Proteomics-based confirmation of protein expression and correction of annotation errors in the *Brucella abortus* genome

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Abstract

**Background:** Brucellosis is a major bacterial zoonosis affecting domestic livestock and wild mammals, as well as humans around the globe. While conducting proteomics studies to better understand *Brucella abortus* virulence, we consolidated the proteomic data collected and compared it to publicly available genomic data.

**Results:** The proteomic data was compiled from several independent comparative studies of *Brucella abortus* that used either outer membrane blebs, cytosols, or whole bacteria grown in media, as well as intracellular bacteria recovered at different times following macrophage infection. We identified a total of 621 bacterial proteins that were differentially expressed in a condition-specific manner. For 305 of these proteins we provide the first experimental evidence of their expression. Using a custom-built protein sequence database, we uncovered 7 annotation errors. We provide experimental evidence of expression of 5 genes that were originally annotated as non-expressed pseudogenes, as well as start site annotation errors for 2 other genes.

**Conclusions:** An essential element for ensuring correct functional studies is the correspondence between reported genome sequences and subsequent proteomics studies. In this study, we have used proteomics evidence to confirm expression of multiple proteins previously considered to be putative, as well as correct annotation errors in the genome of *Brucella abortus* strain 2308.

Background

*Brucella* species bacteria are gram negative alpha proteobacteria superbly adapted for survival in intracellular environments. They infect a wide range of mammals, including essentially all economically important domestic mammals, many wild species, and humans. Brucellosis is the largest bacterial zoonosis in the world [1-3]. In humans, untreated brucellosis is a long lasting disease characterized by recurrent fever episodes and clinical manifestations that include spondylitis, severe headaches, joint or abdominal pain, endocarditis, and meningencephalitis. In severe non-treated cases brucellosis can cause death [1-3].

Seven terrestrial *Brucella* species have been defined: *Brucella melitensis*, *Brucella abortus*, *Brucella suis*, *Brucella ovis*, *Brucella canis*, *Brucella neotomae* and *Brucella microti* which infect goats, cattle, pigs, sheep, dogs, desert wood rats and common voles, respectively [1,4]. Two *Brucella* species infecting marine mammals such as dolphins, whales, seals, sea lions and walrus have also been defined as *Brucella ceti* and *Brucella pinnipedialis* [5-7]. With the exception of *B. suis* biovar 3, the *Brucella* genome is encoded on two chromosomes, containing in total approximately 3,500 genes. Genome sequences from 32 different *Brucella* strains, representing all species, have been published either as complete genomes (10 strains) or as draft assemblies in NCBI (22 strains) [8-14].

The raw genome sequencing data of 78 other strains is also available in the Sequence Read Archive of NCBI. The genome sequences were very highly homologous, although regions of unique genetic material were also observed. It is possible that these regions are involved in establishing the distinct host preferences and biological
behavior of the different Brucella species sequenced to date [15].

Unlike other pathogenic bacteria, Brucella virulence does not appear to be the result of relatively few virulence genes that can be transferred horizontally via plasmids, phages, or assembled in pathogenicity islands. Brucella also lack typical virulence factors such as exotoxins, flagella, capsules, and type III secretion systems. Rather, the pathogen’s virulence appears to be an integrated aspect of its physiology. Therefore, to better understand Brucella virulence, we will need to better understand the Brucella proteome, including how it changes during the different stages of the intracellular and extracellular Brucella life-cycles, and how it interacts with host proteins and processes. Indeed, we have previously demonstrated that Brucella bacteria are capable of extensive, reversible, remodeling of their cell envelopes [16]. Furthermore, during the establishment of an intracellular infection, Brucella bacteria also appear able to carry out extensive, and reversible, modifications to their biosynthetic pathways and respiration in order to adapt to the changing microenvironments encountered in infected host cells [17]. This suggests that the Brucella proteome is considerably more dynamic than previously suspected, and that in depth proteomic analysis of the pathogen, as well as integration of these data with the available genomic information, will result in novel mechanistic and possibly therapeutic insights.

In this work we have generated a synthesis of the proteomic datasets we produced from multiple independent comparisons of Brucella strains either grown in media or retrieved from infected host cells. Some of this data is currently publicly available [16,17];[http://proteomicsresearchsource.org/Default.aspx] with the remainder becoming available as part of this work. These studies were originally designed to identify experimental condition-specific differences in the Brucella proteome. We compiled the experimental evidence for any Brucella protein detected and compared the proteomic data to the available genomic data. We provide the first direct experimental evidence for the expression of 305 Brucella proteins, but also identified experimental evidence for the expression of five genes previously annotated as pseudogenes, and of start site errors in two other genes.

Results and Discussion

First experimental evidence of the expression of 305 proteins in B. abortus 2308

Samples used for the proteomic analysis came from B. abortus either grown extracellularly in media or isolated from infected RAW264.7 macrophages. The extracellular samples included whole bacteria grown directly in trypsic soy broth, outer membrane preparations (blebs) [16] and cytosols. Intracellular samples consisted of viable B. abort-
Table 1: *B. abortus* 2308 proteins for which the expression was demonstrated for the first time

**Cytoplasm**

| BAB1_0002 | DnaN | BAB1_0855 | GRX family | BAB1_1449 | UDP-N-acetylglucosamine transferase | BAB1_2149 | PepS |
|-----------|------|-----------|------------|-----------|-------------------------------------|-----------|------|
| BAB1_0022 | Unknown | BAB1_0856 | BoA-related | BAB1_1450 | acetyl-L-muramidase | BAB1_2168 | RpsO; S15 |
| BAB1_0023 | AroA | BAB1_0857 | FGAM synthase II | BAB1_1508 | L-alanine ligase | BAB1_2173 | FabB |
| BAB1_0035 | KdsB | BAB1_0861 | PurS | BAB1_1508 | CarB | BAB2_0083 | Eda2 |
| BAB1_0063 | Unknown | BAB1_0864 | HpcH/Hpal | BAB1_1512 | CspA | BAB2_0090 | GCNS-related |
| BAB1_0071 | ArgG | BAB1_0874 | AcpP | BAB1_1523 | GreA | BAB2_0090 | N-acetylmuramoyltransferase |
| BAB1_0100 | Putative AsnC family | BAB1_0880 | HAD-like | BAB1_1528 | SseA-1 | BAB2_0109 | Gnd |
| BAB1_0107 | Trs-ABC (P-loop) | BAB1_0886 | NN:DBI PRT | BAB1_1538 | OmpR | BAB2_0160 | Unknown |
| BAB1_0118 | Unknown | BAB1_0896 | ArgS | BAB1_1547 | PepQ | BAB2_0162 | L-carnitine dehydrogenase |
| BAB1_0122 | GyrB | BAB1_0898 | NagZ | BAB1_1549 | PrsA | BAB2_0177 | Gnr |
| BAB1_0139 | NifU | BAB1_0918 | GatB/YqeY | BAB1_1553 | YchF | BAB2_0177 | YafB |
| BAB1_0159 | S30EA | BAB1_0924 | AccC | BAB1_1613 | Unknown | BAB2_0186 | Fumarate hydratase |
| BAB1_0160 | PtsN-like | BAB1_0933 | PCRF 2 | BAB1_1645 | DhaK-1 | BAB2_0187 | Unknown |
| BAB1_0191 | GABAtransms | BAB1_0943 | TyrS | BAB1_1646 | DhaK-2 | BAB2_0191 | HAD-like, dehydrogenase |
| BAB1_0204 | AdhP | BAB1_0949 | SufC | BAB1_1655 | GabD | BAB2_0198 | Pseudouridine |
| BAB1_0215 | ThiE | BAB1_0955 | DeaD | BAB1_1669 | PAS domain | BAB2_0198 | Pseudouridine |
| BAB1_0216 | ThiG | BAB1_0960 | Trs heavy metal | BAB1_1671 | TcaR | BAB2_0198 | Pseudouridine |
| BAB1_0242 | ManR | BAB1_1014 | MetG | BAB1_1687 | Dut | BAB2_0216 | 3-hydroxybutyryl-CoA dehydrogenase |
| BAB1_0285 | HisD | BAB1_1030 | Gor | BAB1_1695 | PurA | BAB2_0216 | 3-hydroxybutyryl-CoA dehydrogenase |
| BAB1_0317 | Trs arginine/ornithine | BAB1_1037 | Mandelate racemase; muconate lactonizing | BAB1_1702 | Phosphoglucomutase | BAB2_0246 | P47K |
| BAB1_0331 | ArgD | BAB1_1070 | MuScA lactonizing | BAB1_1778 | FdxA | BAB2_0337 | RocF |
| BAB1_0344 | Pip | BAB1_1043 | Unknown | BAB1_1719 | ThiE | BAB2_0295 | DgoK |
| BAB1_0353 | Unknown dehydrogenase | BAB1_1050 | FolB | BAB1_1722 | Efp | BAB2_0296 | KdgA |
| BAB1_0416 | DUF85 | BAB1_1077 | Ach1p | BAB1_1751 | Unknown | BAB2_0333 | NADH:flavin oxidoreductase/NADH oxidase |
| BAB1_0429 | Polyprenyl synthetase | BAB1_1098 | PRA-CH | BAB1_1778 | FdxA | BAB2_0337 | RocF |
| BAB1_0446 | DnaJ | BAB1_1121 | DNA gyrase subunit A | BAB1_1781 | Unknown | BAB2_0343 | Trx-2 |
| BAB1_0447 | FabI-1 | BAB1_1130 | ClpA/B | BAB1_1804 | MarR family | BAB2_0358 | Dhp |
| BAB1_0482 | FabD | BAB1_1132 | ClpP | BAB1_1810 | AtpH | BAB2_0361 | TypA |
| BAB1_0484 | AcpP | BAB1_1156 | KdsA | BAB1_1813 | Transaldolase | BAB2_0365 | FbaA |
| BAB1_0489 | Guanylate kinase | BAB1_1157 | PyrG | BAB1_1815 | LeuS | BAB2_0366 | RpiB/LacA/LacB |
| BAB1_0510 | ThrC | BAB1_1161 | TpiA | BAB1_1819 | ACAT | BAB2_0367 | TIM 2 |
| BAB1_0525 | PpdK | BAB1_1164 | TrpC | BAB1_1824 | PurH | BAB2_0370 | EryC |
| BAB1_0532 | Transthretin | BAB1_1167 | GltX | BAB1_1837 | CynT | BAB2_0448 | Unknown |
| BAB1_0540 | Formyl transferase, N-terminal | BAB1_1170 | GltA | BAB1_1840 | MmsA | BAB2_0457 | FoID |
| BAB1_0544 | Degt/DnrJ/EryC1/Srs | BAB1_1174 | FabZ | BAB1_1872 | PrfA | BAB2_0459 | Pgi |
| BAB1_0561 | Man-6-P isomerase type II | BAB1_1177 | Endoribonuclease | BAB1_1874 | LysC | BAB2_0460 | Zwf |
| BAB1_0570 | XylA | BAB1_1205 | ElaB-domain | BAB1_1879 | GrxC | BAB2_0483 | ShuT |
| BAB1_0578 | Unknown | BAB1_1212 | BhbA | BAB1_1887 | HemC | BAB2_0513 | GcvT |
| BAB1_0588 | ATP/GTP-binding | BAB1_1213 | Unknown; conserved | BAB1_1926 | SusC | BAB2_0568 | Unknown |
### Table 1: *B. abortus* 2308 proteins for which the expression was demonstrated for the first time (Continued)

| BAB1_0641 | Alanine aminopeptidase; Neutral zinc metallopeptidase, zinc-binding region | BAB1_1223 | AlaS | BAB1_1936 | GloB | BAB2_0572 | IlvE |
| BAB1_1224 | RecA | BAB1_1946 | SecA | BAB2_0620 | Unknown |
| BAB1_1233 | RpsM; S13 Ads | BAB1_1970 | FadB | BAB2_0642 | Acyl-CoA dehydratase |
| BAB1_1234 | Adk | BAB1_1971 | EtfA | BAB2_0644 | Metal-dependent |
| BAB1_0666 | DapA | BAB1_1241 | RpsH; S8 | BAB1_1988 | HisC | BAB2_0645 | GatC |
| BAB1_0671 | MoaD | BAB1_1246 | RplP; L16 | BAB1_2023 | CipA/cipB | BAB2_0851 | GuaB |
| BAB1_0740 | Unknown | BAB1_1249 | RpsC; S3 | BAB1_2059 | ParB | BAB2_0861 | DapA |
| BAB1_0775 | AspS | BAB1_1256 | RpsJ; S10 | BAB1_2080 | HisU | BAB2_0897 | AldB |
| BAB1_0780 | HemB | BAB1_1266 | RplJ; L10 | BAB1_2081 | HisV | BAB2_0898 | ArgB |
| BAB1_0787 | GlyA | BAB1_1280 | Unknown | BAB1_2087 | HisE | BAB2_0900 | Unknown |
| BAB1_0789 | RibD | BAB1_1286 | GloA | BAB1_2096 | PTS system IIA | BAB2_0991 | DapD |
| BAB1_0790 | RibE | BAB1_1294 | Aminotransferase subunit | BAB2_0993 | DapE |
| BAB1_0813 | CysD | BAB1_1297 | Unknown | BAB2_2109 | AccD | BAB2_1009 | MgsA |
| BAB1_0817 | Unknown; conserved | BAB1_1376 | UreA | BAB2_2133 | Unknown | BAB2_1012 | DapB |
| BAB1_0826 | NuoE | BAB1_1408 | IlvB | BAB2_2134 | SMP-30 | BAB2_1013 | Gpm |
| BAB1_0842 | ProS | BAB1_2135 | Glutathione synthetase | BAB2_1032 | | |

#### Inner membrane

| BAB1_0400 | Unknown | BAB1_1281 | DUF192 | BAB2_0261 | RecA | BAB2_0877 | Binding-protein-dependent transport system inner membrane component |
| BAB1_0425 | NhaA | BAB1_1703 | FtsH | BAB2_0709 | FtsK-alpha | |
| BAB1_0542 | WbkC | BAB1_1712 | MotA; TolQ; ExbB | BAB2_0728 | CydA | |

#### Periplasm

| BAB1_0010 | Trs-ABC oligopeptide | BAB1_1118 | PpiB-1 | BAB2_0427 | Trs-ABC spermidine/putrescine | BAB2_0697 | Unknown; conserved |
| BAB1_0155 | OstA-like | BAB1_1362 | Lac | BAB2_0812 | Trs-ABC oligopeptide | |
| BAB1_0404 | Unknown | BAB1_1413 | DegP | BAB2_0451 | Trs-ABC oligopeptide | |
| BAB1_0444 | PdxH | BAB1_1890 | YciL-like protein | BAB2_0879 | Trs-ABC spermidine/putrescine | |
| BAB1_0739 | ETC complex I | BAB1_1919 | Unknown | BAB2_0593 | Trs-ABC amino acid | |
| BAB1_0776 | Unknown | BAB1_1981 | TlpA | BAB2_0880 | Unknown | |
| BAB1_0881 | Trs-ABC amino acid | BAB2_0374 | Unknown | BAB2_0664 | Trs-ABC peptidase | |

#### Outer membrane

| BAB1_0659 | Omp2a | BAB1_0707 | OtsA | BAB1_0963 | TolC | |

**Table 1:** Summary of proteins for which the expression was demonstrated for the first time in *B. abortus* 2308. This table continues from the previous page, showing additional proteins along with their associated functional annotations.
these ORFs (Figure 1): BAB1_1205, BAB1_1645, BAB1_1646, BAB1_1768 and BAB2_0216. The MSMS spectra of the 18 peptides representing these former pseudogenes were manually validated. We thus investigated the reasons for which these genes had been annotated as pseudogenes. The genomic sequence of the cytoplasmic protein with a conserved DUF 883 domain BAB1_1205 was found to be identical to BMEI0805, its

| Locus tag   | Protein description       | Peptide sequence       | Protein sequence       |
|-------------|---------------------------|------------------------|------------------------|
| BAB1_1205   | Hypothetical protein      | AENINDIQKALEK          | QQLAEHFR               |
|             | (BMEI0805)                |                        |                        |
| BAB1_1645   | Dak phosphatase domain    | AGDWLTMERA               | VGVVTGGGSGHEPAFIGYTGK  |
|             | (BMEI0397)                |                        |                        |
| BAB1_1646   | Dak phosphatase domain    | AATGATLEVR               | LRPGILSDTGTMQF          |
|             | (BMEI0396)                |                        |                        |
| BAB1_1768   | Hypothetical protein      | TAYGGYGGAGAILAGGAAGGGNR | WSVLNMIAVAGLASSCTTIN     |
|             | (BMEI0827)                |                        |                        |
| BAB2_0216   | Enoyl-CoA hydratase       | QQLAEHFR               | VSVKIVVGYGAVTIN         |
|             | (BMEI1021)                |                        |                        |

Locus tags and descriptions of proteins are indicated and proteins are organized by predicted subcellular localization.
The second peptide, "TDLPIMK", was found to match the cytoplasmic Brucella melitensis keto-hydroxylglutarate-aldolase (BMEII0009) and then assigned to BAB2_0083 in B. abortus 2308. This peptide overlaps the region upstream to the currently annotated translation start site and the first three amino acids based on the annotated translation start site (Figure 2B). Alignment of the current B. abortus 2308 protein sequence with its counterparts in other Brucella strains and species indicates that the 2308 protein sequence is falsely truncated. Other start sites lead to proteins having N-terminals longer by 11, 26 or 44 amino acids. Although we cannot clearly indicate the actual start site of BAB1_1926 or BAB2_0083, we can confirm that their N-terminals are longer than currently annotated. Based on the homology of the B. abortus 2308 genome being highest with that of other Brucella strains, one can speculate that the start sites would be identical to those mapped in these strains.

Operons
Since genes that are part of an operon are usually co-transcribed, it is possible that these genes might also be cotranslated [32]. Considering all proteins identified by our studies, we were able to almost fully reconstitute one of the two ribosomal RNA operons, with all but BAB1_1237 found. Additionally, the previously mentioned BAB1_1645 and BAB1_1646 genes are predicted to be part of an operon containing 6 genes, BAB1_1645 to BAB1_1650 http://www.microbesonline.org/operons/gnc359391.html. Four of these proteins were detected in our studies, although only BAB1_1645, -46 and -48 were found in the same experimental condition.

Conclusions
Mass spectrometry has proven to be a valuable tool to identify and correct genomic annotation errors in the study of microorganisms [33-37]. We performed a proteomics analysis of B. abortus 2308 proteins expressed upon extracellular and intracellular growth conditions to validate existing gene predictions at the protein level, to
acquire useful information on B. abortus 2308 expressed proteins and to identify and correct inaccurately annotated ORFs. We were able to confirm the expression of over 300 previously unreported proteins and five pseudogenes, and corrected two wrongly assigned translation start sites. Taken together, these findings further demonstrate that computational genomic annotation errors can be corrected using proteomics. This will lead to improved databases and thus better protein identification and functional annotation.
Methods

Brucella abortus protein preparation for mass spectrometry analysis

Four types of B. abortus 2308 samples were prepared: outer membranes, cytosols, intracellular bacteria isolated from infected RAW264.7 macrophages and extracellular bacteria from overnight cultures. Outer membrane samples were prepared and processed for mass spectrometry analysis as previously described [16]. Cytoplasmic fractions were prepared as described previously [38]. Briefly, bacteria grown in tryptic soy broth (Difco) in 2-liter flasks on an orbital shaker and harvested by centrifugation in sealed cups at 7,000 × g for 20 min. The thick slurry of bacteria were suspended in 10 mM phosphate-buffered saline (pH 7.2) was passed twice through a French press (Pressure Cell 40 K, Aminco; SLM Instruments Inc., Urbana, Ill.) at an internal pressure of 35,000 lb/in². The homogenate was digested with 50 mg of DNase II type V and RNase A per ml (Sigma) for 18 h at 37°C and fractionated by ultracentrifugation. The cell envelopes in the bottom of the tube removed and the cytoplasmic fractions in the supernatant, filtered, lyophilized and characterized as described previously [39]. Intracellular bacteria were isolated from RAW264.7 macrophages 3, 20 and 44 hours post-infection as previously described [17]. Proteins were extracted from intracellular and extracellular bacteria using the same method and digested for mass spectrometry as previously described [17].

Liquid Chromatography - Mass Spectrometry (LC-MS)

Peptide digests were analyzed by liquid chromatography coupled to mass spectrometry (LC-MS) as described [40]. Briefly, the samples were injected onto a reversed-phase column (Jupiter C18, Phenomenex, Torrance, CA) for HPLC separation. For LC-MS survey scans, the mass spectra were acquired over 400-1600 Da at a rate of 1 spectrum/second. Peptide sequencing was achieved by targeted and shotgun LC-MS/MS. For MS/MS scans, the mass range was 50-2000 Da, and each spectrum was acquired in 2 seconds. For LC-MS/MS, the duty cycle was one survey scan followed by one product ion scan (MS/MS).

Protein identification

Protein identification was done by submitting LC-MS/MS spectra to Mascot software (MatrixScience, Boston, MA) and searching against custom protein databases (see below). The parameters used for the Mascot search and protein homology clustering were previously detailed [16]. No multidimensional fingerprinting method was used. Annotation for each protein was performed using ExPASy Proteomics tools http://us.expasy.org/tools/#proteome, Kegg GenomeNet Database Service http://www.genome.jp/ and literature mining of orthologous genes and proteins.

Protein databases

The databases were composed of protein sequences obtained from the National Center for Biotechnology Information (NCBI) protein database (for B. abortus 2308, NC_007618 and NC_007624; for B. melitensis 16 M, NC_003317 and NC_003318; for Mus musculus, all protein sequences contained under taxonomy ID 10090) and of B. abortus 2308 "pseudoproteins" corresponding to the custom translation of pseudogenes. Genomic regions corresponding to the 316 entries annotated as pseudogenes in NCBI were directly translated and added to the database. Additionally, the ORF Finder tool from NCBI was used to determine other possible protein sequences corresponding to the pseudogenes. The ORF search was done by including 0 to 200 bp upstream or downstream from these regions. All resulting ORFs spanning the entire pseudogene sequence were kept. Ribosome binding sites were mapped when possible according to the sequence described in reference [41]. A total of 471 translated protein sequences were added to the NCBI databases.

Validation of mass spectrometry results

Sequences assigned to MS/MS spectra of peptides, which were mapped to pseudogenes or to genomic regions annotated as untranslated regions, were manually validated. For proteins identified by a single peptide, manual validation of the spectra was performed for peptide sequences having a Mascot score below 45.

Prediction of protein localization

The localization of newly demonstrated proteins was predicted using PSORTb version 2.0.4 http://www.psort.org/psortb/index.html, CELLO version 2.5 http://cello.life.nctu.edu.tw/ and PSLpred http://www.imtech.res.in/raghava/pslpred/index.html. For a localization to be assigned, a minimum of 2 of the 3 predictions had to match.

Additional material

Additional file 1 Proteins newly demonstrated in B. abortus 2308.
Each entry is represented by a gene locus tag, description of the protein and the sequences of the peptides measured. Proteins are organized by predicted subcellular localization.

Authors' contributions

JL designed and coordinated the study, analyzed the data and wrote the manuscript. MB participated in the data analysis and manuscript writing. AF performed the mass spectrometry experiments and peptide validations. ACM participated in the data analysis. NN performed the protein identification steps. FT participated in the protein identification steps. IM participated in the data analysis and manuscript writing. EM participated in the data analysis and manuscript writing. EP conceived of the study and participated in manuscript writ-
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