

Biotinylation of the E. chaffeensis surface showed a series of protein bands labeled with Sulfo-NHS-LC-Biotin. Of note, the density of each band of Sulfo-NHS-LC-Biotin-labeled E. chaffeensis surface proteins (Fig. 1) was different from the density of the corresponding band in Sulfo-NHS-SS-Biotin-labeled and streptavidin affinity-purified proteins (Fig. 2A). This is because the band density of biotin blotting (Fig. 1) is determined not only by protein abundance but also by the amounts of surface-exposed free amines in individual proteins. In other words, the larger the surface-exposed regions and the more biotin-reactive free amine residues there are, the more biotin molecules are present per protein, and thus, the denser the band is. On the contrary, proteins with smaller surface-exposed regions and/or few biotin-reactive residues, such as OMP-1/P28 family proteins, are weakly labeled and generate lighter biotin bands. This is consistent with our previous finding by immunogold transmission electron microscopy with the P28-specific antibody, which showed immunogold particles bound very close to the E. chaffeensis outer membrane lipid bilayer (33). This immunoprobe labeling pattern is distinct from the surface antigen labeling of some other rickettsial pathogens such as Orientia tsutsugamushi (43). Considering the 2-D prediction of the amphibial β-barrel structure, the integral OMP-1/P28 outer membrane family proteins may have a key function as porins in bacterial metabolism similar to that of the major β-barrel protein P44 (Msp2s) of A. phagocytophilum (21). In contrast, heavily Sulfo-NHS-LC-Biotin-labeled E. chaffeensis surface proteins such as the 47-, 55-, 60-, 75-, 80-, 90-, 120 (perhaps E. chaffeensis surface protein gp120 [39])- and 200-kDa proteins may play important roles in bacterial binding and internalization of host cells. In fact, gp47 and gp120 have been proposed to function as adhesins in mediating bacterial binding to host cells (13, 39). The GelCode blue-stained band densities of Sulfo-NHS-SS-Biotin-labeled and streptavidin affinity-purified E. chaffeensis surface proteins more or less reflect the abundance of the protein (Fig. 2A). In addition to the major surface proteins identified in this paper, the identities of other, relatively less abundant surface proteins with heavy biotin labeling, such as the 90- and 200-kDa proteins (Fig. 1), deserve further investigation.

In conclusion, the identification of E. chaffeensis major surface proteins purified by the biotinylation method will greatly advance the knowledge of E. chaffeensis surface components and set the foundation for future studies on the Ehrlichia-host interaction. Future studies will be aimed at characterizing the biological functions of the newly identified E. chaffeensis surface proteins.

ACKNOWLEDGMENTS

We appreciate the technical assistance of Kari Green-Church in proteomic analysis. We thank Rikihisa laboratory member Haibin Huang for preparing recombinant PET29a-OMP-1B in E. coli BL21(DE3). We also thank Kate Hayes for copy editing of the manuscript.

This work was supported by National Institutes of Health grant R01 AI 30010.

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