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Extensive Cell Envelope Modulation Is Associated with Virulence in *Brucella abortus*

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Brucella virulence is linked to components of the cell envelope and tightly connected to the function of the BvrR/BvrS sensory-regulatory system. To quantify the impact of BvrR/BvrS on cell envelope proteins, we performed a label-free mass spectrometry-based proteomic analysis of spontaneously released outer membrane fragments from four strains of *Brucella abortus* (wild type virulent, avirulent *bvrR*$^-$ and *bvrS*$^-$ mutants as well as reconstituted virulent *bvrR*$^+$ (*bvrR*$^-$/p*bvrR*$^+$)). We identified 167 differentially expressed proteins, of which 25 were assigned to the outer membrane. Approximately half of the outer membrane proteins decreased in abundance, whereas half increased. Notably, expression of five Omp3 family proteins decreased whereas five lipoproteins increased in the mutant strains. In the periplasmic space, by contrast, approximately 80% of the 60 differentially expressed proteins were increased in at least one avirulent mutant. Periplasmic proteins are primarily involved in substrate uptake and transport, and a uniform increase in this class may indicate a nutritional stress response, possibly a consequence of defective outer membrane function. Virtually all proteins reverted to wild type levels in the reconstituted virulent *bvrR*$^+$ strain. We propose that the wide changes in cell envelope protein expression relate to the markedly avirulent phenotype of *bvrR*$^-$ and *bvrS*$^-$ mutants and that *Brucella* virulence depends on regulatory networks involving cell envelope and metabolism rather than on discrete virulence factors. This model may be relevant to other α-*Proteobacteria* harboring BvrR/BvrS orthologous systems known to be essential for parasitism or endosymbiosis.

Keywords: *Brucella abortus* • virulence • outer membrane • periplasm • proteomics

Introduction

Bacterial pathogenicity is often the result of the expression of virulence factors such as antigenic variation, exopolysaccharides, exotoxins, exoenzymes, fimbriae, flagella, and secretion systems.[1,2] Accordingly, horizontal acquisition of relatively few genes, often carried in plasmids, lysogenic phages, or assembled in pathogenicity islands, may turn otherwise attenuated strains into virulent forms. There are a number of intracellular bacteria, however, in which most or all of those factors are absent and in which virulence cannot be defined in these terms. Among these pathogens stand the members of the genus *Brucella*, the agents of brucellosis. *Brucella melitensis*, *Brucella abortus*, and *Brucella suis* are major causes of economic loss and human suffering as well as potential bioterrorism threats.[3]

In nature, brucellae behave as intracellular parasites that cause insidious and chronic infections, typically without the acute symptoms characteristic of most gram-negative pathogens.[4] Although not fully understood, *Brucella* virulence is linked to molecules that either act as key building blocks of the cell envelope (CE) or are assembled in it.[3,5] The *Brucella* lipopolysaccharide (LPS) lacks the pathogen-associated molecular pattern typical of many *Proteobacteria*, and this thwarts recognition by innate immunity elements like bactericidal peptides, complement, or TLR-4.[6] Phosphatidylcholine, which is typically eukaryotic, is the major *Brucella* phospholipid, and it cannot be replaced by other phospholipids without virulence loss.[7] Also indicative of the role of the *Brucella* CE is that LPS, periplasmic cyclic β-glucans, and the virB type IV secretion system, all localized to the CE, are involved in the control of the intracellular trafficking necessary to reach the endoplasmic reticulum-like vacuole where this pathogen multiplies.[6,8−10]

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B. abortus BvrR/BvrS, a sensory-regulatory system associated to the inner membrane controls the structure of the LPS lipid A[11−14] and the expression of Omp3a and Omp3b, two major *Brucella* outer membrane proteins (Omp). BvrR/BvrS mutants are highly sensitive to bactericidal peptides and complement and are unable to promote entry into host cells or multiply in them.[11,13,14] The fact that no other *Brucella* mutants, including those in LPS, Omp3a, and Omp3b genes, display such a complete loss of virulence demonstrates the critical role of BvrR/BvrS and strongly suggests that it controls other elements important in virulence. Unfortunately, our knowledge of the *Brucella* CE is incomplete and indirect.[5] Typical gram-negative bacterial fractionation procedures are not readily applicable because of the marked resistance the *Brucella* CE has to detergents, chelators, and cationic peptides.[15,16] However, growing *Brucella* cells spontaneously shed outer membrane fragments (OMF) in culture media without loss of viability.[17] These OMF are rich in LPS, native hapten polysaccharide (NH), phosphatidylcholine, group 3 Omp, and other unidentified proteins.[17−19] In the case of the intracellular Gram negative *Legionella* pathogen, which also replicates in compartments resembling the endoplasmic reticulum, it has been demonstrated that the OMF of this bacterium are capable of inhibiting the fusion of phagosomes with lysosomes.[20] Within this perspective, the analysis of OMF is relevant due to the paucity of factors involved in the control of *Brucella* intracellular trafficking. Therefore, to gain insight into the basis of *Brucella* virulence, which is linked to the outer membrane (OM) and requires a functional BvrR/BvrS, we have performed a high-resolution quantitative proteomic study of OMF from virulent *B. abortus*, *bvrR*$^-$, and *bvrS*$^-$ avirulent mutants, and a reconstituted virulent *bvrR*$^+$ (*bvrR*$^-$/p*bvrR*$^+$) strain. The purified OMF were digested by proteolysis to derive peptides which were separated by liquid-phase chromatography and analyzed using quantitative, label-free, mass spectrometry-based methods. We report that a surprisingly large number of proteins are altered in these mutants. Disruption of bvrR or bvrS resulted in a widespread decrease of *Brucella* Omp, many of which were not previously associated with virulence. We also observed an extensive increase in periplasmic proteins in both mutants suggestive of nutritional stress, a fact that was unexpected because the bvrR and bvrS mutants do not display obvious auxotrophic defects.[11] Most of the changes were reversed upon introduction of wild type BvrR. These results provide insights not only into the plasticity of the *Brucella* CE but also into bacterial virulence as a coordinated structural and physiological process.

Methods

OMF Production. OMF were generated and characterized as described.[12,13,17,18] Briefly, supernatants of exponentially growing *B. abortus* strains were clarified, centrifuged at 100 000× g for 6 h at 4 °C, pellets resuspended in double distilled water, and lyophilized. Analysis of the pellets revealed the presence of OMF, as demonstrated by electrophoretical, chemical, and immunochemical analysis, whereas the supernatants did not reveal significant amounts of residual proteins or LPS. Protein content, total fatty acids, NH, phospholipids, and LPS of the OMF were estimated as described.[16,17,21−24] Western blots were carried out as described.[12]

Antibodies. Rabbit anti-BvrR antibodies were generated using BvrR-GST antigen. The antibodies recognize a single polypeptide band in *B. abortus* lysates absent in a *B. abortus* bvrR− mutant.[25] Antibodies against LPS and NH were used as previously described.[18] Antibodies against Omp3a (Omp25) were obtained from Axel Cloeckaert (INRA Nouzilly, France).

OMF Processing. OMF powders were dissolved in 300 μL of water, vortex mixed, and sonicated, each for 30 s. Protein content was determined by BCA protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions. For each sample, 200 μg of protein were suspended in a final volume of 50 μL of water. The sample was sonicated, and 50 μL of 100 mM ammonium bicarbonate (Sigma-Aldrich, St. Louis, MO) containing 2% acid-labile surfactant[26] (ALS; Waters, Milford, MA) and 8 M urea were added. The sample was vortex mixed for 1 h. A sample of 50 μL of the OMF suspension was added to a final volume of 1 mL of a chloroform/methanol solution (2:1 v/v). The sample was vortex mixed and incubated at −20 °C for 2 h. Subsequently, 100 μL of cold methanol (−20 °C) was added, and the sample was cleared by centrifugation for 10 min at 21 000× g. The supernatant was dried under vacuum and resuspended in 4 M urea; 50 mM ammonium bicarbonate pH 8.0. Lys-C (Wako, Richmond, VA) was added at a 1:50 protein ratio for 4 h at room temperature. The samples were then diluted 4:1 with 50 mM ammonium bicarbonate buffer pH 8.0, and trypsin (Promega, Madison, WI) was added at a 1:25 protein ratio for an additional 16 h. Following proteolysis, the samples were distributed into 96-well plates for mass spectrometry analysis and entered into the Laboratory Information Management System (Nautilus LIMS, Thermo Electron, Woburn, MA). Sample plates were then lyophilized and stored at −80 °C.

Liquid Chromatography−Mass Spectrometry (LC−MS). Peptide digests were analyzed by liquid chromatography coupled to mass spectrometry (LC−MS). The LC−MS system consisted of a CapLC (Waters, Milford, MA) with a cooled autosampler and a QTOF Ultima (Waters, Milford, MA) controlled by MassLynx version 4.0 software. Samples were reconstituted in 15 μL of water/10% acetonitrile/0.1% formic acid solution and injected onto a reversed-phase (Jupiter C18, Phenomenex, Torrance, CA) column. For the reversed-phase HPLC separation, buffer A was water/0.2% formic acid, and buffer B was acetonitrile/0.2% formic acid. The gradient started at 10% B and was ramped linearly up to 60% B in 55 min. After holding at 60% B for 2 min, B was decreased to 10% for column re-equilibration before the next injection. For LC−MS survey scans, the mass spectra were acquired over 400−1600 Da at a rate of 1 spectrum/second. For MS/MS scans, the mass range was 50−2000 Da, and each spectrum was acquired in 2 s. For LC−MS/MS, the duty cycle was one survey scan followed by one product ion scan (MS/MS). Inclusion MS/MS spectra were acquired for target peptides selected by expression analysis (described below) and contained in inclusion lists. Tolerances for inclusion MS/MS acquisition of target peptides were ±1 min retention time and ±0.2 Da. The collision energy varied depending on the m/z as well as the precursor ion charge state.

Instrument performance was verified by injecting 10 μL of the peptide standards mixture. To prepare the standard sample, eight trypsin-digested proteins (carboxypeptidase A, deoxyribonuclease, bovine serum albumin, phosphorylase B, beta-galactosidase, peroxidase, glyceraldehyde-3-phosphate dehydrogenase, alcohol dehydrogenase) were obtained from Michrom Bioresources (Auburn, CA) and combined into a stock solution of 40 fmol/μL in 10% acetonitrile/0.2% formic acid. Performance characteristics were automatically generated following each run. The sensitivity was recorded in terms of the number

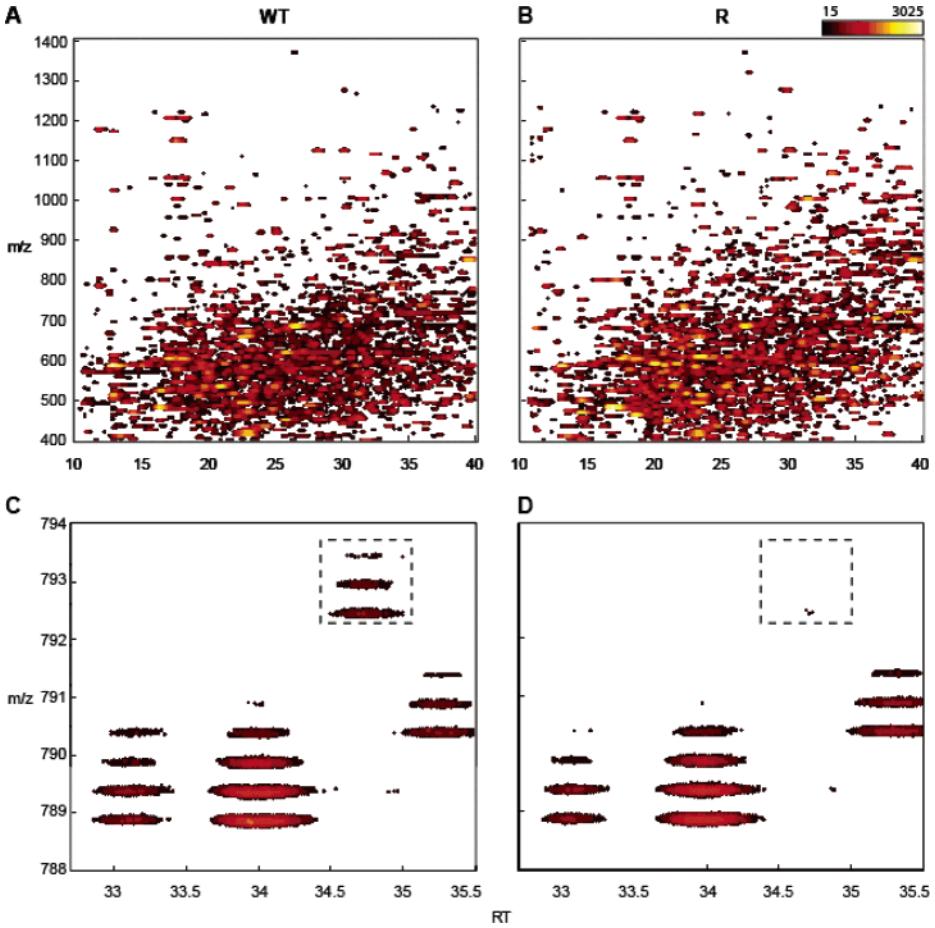


Figure 1. Representative isotope maps of peptides derived from processed OMF of wild type *B. abortus* 2308 (A) and *bvrR*$^{-}$ mutant *B. abortus* 65.21 (B). The *x*-axis indicates the chromatographic retention time (RT) in minutes, and the *y*-axis indicates the mass-to-charge ratio (*m*/*z*). Peak intensity is indicated by the color scale shown above (B). An enlarged area of (A) showing a peptide that is overexpressed in the wild type sample (dashed rectangle) compared to the corresponding area of the mutant sample in (B) are indicated in (C) and (D), respectively.

of multiply charged ions. The retention time and mass accuracy of two peptides in the standard samples were also recorded. Sample lists were generated by the LIMS and imported into MassLynx. Samples were injected sequentially, and replicates of samples to be compared were interleaved during analysis. The same mass spectrometer was used to analyze all the samples. As data were acquired from the mass spectrometer, they were automatically retrieved from the instrument computer to a central database where they were registered. The raw data was then converted into a three-dimensional isotope map format containing *m*/*z*, retention time, and intensity information.

Peptide Detection and Alignment. The first step in the LC−MS data analysis is peak detection, which is the process of detecting isotopic peaks in the LC−MS data. Peak detection is automatically applied to every LC−MS and LC−MS/MS analysis, and the peaks are represented as isotope maps. Examples of isotope maps are shown in Figure 1. Isotope maps are converted into peptide maps by Savitzky−Golay smoothing in both the *m*/*z* and retention time dimensions followed by peak fitting to a four dimensional (*m*/*z*, retention time, charge, and intensity) peptide isotope model. Smoothing parameters were chosen such that isotopic resolution was not diminished. The peptide detection model utilizes the difference in mass between peptide isotope peaks, retention time coincidence of peptide isotopes, and the expected intensity profile of a peptide's isotopes as a function of peptide mass. The peptide map output is a listing of the *m*/*z*, charge, retention time, and intensity of all peptides.

The peptide maps undergo normalization of retention time to correct for analytical variability. A dynamic and nonlinear correction algorithm for normalizing retention time across all comparable LC−MS and LC−MS/MS injections of a study is applied. First, a standard injection is selected by sorting all injections by their overall retention time offset and selecting the injection with median offset. Then the retention times of all of the other injections are normalized to the retention time of the standard injection. This software tool allows tracking between two or more LC−MS or LC−MS/MS injections, independent of the LC column or mass spectrometer, or the time of the analysis. This dynamic function is able to reduce the retention time variability, for each peptide, to less than 7 s.

Following normalization, peptides are matched across all samples in a study. Peptides are clustered according to mass, retention time, and charge using standard hierarchical clustering techniques adapted to the proteomics context. The process of peptide clustering, or grouping, of the same peptide observed in different samples across a study enables the detection of peptides that are differentially expressed. Once peptide clusters have been formed, a representative median mass and median retention time are calculated to represent the peptide cluster.

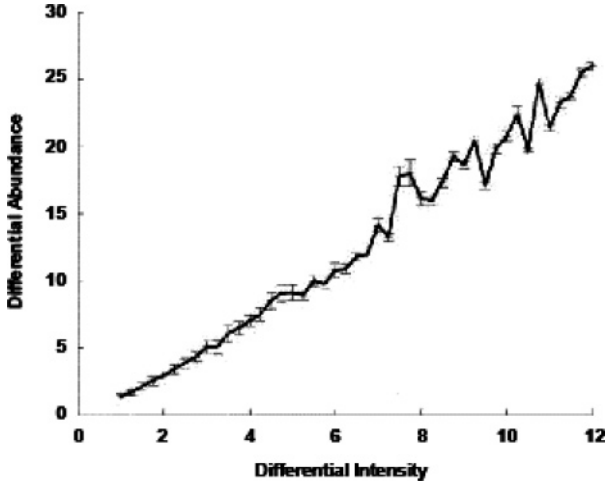


Figure 2. Correlation of peptide differential intensity with protein differential abundance. *x*-axis indicates differential intensity. *y*-axis indicates differential abundance. Presented are means and standard deviations of triplicate LC−MS measurements. The correlation coefficient is 0.99.

Linearity of Relative Differential Expression. An accurate correlation between the observed peptide ion intensity and the actual protein abundance is a fundamental requirement of mass spectrometry-based differential protein expression profiling. An empirical relationship between the observed differential peptide intensity and the actual differential peptide abundance was derived by spiking peptides having a range of concentrations into a complex biological sample. Tryptic digests of phosphorylase B, bovine serum albumin, and alcohol dehydrogenase were obtained from Michrom Bioresources (Auburn, CA). Each protein was diluted to a stock concentration of 1 pmol/μL in 2% acetonitrile/0.2% formic acid. These three proteins were spiked into aliquots of pooled purified plasma membrane isolated from human colon tumor tissue at 14 different final concentrations ranging from 0.25 to 100 fmol/μL. Sixty peptides (20 from each protein) were tracked across three replicates of each of the spiked samples. Differential abundance ratios between all pairs of concentrations were then correlated to all pairs of differential peptide ratios, for all peptides. A linear relationship between actual differential abundance and observed differential intensity was then obtained. The correlation coefficient was 0.99 (Figure 2), suggesting an accurate measurement of differential protein abundance in a complex biological matrix. For a given peptide, the LC−MS instrument response is linear up to the point of detector saturation; i.e. the intensity of the signal reaches the dead time of the time to digital converter. Provided that samples of equivalent complexity are compared, peptide differential intensity corresponds to peptide (and therefore the corresponding parent protein) differential abundance. Thus, relative peptide abundance (dA) can be deduced from the peptide intensity ratios (dI) using the equation dA = 2.2168*dI − 1.7132 for dI values less than 6-fold and using the equation dA = 1.9311*dI − 1.0523 for those greater than 6-fold.

Expression Analysis. The expression analysis involves the selection of significantly and reproducibly differentially expressed peptides. Peptides whose intensity profiles differed between any 2 strains were identified using an unpaired *t*-test and were statistically significant with a *p*-value less than 0.00001. Permutation tests were applied to determine the false discovery rate (FDR)[27] of differentially expressed peptides at this stage. Unsupervised clustering and dimensional reduction analysis tools such as principal component analysis (PCA) were employed to visualize differences between strains and to detect bias.[28] The greater the dissimilarity between samples, the larger is the distance between them. These calculations were performed using GeneLinker software (Improved Outcomes Software, ON, Canada). Peptides demonstrating a statistically significant change in expression level were targeted for sequencing by LC−MS/MS. For this purpose, the target peptides were compiled into inclusion lists containing retention time, charge state, and m/z for each target peptide. By comparing the sequenced peptides in the MS/MS analyses with the peptides shown to be differentially expressed in the MS survey scans, a match can be made between the two. Peptides identified by LC−MS/MS were thus linked to the target peptide.

Intensity values for the 4 strains (WT, *bvrR*$^-$, *bvrS*$^-$, *bvrR*$^-$/*pbvrR*$_+$) were calculated for each differentially expressed peptide successfully acquired by LC−MS/MS. First, a baseline noise value was removed from all the raw intensities. The median peptide intensity was calculated per strain and in each case normalized to the median intensity of the same peptide in the wild-type strain, *B. abortus* 2308. Relative abundance (dA) can be deduced from the intensity ratios (dI) using an experimentally determined formula as described earlier.

Protein Identification. All LC−MS/MS spectra were submitted to Mascot (MatrixScience, Boston, MA) for database searching against the National Center for Biotechnology Information protein database (NCBI) to assign sequences to peptide MS/MS spectra. Mascot parameters specify trypsin proteolysis with one allowed missed cleavage and with variable modification of methionine (oxidation) and glutamine (deamidation). Mass tolerances were 0.25 Da for both precursor and fragment ions. The identified proteins were clustered by sequence homology using BlastClust at 95% homology over 50% of the sequence length. Homology clustering groups proteins that are likely redundant but are not differentiated by the identified peptides. The correlation coefficients of expression patterns of peptides belonging to the same protein were calculated. To evaluate the significance of the correlation, *P*-values were calculated based on the distribution of correlation coefficients for all sequenced peptides in the study. This information was used to remove any peptides that were potentially incorrectly assigned to a protein.

Results

OMF Peptide Profiles Segregated Virulent and Avirulent Strains. OMF from virulent *B. abortus* 2308 (WT), avirulent *bvrR*::Tn5 (*bvrR*$^-$) and *bvrS*::Tn5 (*bvrS*$^-$) mutants, the reconstituted virulent *bvrR*$^+$ strain (*bvrR*$^-$ carrying WT *bvrR*),[13] and *B. abortus* S19, a vaccine strain not derived from 2308, were digested and analyzed by quantitative label-free mass spectrometry. All of the OMF from the different *B. abortus* strains analyzed showed similar overall composition, containing 40−42% LPS, 17−18% NH, 23−26% lipids, and 15−17% protein. Enzymatic, immunochemical and two-dimensional gel analysis of all the OMF revealed major quantities of OM molecules but failed to demonstrate significant quantities of cytoplasmic components[12] (Guzmán-Verri 2002 Ph.D. Thesis, available at http://diss.kib.ki.se/2002/91−7349-114−4/; Sola-Landa, A. 2000 Ph.D. Thesis, University of Navarra, Pamplona, Spain). Mass spectrometry revealed approximately 8680 peptidic ions. Each ion was characterized by its m/z (mass/charge) ratio, its charge, its chromatographic retention time, and importantly, its in-

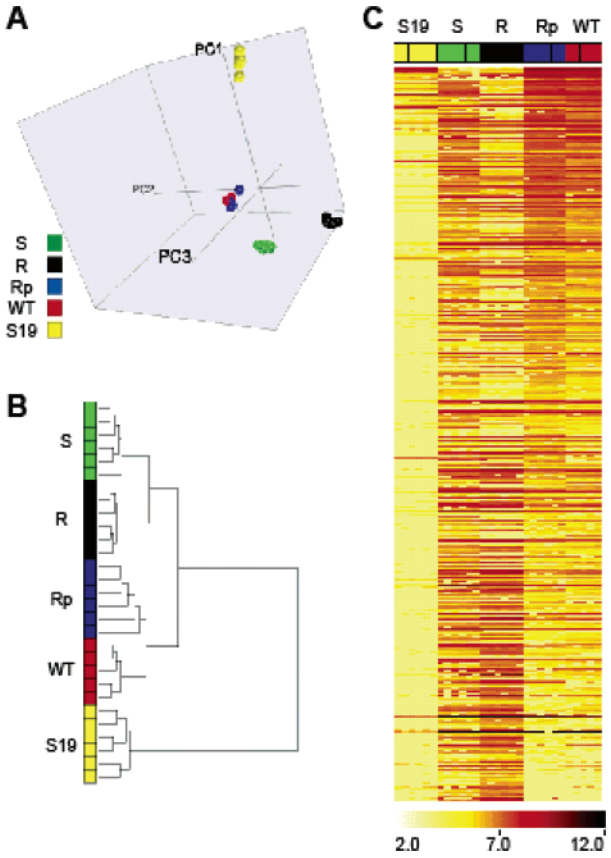


Figure 3. Comparative proteomic analysis. Peptides derived from the OMF of *B. abortus* strains were analyzed by mass spectrometry ($N = 5-6$ replicates per strain). (A) Principle component analysis was performed using the intensity values of all the peptide ions detected. Unsupervised sorting of blinded replicates along 3 axes of variance (PC1−PC3) was conducted. (B) Dendrograms were generated by hierarchical clustering using Pearson correlation and average linkage on the intensities of all the peptides detected. (C) Heat map analysis. Replicates are indicated along the *x*-axis and peptides are indicated along the *y*-axis. Log2 values of the intensities of 544 peptides that were differentially expressed in any two-strain combinations are displayed. WT, *B. abortus* 2308; S, *bvrS*$^-$; R, *bvrR*$^-$; Rp, *bvrR*$^+$; S19, *B. abortus* S19.

tensity. Unsupervised principle component analysis of the peptides clearly separated the different strains, indicating that the compositions of the OMF were distinct (Figure 3A). Dendrogram analysis showed that the OMF from the 2308-derived strains were more closely related to each other than to S19 OMF. Moreover, the OMF of the two avirulent mutants (*bvrR*$^-$ and *bvrS*$^-$) were more closely related to each other than those of the two virulent strains (WT and *bvrR*$^+$), which were also closely related (Figure 3B). Comparison of peptide intensities across strains identified those that were differentially expressed (Figure 3C). The average FDR for each pairwise comparison was 1.5%. These data suggested that disruption of *bvrR* or *bvrS* resulted in large scale but non-identical OMF protein changes and that reintroduction of WT *bvrR* into the *bvrR*$^-$ mutant reversed most of these changes.

CE Protein Changes in Avirulent *bvr*$^-$ Mutants. Protein sequences identified by Mascot search of the NCBI database were clustered together based on pairwise sequence alignment using the NCBI BlastClust. This resulted in the identification of 167 differentially expressed proteins (full list in Supplemental Table 1, Supporting Information). The changes were not identical for *bvrS*$^-$ and *bvrR*$^-$ mutants. Overall, 88% of the proteins that changed in the *bvrS*$^-$ strain also changed in the *bvrR*$^-$ strain indicating that, as expected, BvrS and BvrR belong to the same pathway. However, only 51% of the proteins that varied in the *bvrR*$^-$ strain also changed in the *bvrS*$^-$ strain, suggesting a broader impact for the regulatory element. Among the differentially expressed proteins, 25 were assigned to the OM, 60 to the periplasmic space, 13 to the inner membrane (IM), 56 to the cytoplasm, and 13 were of unknown localization. The average peptide coverage for the differentially expressed OM or periplasmic proteins was approximately 3 peptides, almost twice the average coverage obtained for IM and cytoplasmic proteins. There were, however, some differentially expressed proteins identified that could be found in multiple compartments. For example, TufA (EF-Tu) and DnaK have been reported to be in both the cytoplasm and the periplasm of *E. coli*, where they appear upon translocation via the MscL channel,[30] which has a *Brucella* homologue (ORF BR0318). Similarly, GroEL has been found in the periplasm and surface of *Legionella* and *Brucella*[31,32], and GroES homologues in the periplasm.[33] Also worth noting, 26 of the 56 cytoplasmic proteins identified were ribosome-related and may represent membrane-docked ribosomes involved in CE biogenesis.

As expected, BvrR was not detected in the *bvrR*$^-$ strain and was at comparable levels in the OMF of WT and *bvrS*$^-$ strains (Figure 4). In the *bvrR*$^+$ strain, which contains a multi-copy *bvrR*$^+$ plasmid, BvrR peptide intensities were fivefold higher than in the WT. These results were verified by Western blot (Figure 4). Among the differentially expressed OM proteins, particularly interesting were the group 3 Omp, comprised of several highly immunogenic proteins[34−36] that have been predicted to be highly expressed in *Brucella* and other α-*Proteobacteria*.[37] Omp3a and Omp3b peptide intensities were 5 to 10 times lower in the avirulent strains, consistent with previous results.[12] The changes in Omp3a levels were also confirmed by Western blot (Figure 4). Omp31b was also reduced by a factor of 3 in both avirulent mutants, while Omp3c and OmpW were reduced mainly in the *bvrS*$^-$ strain (Figure 4). In total, 12 of the 25 OM assigned proteins were reduced in one or both avirulent mutants (Figure 5), including Omp groups 1 to 3, OmpA, and the metal uptake FrpB-like protein. Conversely, lectin-like BA14[38] and most lipoproteins were augmented in both mutants (Figure 5). Virtually all Omp reverted to WT levels in the reconstituted *bvrR*$^+$ strain.

In contrast to the Omp changes in the mutant strains, most of the differentially expressed proteins assigned to the periplasm were increased (Figure 6). Of the 60 differentially expressed periplasmic proteins identified, approximately 80% were increased in at least one avirulent mutant, and 75% returned to WT levels in the reconstituted *bvrR*$^+$ strain. Avirulent mutants showed a substantial increase in chaperones, peptidoglycan processing proteins, and transporters such as the inorganic ABC transporter family (Figure 7). Most of the members of this family were increased in both avirulent strains compared to the virulent strains, and a minority appeared to be substantially increased in only one of the two avirulent strains (Figure 7). A number of periplasmic proteins without predicted function displayed similar expression patterns. Metal uptake and oxidative stress proteins, however, appeared to be reduced in the avirulent strains.

Some known OM and periplasmic *Brucella* proteins were not detected. This was expected for Omp25b, Omp31, and Omp2a because their genes are truncated or deleted in *B. abortus*.[36,39,40]

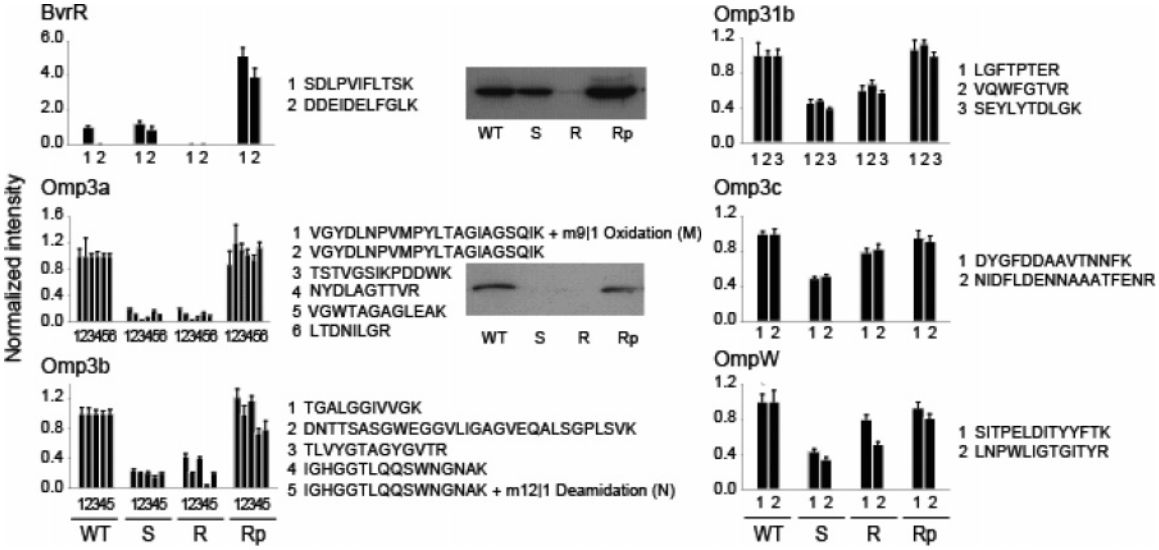


Figure 4. Expression changes in BvrR and group 3 Omp. The intensities of each indicated peptide were normalized to the WT strain. Median intensities were calculated using from 5 to 6 replicates. All peptides were differentially expressed with a statistical significance of $p < 0.05$ when compared between *bvrS*$^-$ and *bvrR*$^-$ and virulent WT and *bvrR*$^+$ strains. Western blots for BvrR and Omp3a were performed using aliquots of identical samples used for the proteomic analysis. WT, *B. abortus* 2308; S, *bvrS*$^-$; R, *bvrR*$^-$; Rp, *bvrR*$^+$.

For other proteins, such as the VirB system and flagella-like components, Omp25d, Omp10, Omp85-like, their absence may relate to limited expression or to exclusion during OMF genesis. However, because Omp10 and VirB8 were detected in all OMF by Western blot (not shown), it is likely that these proteins were insufficiently differentially expressed to be targeted by this analysis. A list of all the differentially expressed proteins identified in this analysis, including the sequences and intensities of the individual peptides measured, are listed in Supplemental Table 1 (see Supporting Information).

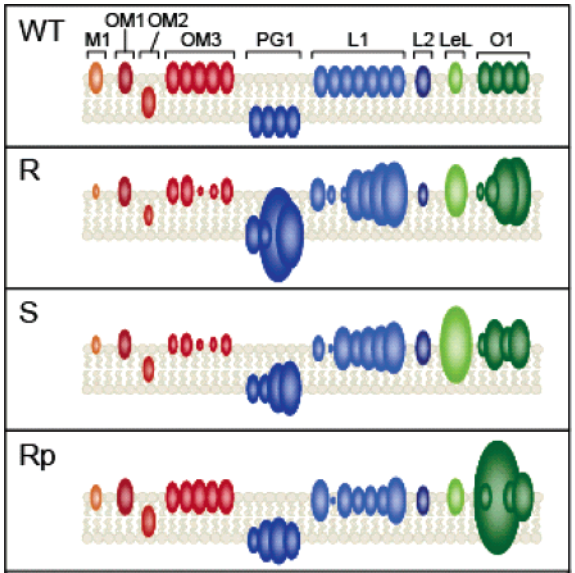
Discussion

Bacterial OMF are released by a spontaneous process that seems to be related to the turnover of the cell wall, generating blebbing of the OM and the concomitant capture of most surface and periplasmic components.[42] Previous studies have demonstrated that *Brucella* OMF are suitable for characterizing differences in LPS and NH structures, phospholipids, Omp, and periplasmic components.[12,13,17−19] Moreover, it seems that OMF are not only released during growth in bacteriological media but also inside cells and that some of their components may play a significant role in virulence by inhibiting the fusion of phagosomes with lysosomes.[20,43] This suggests that the OMF are physiologically relevant structures with a role in the establishment of a *Brucella* infection.

Several lines of evidence indicate that the cell envelope, and the OM in particular, is a structure of central importance for *Brucella* virulence. In this study, we performed large scale quantitative proteomic analysis of the protein expression changes in cell envelopes of virulent and non-virulent *B. abortus* strains. We selected strains defective in the BvrR/BvrS system, which is orthologous to systems essential for parasitism or endosymbiosis in bacteria such as *Bartonella*, *Agrobacterium*, *Mesorhizobium*, and *Sinorhizobium*.[12,44] In *Brucella*, BvrR/BvrS dysfunction generates attenuation that appears to be linked to OM defects. Therefore, an extensive comparative proteomic study of the outer layers of virulent and attenuated *bvrS*$^-$ and *bvrR*$^-$ mutants should help better understand intracellular persistence, not only of *Brucella*, but also in other α-*Proteobacteria* associated with eukaryotic cells. Furthermore, to better correlate the differentially expressed proteins to virulence, cell envelopes from wild type *B. abortus*, as well as from a reconstituted virulent *bvrR*$^-$ strain in which wild type BvrR was introduced, were also analyzed.

We identified 25 Omp and 60 periplasmic proteins whose expression levels are influenced by BvrR/BvrS. Half of the differentially expressed Omp decreased in one or both avirulent mutants. The other half, mainly lipoproteins, had almost an opposite pattern of expression. These changes produce an altered OM topology in the BvrR/BvrS mutants that could modify surface properties relevant for virulence. For example, the augmented binding of *bvrS*$^-$ and *bvrR*$^-$ to cells[11] may be due to the higher expression of lectin-like BA14. The inability of the mutants to promote their entry into some cells and to recruit small GTPases[45] possibly relates to the severe reduction in group 3 Omp and the increased amounts of lipoprotein ligands, respectively. The sensitivity to complement and bactericidal peptides, and the altered lipid A patterns of BvrR/BvrS mutants may result from a disruption of group 3 Omp-LPS interactions and LPS synthesis/translocation impairment.[11,13,16]

Group 3 Omp have been proposed to be essential for *Brucella* OM integrity due to their tight association with LPS.[5] They are predicted to be highly expressed[37] and generate a strong antibody response in infected animals.[34,45] Interestingly, the absence of a single group 3 outer membrane protein seems to be compensated by an increase in other family members,[36] and thus, the absence of a single group 3 outer membrane protein does not generate the attenuation of the BvrR/BvrS mutants.[14,46] Omp3a and Omp3b are known to be transcriptionally regulated by BvrR/BvrS.[12] The expression changes reported here for the whole family suggests that the expression of the whole family may be coordinated. It is thus possible that the redundancy noted for the group 3 Omp may be an adaptation to preserve a function required for entry into host cells and intracellular persistence. Therefore, it is possible that the function accomplished by group 3 Omp is critical for *Brucella* virulence despite the results obtained with single member mutants.[14,46] Other α-*Proteobacteria* with BvrR/BvrS orthologues also contain



Family	Accession	Protein description
Metal uptake		
M1	62318005	FrpB-like protein
Major outer membrane proteins		
OM1	62290067	Omp1
OM2	62289600	Omp2b porin
OM3	62290446	Omp W
	62289100	Omp3c
	62289663	Omp3a
	62290190	Omp3b
	62290498	Omp31b
Peptidoglycan binding / processing		
PG1	62289532	Ybis-like protein 2
	62289036 / 62289117	Ybis-like protein 1
	62290567	Omp16
	62290315	ComL-like protein
Lipoproteins		
L1	62289693	Omp 8K
	62290116	Omp 22K
	62289833	NlpD-like protein
	62289568	LipA
	62290784	Omp19
	62289467	Omp 21K
	62289724	SmpA-like protein
L2	62290115	OmpA family protein
Other		
LeL	62317424 / 62317556	BA14
O1	62289670	Omp16K
	62289035	Omp160
	62289332	InvB-Like protein 1
	62289451	InvB-Like protein 2

Figure 5. Outer membrane protein changes. Differentially expressed outer membrane proteins are represented as ellipses in each of the 4 strains analyzed. The relative expression of each protein is normalized to WT and is indicated by the area of the ellipses, which was calculated per protein using the median intensity of the individual peptides measured. The proteins are color-coded into the families shown. Names and accession numbers for each protein are indicated. The names of the proteins correspond from top to bottom to the ellipses from left to right per family. The proteins in the O1 family are not related. The sequences and intensities of the peptides detected for each protein are listed in Supplemental Table 1 (see Supporting Information). WT, *B. abortus* 2308; S, *bvrS*$^{-}$; R, *bvrR*$^{-}$; Rp, *bvrR*$^{+}$.

group 3 Omp orthologues. These genes have high $E(g)$ values, defined as the estimated expression level of gene g according to the frequency of its codon usage.[37] It is therefore feasible to propose a similar regulation and critical role in the interaction with host cells in those *Brucella* relatives that are intracellular symbionts or pathogens.

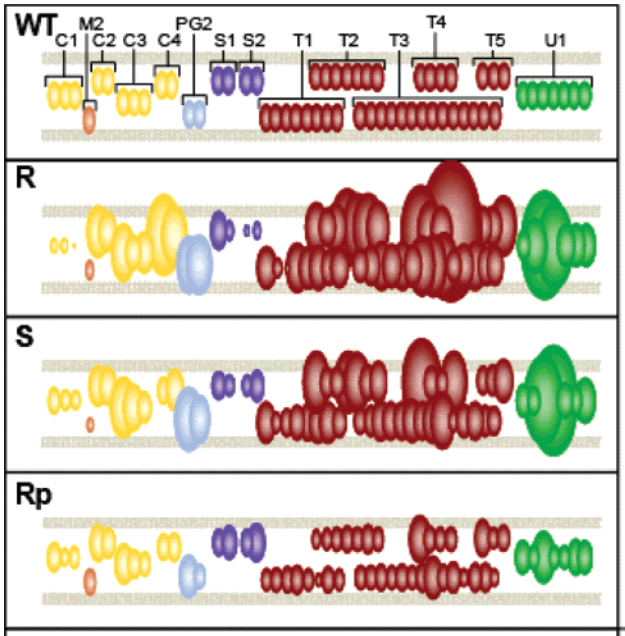
In contrast to the changes in Omp expression, the majority of periplasmic proteins, ABC transporters and chaperones in particular, increased in the *bvrR*$^{-}$ and *bvrS*$^{-}$ mutants. These changes suggest a state that mimics nutrient deprivation, even though the *bvrR*$^{-}$ and *bvrS*$^{-}$ mutants do not demonstrate obvious auxotrophic defects. Therefore, in addition to its defects affecting entry into host cells, the compromised OM may also be defective in nutrient transport, which drives a compensatory response in the periplasm of cultured bacteria. Consequently, in addition to an important role in the control of OM changes related to adherence and invasion, it seems likely that the BvrR/BvrS controlling elements are directly or indirectly involved in adjusting the metabolism of *Brucella* to the nutrient shift expected to occur during the transit to the intracellular niche. *In vitro* and genomic analyses indeed suggest a connection of BvrR/BvrS with metabolism.[47] Moreover, the genes corresponding to ABC transporters have high $E(g)$ values in *Brucella* and several α-*Proteobacteria*[37] indicative that they are readily expressed in response to environmental changes. So far, we have not detected polar effects on genes downstream of *bvrS* and *bvrR* (ref 11 and unpublished results). Therefore, it is unlikely that other genes located downstream of *bvrS*, such as *hprK* involved in carbohydrate metabolism in other bacteria, are directly affected in the *B. abortus* Bvr mutants. On this basis, we propose that BvrR/BvrS is directly or indirectly involved in the regulation of *Brucella* metabolic networks.

A number of other differentially expressed proteins were identified that may be present in multiple bacterial compartments or be loosely associated with the CE. The differentially expressed ribosomal proteins identified in the OMF are likely to represent ribosomes actively associated with the CE because OMF generation seems related to CE biogenesis.[17,42] Others, like some chaperones, may in fact be in several compartments, including the periplasm.[30−33] Overall, however, the amount of IM and cytoplasmic components present in OMF was relatively low as judged by the quantities of OM LPS and NH. Previous studies failed to detect inner membrane markers like NADH-cytochrome C-oxidoreductase or succinate dehydrogenase,[17,34] and the spontaneous release of OMF and gentle isolation procedure should ensure minimal cytoplasmic leakage and prevent the contamination that follows cell disruption.

Although our results do not distinguish proteins directly or indirectly regulated by BvrR/BvrS, the extent of CE changes was surprising and far larger than previous observations.[11−14] The results suggest that the expression of a significant proportion of OM and periplasmic proteins can be influenced by the BvrR/BvrS system. A recent proteomic study identified 6 Omp and 4 periplasmic proteins in *B. abortus* 2308 CE extracts.[48] The expression levels of 5 of the Omp and 2 of the periplasmic proteins, respectively, were shown to be modulated by the BvrR/BvrS system in this study. Thus far, however, only 4 of the 25 differentially expressed OM proteins[14,36,50] and 6 of the 60 differentially expressed periplasmic proteins[51−56] reported here have been tested for a connection with virulence and in no case an effect as marked as that of BvrR/BvrS dysfunction has been noted.

Conclusions

These results, and previous findings,[11−14] support the proposal that the role of the BvrR/BvrS system is to tune the CE



Family	Accession	Protein description
Chaperones		
C1	62317142	GroEL
	62290965	DnaK
	62317143	GroES
C2	62290796	PpiI
	62289646	PpiI SurA-like protein
C3	62289573	Protease Do
	62290699	CtpA-like protein
	62289477	DsbA-like protein
C4	62290565	TPR domain protein 2
	62317211	TPR domain protein 1
Metal binding		
M2	62290360	Iron binding
Peptidoglycan binding / processing		
PG2	62289715	Peptidoglycan processing
	62290691	MltB
Oxidative stress		
S1	62317454	SodC
	62289534	SodA
S2	62289459	AhpC
	62317450	AhpD
ABC transporters - sugars, polyalcohols and related		
T1	62317463	P39 sugar binding
	62289205	Sugar binding
	62317414	Sugar binding
	62289086	Sugar binding
	62317809	D-galactose binding
	62290506	Rhizopine or inositol-related binding
	62289515	D-ribose binding
	62317312	D-ribose binding
ABC transporters - inorganic		
T2	62317456	Fe binding
	62317071	Fe binding
	62290977	PstS phosphate binding
	62289088	Sulfate binding
	62290231	Sulfate binding
	62317047	Molybdenum binding
	62317215	NlpA metal ion binding

Family	Accession	Protein description
ABC transporters - oligopeptides, amino acids, amines		
T3	62317600	AgpA oligopeptide binding
	62317599	Oligopeptide binding
	62317842	Peptide binding
	62316984	Branched amino acid binding
	62318008	Branched amino acid binding
	62317222	Branched amino acid binding
	62290651 / 62290653	Branched amino acid binding
	62290491	Spermidine-putrecine binding
	62290628	Thiamine binding
	62290460	Glycine-betaine-L-proline binding
	62317421	Glycine-betaine-L-proline binding
	62317472	Amino acid binding
	62289197	Glycine-betaine-L-proline binding
	62317516	Oligopeptide-glutamate binding
Non-ABC transporters		
T4	62290245	TonB component
	62317478	Fe-aguibactin binding
	62317721	Trs-Metal Fe binding
	62290235	Metal Ca binding
Transporters - Tol components and non predicted		
T5	62290569	TolB
	62290105	Trap-like protein
	62290897	ABC, unknown substrate
Unknown function		
U1	62290366 / 62196498	BP26
	62290196	Conserved protein, α-P
	62289748	Conserved protein, α-P
	62289425	Conserved protein, α-P
	62290537	Conserved protein, α-P
	62289776	Brucella protein
	62290367	Brucella protein

Figure 6. Periplasmic protein changes. Differentially expressed periplasmic proteins are represented as ellipses in each of the 4 strains analyzed. The relative expression of each protein is normalized to WT and is indicated by the area of the ellipses which was calculated per protein using the median intensity of the individual peptides measured. The proteins are color-coded into the families shown. Names and accession numbers for each protein are indicated. The names of the proteins correspond from top to bottom to the ellipses from left to right per family. The proteins in the U1 family are not related. Conserved protein α-P denotes proteins conserved in α-*Proteobacteria*. The sequences and intensities of the peptides detected for each protein are listed in Supplemental Table 1 (see Supporting Information). WT, *B. abortus* 2308; S, *bvrS*$^{-}$; R, *bvrR*$^{-}$; Rp, *bvrR*$^{+}$.

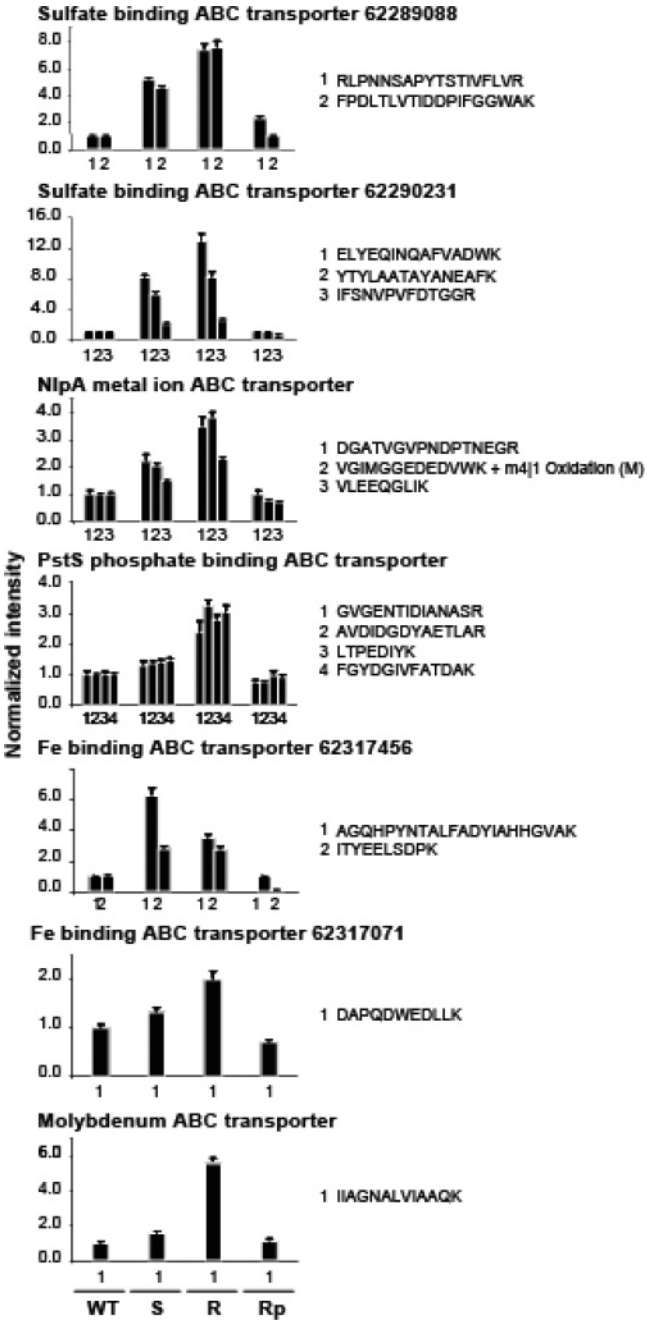


Figure 7. Expression changes in inorganic ABC transporter periplasmic proteins. The intensities of each indicated peptide were normalized to the WT strain. Median intensities were calculated using from 5 to 6 replicates. All peptides were differentially expressed with a statistical significance of $p < 0.05$ when compared between *bvrS*$^-$ and *bvrR*$^-$ and virulent WT and *bvrR*$^+$ strains. WT, *B. abortus* 2308; S, *bvrS*$^-$; R, *bvrR*$^-$; Rp, *bvrR*$^+$.

structure and physiology to the environmental changes occurring in the transit to the intracellular niche. This perspective suggests that *Brucella* virulence should not be related to single molecules but viewed as a multicomponent phenomenon in which many discrete elements participate in a coordinated adaptation to the intracellular environment. Our results, therefore, shed light on *Brucella* virulence but may also provide a model of structural and physiological elements relevant for the adaptation of other eukaryotic cell-associated α-*Proteobacteria* to their respective hosts.

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Supporting Information Available: Supplemental Table 1 lists the sequence of each peptide identified per differentially expressed protein and includes median peptide intensity and standard deviation in each of the four bacterial strains analyzed. This material is available free of charge via the Internet at http://pubs.acs.org.

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