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Global analysis of the *Brucella melitensis* proteome: Identification of proteins expressed in laboratory-grown culture

Brucella melitensis is a facultative intracellular bacterial pathogen that causes brucellosis, a zoonotic disease primarily infecting sheep and goats, characterized by undulant fever, arthritic pain and other neurological disorders in humans. A comprehensive proteomic study of strain 16M was conducted to identify and characterize the proteins expressed in laboratory-grown culture. Using overlapping narrow range immobilized pH gradient strips for two-dimensional gel electrophoresis, 883 protein spots were detected between pH 3.5 and 11. The average isoelectric point and molecular weight values of the detected spots were 5.22 and 46.5 kDa, respectively. Of the 883 observed protein spots, 440 have been identified by matrix-assisted laser desorption/ionization-mass spectrometry. These proteins represent 187 discrete open reading frames (ORFs) or 6% of the predicted 3197 ORFs contained in the genome. The corresponding ORFs of the identified proteins are distributed evenly between each of the two circular *B. melitensis* chromosomes, indicating that both replicons are functionally active. The presented proteome map lists those protein spots identified to date in this study. This map may serve as a baseline reference for future proteomic studies aimed at the definition of biochemical pathways associated with stress responses, host specificity, pathogenicity and virulence. It will also assist in characterization of global proteomic effects in gene-knockout mutants. Ultimately, it may aid in our overall understanding of the cell biology of *B. melitensis*, an important bacterial pathogen.

Keywords: *Brucella melitensis* / Brucellosis / Mass spectrometry / Protein identification

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1 Introduction

Brucella melitensis is a pathogenic, facultative intracellular, Gram negative, aerobic, nonmotile, coccobacillus that causes brucellosis, a chronic infectious disease in humans characterized by undulant fever, arthritic pain and other neurological disorders [1]. Brucellosis frequently causes abortion and sterility in domestic and wild animals [2]. Because they are sequestered inside macrophages where they replicate and evade the immune system, *brucellae* can live inside their host indefinitely causing chronic infection [3]. *Brucellae* belong to the family *Rhizobiaceae* of the α -2 subgroup of the class Proteobacteria [4, 5]. Based on pathogenicity and host preference, six species were identified within the genus: *B. abortus*, *B. canis*, *B. melitensis*, *B. neotomae*, *B. ovis* and *B. suis* [6].

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Abbreviations: CFR, curved field reflectron; O-MU, O-methylisourea

The genome of *B. melitensis* strain 16M (Biovar 1, ATCC 23456) has been sequenced, closed and annotated. The genome is composed of 3.29 Mb predicted to encode for 2920 ORFs, distributed over two circular chromosomes of 2.11 Mb and 1.18 Mb [7]. More recent annotation of the genome placed the predicted number of ORFs at 3197 [8]. The availability of this genomic data has provided a core of information essential in performing a comprehensive proteomic study of this organism. Thus, identification of the ORFs expressed by strain 16M under laboratory conditions is aimed at generating a baseline signature or cartographic reference of the *B. melitensis* proteome.

Traditionally, Western blotting and Edman degradation have been used in the identification of proteins on 2-D gels of extracts from various *Brucella* species [9–13]. Using these techniques, a combined total of 79 *Brucella* proteins were identified from all previous studies. These studies were limited because of the inability to perform large-scale and high throughput data analysis. Further, the use of only broad pH ranges greatly limited protein resolution and identification since several proteins with similar p/s and molecular masses tend to overlap and appear as one spot on 2-D gels. Thus, we have combined IPG strips-based 2-D PAGE for increased resolution and

MALDI-MS for the rapid and large scale identification of *B. melitensis* proteins. Several IPG strips of overlapping, narrow pH range were used for IEF to maximize protein resolution. In addition, protein spots were stained with recently introduced SYPRO® Ruby which has a far superior linear range of sensitivity than either silver or Coomassie blue stains and whose chemistry is compatible with MALDI-MS [14].

Herein, we present a global overview of the protein complement of the *B. melitensis* genome. The identification and characterization of all the proteins expressed under laboratory conditions are valuable in generating a reference map for future studies aimed at elucidating biochemical pathways affected in *Brucella* during *in vivo* infection, under conditions of acid or oxidative stress, as well as those associated with pathogenicity, virulence and host specificity. Likewise, these data will be important for future studies involving human vaccine development, diagnostic procedures, and design of novel antimicrobial and antibacterial drugs. The information presented here will also lead to a better understanding of the overall cell biology of this important bacterial pathogen.

2 Materials and methods

2.1 Materials

The following were purchased from Genomic Solutions (Ann Arbor, MI, USA): sample buffer 1 (0.3% SDS, 0.2 M DTT, 50 mM Tris-HCl, pH 8.0); sample buffer 2 (50 mM MgCl₂, 8 U Dnase I, 3 U Rnase A, 0.5 M Tris-HCl, pH 8.0); loading buffer (8 M urea, 4% CHAPS, 40 mM Tris base, 65 mM DTT, 0.01% bromophenol blue); rehydration buffer (8 M urea, 2% CHAPS, 10 mM DTT, 2% carrier ampholytes, 0.01% bromophenol blue); equilibration buffer 1 (6 M urea, 133 mM DTT, 30% glycerol, 50 mM Tris-acetate, pH 7.0); equilibration buffer 2 (6 M urea, 2.5% iodoacetamide, 30% glycerol, 50 mM Tris-acetate, pH 7.0); non-conductive oil, precast 10% homogenous Duracryl™ gels and trypsin digestion kits. SYPRO® Ruby fluorescent stain was purchased from Molecular Probes (Eugene, OR, USA). IPG strips were from Amersham Pharmacia Biotech (Uppsala, Sweden). Ammonium bicarbonate and formic acid were obtained from Sigma (St. Louis, MO, USA). ZipTips with C₁₈ media were manufactured by Millipore (Bedford, MA, USA). Iodoacetamide and O-methylisourea (O-MU) were from Acros (a division of Fisher, Pittsburgh, PA, USA). Dithiothreitol and acetonitrile were from Fisher. α -Cyanohydroxycinnamic acid was purchased from Aldrich (Milwaukee, WI, USA). Sequencing grade modified porcine trypsin was manufactured by Promega (Madison, WI, USA). All nonconsumable equipment required for 2-DE, *i.e.* gel boxes, power supplies, robotic

systems, *etc.*, were purchased from Genomic Solutions. The Axima CFR (curved field reflectron) [15] mass spectrometer was purchased from Kratos Analytical (Manchester, UK).

2.2 Bacterial cultures

Brucella melitensis strains 16M and Rev 1 were grown on Schaedler blood agar in petri dishes under biosafety level 3 conditions. After 3 d at 37°C, the cells were collected by scraping and suspended in PBS buffer, pH 7.2. An equal volume of chloroform was then added to kill the cells. The centrifuged pellet of bacterial cells was resuspended in PBS and stored at -20°C until use.

2.3 Total protein determination

To quantify the amount of protein, 10 μ L of cell suspension was centrifuged at 10 000 rpm for 3 min at 4°C. The pellet was resuspended in 40 μ L of sample buffer 1 plus 4 μ L of sample buffer 2, and incubated at 4°C for 10 min. Then, 5 μ L of this solution was used to quantify the proteins according to Bradford [16], using the Bio-Rad (Hercules, CA, USA) protein stain with BSA as a standard. All samples, including the BSA standards, contained 10 μ L of a 10:1 mixture v/v of sample buffers 1 and 2.

2.4 Protein extraction

Protein extraction was performed using a modified protocol of Rafie-Kolpin *et al.* [13]. All operations were performed on ice with ice-cold reagents, and all centrifugations were for 3 min, 10 000 rpm, at 4°C. Chloroform-killed cells (40 μ L containing 40 μ g protein) were mixed with an equal volume of 10% TCA, incubated for 5 min and then centrifuged. The pellet was then resuspended in 40 μ L of 5% TCA, incubated for 5 min and centrifuged. Once more, the pellet was washed with 40 μ L of acetone, centrifuged, and resuspended in 40 μ L of sample buffer 1 and 4 μ L of sample buffer 2. The mixture was incubated for 10 min, after which 160 μ L of loading buffer and 200 μ L of rehydration buffer were added.

2.5 Isoelectric focusing

IPG strips of linear pH gradients (3.5–4.5, 4–5, 4.5–5.5, 5–6, 5.6–6.7, 6–11, 4–7, 3–10) were placed gel-side down in the pHaser™ tray, with the acidic end toward the anode. The 18 cm strips were rehydrated overnight at ambient temperature with all 400 μ L of prepared sample, containing 40 μ g total protein. To prevent dehydration, strips were overlaid with 2 mL of nonconductive oil. Moist

cotton wicks were added to each end of the strips, ensuring thorough contact between the gel and the electrodes. IEF was performed at 20°C. Within 24 h, a total of 80 000 Vh was delivered to the gels using the Investigator™ power supply (maximum of 5000 V, maximum current of 80 µA/gel, end of run hold at 125 V).

2.6 SDS-PAGE

After IEF, each IPG strip was washed first for 15 min in 10 mL of equilibration buffer 1, and then for 15 min in 10 mL of equilibration buffer 2. The IPG strips were loaded onto precast 10% homogeneous Duracryl™ gels (22 cm × 23 cm × 1 mm; Tris/Tricine/SDS chemistry). Electrophoresis was performed at 4°C for 18–19 h at 500 V and 1600 mW/gel, or for 5–6 h at 500 V and 14 000–20 000 mW/gel. All gels were stained with SYPRO® Ruby for 12–15 h in the dark using an automated stainer. Briefly, gels were first fixed for 30 min in a solution of 40% methanol with 10% acetic acid and then rinsed for 5 min with deionized water before adding SYPRO® Ruby. After staining, the gels were submerged in deionized water for 5 min and destained for 30 min with a solution of 10% methanol and 6% acetic acid. The gels were rinsed with deionized water and stored in 2% glycerol in the dark at 4°C. Gel images were captured under 470 nm light with a Fujifilm LAS-1000Plus imager. Protein spots (1.2 mm od) were excised from the SYPRO Ruby stained 2-D gels using a UV box equipped ProPic™ robot. Plugs were frozen at –80°C and thawed prior to trypsin digestion.

2.7 Gel analysis

The experimental *pI* and *M_r* values of each protein were estimated using the Investigator HT Analyzer program developed by Nonlinear Dynamics (Newcastle upon Tyne, UK). Each sample was run in triplicate for each pH range. An average gel was generated using all these gels. Only those spots present in at least two of the three gels were included in the average gel.

2.8 In-gel trypsin digestion with O-methylisourea modification

Digestion with trypsin was performed according to the ProGest™ Digestion Station default long trypsin digestion protocol as described by the manufacturer, but was altered to include modification of the tryptic peptides with O-methylisourea (O-MU). Throughout the procedure, gel plugs were shrunk with ACN and rehydrated with each subsequent reagent solution, as described [17]. The pH of

all solutions was adjusted to pH 7.9, unless otherwise stated. Reagents were from the trypsin digestion kit, with exception of those required for modification with O-MU.

Essentially, proteins were reduced with 10 mM DTT in 25 mM ammonium bicarbonate (NH₄HCO₃) for 10 min at 65°C before alkylation at ambient temperature with 100 mM iodoacetamide in 50 mM NH₄HCO₃. A 7.5 h digestion with 15 µL of trypsin (16.7 ng/µL stock solution; 250 ng/sample) was performed at 37°C. Tryptic peptides were modified for 1 h at 37°C with 1 M O-MU in 100 mM NH₄HCO₃ at pH 10.0, as described [18]. Modified tryptic peptides were recovered in a mixture of 10% formic acid and ACN.

2.9 Preparation of tryptic peptides for MALDI analysis

Tryptic peptides generated using the ProGest™ were dried under vacuum and resuspended in 100 µL of 10% formic acid. Sample cleanup was performed using the ProMS™ Sample Preparation Station, following the acetonitrile elution protocol as described by the manufacturer. ZipTips (C₁₈ media) conditioned with a 1:1 mixture of ACN and water were used to remove salts from the peptide mixtures. Desalted peptides were concentrated as they were eluted from the ZipTips in a top-to-bottom manner with 3 µL of matrix (10 mg α-cyanohydroxycinnamic acid per 1 mL of 50% ACN, 0.1% TFA, in water). Finally, 1 µL of the peptide/matrix mixture was spotted to the stainless steel 384-well Kratos MALDI target plate.

2.10 Mass spectral analysis

All spectra were obtained using the Kratos Axima CFR, running in reflectron mode. The most abundant trypsin autolysis peaks at 842.509 and 2254.12 Da (the 2212.11 peak, modified by O-MU) were used as internal calibrants. Spectra, each an average of 200 profiles, were collected in automated mode using the Kompact software package. Spectra were collected manually from those samples which were not amenable to automated collection (*vide infra*). Monoisotopic peaks were manually selected. Peptide mass fingerprints were searched in-house against all 3197 ORFs of the *B. melitensis* 16M genome using the Mascot and Mascot Daemon software packages from Matrix Science [19]. The search parameters were: maximum of one missed cleavage by trypsin; fixed modifications of oxidized methionine and carbamidomethylated cysteine; variable modification of acetylated lysine (this modification is of the same mass as guanidination, 42.02 Da); charge state of +1; and mass tolerance of ± 0.2 Da.

3 Results

Total cellular proteins extracted from *B. melitensis* 16M grown under laboratory conditions were separated on 2-D gels of various pH ranges. The second dimension separation on 10% Duracryl™ gels allowed observation of proteins between 6.5 and 205 kDa, with optimal resolution between 29 and 116 kDa. Images of three SYPRO® Ruby stained 2-D gels were computer analyzed to create an average gel for each pH range.

Using a broad range IPG strip of pH 3–10, about 500 protein spots were detected (Fig. 1). To increase the number of observed proteins, an assortment of overlapping IPG strips with narrow, linear pH gradients were used to separate proteins according to charge in the first dimen-

sion. This approach effectively increased resolution, as now 1162 spots were detected from pH 4 to 11 on the average gels. However, this number drops to 883 when each spot is counted only once. The results are shown in Fig. 2A–E and summarized in Table 1. In addition, there were approximately 35 spots resolved on 2-D gels generated using pH 3.5–4.5 (data not shown). The average mass of all detected spots was 46.5 kDa, and the average pI was 5.22.

After imaging, SYPRO® Ruby stained proteins were excised and in-gel digested with modified porcine trypsin. The ϵ -amino groups of Lys residues in the tryptic peptide mixture were guanidinated with O-MU to enhance detection of these peptides by MALDI-MS [18]. Manual collection of MALDI-MS spectra was necessary only for those

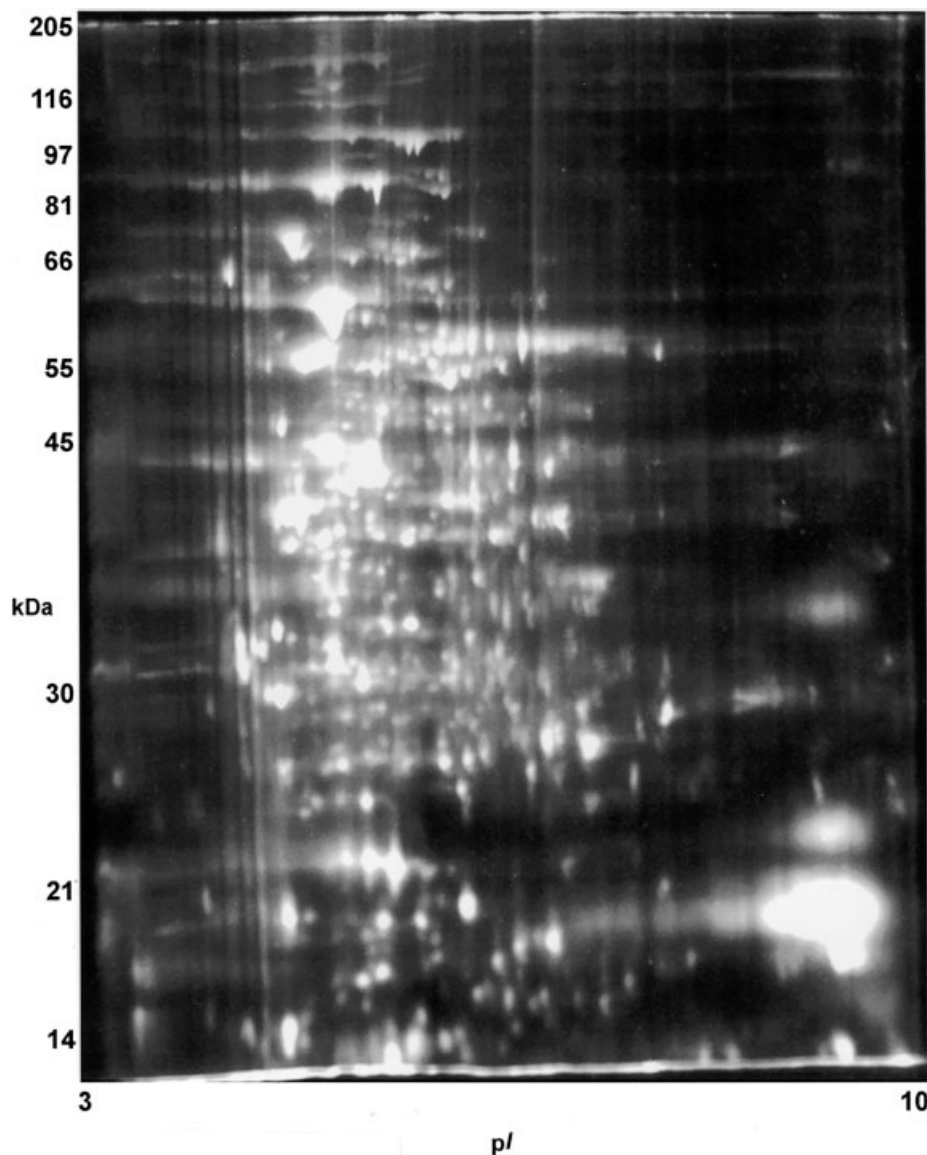


Figure 1. 2-D gel image of *B. melitensis* 16M proteins focused using a pH 3–10 IPG strip, and stained with SYPRO® Ruby.

Table 2. List of *B. melitensis* 16M proteins identified by peptide mass fingerprinting. Experimental pI and M_r values were obtained from analysis of the picked gel images, performed with the HT Investigator Analyzer software. For proteins identified more than one time, the high and low experimental pI and M_r values are indicated. The respective theoretical values, as well as the percent sequence coverage, were calculated by the Mascot search engine. The chromosome on which each ORF is located is included in the ORF name, where I refers to the 2.1 Mb chromosome, and II to the 1.2 Mb chromosome. Superscripts denote the corresponding reference for proteins which have previously been identified. Protein names followed by "(S)" designate presence of a secretory signal sequence.

Protein ID	ORF	pI (exp/theor)	kDa (exp/theor)	Sequence coverage (%)	Coding strand
10 kDa Chaperonin GroES ^{10, 11}	BMEII1047	5.46/5.41	23.6/10.4	53	+
16 kDa Heat Shock Protein A	BMEII0042	6.58/6.10	25.4/17.5	24	–
25 kDa Outer-Membrane Immunogenic Protein Precursor (S)	BMEI1007	4.50/4.72	29.3/25.3	16	–
25 kDa Outer-Membrane Immunogenic Protein Precursor (S)	BMEI1249	6.73–6.76/8.85	26.5–28.3/23.2	26	–
25 kDa Outer-Membrane Immunogenic Protein Precursor (S)	BMEI1829	4.40–4.60/4.79	27.8–33.6/24.6	25	+
26 kDa Periplasmic Immunogenic Protein Precursor (S)	BMEI0536	5.07–5.32/6.40	27.5–33.5/26.7	26	–
2-Dehydro-3-Deoxyphosphooctonate Aldolase (EC 4.1.2.16)	BMEI0850	6.63–6.88/6.12	29.5–30.1/29.8	22	+
2-Hydroxyhepta-2, 4-Diene-1,7-Dioate Isomerase (EC 5.3.3.-)	BMEI1708	4.95–5.03/6.45	34.0–34.2/37.0	19	–
5-Carboxymethyl-2-Oxo-Hex-3-Ene-1,7-Dioate Decarboxylase (EC 4.1.1.-) ¹¹					
2-Hydroxymuconic Semialdehyde Hydrolase (EC 3.1.1.-)	BMEI2011	5.29/5.92	33.4/30.3	18	+
2-Octaprenyl-3-Methyl-6-Methoxy-1,4-Benzoquinol Hydroxylase (EC 1.14.13.-)	BMEI0165	6.07/6.29	38.2/46.9	13	–
2-Oxoisovalerate Dehydrogenase Alpha Subunit (EC 1.2.4.4)	BMEII0748	6.35/6.29	35.4/46.0	19	–
2-Oxoisovalerate Dehydrogenase Beta Subunit (EC 1.2.4.4)	BMEII0747	4.82/5.50	26.7/37.3	10	–
31 kDa Immunogenic Protein Precursor (S)	BMEI0796	5.37–5.74/6.92	28.3–34.3/34.3	22	+
31 kDa Outer-Membrane Immunogenic Protein Precursor	BMEII0844	4.62–5.10/5.21	27.2–35.8/23.3	25	+
3-Deoxy-Manno-Octulosonate Cytidyltransferase (EC 2.7.7.38)	BMEI1904	6.76/9.42	29.1/32.6	17	+
3-Hydroxybutyrate Dehydrogenase (EC 1.1.1.31) ¹⁰	BMEI1024	6.70/6.45	29.5/31.2	24	+
3-Hydroxybutyryl-CoA Dehydrogenase (EC 1.1.1.157)	BMEI0099	5.91/5.73	31.4/31.9	15	+
3-Hydroxyisobutyrate Dehydrogenase (EC 1.1.1.31) (S)	BMEI0688	5.38/5.80	29.2/32.4	14	–
3-Oxoacyl-(Acyl-Carrier Protein) Reductase (EC 1.1.1.100) (S)	BMEII0816	7.22/6.85	28.6/26.5	32	+
3-Oxoacyl-(Acyl-Carrier Protein) Reductase (EC 1.1.1.100)	BMEI0032	4.49/4.47	27.3/27.2	12	+
4-Hydroxybutyrate Dehydrogenase (EC 1.1.1.61)	BMEII1094	5.28–5.31/5.23	30.3–35.9/34.2	26	+
60 kDa Chaperonin GroEL ^{9, 10, 11, 12, 13}	BMEII1048	4.90–7.63/5.04	49.6–66.7/57.8	25	+
6-Phosphogluconate Dehydrogenase, Decarboxylating (EC 1.1.1.44)	BMEII1124	5.62/5.60	37.8/50.7	19	–
7-Alpha-Hydroxysteroid Dehydrogenase (EC 1.1.1.159)	BMEI0405	6.94/6.38	28.8/28.3	23	–
ABC Transporter Periplasmic Binding Protein (S)	BMEII0702	5.29–5.32/5.85	32.1–37.1/40.1	20	+

Table 2. Continued

Protein ID	ORF	pI (exp/theor)	kDa (exp/theor)	Sequence coverage (%)	Coding strand
ABC Transporter Substrate-Binding Protein (S)	BMEI0015	4.95/5.00	32.1/32.3	27	–
Acetate CoA-Transferase Alpha Subunit (EC 2.8.3.8)	BMEI0928	5.63–5.71/5.54	44.7–45.8/54.5	13	+
Acetolactate Synthase Large Subunit (EC 4.1.3.18)	BMEI0617	5.60/5.62	54.5/67.0	13	+
Acetolactate Synthase Small Subunit (EC 4.1.3.18)	BMEI0618	7.44–7.52/7.15	26.0–26.0/20.8	32	+
Aconitate Hydratase (EC 4.2.1.3)	BMEI1855	4.97–5.62/5.55	107.3–120.3/97.9	6	+
Acriflavin Resistance Protein A Precursor (S)	BMEI1630	5.18–5.20/5.53	39.4–43.7/42.5	23	–
Adenylate Kinase (EC 2.7.4.3)	BMEI0778	6.69–6.73/6.63	26.5–28.3/21.0	38	+
Adenylosuccinate Synthetase (EC 6.3.4.4)	BMEI0351	5.90/8.19	35.3/57.0	13	+
Aldehyde Dehydrogenase (EC 1.2.1.3)	BMEI1740	5.99/5.99	43.2/51.5	14	–
Aliphatic Sulfonates-Binding Lipoprotein (S)	BMEII0109	5.15/5.62	29.3–35.1/34.6	26	+
Amidase (EC 3.5.1.4)	BMEII1134	5.43/5.40	36.6/42.6	15	+
Aminomethyltransferase (EC 2.1.2.10)	BMEII0559	5.43/5.33	34.3/39.1	15	+
Aminopeptidase N (EC 3.4.11.2)	BMEI1324	5.74/5.72	126.6/98.5	12	+
Argininosuccinate Synthase (EC 6.3.4.5)	BMEII1870	6.14/6.06	39.5/45.4	15	+
Aspartate Aminotransferase A (EC 2.6.1.1)	BMEI0516	6.04/8.08	35.8/46.5	21	–
ATP Synthase Alpha Chain (EC 3.6.1.34)	BMEI0249	5.91–6.10/6.10	46.9–52.4/55.2	22	+
ATP Synthase Beta Chain (EC 3.6.1.34)	BMEI0251	8.93/5.48	46.9/55.0	28	+
ATP Synthase Delta Chain (EC 3.6.1.34)	BMEI0248	8.22/7.90	25.9/19.9	27	+
ATP-Dependent CLP Protease, ATP-Binding Subunit CLPB	BMEI0195	5.35/5.41	113.4/103.8	8	+
Bacterioferritin ^{9, 11}	BMEII0704	4.08–4.77/4.74	23.8–26.8/20.2	22	–
Branched-Chain Amino Acid ABC Transporter, Periplasmic Amino Acid-Binding Precursor (S)	BMEII0344	5.21/5.31	33.9/43.5	11	–
Carbamoyl-Phosphate Synthase Large Chain (EC 6.3.5.5) (S)	BMEI0522	5.35/5.07	146.1/127.4	12	–
Catalase (EC 1.11.1.6)	BMEII0893	5.76–6.99/6.43	45.1–57.0/57.7	16	–
Channel Protein VIRB8 Homolog	BMEII0032	8.82/8.60	29.3/26.5	24	+
Channel Protein VIRB9 Homolog	BMEII0033	9.65/9.72	30.2/27.5	17	+
Chaperone Protein DnaJ	BMEI1513	9.90/8.89	31.4/33.7	29	–
Choloylglycine Hydrolase (EC 3.5.1.24) (S)	BMEI0543	5.51–5.73/6.33	31.3–33.1/40.6	21	+
Chromosome Partitioning Protein ParB	BMEI0010	5.79–6.26/5.81	30.3–31.9/32.7	19	+
COBS Protein	BMEI0049	5.44/5.41	31.9/38.4	14	+
CTP Synthase (EC 6.3.4.2) (S)	BMEI0849	5.11/5.39	76.6/60.5	7	+
Cysteine Synthase A (EC 4.2.99.8)	BMEI0101	5.82–6.38/5.72	32.5–34.3/35.5	18	–
Cystine-Binding Periplasmic Protein Precursor (S)	BMEII0601	7.31–7.41/7.74	26.9–28.7/27.8	55	–
D-3-Phosphoglycerate Dehydrogenase (EC 1.1.1.95)	BMEI0349	5.67/5.58	62.2/57.9	27	+
D-Alanine Aminotransferase (EC 2.6.1.21)	BMEII0363	5.69/5.62	33.3/32.7	20	–
Deoxyuridine 5'-TriPhosphate Nucleotidohydrolase (EC 3.6.1.23)	BMEI0358	6.83/6.98	24.8/18.7	30	+
D-Galactose-Binding Periplasmic Protein Precursor (S)	BMEII0983	5.18–5.20/5.74	33.3–36.0/36.9	34	–
Dihydrolipoamide Dehydrogenase (EC 1.8.1.4)	BMEI0145	6.37/6.21	45.4/49.3	35	+
Dihydrolipoamide Succinyltransferase Component (E2) of 2-Oxoglutarate Dehydrogenase Complex (EC 2.3.1.61)	BMEI0141	5.39/5.41	44.9/43.2	20	+
Dihydroorotase (EC 3.5.2.3)	BMEI1281	6.44/6.38	38.2/52.0	12	–
Dihydroorotate Dehydrogenase (EC 1.3.3.1)	BMEI1611	4.91/8.42	41.9/39.5	11	+
DNA Gyrase Subunit B (EC 5.99.1.3)	BMEII0676	6.53/6.30	28.8/77.7	9	–

Table 2. Continued

Protein ID	ORF	p/ (exp/theor)	kDa (exp/theor)	Sequence coverage (%)	Coding strand
DNA Polymerase III, Beta Chain (EC 2.7.7.7)	BMEI1942	4.92/5.32	40.2/43.3	21	–
DNA Protection During Starvation Protein ^{10, 11}	BMEI1980	4.93–5.52/5.43	24.6–27.8/20.0	25	+
DNA-Directed RNA Polymerase Alpha Chain (EC 2.7.7.6)	BMEI0781	4.40–5.04/4.85	36.8–42.6/37.6	19	+
DnaK Protein ^{9, 10, 11, 12, 13}	BMEI2002	4.86–4.92/4.86	69.5–80.6/69.0	21	–
D-Ribose-Binding Periplasmic Protein Precursor (S)	BMEI1390	4.48/4.61	30.4/33.3	28	–
D-Ribose-Binding Periplasmic Protein Precursor	BMEI0435	4.65–4.77/5.6	26.9–33.3/31.1	29	+
D-Xylose-Binding Periplasmic Protein Precursor	BMEI0146	4.99/8.91	36.0/42.3	40	–
Electron Transfer Flavoprotein Alpha-Subunit ⁹	BMEI0097	4.68–4.75/5.10	28.8–34.7/36.3	19	+
Electron Transfer Flavoprotein Beta-Subunit ¹¹	BMEI0096	4.76–7.98/7.77	27.1–30.1/26.5	48	+
Enolase (EC 4.2.1.11)	BMEI0851	4.83–5.07/4.99	37.8–43.6/45.6	29	+
Ferric Anguibactin-Binding Protein Precursor	BMEI0607	5.41–5.43/6.02	29.4–34.8/33.8	26	–
General L-Amino Acid-Binding Periplasmic Protein AAPJ Precursor ^{9, 10, 11} (S)	BMEI1211	4.93–5.11/5.30	30.0–34.7/37.4	19	–
Glucosamine-6-Phosphate Isomerase (EC 5.3.1.10)	BMEI0384	8.05/7.96	32.7/36.3	22	+
Glucose-6-Phosphate 1-Dehydrogenase (EC 1.1.1.49)	BMEI0513	5.82–5.84/5.74	43.1–50.2/55.3	18	–
Glutamate N-Acetyltransferase (EC 2.3.1.35)/ Amino-Acid Acetyltransferase (EC 2.3.1.1)	BMEI0124	7.70/6.42	28.3/45.3	14	+
Glutathione S-Transferase (EC 2.5.1.18)	BMEI1316	7.39/6.78	29.0/27.2	16	–
Glutathione-Dependent Formaldehyde Dehydrogenase (EC 1.2.1.1)	BMEI1819	4.95/5.72	33.8/40.4	14	+
Glyceraldehyde 3-Phosphate Dehydrogenase (EC 1.2.1.12)	BMEI0310	6.51–6.79/6.13	32.1–35.1/36.5	29	–
Glycerol-3-Phosphate-Binding Periplasmic Protein Precursor (S)	BMEI0115	4.95/5.14	35.0/45.8	18	+
Glycerol-3-Phosphate-Binding Periplasmic Protein Precursor (S)	BMEI0625	5.33/5.51	39.8/48.6	13	–
Glycine Betaine/L-Proline-Binding Protein PROX (S)	BMEI0550	4.83–5.20/5.57	33.6–33.6/32.1	22	+
Glycine Betaine/L-Proline-Binding Protein PROX (S)	BMEI0441	5.09/5.23	33.9/35.8	35	–
GrpE Protein	BMEI1777	4.57–4.66/4.86	28.8–34.0/19.4	29	+
Hypothetical Pyridoxal Phosphate Biosynthesis Protein	BMEI1091	5.28/5.23	36.7/45.5	15	+
Inosine-5'-Monophosphate Dehydrogenase (EC 1.1.1.205)	BMEI0896	6.66–7.41/6.88	43.2–48.8/52.8	17	–
Invasion Protein B ¹¹ (S)	BMEI1584	8.46/8.98	27.7/18.3	29	+
Iron(III)-Binding Periplasmic Protein Precursor (S)	BMEI1120	4.77–4.85/4.88	33.0–38.4/39.2	36	+
Isocitrate Dehydrogenase (NADP) (EC 1.1.1.42)	BMEI0791	5.82–6.25/5.85	35.7–41.7/45.8	15	–
Isovaleryl-CoA Dehydrogenase Precursor (EC 1.3.99.10)	BMEI1923	5.27–5.40/5.36	35.2–39.4/42.1	17	+
Leu/Ile/Val-Binding Protein Precursor (S)	BMEI0103	4.93–5.23/5.66	34.9–40.0/48.4	22	–
Leucine Aminopeptidase (EC 3.4.11.1)	BMEI1261	5.58–5.69/6.65	56.3–57.3/52.2	16	–
Leucine-, Isoleucine-, Valine-, Threonine-, and Alanine-Binding Protein Precursor ¹⁰ (S)	BMEI1930	5.16/6.13	35.2/40.2	23	+
Leucine-, Isoleucine-, Valine-, Threonine-, and Alanine-Binding Protein Precursor ¹⁰ (S)	BMEI0633	5.26–5.41/5.54	34.1–38.4/42.7	28	–
Leucine-, Isoleucine-, Valine-, Threonine-, and Alanine-Binding Protein Precursor ¹⁰ (S)	BMEI0263	4.95–5.18/5.21	33.8–39.1/40.0	18	+
LexA Repressor (EC 3.4.21.88)	BMEI0840	7.40/7.04	27.6/26.2	26	–

Table 2. Continued

Protein ID	ORF	p/ (exp/theor)	kDa (exp/theor)	Sequence coverage (%)	Coding strand
Lipoprotein (S)	BMEI1954	4.96/5.24	33.1/29.2	12	–
LSU ribosomal protein L1E (= L4P)	BMEI0758	5.14–7.70/10.21	28.5–32.9/22.2	32	+
LSU ribosomal protein L1P	BMEI0746	9.65/9.42	28.9/24.8	31	+
LSU ribosomal protein L25P	BMEI0481	6.44/5.92	26.7/25.1	28	–
LSU ribosomal protein L9P	BMEI1483	4.93–4.96/4.79	25.4–27.4/21.0	24	+
Malate Dehydrogenase (EC 1.1.1.37) ^{9, 10} (S)	BMEI0137	5.29/5.39	33.1/34.8	25	+
Malonate-Semialdehyde Dehydrogenase [Acylating] (EC 1.2.1.18) / Methylmalonate-Semialdehyde Dehydrogenase [Acylating] (EC 1.2.1.27)	BMEI0219	5.77/5.74	45.8/54.1	12	+
Maltose/Maltodextrin Transport ATP-Binding Protein MALK	BMEI1713	8.02/7.88	33.9/39.9	15	–
Maltose-Binding Periplasmic Protein (S)	BMEI0945	4.77–4.81/4.82	40.6–45.3/45.9	20	–
Membrane-Bound Lytic Murein Transglycosylase B Precursor (EC 3.2.1.-)	BMEI0223	9.69/9.23	28.5/27.6	13	–
Methylenetetrahydrofolate Dehydrogenase (EC 1.5.1.5) / Methenyltetrahydrofolate Cyclohydrolase (EC 3.5.4.9)	BMEI0510	6.79/5.68	30.1/33.6	25	+
Molybdopterin Biosynthesis MoeB Protein	BMEI1940	8.03/8.65	29.1/28.9	13	–
NAD(P) Transhydrogenase Subunit Alpha (EC 1.6.1.2)	BMEI0323	6.69/8.99	40.0/44.8	20	+
Nitrogen assimilation Regulatory Protein NtrX	BMEI0868	5.67/5.58	52.6/50.3	33	+
Non-Motile and Phage-Resistance Protein (EC 2.7.1.-)	BMEI0417	5.05–5.30/4.97	147.1–148.1/ 112.2	8	–
Oligopeptide Transport ATP-Binding Protein OPPD	BMEI1938	6.69/9.48	54.2/65.3	8	+
Oligopeptide-Binding Protein APPA Precursor	BMEI0859	5.68/5.71	40.3/54.8	10	+
Omega-Amino Acid-Pyruvate Aminotransferase (EC 2.6.1.18)	BMEI1757	5.94/5.99	38.2/50.0	14	–
Outer Membrane Protein	BMEI1060	7.37/6.53	27.8/27.6	34	+
Outer Membrane Protein	BMEI1895	5.55/5.57	75.0/67.3	29	–
Outer Membrane Protein (S)	BMEI0830	5.05–5.42/5.37	89.9–94.8/86.0	21	+
Outer Membrane Protein Precursor (S)	BMEI0338	4.94/5.44	33.3/30.6	16	–
Outer Membrane Protein TOLC Precursor (S)	BMEI1029	9.10/9.16	38.3/48.6	14	–
Periplasmic Oligopeptide-Binding Protein Precursor ¹¹ (S)	BMEI0735	4.91–4.98/4.97	44.0–55.1/59.3	15	+
Periplasmic Oligopeptide-Binding Protein Precursor ¹¹	BMEI0734	5.08–5.11/4.97	52.2–55.3/56.6	22	+
PHOH Protein	BMEI1975	7.34–7.44/8.63	32.8–33.0/34.7	18	–
Phosphoglycerate Kinase (EC 2.7.2.3)	BMEI0309	5.68/5.83	36.4/43.2	20	–
Phosphoglycerate Mutase (EC 5.4.2.1)	BMEI0248	6.44–6.55/6.24	26.4–28.3/24.2	31	–
Phosphoribosylaminoimidazolecarboxamide Formyltransferase (EC 2.1.2.3) / IMP Cyclohydrolase (EC 3.5.4.10)	BMEI0233	5.84/5.77	48.8/56.8	14	–
Pleiotropic Regulatory Protein	BMEI0421	5.41/5.50	35.7/41.9	17	+
Polyribonucleotide Nucleotidyltransferase (EC 2.7.7.8)	BMEI1961	4.92–5.05/4.99	84.4–97.7/78.1	12	–
Protein Translation Elongation Factor G (EF-G)	BMEI0754	4.92–5.08/5.05	84.4–97.7/76.8	16	+
Protein Translation Elongation Factor Ts (EF-Ts)	BMEI0824	4.95/5.03	32.1/32.2	33	+
Protein Translation Elongation Factor Tu (EF-TU)	BMEI0755	4.51–7.63/5.29	34.6–42.7/42.9	22	+
Protein Translocase Subunit SecB	BMEI2055	4.71/4.89	24.8/18.0	20	+
Protein-L-Isoaspartate O-Methyltransferase (EC 2.1.1.77)	BMEI1030	4.43–4.43/4.39	27.4–28.7/24.0	23	–

Table 2. Continued

Protein ID	ORF	p/ (exp/theor)	kDa (exp/theor)	Sequence coverage (%)	Coding strand
Putative Binding Protein YDDS Precursor (S)	BMEI0203	4.68–4.72/4.92	48.5–50.1/57.3	23	–
Putative Protease IV	BMEI1793	5.94/5.81	43.4/32.4	10	+
Putative Thiosulfate Sulfurtransferase (EC 2.8.1.1)	BMEI0931	4.98–5.41/5.39	28.2–34.3/31.3	21	–
Putrescine-Binding Periplasmic Protein Precursor (S)	BMEI0411	4.84/4.96	32.4/41.2	32	+
Pyruvate Dehydrogenase E1 Component, Beta Subunit (EC 1.2.4.1) ⁹	BMEI0855	4.73–6.60/4.73	59.7–65.4/49.3	22	+
Riboflavin Synthase Alpha Chain (EC 2.5.1.9)	BMEI1188	4.92/5.02	27.8/22.5	16	–
Ribose 5-Phosphate Isomerase (EC 5.3.1.6)	BMEI0424	4.88/5.90	26.7/17.4	41	–
Ribose 5-Phosphate Isomerase (EC 5.3.1.6)	BMEI0974	5.31/5.74	32.9/25.8	28	–
Ribosome Recycling Factor (RRF) ^{9, 11}	BMEI0826	6.44–7.09/6.34	26.0–28.3/20.9	28	+
S-Adenosylmethionine Synthetase (EC 2.5.1.6)	BMEI1970	6.67–8.15/8.07	34.6–37.6/48.9	17	–
Short-Chain Dehydrogenase	BMEI1477	6.80/6.44	28.5/26.2	48	–
SSU ribosomal protein S1P ⁹	BMEI1915	4.93–5.24/5.19	64.4–73.5/63.8	18	–
SSU ribosomal protein S6P	BMEI1480	7.50/9.29	24.9/17.1	25	+
Stationary-Phase Survival Protein SURE	BMEI1081	5.40/5.72	33.4/29.1	24	–
Succinyl-CoA Synthetase Beta Chain (EC 6.2.1.5)	BMEI0138	4.93/4.90	37.8/42.8	20	+
Sugar-Binding Protein	BMEI0590	4.79–5.42/4.97	33.0–39.6/43.5	27	+
Sugar-Binding Protein	BMEI0542	4.70–4.75/4.88	33.3–33.7/45.2	15	–
Tail-Specific Protease Precursor (EC 3.4.21.-) (S)	BMEI0214	5.39–5.41/6.30	41.1–45.5/50.9	36	+
Tetratricopeptide Repeat Family Protein	BMEI1531	5.71/5.75	56.6/68.1	16	–
Thiamin-Phosphate Pyrophosphorylase (EC 2.5.1.3)	BMEI0329	4.71/4.76	25.5/23.4	20	–
Thioredoxin	BMEI0401	4.86–4.86/4.94	31.3–32.4/35.3	15	+
ToIB Protein Precursor (S)	BMEI0339	6.48–6.55/8.75	34.2–35.5/48.8	30	+
Transcription Antitermination Protein NusG	BMEI0744	6.81/6.64	25.9/19.7	49	+
Transcription Termination Factor Rho	BMEI0003	5.89/5.87	39.6/47.2	23	+
Transcriptional Regulator, GNTR Family	BMEI0757	9.10/9.43	29.2/32.2	29	+
Transporter	BMEI1890	6.71–7.08/6.34	30.2–31.1/35.1	23	+
Trehalose/Maltose Binding Protein ^{9, 11}	BMEI1716	4.92–5.12/5.09	37.0–42.1/46.4	26	–
tRNA Pseudouridine Synthase A (EC 4.2.1.70)	BMEI1039	5.89/10.47	34.4/62.4	11	–
Two Component Response Regulator	BMEI0066	5.39/5.25	33.1/25.4	18	+
UDP-3-O-[3-Hydroxymyristoyl] N-Acetyl- glucosamine Deacetylase (EC 3.5.1.-)	BMEI0586	4.92/4.94	31.0/31.1	27	+
Universal Stress Protein UspA and Related Nucleotide-Binding Proteins	BMEI0938	4.94–5.36/5.24	30.3–35.6/30.9	18	+
UTP-Glucose-1-Phosphate Uridyltransferase (EC 2.7.7.9) (S)	BMEI0023	5.87/5.73	29.2/33.0	19	–
Hypothetical Protein	BMEI0443	7.21/6.90	27.7/17.0	33	–
Hypothetical Protein (S)	BMEI1494	4.69/4.65	24.3/12.0	49	–
Hypothetical Protein (S)	BMEI1033	4.81/5.01	33.3/23.3	18	–
Hypothetical Protein	BMEI1843	4.43–4.46/7.67	24.3–26.7/25.8	24	+
Hypothetical Protein	BMEI1092	4.86/4.98	26.7/24.9	16	–
Hypothetical Protein	BMEI0821	5.71/5.62	29.7/29.9	21	+
Hypothetical Protein (S)	BMEI0542	4.74–4.74/4.83	27.5–28.6/30.1	16	–
Hypothetical Protein (S)	BMEI0507	5.17/5.69	35.8/37.8	14	+
Hypothetical Protein	BMEI0291	4.78/4.81	27.0/16.3	27	+
Hypothetical Cytosolic Protein	BMEI1075	7.19/6.87	29.3/31.0	23	–
Hypothetical Cytosolic Protein	BMEI1541	4.71/9.45	61.8/43.9	9	+
Hypothetical Cytosolic Protein	BMEI0092	6.84/6.85	28.3/24.3	21	+

Table 2. Continued

Protein ID	ORF	p/ (exp/theor)	kDa (exp/theor)	Sequence coverage (%)	Coding strand
Hypothetical Cytosolic Protein	BMEI1121	4.71–4.84/4.84	23.0–24.3/12.2	45	–
Hypothetical Cytosolic Protein	BMEI1201	4.64–4.91/4.94	30.6–40.9/40.0	17	+
Hypothetical Cytosolic Protein	BMEI1968	4.69–4.73/4.75	30.5–31.1/24.1	29	–
Hypothetical Cytosolic Protein	BMEI0313	4.75/4.78	26.6/13.5	35	+
Hypothetical Membrane Associated Protein	BMEI1551	4.85/4.96	33.3/23.7	43	–
Hypothetical Periplasmic Protein	BMEI1791	9.02/9.17	28.2/18.1	19	–

Table 3. Distribution of those ORFs which encode identified proteins between the two chromosomes of *B. melitensis* 16M, listed also with respect to coding strand.

Chromosome	Theoretical ORFs	Identified ORFs	% Identified
21.1 Mb (I)	2059	1053 (+)	51.0
		1006 (–)	49.0
1.2 Mb (II)	1138	549 (+)	48.2
		590 (–)	51.8

The hidden Markov model algorithm of SignalP V2.0 [20, 21] was used to predict that 460 ORFs in the 16M genome contain secretory signal sequences. The 44 proteins observed in this study which contain signal sequences are designated in Table 2; 15 of these have been annotated as periplasmic proteins.

Table 3 displays the distribution of the identified ORFs over each of the two circular chromosomes in the *B. melitensis* genome. The ratio of expressed genes identified under the conditions defined, relative to the number of ORFs predicted on each chromosome, is 6.5% for chromosome I and 4.7% for chromosome II. Additionally, 56% of the identified proteins encoded on chromosome I originated on the positive strand, while 47% of those on chromosome II originated on the positive strand.

4 Discussion

The genomes of the different *Brucellae* species are greater than 87% similar, as estimated by DNA-DNA hybridization studies [22]. A more recent study which used PFGE to generate macrorestriction maps of each species also showed this genus to be highly conserved [23]. Due to the conserved nature of the genomes, it is likely that host specificity of the different species stems from proteomic variation arising from subtle genomic differences. This idea has been suggested to explain differences observed in 2-D gel patterns of vaccinal and virulent strains of *B. abortus*

[24]. Additionally, genomic analysis of the recently published *Brucella melitensis* 16M genome [8] has not revealed any traditional virulence factors, toxin genes, or pathogenicity islands. It is hoped that virulence mechanisms of the *Brucellae* will be deciphered through comparative proteomic studies which focus on expression of proteins involved in secretion pathways, transcriptional activation, and response to environmental stress.

The number of ORFs in the genome is greater than the 883 protein spots observed in this study. The majority of identified proteins are cytoplasmic; only 44 were determined to have *N*-terminal secretory signal sequences, and 15 of these are targeted to the periplasmic space. It is possible that the sample preparation methods used in this study were biased toward hydrophilic cytosolic proteins, rather than more hydrophobic membrane proteins. None of the membrane proteins identified here contain more than one transmembrane domain, as determined using SOSUI/G version 1.1 (<http://sosui.proteome.bio.tuat.ac.jp>). Furthermore, very high and low molecular weight proteins are not effectively resolved in our system; the 10% Duracryl™ concentration of the second dimension gels optimally resolves proteins between 29 and 116 kDa. Thus, it is emphasized that membrane-bound, hydrophobic, very high and very low molecular mass proteins are under-represented and will be the subject of a separate study. Certainly, varied sets of proteins are expressed under particular environmental conditions [9–13] or at different phases of growth; not all coding regions are expressed simultaneously. It may also be that some of the ORFs predicted in the genome are silent. Most likely, the number of observed proteins will become closer to the number predicted in the genome as the proteins identified in 2-D studies performed under several different conditions are compiled. It is worth noting the identified proteins are not biased with respect to chromosome of origin or coding strand, as summarized in Table 3.

This study increased the number of *Brucella* proteins observed on 2-D gels by combining two recently developed technologies. The first is SYPRO® Ruby gel stain,

which has greater sensitivity, linearity and reproducibility than silver stain and does not interfere with MALDI-MS [14]. The second is overlapping narrow pH range IPG strips, which have been shown to increase the number of protein spots observed in the proteomes of *Staphylococcus aureus* [25], *Saccharomyces cerevisiae* [26], and *Escherichia coli* [27]. In past studies, *Brucella* proteins were resolved on 2-D gels after IEF using a single medium pH range of 4.7–7, and detected by either radiolabelling with [³⁵S]-methionine and [³⁵S]-cysteine [9] or silver staining [10, 11]. This allowed observation of 676 or 595 spots, respectively. In contrast, this study observed 513 SYPRO® Ruby stained protein spots on the average 2-D gel spanning pH 4–7. This number increased to 883 when considering gels arising from the use of overlapping, narrow pH range IPG strips covering pH 4 to 11, as described above.

Although not frequently encountered, incidences of false positives in data mining can be problematic [28]. In addition to increasing the ionization of C-terminal Lys tryptic peptides, guanidination by O-MU served to aid in evaluation of our peptide mass fingerprint data. A recent report demonstrated that consideration of chemical modification of peptides by O-MU increases the power of database searching [29]. In this study, observed peptide masses were expected to match theoretical peptide masses which reflected both a low occurrence of missed cleavages by trypsin and a high occurrence of Lys residues modified by O-MU. Therefore, statistically significant results which did not agree with experimental setup, *i.e.* a high incidence of missed cleavages or lack of modified Lys residues, were discarded.

To date, Western blotting and Edman degradation have been used to identify 48 spots on 2-D gels of protein extracts from *B. melitensis* [9, 11], 25 from *B. ovis* [10], and 6 from *B. abortus* [12, 13]. This study has benefited from the technique of peptide mass fingerprinting using automated equipment. However, the true potential of this method was realized only through availability of the finished genome of the 16M strain. For example, the N-terminal sequences of some tryptic peptides obtained from *B. melitensis* and *B. ovis* proteins in previous studies did not yield identifications since the information was not yet available in the databases. A blast of these sequences against the annotated 16M genome found the following proteins were previously observed in *B. melitensis*, but not yet identified: Peptidyl-prolyl cis-trans isomerase A precursor (EC 5.2.1.8) [9]; trehalose/maltose binding protein, 19 kDa periplasmic protein [9, 11]; periplasmic oligopeptide-binding protein precursor, 2-hydroxyhepta-2,4-diene-1,7-dioate isomerase (EC 5.3.3.-)/5-carboxymethyl-2-oxo-hex-3-ene-1,7-dioate decarboxylase (EC 4.1.1.-), and DNA protection during starvation protein

[11]. Similarly, the following were observed for *B. ovis*, although not previously identified: Iron(III)-binding periplasmic protein precursor, protein translation elongation factor P (EF-P), branched-chain amino acid aminotransferase (EC 2.6.1.42), and DNA protection during starvation protein [10]. In total, proteins encoded by 16 different ORFs observed in this study have been previously identified in *B. melitensis*, 8 in *B. ovis*, and 2 in *B. abortus*. These are designated in Table 2.

The experimental *pI* and *M_r* values are in agreement with the corresponding theoretical values for the majority of the proteins identified. Divergent theoretical and experimental *pI* values might be explained by: (1) post-translational modifications which alter the charge of the protein; (2) theoretical *pI* values calculated by algorithms using mathematical and physical considerations which do not approximate actual conditions, and (3) incomplete focusing during IEF. Theoretical *M_r* values which are greater than experimental may arise from observation of the mature forms of precursor proteins, and also for proteins which have undergone degradation. In the opposite case, where the experimental *M_r* is greater than the theoretical, observed proteins may contain post-translational modifications such as lipidations which increase their apparent mass, or may be covalently bound to cofactors or ligands. One must also consider that determination of *M_r* by SDS-PAGE is not definitively accurate. An in-depth investigation of the post-translationally modified forms of the identified protein spots is beyond the scope of this paper and is the subject of a future investigation.

5 Concluding remarks

In this study, only proteins expressed under laboratory conditions of growth on Schaedler blood agar were considered. This baseline proteomic signature will serve as a reference to which protein patterns arising from growth *in vivo* or under experimental conditions can be compared. Correlation of this proteomic data with the genomic sequence should lead to identification of conserved stress- and virulence-related promoter sequences. Furthermore, comparison of the proteomic signatures of *Brucella* grown in the laboratory with those isolated from the natural host will lead to elucidation of those proteins important in host specificity.

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