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Proteome analysis of *Rickettsia conorii* by two-dimensional gel electrophoresis coupled with mass spectrometry

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Abstract

The availability of genome sequence offers the opportunity to further expand our knowledge about proteins expressed by *Rickettsia conorii*, strictly intracellular bacterium responsible for Mediterranean spotted fever. Using two-dimensional polyacrylamide gel electrophoresis combined with MALDI-TOF mass spectrometry, we established the first reference map of *R. conorii* proteome. This approach also allowed identification of GroEL as the major antigen recognized by rabbit serum and sera of infected patients. Altogether, this work opens the way to characterize the proteome of *R. conorii*, to compare protein profiles of different isolates or of bacteria maintained under different experimental conditions and to identify immunogenic proteins as potential vaccine targets.
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Keywords: *Rickettsia*; Proteomics; GroEL; ORFan

1. Introduction

Rickettsia conorii is a gram-negative bacterium responsible for Mediterranean spotted fever [1]. This bacterium, transmitted to humans by the bite of infected arthropods, is characterized by its strictly intracellular location which has long prohibited its detailed study. Completion of the 1.2 Mb *R. conorii* genome sequence [2] should accelerate progress in this field. One of the most surprising findings resulting from genomic data analysis was that about one third of the predicted proteins identified in *R. conorii* displays no sequence similarity to known genes from any other organisms. Such species-specific unique sequences, which are not exclusive to *R. conorii* but are observed in each new sequenced genome of rickettsia and of other bacteria [3], have been termed "ORFans" [4]. It has been suggested that ORFans might not correspond to real genes [5–7] a hypothesis currently under discussion [8]. Another interesting point highlighted by the *R. conorii* genome analysis is the presence of repeated palindromic elements, ranging from 106 to 150 nucleotides, found in open reading frames (ORFs) of the genome [9]. In the course of evolution, and in contrast with the general tendency of intracellular bacteria to minimize their genome size, *Rickettsia* has also acquired genomic features such as the retention of multiple copies of genes which could

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be involved in adaption to extreme environmental conditions within the tick (pH, temperature, nutrient limitation) [10]. The major challenge in the post-genomic era will be to determine whether these predicted proteins are expressed. Proteomic approach, which relies on two-dimensional gel electrophoresis (2-D PAGE) coupled with high-throughput matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry, is a powerful tool. Although widely used, it is time-consuming, laborious and has some limitations in dealing with very large or small proteins, proteins having extreme p*I*, and membrane or low-abundance proteins [11]. One way to overcome these limitations is to use two-dimensional liquid chromatography coupled to tandem mass spectrometry [12]. This technique allowed identification of 252 proteins out of 834 predicted protein-coding genes of *R. prowazekii* [13]. Therefore, we believe that comprehensive proteome analysis of microorganisms must integrate both approaches. Quantitative comparative analysis is one of the advantages offered by 2-D PAGE. It can be applied to compare different clinical isolates [14], as well as bacteria maintained under various experimental conditions, providing a comprehensive view of the physiology of growing or starving cell populations [15,16]. Analysis of the protein profiling following incubation with antibacterial agents could also permit the designing of novel antibiotic strategies [17]. Another powerful application is immunoblotting which would permit characterization of antigenic proteins with possible diagnostic applications [18–22].

Rickettsia genome sequencing has already had immediate impact on biomedical research such as the design of oligonucleotide primers suitable for suicide PCR applicable especially to skin biopsy specimens [23]. Genome information, particularly the polymorphisms of genes encoding membrane proteins should also be exploited to provide rapid and accurate diagnostics to identify related pathogens that exhibit strong serological cross-reactions with each other, such as *R. typhi* and *R. prowazekii* [1,24]. In this paper, we report the first 2-D reference map of *R. conorii* proteome. The application of Western immunoblotting for identification of immunogenic antigens is also described.

2. Materials and methods

2.1. Rickettsiae

Rickettsia conorii (strain Seven Malish, ATCC VR-613^{T}) were propagated in Vero cells and purified onto a discontinuous renographin gradient as described [25]. Purified bacteria were washed in PBS (10,000*g*, 10 min) and stored at −80 °C until use. All purification steps were checked by staining the samples by the Gimenez technique [26].

2.2. Crude extract sample preparation for 2-D gel electrophoresis

The *R. conorii* pellet was resuspended in 5 ml of Milli-Q water, and cells were lysed by two passes through a Constant Cell Disruption System (2 kBars, Constant Systems Ltd., Daventry, UK). After centrifugation at 5600*g* for 30 min (4 °C) to eliminate cells debris, the supernatant was collected and lyophilized. The proteins extract (crude extract) was quantified using Biorad DC assay (Bio-Rad) and resuspended in Milli-Q water. Proteins were then precipitated by using PlusOne 2-D Clean-Up Kit (Amersham) and directly resuspended in rehydration solution [7 M urea, 2 M thiourea, 4% (w/v) Chaps, 40 mM DTT, 0.5% (v/v) IPG buffer (Amersham Biosciences)]. Samples were stored at −80 °C until they were subjected to isoelectric focusing (IEF).

2.3. Membrane-enriched sample preparation for 2-D gel electrophoresis

The *R. conorii* pellet was resuspended in 5 ml of 5 mM Tris–HCl buffer (pH 7.6) and lysed by two passages through a Constant Cell Disruption System (2 kBars). Cell debris and unbroken cells were discarded following a centrifugation at 5600*g* for 20 min. The supernatant was transferred into a new tube and ultracentrifuged (100,000*g*; 2 h; Beckman MLS-50 rotor). After washing in 5 mM Tris–HCl, pH 7.6, membrane-enriched pellet was resuspended in rehydration solution and stored at −80 °C. The protein concentration was determined as described above.

2.4. 2-D electrophoresis and silver staining

Immobiline™ DryStrips (18 cm, pH 4–7 or pH 3–11 NL, Amersham) were rehydrated overnight with various amounts (30–300 μg) of proteins from either crude extract or membrane-enriched fraction diluted in rehydration solution supplemented with 2% (v/v) appropriate IPG buffer (Amersham Biosciences). IEF was carried out according to the manufacturer's protocol (Multiphor II, Pharmacia). Prior to electrophoresis in the second dimension, strips were equilibrated for 15 min in equilibration buffer [30% (v/v) glycerol, 2% (w/v) SDS, 6 M urea, 50 mM Tris–HCl, bromophenol blue, pH 8.8] containing 65 mM DTT. This step was repeated using of equilibration buffer supplemented with 100 mM iodoacetamide. The strips were then embedded in 0.5% agarose and the proteins resolved by electrophoresis through 10% SDS–PAGE (Ettan™ DALT, Amersham) at 5 W/gel for 30 min, followed by 17 W/gel for 4–5 h. For protein identification, following electrophoresis, the gels were silver-stained [27] and digitized by transmission scanning (ImageScanner, Amersham).

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Spots excised from the gel were stored at −20 °C until identification by MALDI-TOF mass spectrometry.

2.5. Western-blot assays

Rickettsial proteins resolved by 2-D gel electrophoresis were transferred onto nitrocellulose membranes (Semi-Phor unit, Hoefer Scientific, San Francisco, CA). Membranes were then blocked in PBS supplemented with 0.2% Tween-20 and 5% non-fat dried milk (PBS-Tween-Milk) for 1.5 h before incubation with serum of a rabbit infected with *R. conorii* and diluted in PBS-Tween-Milk (1:6400). Alternatively, membranes were probed with sera of infected patients sent to the Unité des Rickettsies for diagnosis. Following 1 h incubation, membranes were washed 3 times in PBS-Tween and probed with horseradish peroxidase-conjugated goat anti-rabbit (Amersham, 1:1000) or anti-human (Southern Biotechnology, 1:1000) secondary antibody. Detection was done by chemiluminescence (ECL, Amersham).

2.6. In-gel digestion and MALDI-TOF mass spectrometry

Protein spots excised from silver-stained gels were destained and subjected to in-gel digestion with trypsin (Sequencing grade modified porcine trypsin; Promega, Madison, WI). Tryptic peptides were then extracted from the gel by successive treatment with 5% formic acid and 50% acetonitrile/5% formic acid. Extracts were pooled and dried in a Speedvac evaporator. Peptides resuspended in an α-cyano-4-hydroxycinnamic acid matrix solution (prepared by diluting 3 times a saturated solution in 50% acetonitrile/0.3% TFA), were then spotted on the metal target. Mass analyses were performed with a MALDI-TOF Bruker Ultraflex spectrometer (Bruker Daltonics, Wissembourg, France). Mass spectra were internally calibrated using autolytic peptides from trypsin.

2.7. Database searching and data interpretation

The peptide mass lists were used to identify the protein using Mascot software available on site.

3. Results

3.1. Determination of optimal conditions for 2-D PAGE of R. conorii crude extracts

To extract *R. conorii* proteins, several procedures such as ultrasonication and disruption in a French press cell were tested. In our hands, the best results were obtained by explosive nitrogen decompression, a new method avoiding heat damage usually generated by other disruptive techniques. An important step that greatly improved the quality of the protein profiles was the sample treatment with the PlusOne 2-D Clean-Up Kit (Amersham), a process allowing removal of non-proteic contaminants interfering with the IEF. As illustrated in Fig. 1, within a p*I* range from 4 to 7, good resolution of proteins was obtained with 200 μg of proteins from the crude extract of purified rickettsiae. Under these conditions, gels were found to be highly reproducible. The resulting pattern within a p*I* range from 4 to 7 consists of 651 well-resolved protein spots of varying intensities with molecular mass ranging from 20 to100 kDa (Fig. 1).

3.2. Identification of rickettsial proteins by MALDI-TOF mass spectrometry

The most intense protein spots detected after silver-staining were excised from the gel for analysis by MALDI-TOF mass spectrometry. Twenty-nine proteins were identified with sequence coverages ranging from 13% to 53% (Table 1). For two thirds of assigned proteins, the identification was based on at least two and mostly three independent sets of data mapping to the same ORF. In all cases, observed p*I* and molecular mass values were in agreement with those expected deduced from *in silico* analysis. Several spots resulted in the same protein identification, suggesting the presence of numerous isoforms.

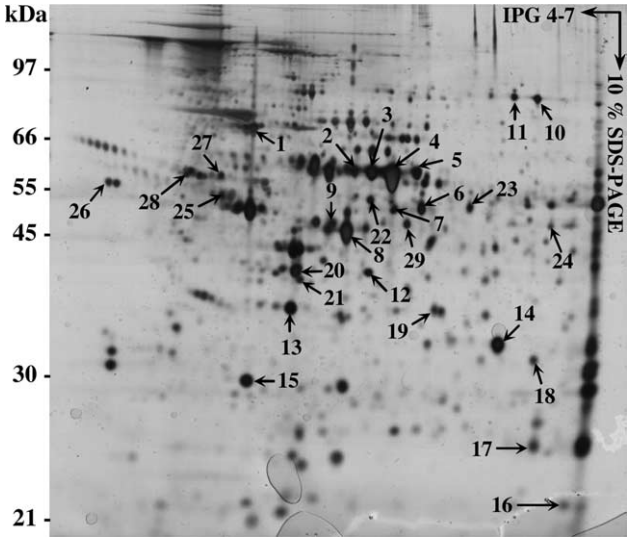

Fig. 1. 2-D PAGE reference map of *R. conorii* proteome. The crude extract of *R. conorii* was separated using a pH 4–7 IPG strip in the first dimension, followed by a 10% SDS–PAGE in the second dimension. The 2-D gel was silver-stained. Protein spots successfully identified in Table 1 are numbered. Relative molecular masses (in kDa) are indicated on the left side.

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Table 1
Identification of rickettsial proteins resolved by 2-D electrophoresis

Spot number		Gene	Protein description	Score	*E* value	Coverage (%)	In silico data		2-D PAGE data	
							MW (kDa)	p*I*	MW (kDa)	p*I*
1	RC0233	*dnaK**	DnaK-Hsp-70 protein	195	1.5e − 014	40	67.98	5.02	70.92	5.14
2	RC0968	*groEL**	groEL; 60 kD chaperonin	73	0.022	18	58.67	5.62	59.07	5.4
3	RC0968	*groEL**	groEL; 60 kD chaperonin	82	0.0028	17	58.67	5.62	59.06	5.80
4	RC0968	*groEL**	groEL; 60 kD chaperonin	113	2.4e − 0006	30	58.67	5.62	51.73	5.91
5	RC0968	*groEL**	groEL; 60 kD chaperonin	75	0.014	23	58.67	5.62	58.74	5.97
6	RC0184	*pepA**	Aminopeptidase A	134	1.9e − 008	30	54.22	5.69	55.28	6.07
7	RC0184	*pepA*	Aminopeptidase A	170	4.7e − 012	38	54.22	5.69	54.76	5.93
8	RC1008	*tuf**	Elongation factor EF-tu	121	3.7e − 007	44	42.95	5.42	46.70	5.67
9	RC1008	*tuf*	Elongation factor EF-tu	116	1.2e − 006	43	42.95	5.42	50.31	5.57
10	RC0663	*pnp*	Polyribonucleotide nucleotidyltransferase	121	3.7e − 007	22	82.35	6.15	82.10	6.68
11	RC0663	*pnp*	Polyribonucleotide nucleotidyltransferase	73	0.024	13	82.35	6.15	82.10	6.55
12	RC0599	*sucC*	Succinate-CoA ligase (ADP forming)	68	0.068	25	42.02	5.51	42.98	5.80
13	RC0113	*tsf*	Elongation factor EF-Ts	118	7.5e − 007	41	33.78	5.21	38.47	5.38
14	RC0520	*mdh*	Malate dehydrogenase	122	2.4e − 007	51	33.67	6.01	35.61	6.52
15	RC0497	*ampD*	AmpD Negative regulator of β-lactamase expression	66	0.12	21	29.63	5.11	31.28	5.15
16	RC1295	*ssb*	Single-strand binding protein	82	0.0032	53	17.42	6.07	22.72	6.92
17	RC0778	*sodB*	Superoxide dismutase	90	0.0005	44	24.8	6.25	26.81	6.74
18	RC0259	*lmp*	TRAP-type uncharacterized transport system	114	1.9e − 006	44	37.54	6.79	34.15	6.72
19	RC0347	*pdhA*	Pyruvate dehydrogenase pdhA	60	0.44	22	36.99	5.76	38.88	6.16
20	RC0982	*rpoA*	Hypothetical protein rpoA	68	0.078	21	38.51	5.16	42.80	5.40
21	RC1198	*mreB*	Rod shape-determining protein mreB	81	0.034	26	37.5	5.29	41.74	5.42
22	RC0193	*gatB*	Glutamyl-tRNA amidotransferase subunit B	95	0.00016	29	54.38	5.49	56.58	5.78
23	RC0194	*gatA*	Glutamyl-tRNA amidotransferase subunit A	129	5.9e − 008	34	53.34	5.97	55.85	6.34
24	RC0760	RC0760	Unknown	124	1.9e − 007	37	51.03	6.24	53.24	6.77
25	RC1235	*atpD**	ATP synthase β chain	186	1.2e − 013	47	54.49	4.93	55.52	4.94
26	RC1235	*atpD*	ATP synthase β chain	73	0.023	21	54.49	4.93	55.02	4.58
27	RC1015	*ftsZ*	Cell division protein ftsZ	56	1.1	20	48.48	4.86	57.84	4.93
28	RC0166	*htrA*	Periplasmic serine proteinase	164	1.9e − 011	40	55.98	4.72	57.80	4.74
29	RC1216	*serS*	Serine-tRNA ligase	49	6.2	13	48.66	5.62	47.10	6

Protein spots were excised from the 2-D gel shown in Fig. 1 and analyzed by MALDI-TOF mass spectrometry. The resulting mass mappings were searched against Swiss-Prot/TrEMBL or against NCBI databases using Mascott software. All theoretical molecular weight (MW) and isoelectric points (p*I*) values were calculated using the ExPASy Compute MW/p*I* tool online. Experimental molecular masses and p*I* values were estimated using the software ImageMaster™ 2D Platinium Version: 5.0.0.0 software (Amersham). The symbol * indicates that a similar identification was obtained from the 2-D gel (p*I* 3–10) performed with membrane-enriched fraction.

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3.3. 2-D PAGE of R. conorii membrane-enriched proteins and identification of reactive antigens

In order to characterize antigenic proteins, membrane-enriched rickettsial extracts were prepared as detailed in Section 2 and compared to that obtained following the mechanical agitation procedure previously described by Dasch [28]. As illustrated in Fig. 2, silver-stained SDS–PAGE (a) and antigenic recognition profiles obtained with serum of immunized rabbit (b) were similar. Fig. 3(a) shows the membrane-enriched proteome profile obtained by 2-D PAGE, the first dimension being obtained using pH 3–11 NL strips. Anti-*R. conorii* rabbit serum tested in SDS–PAGE was used to probe the resolved rickettsial membrane proteins (Fig. 3(b)). We also investigated reactivity of sera from infected patients (Fig. 3(c) and (d)). In all cases, one species of proteins at 60 kDa was recognized. This recognition is specific since neither pre-immune rabbit serum nor serum from healthy blood donors were positive (not shown). From the corresponding silver-stained gel, the immunodetected protein was identified as heat-shock protein (Hsp-60/GroEL) using MALDI-TOF mass spectrometry. Other identified spots excised from this gel were PepA, EF-Tu, AtpD, DnaK and RC0627. With the exception of RC0627 which presents a basic p*I* (Fig. 4), all these proteins were previously identified and positionned on p*I* 4–7 2-D gel performed using crude exctracts of *R. conorii*.

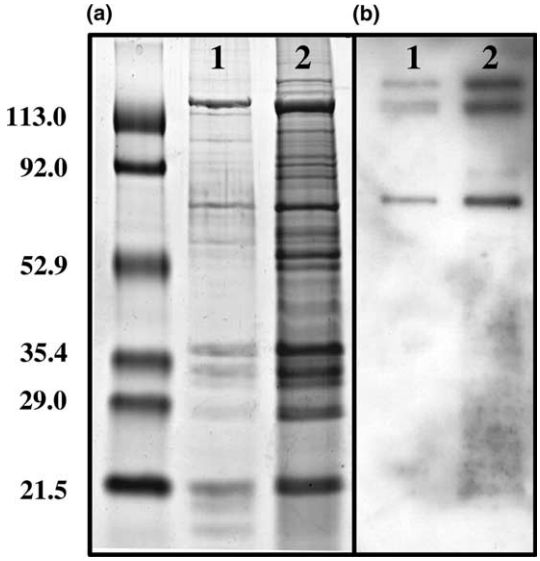


Fig. 2. Western-blot recognition of *R. conorii* membrane antigens by serum from immmunized rabbit. Membrane extracts were separated by SDS–PAGE and stained with Coomassie blue (a) or transferred onto nitrocellulose membranes before Western-blot analysis with rabbit serum (1:6400, b). Lane 1, membranes prepared by mechanical agitation; lane 2 membranes obtained following pressure disruption. Molecular mass standards (MW) are indicated on the left.

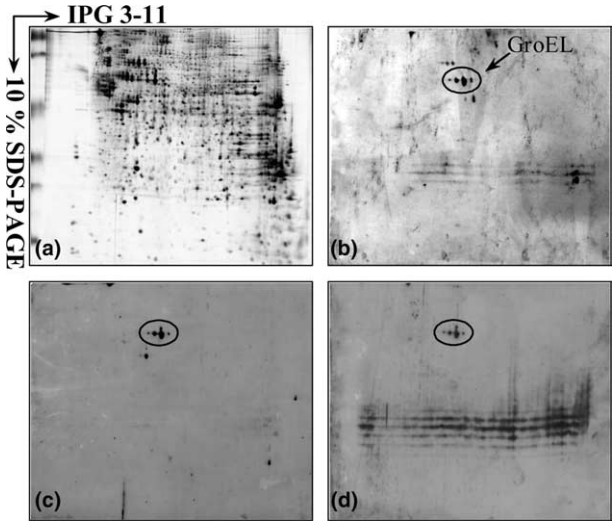
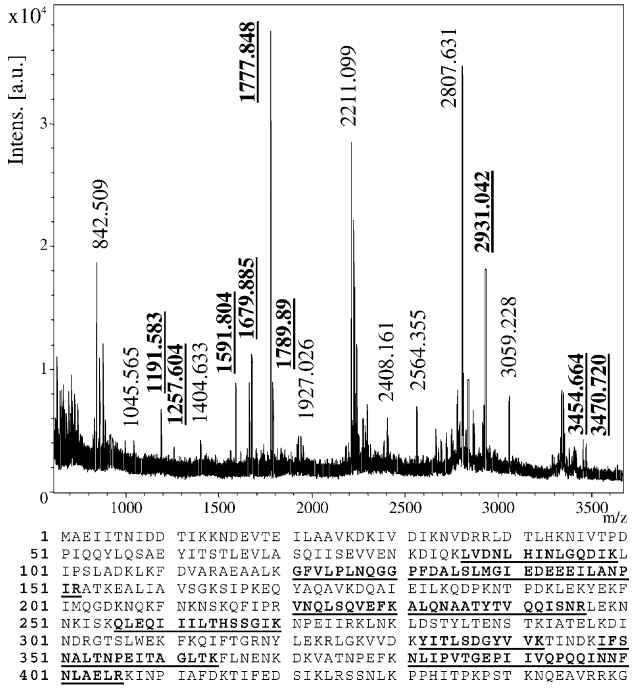


Fig. 3. 2-D PAGE and Western-blotting of rickettsial membrane proteins. Membranes proteins of *R. conorii* were separated using a pH 3–11 NL IPG strip in the first dimension followed by a 10% SDS–PAGE in the second dimension. The 2-D gel was either visualized by silver staining (a) or transferred to nitrocellulose and probed with serum from immunized rabbit (b) or from *R. conorii*-infected patients (c and d).



Score: 97 Sequence coverage: 31%

Fig. 4. MALDI-TOF mass spectrometry spectra of peptides matching with the ORFan RC0627. Spectra corresponding to tryptic peptides resulting from in-gel digestion of the spot (MW 55.83 kDa, p*I* 8.97) excised from p*I* 3–11 gel. Mass and sequences of tryptic peptides mapping to the putatively encoded-protein sequence are underlined. This result is representative for another experiment.

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4. Discussion

In this paper, we present the first reference proteome map of *R. conorii*. One of the most important technical challenges derived from the strictly intracellular nature of these bacteria. Spots excised from 2-D gels were identified by MALDI-TOF mass spectrometry mapping of corresponding tryptic peptide digests. Obtained data indicated that our samples were mostly devoid of eucaryotic proteins, apart from actin and vimentin. Both of these are major constituents of the complex network of filamentous proteins that maintains the cytoskeleton architecture of eukaryotic cells [29]. They are probably closely associated with the bacteria, and consequently difficult to remove during purification. This is particularly true concerning actin which can be polymerized by *R. conorii* through a mechanism that relies on entry of these microorganisms into non-phagocytic cells [30,31]. Identified rickettsial proteins belong to several functional categories, including replication (Ssb), transcription (RpoA and PnP) and translation (SerS, GatA, GatB, EF-Ts, EF-Tu, HtrA, and PepA). A large proportion of the identified proteins are involved in energy metabolism. Two key enzymes of the TCA cycle, which have been shown to be complete in *Rickettsia* (KEGG, MAP00020), were found, namely malate dehydrogenase (Mdh) and succinyl-CoA synthetase (SucC). Pyruvate dehydrogenase (PdhA) was also found. This identification, coupled with the fact that metabolic pathways necessary for pyruvate synthesis are lacking in rickettsiae, confirms exogenous uptake of this major glycolysis product [32]. Another protein expressed at high levels is the β chain of ATP synthase (AtpD). This finding illustrates the importance of oxidative phosphorylation required for ATP synthesis, necessary to compensate for glycolysis defect. Other proteins classified in cellular processes such as cell division (MreB, FtsZ) or heat shock-induced proteins (Hsp) (Hsp-70/DnaK; Hsp-60/GroEL), were also identified. Hsp are essential components ubiquitously distributed, and ensure that genome encoded proteins are expressed as functional molecules [33,34]. The role of these evolutionarily conserved proteins is particularly important in adverse conditions, as they prevent misfolding and protein aggregation [35]. *R. conorii* is transmitted to human by ticks, and its survival requires adaptation to different environments, including physiological and nutrient changes between hosts. The relative high intensity of Hsp-60 spots in the *R. conorii* proteome is likely to be correlated with the requirement of an elaborated machinery to resist to lethal conditions, a liable to be encountered by these bacteria. Proteins implicated in drug and analogue sensitivity were also detected (AmpD, SodB). The high abundance of superoxide dismutase, which confers anti-active oxygen sensitivity, is not surprising in view of the intracellular proliferation of these bacteria. Finally, this analysis led to the identification of one candidate with unknown function (RC0760).

Identification and characterization of cell-surface proteins expressed by *Rickettsia* is of great interest as immunoreactive antigens are putative candidates for diagnosis or vaccination as well as targets for therapy [36]. One-dimensional immunoblots are routinely used in diagnosis of Mediterranean Spotted Fever [37]. The two major immunodominant surface proteins of *R. conorii* are rOmpA (116 kDa) and rOmpB (120 kDa) [38–40]. Recognition of another non-assigned 60 kDa protein by sera from patients suffering from a malignant form of Mediterranean Spotted Fever has also been reported [37]. As demonstrated by immunoblots with a serum of immunized rabbit, all these candidate antigens are present in our *R. conorii* membrane-enriched extracts. Membrane proteins tend to be basic [11] thus pH 3–11 strips were used for IEF of these samples. Only a few sets of proteins were excised from resulting 2-D gels and analysed by MALDI-TOF mass spectrometry. Excepted for RC0627 discussed above, all assigned spots correspond to proteins identified from cude extracts and described as being associated with the outer membrane of bacteria, such as GroEL [41], DnaK [42], and PepA [43]. Transfer of EF-Tu from the cytoplasm to the periplasm has also been described [44]. From these 2-D PAGE, we characterized *R. conorii* immunoproteome. In contrast to what was observed in one-dimensional gels, rOmpA and rOmpB were not recognized. The size and hydrophobicity of these large membrane proteins most probably hampered their separation [45]. In fact, the most prominent spots reacting with the rabbit serum as well as with sera of infected patients corresponded to the 60 kDa protein identified as GroEL. Multiple isoforms that are immunoreactives can be visualized on the gel. Antigenic properties of GroEL homologs were described for several microbial pathogens [46–49], these proteins being usually recognized by the immune system of infected hosts. Detection and identification of putative GroEL homologs from four rickettsia species (*R. akarii*, *R. australis*, *R. bellii* and *R. rickettsii*) has been previously investigated using polyclonal antibodies raised against recombinant *Escherichia coli* proteins [50]. Genome sequence availability recently allowed comparison of the amino acid sequence of GroEL from *R. typhi* with those from *R. prowazekii* and *R. conorii* and to provide evidence of strong similarities [51]. Despite the high degree of amino acid conservation, this protein was shown as a promising candidate vaccine against several microbial infections, namely for *Mycobacterium tuberculosis* [47] and *Helicobacter pylori* [48,49]. Consequently, possible protection of rickettsiosis by GroEL merits further investigation.

Another interesting feature resulting from the analysis of rickettsial membrane proteins is the detection of an ORFan-encoded protein, namely RC0627, that is

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exclusive for *Rickettsia* genus. Several explanations such as low database enrichment, annotation artifacts or sequencing errors leading to frame shifts have been evoked to explain the existence of genome-specific ORFs [52]. It has been established that ORFans were, on average, shorter than non-ORFans [4,53]. This observation was extended to *R. conorii* genome with the conclusion that some ORFans corresponded to pseudo-genes or decaying genes [7]. Identification of the 50 kDa protein encoded by ORFan gene RC0627 is in contradiction with this interpretation. While RC0627 function remains to be characterized, this finding demonstrates that ORFans can be expressed. A recent structural ORFans analysis published by Siew and Fisher [8] has demonstrated that 3-D structures of many of them clearly have previously observed folds. This is an agreement with the hypothesis that membrane proteins may evolve to conserve structure rather than sequence [54] thus impairing classical homology search tools. A clear example has been recently reported for a viral capsid, for which structure analysis allowed identification of a level of conservation that was not found by genetic sequence analysis [55]. Expression and structural analysis of RC0627-encoded protein would provide additional information about its putative functional activity.

In summary, this approach constitutes a valuable tool for structural and functional proteome study of *Rickettsia* and should be useful to evaluate differences in expressed proteome content within different physiological states or among different strains. This method would be also helpful to characterize antigenic proteins for further improvement in the specificity of serological tests. Finally, the proteomic approach could shed light on the functions and origins of ORFans, thereby serving to elucidate the genetic diversity of sequenced genomes.

Acknowledgments

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