Serological characterization of surface-exposed proteins of *Coxiella burnetii*

Jun Jiao,¹ Xiaolu Xiong,¹ Yong Qi,² Wenping Gong,¹ Changsong Duan,¹ Xiaomei Yang¹ and Bohai Wen¹

¹State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing 100071, PR China
²Department of Medical and Pharmaceutical Biotechnology, Huadong Research Institute for Medicine and Biotechniques, Nanjing 210002, PR China

The obligate intracellular Gram-negative bacterium *Coxiella burnetii* causes Q fever, a worldwide zoonosis. Here we labelled *Cox. burnetii* with biotin and used biotin-streptavidin affinity chromatography to isolate surface-exposed proteins (SEPs). Using two-dimensional electrophoresis combined with mass spectrometry, we identified 37 proteins through bioinformatics analysis. Thirty SEPs expressed in *Escherichia coli* (recombinant SEPs, rSEPs) were used to generate microarrays, which were probed with sera from mice experimentally infected with *Cox. burnetii* or sera from Q fever patients. Thirteen rSEPs were recognized as seroreactive, and the majority reacted with at least 50 % of the sera from mice infected with *Cox. burnetii* but not with sera from mice infected with *Rickettsia rickettsii*, *R. heilongjiangensis*, or *R. typhi*. Further, 13 proteins that reacted with sera from patients with Q fever did not react with sera from patients with brucellosis or mycoplasma pneumonia. Our results suggest that these seroreactive SEPs have potential as serodiagnostic antigens or as subunit vaccine antigens against Q fever.

INTRODUCTION

The obligate intracellular Gram-negative bacterium *Coxiella burnetii*, a member of the order *Legionellales*, causes Q fever. Its broad host range includes domestic and wild animals as well as humans, and its pathogenicity is maintained by its extraordinary resistance to desiccation and the effects of physical and chemical factors encountered in the environment. Acute Q fever is usually transmitted to humans by inhalation of aerosols generated by infected animals, and the disease is treated effectively using antibiotics (Kokkini et al., 2013). However, acute Q fever may progress to chronic disease complicated by endocarditis, chronic hepatitis, and/or osteomyelitis (Kampschreur et al., 2013; Varghees et al., 2002), which are sometimes incurable (Almogren et al., 2013; Zhang et al., 2005). Humans typically contract Q fever by breathing contaminated aerosols released by infected sheep or goats (Kampschreur et al., 2013; Kwak et al., 2013). Large outbreaks of Q fever occurred in the Netherlands from 2007 to 2010, and *Cox. burnetii*-infected dairy sheep or goats were identified as the source (Roest et al., 2011). Reducing such major impacts of Q fever on public health requires the development of accurate diagnostic techniques and effective vaccines.

Although an inactivated *Cox. burnetii* phase I whole-cell vaccine effectively controls Q fever in humans and domestic animals, strong side effects, such as sterile abscesses and granulomas at the inoculation site in humans previously sensitized by natural infection of *Cox. burnetii*, limit its use in humans (Ackland et al., 1994). Immunization with bacterial surface proteins such as Com1, Mip, and SecB confer partial protection against *Cox. burnetii* infection in murine models (Wei et al., 2011; Xiong et al., 2012). A *Cox. burnetii* whole-cell antigen (WCA) vaccine induces enhanced immunity compared with individual antigens (Wei et al., 2011; Xiong et al., 2012a).

Correct diagnosis of Q fever is challenging, because its clinical signs are not pathognomonic (Anastácio et al., 2013). Specialized laboratories use diagnostic techniques employing antibodies, and the most commonly used and reliable methods include complement fixation (Houpikian & Raoult, 2003), indirect immunofluorescence assay (IFA) (Peacock et al., 1983), and enzyme linked immunosorbent assay (ELISA) (Fournier et al., 1998). Moreover, these methods require considerable time, and preparing the
diagnostic antigens (Cox. burnetii WCA) is hazardous and laborious (Watanabe & Takahashi, 2008).

Proteomics analysis may identify bacterial surface-exposed proteins (SEPs) that have potential as serodiagnostic antigens or as subunit vaccine antigens against corresponding bacterial infection. In the present study, we performed a proteomics analysis of Cox. burnetii cell-surface proteins and identified 37 SEPs. Thirty were expressed in Escherichia coli and were used to fabricate microarrays that were probed with sera from mice experimentally infected with Cox. burnetii and from patients with Q fever.

METHODS

Cultivation and purification of Cox. burnetii. Cox. burnetii Xinqiao strain (phase I) propagated in fertilized eggs was purified using differential centrifugation and renograin density-gradient centrifugation as described previously (Xiong et al., 2012b). The purified bacteria were diluted in PBS buffer (8.1 mM Na2HPO4, 1.9 mM NaH2PO4 and 154 mM NaCl; pH 7.4).

Mouse and human sera. Thirty-two male BALB/c mice aged 6 weeks (Laboratory Animal Center of Beijing, China) were injected intraperitoneally with Cox. burnetii Xinqiao strain (1 x 10^8 cells per mouse) in a biosafety level-3 laboratory. Sera were collected and pooled from eight mice that were chosen at random on days 7, 14, 21 and 28 post-infection (pi). Sera were collected individually from another 10 individual BALB/c mice 28 days pi, as well as from BALB/c mice infected with Rickettsia rickettsii (Sheila Smith strain), R. heilongjiangensis (054 strain) or R. typhi (Wilmington strain) 28 days pi as described previously (Qi et al., 2013). Sera from 10 individual normal BALB/c mice were used as negative controls.

We collected sera with IgG IFA titres ≥ 1:800 and 1:400 against Cox. burnetii phase I or II antigens, respectively, from nine inpatients being treated for Q fever at the Peking Union Medical College Hospital. Sera from 10 patients with brucellosis, with Brucella-specific IgG titres determined using the Rose Bengal plate test, were provided by Professor Cui at the Brucellosis Laboratory of the Chinese Centers for Disease Control. Sera from 10 patients with mycoplasma pneumonia, with positive IgG titres against Mycoplasma pneumoniae determined using the passive particle agglutination test, were provided by Dr Sun at the 463 Hospital of the People’s Liberation Army (PLA), Shenyang, China. Sera from 10 healthy blood donors were obtained from the 307 Hospital of the PLA, Beijing, China. Serum samples were collected as part of the routine management of patients whose sera were anonymized without additional sampling.

Separation of surface-exposed proteins. Purified Cox. burnetii were biotinylated using EZ-Link Sulfo-NHS-SB-Biotin (Pierce), sonicated (300 W, 3 s on and 9 s off) for 30 min at 4 °C using an ultrasonic processor (Sonics & Materials), and centrifuged at 20000 g for 1 h at 17 °C to remove any insoluble material, before performing streptavidin-agarose affinity purification (Pierce) (Qi et al., 2013). The isolated proteins were eluted with 500 mM DTT and precipitated with a 2D-Cleanup kit (GE Healthcare). The pellets were resuspended in rehydration buffer (7 M urea, 2 M thiourea and 4 % CHAPS) and separated on a 17 cm pH 3–10 NL IPG strip (Bio-Rad) in the first dimension followed by 12 % SDS-PAGE (Qi et al., 2013).

Identification of SEPs. The silver-stained spots in the polyacrylamide gel were excised, digested with trypsin, and subjected to electrospray ionization tandem mass spectrometry (ESI-MS/MS), which was performed by the National Center of Biomedical Analysis (Beijing, China). Using the Mascot search engine (http://www.matrixscience.com), peptides that generated mass fingerprints were compared with sequences in the nonredundant databases of the United States National Center for Biotechnology Information (NCBI). Amino acid compositions and N-terminal signal peptide cleavage sites were predicted using the SignalP4.1 web server (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004), and the signal peptides of lipoproteins and N-terminal membrane helices were analysed using the LipoP 1.0 web server (http://www.cbs.dtu.dk/services/LipoP/) (Juncer et al., 2003). The subcellular location of each protein was predicted using the PSORTb 3.0.2 (http://psort.org/psortb/) and SOSUI-GramN servers (http://bp.nuap.nagoya-u.ac.jp/sosui/sosuigrann/sosuigrann_submit) (Qi et al., 2013). The proteins identified were classified into Clusters of Orthologous Groups of proteins (COGs), and their functions were assigned using NCBI’s COGnitor server (http://www.ncbi.nlm.nih.gov/COG/).

Preparation of recombinant SEPs. The genes encoding the SEPs identified by mass spectrometry were amplified using their cognate primer pairs (Table S1, available in the online Supplementary Material), designed according to the genomic sequence of Cox. burnetii (GenBank accession number: NC_002971), using PCR. The SEP amplicons were inserted into the plasmids pET32a(+) or pQE30 (Novagen), and the constructs were used to transform competent E. coli (Novagen). The proteins expressed in E. coli are designated recombinant surface-exposed proteins (rSEPs). They are referred to in the text, tables, and figures according to their NCBI Gene symbol/Locus tags (e.g. CBU_0067). The rSEPs were purified using a Ni-NTA affinity resin according to procedures described previously (Li et al., 2005).

Fabrication of protein microarrays. Each of the purified rSEPs was adjusted to 400 μg ml⁻¹ using elution buffer (Qiagen) and printed as five spots on OPEpoxy slides (CapitalBio). Lysates of E. coli transformed with pET-32a(+) plasmids were printed on the slides as negative controls. Mouse and human IgG molecules printed on the slides served as positive controls. The protein arrays were incubated with Cy5-labelled anti-His-tag IgG (SBA) as a quality control measure.

Serological analysis of rSEP microarrays. Each serum was diluted 1:100 in PBS and mixed with an equal volume of an E. coli lysate (5 mg ml⁻¹) overnight at 4 °C to sequester the antibodies against E. coli (Beare et al., 2008). The protein arrays were blocked with PBS containing 1 % (w/v) BSA overnight at 4 °C. After washing with deionized water, each protein on the array was incubated with an individual serum for 1 h at room temperature. After five washes in PBS containing 0.05 % (v/v) Tween 20 and one wash with deionized water, the air-dried arrays were incubated with goat anti-human IgG-Cy5 or goat anti-mouse IgG-Cy5 (SBA) (1 : 500 dilution) antibodies for 1 h in the dark at room temperature. After six more washes, the air-dried arrays were scanned using a GenePix Personal 4100A scanner (Molecular Devices), and fluorescence intensity (FI) was determined using GenePix Pro 6.0 software.

Data analysis. The FI value of each protein was calculated by averaging the FI values of five replicate spots after subtracting background. FI values for proteins probed using sera from Cox. burnetii-infected mice or patients were analysed using the Kruskal–Wallis test followed by the Student–Newman–Keuls multiple range test (SAS 9.1, SAS Institute) (Qi et al., 2013).

RESULTS

Identification of SEPs. Cox. burnetii SEPs purified by biotin-streptavidin affinity system were separated by 2D-PAGE and finally stained
with silver (Fig. 1). ESI-MS/MS was used to analyse 82 silver-stained spots, corresponding to molecular masses from 7 to 71 kDa and isoelectric points (pIs) from 4 to 11, which were excised from the gel. Thirty-seven proteins were identified (Table S2). Certain single spots (e.g. 2, 4 and 12) contained several different proteins, certain single spots (such as spots 1, 5 and 6) were identified as a unique protein, and certain proteins (such as heat-shock proteins DnaK, GroEL and GroES) were present in multiple spots (Fig. 1, Table 1). The 37 SEPs were classified into 11 categories according to the results of a search for COGs (Table 1). Analysis using the SignalP4.1/LipoP 1.0 web server predicted that nine spots contained a signal peptide cleavage site.

**Analysis of rSEPs with mouse sera**

Thirty of the 37 SEPs were expressed in *E. coli*, and these rSEPs were used to generate a protein microarray. The microarray was probed with the mouse sera listed in Table S3, and a reaction was considered positive if the average FI value of any rSEP probed with each serum sample was $>3$ sds above the average FI value of the same protein probed with normal mouse sera (Qi *et al.*, 2013). The pooled sera obtained from mice infected with *Cox. burnetii* on days 7, 14, 21 and 28 pi reacted with 0, 16, 25 and 27 of the 30 rSEPs, respectively (Figs 2 and 3).

Twenty-three of the 30 rSEPs reacted with at least 5 of the 10 sera collected from *Cox. burnetii*-infected mice, and the average IF values of eight rSEPs (CBU_0067, CBU_0227, CBU_0630, CBU_1078, CBU_1290, CBU_1385, CBU_1594 and CBU_1706) were significantly higher compared with those of sera from mice infected with *R. rickettsia, R. heilongjiangensis* or *R. typhi* ($P<0.05$) (Fig. 3). Further, rSEP CBU_0067 reacted with nine of the 10 sera collected from *Cox. burnetii*-infected mice. The average FI value for rSEP CBU_0630 was the highest (Table 2).

**Fig. 1.** 2D PAGE reference map of surface-exposed proteins of *Cox. burnetii*. The biotin-labelled proteins were enriched using streptavidin affinity chromatography and were separated on a 17 cm pH 3–10 NL IPG strip (Bio-Rad) in the first dimension followed by 12% SDS-PAGE. Numbers refer to the silver-stained protein spots excised from 2D-PAGE gels that were subjected to ESI-MS/MS analysis. The relative molecular masses of the marker proteins are indicated in kDa on the left side of the figure, and the protein spots identified using ESI-MS/MS analysis are listed in Table S2.
**Table 1.** Classification of COGs and bioinformatics analysis of *Cox. burnetii* surface-exposed proteins identified using ESI-MS/MS

<table>
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<tr>
<th>COG</th>
<th>Annotation</th>
<th>Locus tag</th>
<th>Gene symbol</th>
<th>Signal peptide (SignalP/LipoP)*</th>
<th>Subcellular location (PSORTb/SOSUI-GramN)</th>
<th>Spot no.</th>
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<td>Antioxidant AhpCTSA family protein</td>
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<td>OM/OM</td>
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<td>CYT/CYT</td>
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<td>CYT/Unknown</td>
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<td>No/No</td>
<td>CYT/CYT</td>
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<td>No/No</td>
<td>Unknown/EC</td>
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<td>Hypothetical protein CBU_2076</td>
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<td>No/No</td>
<td>Unknown/PP</td>
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<td>Hemerythrin HHE cation binding domain protein</td>
<td>CBU_1677</td>
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<td>No/No</td>
<td>CYT/CYT</td>
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<td>Dihydrolipoamide-residue succinyltransferase</td>
<td>CBU_1398</td>
<td>sucB</td>
<td>No/No</td>
<td>CYT/CYT</td>
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Analysis of rSEPs with human sera

Microarrays of the 30 rSEPs were probed with sera from patients with Q fever ($n=9$), brucellosis ($n=10$) and mycoplasma pneumonia ($n=10$) (Table S4). A reaction was considered positive if the average FI value of any rSEP was $>3$ SDs compared with the average FI value of the same protein probed with sera from healthy people. Fifteen rSEPs (CBU_0229, CBU_0236, CBU_0337, CBU_0510, CBU_0630, CBU_0867, CBU_0952, CBU_1169, CBU_1320, CBU_1385, CBU_1519, CBU_1594, CBU_1718, CBU_1719 and CBU_1910) reacted with at least five of nine sera collected from patients with Q fever (Table 3). The average FI value of these proteins probed with Q fever sera was significantly higher compared with those probed with the sera from patients with brucellosis or mycoplasma pneumonia ($P<0.05$). Further, 10 rSEPs (CBU_0229, CBU_0337, CBU_0867, CBU_0952, CBU_1169, CBU_1320, CBU_1385, CBU_1718, CBU_1719 and CBU_1910) reacted with greater than seven Q fever sera (Figs 4 and 5), and the IF value for rSEP CBU_1718 was the highest. None of the 15 rSEPs reacted detectably with sera from patients with brucellosis or mycoplasma pneumonia (Table 3).

**DISCUSSION**

*Cox. burnetii* SEPs may mediate crucial interactions between *Cox. burnetii* and host cells, such as initial attachment of *Cox. burnetii* and subsequent contact with host cytosolic proteins (Sears et al., 2012). Moreover, SEPs may interact with the host’s immune cells and induce the production of pathogen-specific humoral antibodies and cell-mediated immune responses (Wei et al., 2011; Xiong et al., 2012a). Here we describe the identification of 37 SEPs and show that 30 react with sera from mice immunized with *Cox. burnetii* and sera from patients with Q fever.

Eight SEPs were predicted to contain a signal peptide cleavage site, which strongly suggests that they are membrane-associated or secreted proteins as follows (ID, protein symbol or hypothetical): CBU_1910 (Com1), CBU_0630 (Mip), CBU_0311 (P1), CBU_0612 (OmpH), CBU_0092 (YbgF), CBU_0370 (hypothetical), CBU_0952 (hypothetical) and CBU_1902 (hypothetical). Com1 is a 27 kDa outer-membrane protein (Omp) of *Cox. burnetii* (Hendrix et al., 1990) and catalyses the formation of disulfide bonds in extracytoplasmic proteins, including the components of the Dot/Icm type IV secretion system (Jameson-Lee et al., 2011). Mip is a 25.5 kDa surface peptidylprolyl-isomerase that potentiates infection of macrophages by *Cox. burnetii* (Mo et al., 1995). Com1 was identified in nine spots (spots 38, 39, 41, 43, 45, 47, 48, 50) with the same molecular mass but different pIs, and Mip was detected in four of nine Com1 spots (spots 39, 47, 48, 50), which reveals that the molecular masses of Com1 and Mip are similar and that these proteins are abundant on the cell surface. OmpH is a 16 kDa outer-membrane protein (Omp) of *Cox. burnetii* (Hendrix et al., 1990) and catalyses the formation of disulfide bonds in extracytoplasmic proteins, including the components of the Dot/Icm type IV secretion system (Jameson-Lee et al., 2011). Mip is a 25.5 kDa surface peptidylprolyl-isomerase that potentiates infection of macrophages by *Cox. burnetii* (Mo et al., 1995). Com1 was identified in nine spots (spots 38, 39, 41, 43, 45, 47, 48, 50) with the same molecular mass but different pIs, and Mip was detected in four of nine Com1 spots (spots 39, 47, 48, 50), which reveals that the molecular masses of Com1 and Mip are similar and that these proteins are abundant on the cell surface. OmpH is a 16 kDa Omp of *Cox. burnetii* (Papadioti et al., 2011), which serves as a serodiagnostic
Serological characterization of SEPs of Cox. burnetii

Marker for Q fever (Sekeyová et al., 2009) and functions to catalyse protein folding or as a chaperone in the extracytoplasmic compartments of E. coli (Missiakas et al., 1996). P1 is a 29 kDa Omp that forms ion-permeable channels in a planar lipid bilayer membrane and is highly expressed in large-cell variants, and its expression is downregulated in small-cell variants of Cox. burnetii (Varghees et al., 2002). YbgF is a 34 kDa Omp component of the Tol-Pal complex that stabilizes the bacterial outer membrane (Lazzaroni et al., 1999). The hypothetical protein CBU_1902 is identified as a β-subunit of an M16-family peptidase that cleaves nuclear-encoded proteins that are transported from the cytosol to the mitochondria in eukaryotic cells (Ohtsuka et al., 2009). The hypothetical proteins CBU_0370 and CBU_0952 with unknown function were identified in four and three spots, respectively, indicating their abundant distribution over the surface of Cox. burnetii.

The heat-shock proteins (HSPs) HSP70/DnaK, HSP60/GroEL, HSP20 and HSP10/GroES, were identified in 11, 9, 1 and 10 spots, respectively, suggesting that except for HSP20, they are abundant on the surface of Cox. burnetii. GroES and GroEL are present on the surface of Lactobacillus acidophilus (Du & Ho, 2003). GroEL, which resides on the surface of Plesiomonas shigelloides, promotes the attachment of bacteria to cells of the human tumour colon carcinoma cell line Caco-2 (Tsugawa et al., 2007). DnaK and GroEL are recruited to ethanol-stressed membranes of Bacillus subtilis to restore membrane fluidity (Seydlová et al., 2012). Further, the pi values and/or molecular masses of these HSPs in the SEP profile differed from their theoretical values, which may be attributed to post-translational modifications or sulfo-NHS-SS-biotin residues that were not removed during purification. HSP20 (CBU_1169) is similar to a surface protein of Helicobacter pylori and may act as a virulence factor (Du & Ho, 2003).

The ribosomal proteins RplA (L1), RplL (L7/12) and RplI (L9), were identified among SEPs and lack a predicted signal peptide cleavage site. Certain rickettsial ribosomal proteins are recognized as membrane-associated proteins or SEPs of rickettsiae (Ogawa et al., 2007; Pornwiroon et al., 2009; Qi et al., 2013) or other bacteria (Mendum et al., 2009; Spence & Clark, 2000). RplL is associated with bacterial pathogenicity (Spence & Clark, 2000; Stoll et al., 2005). Moreover, the elongation factors Ts and Tu were identified as SEPs of Cox. burnetii in the present study and were found to be more abundant in large-cell, compared with small-cell variants (Seshadri et al., 1999). Ts is expressed on the surface of Streptococcus spp. and is a major immunogen (Cole et al., 2005; Wilkins et al., 2003; Wu et al., 2008). Tu localizes to the surface of Francisella tularensis, and its ability to invade host cells is mediated by its interaction with the surface-associated nucleolin of monocytes (Barel et al., 2008).

SEPs CBU_1519 and CBU_1143 were identified as the preprotein translocase subunits SecB and SecDF (YajC), respectively, that play important roles in transporting secretory proteins through the membrane (Economou, 1998; Randall & Hardy, 2002). SEP CBU_0067 was identified as ZapA, which enhances the lateral associations between FtsZ fibres that assemble into a ring (Z-ring) in

![Fig. 2. Analysis of recombinant surface-exposed proteins on the microarray using sera from mice infected with Cox. burnetii. Thirty rSEPs on the microarray were probed with sera obtained at days 7, 14, 21 and 28 pi from infected and uninfected mice (negative controls), respectively. The fluorescence intensity (FI) of each protein was calculated by averaging the FIs of five replicate spots after subtracting background fluorescence. The FIs were analysed using Cluster 3.0 and TreeView (Wang et al., 2013).](http://mic.sgmjournals.org/2723)
Fig. 3. Major seroreactive recombinant surface-exposed proteins probed with individual mouse sera. Sixteen major seroreactive SEPs were probed with the sera from mice infected with Cox. burnetii (C. bur), R. rickettsii (R. ric), R. heilongjiangensis (R. hei) or R. typhi (R. typ) on day 28 pi. The reaction was considered positive if the average FI value of any rSEP probed with each serum sample was >3 SDs above the average FI value of the same protein probed with normal mouse sera. The distribution of FI values was analysed using GraphPad Prism 5 (Qi et al., 2013). The short solid line represents the average FI value of each group, and the dotted line represents the cut-off point to determine whether the reaction was positive or negative. Symbols (+, *, $ and/or/!)) indicate whether the average FI value was significantly different from that of the other groups (P<0.05). The average FI values of the groups with the same symbols were not significantly different (P>0.05).

Table 2. Average fluorescence intensity and standard deviation of SEPs probed with mouse sera

<table>
<thead>
<tr>
<th>Protein</th>
<th>Average fluorescence intensity ± sd (positive serum no./total serum no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>CBU_0067</td>
<td>109.6 ± 31.8</td>
</tr>
<tr>
<td>CBU_1718</td>
<td>238.4 ± 108.6</td>
</tr>
<tr>
<td>CBU_1594</td>
<td>264.1 ± 142.3</td>
</tr>
<tr>
<td>CBU_0227</td>
<td>173.7 ± 77.7</td>
</tr>
<tr>
<td>CBU_0236</td>
<td>154.1 ± 66.7</td>
</tr>
<tr>
<td>CBU_0510</td>
<td>82.2 ± 27.4</td>
</tr>
<tr>
<td>CBU_0921</td>
<td>105.4 ± 48.1</td>
</tr>
<tr>
<td>CBU_0630</td>
<td>236.2 ± 142.0</td>
</tr>
<tr>
<td>CBU_1078</td>
<td>193.2 ± 106.9</td>
</tr>
<tr>
<td>CBU_1910</td>
<td>240.0 ± 99.9</td>
</tr>
<tr>
<td>CBU_1706</td>
<td>266.3 ± 138.8</td>
</tr>
<tr>
<td>CBU_1719</td>
<td>243.0 ± 89.7</td>
</tr>
<tr>
<td>CBU_1290</td>
<td>84.9 ± 54.1</td>
</tr>
<tr>
<td>CBU_1385</td>
<td>121.1 ± 54.3</td>
</tr>
<tr>
<td>CBU_0952</td>
<td>211.5 ± 58.0</td>
</tr>
<tr>
<td>CBU_2076</td>
<td>103.1 ± 48.6</td>
</tr>
</tbody>
</table>
the induction of expression of genes in vascular endothelial cells by \textit{R. rickettsii} (Clifton et al., 1998). An SSB was identified as an SEP of \textit{R. rickettsii} (Gong et al., 2014). SEP CBU_1320 is the alpha subunit of the integration host factor (IHF). IHF forms heterodimers between IHF$\alpha$ and IHF$\beta$ and plays an important role in bacterial adaptive processes (Stonehouse et al., 2008). \textit{Legionella pneumophila} IHF mutants fail to replicate in \textit{Acanthamoeba castellanii},

\begin{table}[h]
\centering
\caption{Average fluorescence intensities and standard deviations of SEPs probed with human sera}
\begin{tabular}{lcccc}
\hline
Protein & Normal & Q fever & Brucellosis & Mycoplasma pneumonia \\
\hline
CBU_0229 & 255.1 $\pm$ 132.0 & 5370.6 $\pm$ 5327.4 (8/9) & 143.5 $\pm$ 68.9 (0/10) & 639.9 $\pm$ 307.2 (0/10) \\
CBU_1320 & 319.6 $\pm$ 225.7 & 4811.0 $\pm$ 5879.0 (8/9) & 33.4 $\pm$ 28.4 (0/10) & 73.0 $\pm$ 69.2 (0/10) \\
CBU_1719 & 381.8 $\pm$ 349.8 & 2111.6 $\pm$ 1308.9 (7/9) & 48.8 $\pm$ 24.2 (0/10) & 231.3 $\pm$ 128.9 (0/10) \\
CBU_1169 & 111.9 $\pm$ 69.7 & 580.9 $\pm$ 311.2 (8/9) & 2.1 $\pm$ 14.6 (0/10) & 49.8 $\pm$ 47.8 (0/10) \\
CBU_1718 & 1012.7 $\pm$ 1196.8 & 13086.9 $\pm$ 9381.3 (7/9) & 122.4 $\pm$ 9.9 (0/10) & 73.0 $\pm$ 69.2 (0/10) \\
CBU_0337 & 198.1 $\pm$ 131.7 & 2286.0 $\pm$ 3012.0 (7/9) & 25.5 $\pm$ 28.5 (0/10) & 32.2 $\pm$ 14.3 (0/10) \\
CBU_1910 & 960.4 $\pm$ 978.1 & 781.2 $\pm$ 501.2 (5/9) & 18.7 $\pm$ 24.4 (0/10) & 24.4 $\pm$ 24.8 (0/10) \\
CBU_0236 & 250.5 $\pm$ 200.4 & 1111.2 $\pm$ 683.4 (5/9) & 8.9 $\pm$ 23.6 (0/10) & 73.2 $\pm$ 56.3 (0/10) \\
CBU_0630 & 291.2 $\pm$ 169.5 & 9439.1 $\pm$ 1208.4 (6/9) & 204.9 $\pm$ 99.1 (0/10) & 358.4 $\pm$ 243.7 (0/10) \\
CBU_1519 & 188.2 $\pm$ 120.1 & 781.2 $\pm$ 501.2 (5/9) & 18.7 $\pm$ 24.4 (0/10) & 24.4 $\pm$ 24.8 (0/10) \\
CBU_0510 & 169.5 $\pm$ 96.7 & 719.6 $\pm$ 565.5 (5/9) & 8.75 $\pm$ 10.8 (0/10) & 89.2 $\pm$ 97.7 (0/10) \\
CBU_1594 & 229.5 $\pm$ 114.1 & 586.4 $\pm$ 358.4 (3/9) & 24.0 $\pm$ 16.5 (0/10) & 60.7 $\pm$ 35.5 (0/10) \\
\hline
\end{tabular}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Recombinant surface-exposed proteins probed with sera of patients with Q fever. The rSEPs on the microarray were probed with nine individual sera from patients with Q fever or the pooled sera from healthy individuals (negative controls). The FI of each protein was calculated by averaging the FIs of five replicate spots after subtracting background fluorescence. The FIs of the rSEPs on the microarray probed with various mouse sera were analysed using Cluster 3.0 and TreeView (Wang et al., 2013).}
\end{figure}
Serological characterization of SEPs of *Cox. burnetii*
suggesting that IHF is essential for parasitization of amoebas by *L. pneumophila* (Morash *et al.*, 2009).

SEP CBU_0648 was identified as 6,7-dimethyl-8-ribityllumazine synthase (RibH) that catalyses the biosynthesis of riboflavin, which is essential for intracellular survival of *Brucella abortus* (Bonomi *et al.*, 2010). Lumazine synthase from *Brucella* spp. is highly immunogenic and confers partial protection against infection with these bacteria (Esten *et al.*, 2009). SEP CBU_1275 is the starvation sensing protein RspA, which is essential for bacterial survival when the concentration of nutrients is growth-limiting (Huismann & Kolter, 1994).

Certain bacterial cytoplasmic proteins were identified here as SEPs. For example, UDP-glucose 4-epimerase (CapD) and dTDP-4-dehydroamnose reductase (RfbD) contribute to exopolysaccharide synthesis in Gram-negative bacteria (Santhanagopalan *et al.*, 2006). Succinate dehydrogenase iron–sulfur subunit (SdhB) and methylenetetrahydrofolate dehydrogenase or cyclohydrolase (FoI)D are associated with the synthesis of membrane polysaccharides or carbohydrates required for energy metabolism in bacterial membranes, and they were identified as SEPs of *R. heilongjiangensis* (Qi *et al.*, 2013). In *Rickettsiae*, cytosol aminopeptidase resides in the outer membrane fraction of *Anaplasma marginale* (Santhanagopalan *et al.*, 2006), and disulfide oxidoreductase is present on the surface of *Ehrlichia chaffeensis* (McBride *et al.*, 2002). The ATP synthase F1 alpha and beta subunits reside on the cell envelope of *S. aureus* (Gatlin *et al.*, 2006).

Therefore, the cytoplasmic bacterial proteins identified here as SEPs may normally reside on the surface of *Cox. burnetii* or other bacteria and play unexpected roles in bacterium–host interactions. Alternatively, cell lysis may have released small amounts of these highly abundant proteins, which were labelled with biotin. Therefore, their surface location remains to be verified using other techniques such as immunoelectron microscopy. The remaining five SEPs (CBU_0110, CBU_0510, CBU_0921, CBU_1078 and CBU_2076) are hypothetical proteins with unknown functions.

The number of rSEPs that reacted with mouse sera increased with time after infection, indicating that most of the SEPs evoked a humoral immune response. Further, the average FI values of nine rSEPs were significantly higher compared with those of mice infected with *R. rickettsii, R. heilongiangensis* or *R. typhi*. In particular, the average FI value was highest for rSEP Mip.

The average FI values of rSEPs that reacted with sera from patients with Q fever patient sera were significantly higher compared with those of sera from patients with brucellosis or mycoplasma pneumonia. The average FI value was highest for HSP60, which is consistent with the results of a microarray assay conducted by others (Wang *et al.*, 2013) and confirms that HSP60 may serve as a marker of Q fever.

Similar to Q fever, brucellosis is a classical zoonosis and is transmitted from livestock to humans through the consumption of unpasteurized dairy products, contact with diseased animals and carcasses or with products of livestock births and abortions (Esmaeili *et al.*, 2014). An epidemiological investigation shows that brucellosis and Q fever appeared to affect livestock production in Togo, and the seroprevalence of both diseases was higher among the Fulani who have greater ownership or management of livestock and consume higher quantities of dairy products that are not boiled compared with the rest of the population (Dean *et al.*, 2013).

Mycoplasma pneumonia is a common cause of community-acquired pneumonia (CAP) (Arnold *et al.*, 2007). Atypical CAP may be caused by *Cox. burnetii*, and the high seroprevalence of *M. pneumoniae*IgM may occur in outbreaks of acute Q fever that lead to misdiagnosis (Lai *et al.*, 2013). When we analysed 15 rSEPs that reacted with more than half of the sera from patients with Q fever patient sera, they did not react with the 10 sera from patients with brucellosis or mycoplasma pneumonia, suggesting these rSEPs may serve as antigens for the specific serological diagnosis of Q fever. Moreover, many SEPs are protective antigens against rickettsial infection, such as OmpA, OmpB, YbgF, Adr1 and Adr2 for *Rickettsia* spp. (Gong *et al.*, 2014; Qi *et al.*, 2013) as well as Com1, Mip, P1 and HSPB for *Cox. burnetii* (Li *et al.*, 2005; Wei *et al.*, 2011; Xiong *et al.*, 2012a). Therefore, the seroreactive SEPs identified here have potential as subunit vaccine antigens against Q fever.

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Serological characterization of SEPs of *Cox. burnetii*

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