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Analyses of *Brucella* pathogenesis, host immunity, and vaccine targets using systems biology and bioinformatics

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Brucella is a Gram-negative, facultative intracellular bacterium that causes zoonotic brucellosis in humans and various animals. Out of 10 classified *Brucella* species, *B. melitensis*, *B. abortus*, *B. suis*, and *B. canis* are pathogenic to humans. In the past decade, the mechanisms of *Brucella* pathogenesis and host immunity have been extensively investigated using the cutting edge systems biology and bioinformatics approaches. This article provides a comprehensive review of the applications of Omics (including genomics, transcriptomics, and proteomics) and bioinformatics technologies for the analysis of *Brucella* pathogenesis, host immune responses, and vaccine targets. Based on more than 30 sequenced *Brucella* genomes, comparative genomics is able to identify gene variations among *Brucella* strains that help to explain host specificity and virulence differences among *Brucella* species. Diverse transcriptomics and proteomics gene expression studies have been conducted to analyze gene expression profiles of wild type *Brucella* strains and mutants under different laboratory conditions. High throughput Omics analyses of host responses to infections with virulent or attenuated *Brucella* strains have been focused on responses by mouse and cattle macrophages, bovine trophoblastic cells, mouse and boar splenocytes, and ram buffy coat. Differential serum responses in humans and rams to *Brucella* infections have been analyzed using high throughput serum antibody screening technology. The Vaxign reverse vaccinology has been used to predict many *Brucella* vaccine targets. More than 180 *Brucella* virulence factors and their gene interaction networks have been identified using advanced literature mining methods. The recent development of community-based Vaccine Ontology and Brucellosis Ontology provides an efficient way for *Brucella* data integration, exchange, and computer-assisted automated reasoning.

Keywords: *Brucella*, pathogenesis, host immunity, vaccine, systems biology, bioinformatics

INTRODUCTION

Brucella abortus is a Gram-negative, facultative intracellular bacterium that causes brucellosis in humans and many animals (Corbel, 1997). The brucellae are taxonomically placed in the alpha-2 subdivision of the class Proteobacteria. There are 10 species of *Brucella* based on preferential host specificity: *B. melitensis* (goats), *B. abortus* (cattle), *B. suis* (swine), *B. canis* (dogs), *B. ovis* (sheep), *B. neotomae* (desert mice), *B. cetacea* (cetacean), *B. pinnipedia* (seal), *B. microti* (voles), and *B. inopinata* (unknown) (O'Callaghan and Whatmore, 2011). Of 10 recognized species of *Brucella*, *B. abortus, B. melitensis, B. suis*, and *B. canis* are pathogenic to humans. Human infections with *B. canis* are rare. *B. abortus, B. melitensis,* and *B. suis* are the most pathogenic to humans, have been identified as agents amenable for use in bio-terrorism, and are listed as category B priority pathogens by the US Center for Disease Control (CDC). Brucellosis is one of the most common zoonotic diseases. It infects annually approximately 500,000 humans worldwide. Upon entry into human or animals, the bacteria invade the blood stream and lymphatics where they multiply inside phagocytic cells and eventually cause septicemia. Symptoms include undulant fever, abortion, asthenia, endocarditis, and encephalitis.

Brucella lacks well-known bacterial virulence factors such as cytolysins, capsules, exotoxins, secreted proteases, fimbriae, phage-encoded toxins, and virulence plasmids (DelVecchio et al., 2002; Paulsen et al., 2002). The brucellae infect phagocytic macrophages and non-phagocytic epithelial cells (e.g., HeLa cells) *in vivo* and *in vitro* (Ko and Splitter, 2003; Kohler et al., 2003; Roop et al., 2004). *Brucella* virulence relies on the ability to survive and replicate in the vacuolar phagocytic compartments of macrophages. Many *Brucella* virulent factors, such as lipopolysaccharide (LPS; Lapaque et al., 2005), type IV secretion system (T4SS; O'Callaghan et al., 1999; de Jong et al., 2008), and the BvrR/BvrS two-component system (Guzman-Verri et al., 2002), have been identified to be critical in the intracellular process of *Brucella* inside macrophages (Xiang et al., 2006). While these virulence factors may not directly mediate clinical manifestations of brucellosis, they are critical for *Brucella* to survive and replicate inside host cells. While prolonged persistence of the brucellae in macrophages leads to the chronic infection, extensive replication of the bacteria in placental trophoblasts results in acute reproductive tract pathology and abortion in natural hosts (Roop et al., 2009). Specifically, the *Brucella* lifecycle contains two phases: (i) chronic infection

of phagocytic macrophage leading to *Brucella* survival and replication, and (ii) acute infection of non-phagocytic epithelial cells leading to reproductive tract pathology and abortion. Spleen and liver are the organs that contain many bacterial cells after *Brucella* invasion. After a majority of *Brucella* cells are killed *in vivo*, the remaining *Brucella* cells will persist and live for a long time *in vivo* (Hort et al., 2003).

Although antibodies specific for the O-antigen (i.e., O polysaccharide or O-side chain) of the lipopolysaccharide can confer partial protection in some host species, cell-mediated immunity (CMI) plays a critical role in protection against virulent *Brucella* infection. The maturation and proinflammatory production of cytokines of dendritic cells is critical for controlling *Brucella* infections (Macedo et al., 2008). Recently we found that *B. abortus* vaccine strain RB51 and *B. suis* vaccine candidate VTRS1 induce caspase-2-mediated apoptotic and necrotic macrophage cell death (Chen and He, 2009; Chen et al., 2011). The programmed cell death is inhibited by virulent *Brucella* strains. Caspase-2-mediated cell death induced by vaccine strain RB51 may promote an effective *Brucella* antigen presentation by a cross-priming mechanism (Bevan, 2006; Chen and He, 2009). Passive transfer assays with mice suggest that both $CD4^+$ and $CD8^+$ T cells are important in protective immunity against brucellosis (Araya et al., 1989; Araya and Winter, 1990). To confer protection against *B. abortus* infection, immune $CD4^+$ T cells secrete many cytokines, including gamma interferon (IFN-γ) that stimulates the antimicrobial activity of macrophages (Jiang and Baldwin, 1993; Zhan and Cheers, 1993; He et al., 2001). A crucial role of IFN-γ in the resistance to *Brucella* infection was demonstrated in mice by *in vivo* antibody neutralization experiments (Zhan and Cheers, 1993) and an IFN-γ knockout mouse study (Murphy et al., 2001). $CD8^+$ cytotoxic T lymphocytes (CTL) are critical in killing *Brucella*-infected target cells (Oliveira and Splitter, 1995; He et al., 2001).

Brucella abortus strain RB51 and strain 19 and *B. melitensis* strain Rev. 1 have been used as commercial animal brucellosis vaccines (Schurig et al., 2002). Strain 19 is the first effective live attenuated *Brucella* vaccine widely used in the world. This smooth strain induces anti-O-antigen antibody in the host. Since this serological response is used for brucellosis diagnosis in the field, Strain 19-induced antibody response is often misdiagnosed as the sign of virulent *Brucella* infection. Cattle brucellosis vaccine strain RB51 is a rough live attenuated *B. abortus* strain derived from smooth virulent strain 2308 (Schurig et al., 1991). RB51 does not induce an anti-O-antigen serological antibody response, thus does not interfere with serological diagnosis. Rev. 1 protects sheep and goats from infection with *B. melitensis*. However, these vaccine strains cannot be used in humans due to their pathogenicity. There is no safe, effective human brucellosis vaccine. However, such a human vaccine is desired for improving public health and biosafety. To rationally design a safe and effective brucellosis vaccine, it is important to further understand the mechanisms of *Brucella* pathogenesis and protective *Brucella* immunity.

Systems biology aims to understand biological systems on a system level using interdisciplinary technologies. In contrast to the traditional reductionist molecular approach, which focuses on understanding the roles of single genes or proteins, systems biology applies a more holistic approach by studying networks and the interactions between individual components of networks (Kuster et al., 2011). The goal of systems biology is to understand the structure, dynamics, and interactions of whole cells rather than portions thereof. Systems biology treats an organism (e.g., *Brucella* and human) as an integrated cellular system consisting of an interacting network of genes, proteins, and molecular cellular components including their biochemical/biophysical reactions. Biological data and software tools for data analysis are two basic ingredients in systems biology. The high throughput experimental "omics" (Omics) technologies, including genomics, transcriptomics, proteomics, and metabolomics, are major driving forces of systems biology (Kay and Wren, 2009; Zhang et al., 2010). The development of genome-scale computational and bioinformatic tools allows analysis and modeling of metabolic, regulatory and signaling networks of the cell at the systems-level.

Bioinformatics is the application of a combination of computer science, statistics, mathematics, and information technology to the field of biology and medicine. Bioinformatics enables the discovery of new biological insights and creates a global perspective of unifying principles in biology. Bioinformatics emerged as a scientific field in 1990s when large amounts of nucleotide and amino acid sequences were generated. At the time, bioinformatics took a role of generating and maintaining databases to store biological information and to support sequence data analyses. Subsequently, bioinformatics has evolved leading to the development of new computational algorithms, statistics methods, and tools to integrate, manage, and analyze various biological data including high throughput Omics data and literature data.

Brucella research has benefited from the application of cutting edge systems biology and bioinformatics technologies. The availability of *Brucella* and host (e.g., human and mouse) genomes allow comparative genomic analyses of host specificity, virulence analysis, and rational vaccine target design. High throughput array technologies have been developed for analyses of transcriptomics and proteomics gene expression profiles of host and *Brucella* *in vitro* and *in vivo*. These analyses have resulted in a better understanding of host–*Brucella* interactions and *Brucella* pathogenesis. Advanced literature mining approaches are also used to identify *Brucella* virulence factors and genetic interaction pathways. Various *Brucella* databases are publicly accessible for query and analysis of structured data. Recently, ongoing ontology studies have facilitated *Brucella* data integration and computer-assisted automated reasoning. This article provides a comprehensive review of the applications of advanced systems biology and bioinformatics to the study of *Brucella* pathogenesis, host–*Brucella* interactions, and for the development of *Brucella* vaccines.

COMPARATIVE *BRUCELLA* GENOMICS FOR UNDERSTANDING *BRUCELLA* GENETIC CONSERVATION, VARIABILITY, AND HOST SPECIFICITY

While the mechanism of *Brucella* host specificity is still unclear, comparative *Brucella* genomics has permitted identification of gene variability among different *Brucella* species and strains, resulting in a better understanding of *Brucella* virulence and adaptation in different hosts.

The genome of *B. melitensis* strain 16 M was first sequenced and published in 2002 (DelVecchio et al., 2002). Since then, *B. suis*

strain 1330 (Paulsen et al., 2002), and *B. abortus* strains 2308 (Chain et al., 2005) and 9-941 (Halling et al., 2005), and vaccine *B. abortus* strain 19 (Crasta et al., 2008) have been sequenced and published in peer-reviewed journals. As of October 11, 2011, the NCBI genome sequence site has been found to contain 14 sequenced *Brucella* genomes[1]. Furthermore, 25 additional *Brucella* genomes have been sequenced by the Broad Institute. These are available for public query, download, and further analysis at URL: http://www.broadinstitute.org/annotation/genome/brucella_group/GenomeStats.html. Therefore, at least 39 *Brucella* strains have been sequenced. With the increasing number of sequenced bacterial genomes, it becomes possible to conduct a systematic comparative analysis of whole genomes of different *Brucella* strains and to dissect their genetic conservation and variability.

Each *Brucella* genome contains two circular chromosomes. The size of Chromosome I and II approximates 2.2 and 1.1 Mb, respectively. There are about 3200–3400 genes in each genome. Based on DNA–DNA hybridization studies, the genus *Brucella* is a highly homogeneous group (>90% DNA identity among all nomenspecies; Verger et al., 2000). Ratushna et al. (2006) compared the genome sequences of *B. abortus* strain 9-941, *B. melitensis* strain 16 M, and *B. suis* strain 1330. A majority (>90%) of annotated genes in these three genomes share 98–100% sequence identity at a nucleotide level. A majority of differentiating genes among these three species are located in large (∼20 kb) regions (Ratushna et al., 2006). Whatmore et al. (2007) examined nine discrete genomic loci that correspond to 4396 bp of sequence from 160 *Brucella* isolates. A phylogeny analysis using the multilocus sequences showed that four classical *Brucella* species, *B. abortus*, *B. melitensis*, *B. ovis*, and *B. neotomae* are well-separated clusters in the phylogenic tree structure. With the exception of biovar 5, *B. suis* isolates cluster together. *B. canis* isolates are located on a phylogenic branch closely related to *B. suis* biovar 3 and 4 isolates. Marine mammal isolates represent a distinct cluster (Whatmore et al., 2007). The major conclusion of the phylogenic tree analysis was verified by another maximum likelihood phylogenetic analysis of the 10 *Brucella* strains (Wattam et al., 2009). That *B. suis* is a single species has been questioned since it has a broader host specificity but does not have any identified species-specific markers (Moreno et al., 2002).

Since limited genome diversity exists among different *Brucella* species, a comparison of *Brucella* species whole genomes is a powerful tool to identify *Brucella* gene variability that is responsible for differences in host preference and virulence restriction. The sequence insertion/deletion events may contribute to host specificity between different *Brucella* species. Rajashekara et al. (2004) used the complete genome sequence of *B. melitensis* 16 M, a strain highly pathogenic to humans, to construct a genomic microarray. Hybridization of labeled genomic DNA from different *Brucella* strains to this microarray identified 217 open reading frames (ORFs) that were altered in five *Brucella* species, including *B. abortus, B. suis, B. canis, B. ovis*, and *B. neotomae*. Many of the ORFs are located in the 16 M genome in nine regions (genomics islands) ranging in size from 5 to 44 kb. Genomic islands lost in a given species are often restricted to that particular species (Rajashekara et al., 2004). The genomic islands missing in *B. ovis* are present in *Brucella* species pathogenic to humans. However, *B. neotomae*, a non-pathogenic species in humans and domestic animals, also possesses these islands. Interestingly, the genetic islands identified do not encode adhesins or secreted virulence factors that contribute to host specificity in other bacterial species (Moon et al., 1977; Tsolis et al., 1999; Inatsuka et al., 2005). It is likely that adhesins and secreted virulence factors are encoded in conserved loci where they are differentially expressed or inactivated by point mutations. As seen in *Bordetella* species (Parkhill et al., 2003), gene inactivation or altered gene regulation may contribute to differences in host range and virulence of *Brucella* species in humans.

Table 1 lists studies published on *Brucella* pathogenesis and host immunity using high throughput transcriptomics and proteomics methods. These diverse studies are described in detail in the following sections.

ANALYSIS OF *BRUCELLA* GENE EXPRESSION PROFILES AND REGULATORY RESPONSES FROM *IN VITRO* CULTURES USING DNA MICROARRAYS

DNA microarrays have been used to delineate *Brucella* pathogenesis mechanisms. Viadas et al. (2009) generated a *Brucella* whole-genome DNA microarray based on a comprehensive collection of *B. melitensis* ORF clones or ORFeomes. The *Brucella* DNA microarray was used to determine the global transcriptional profile of *B. abortus* grown under laboratory conditions. Ribosomal proteins, Krebs cycle, and oxidative phosphorylation enzymes were found to have overexpressed transcripts. T4SS *virB* operon, flagellar components, and other genes related to virulence and intracellular growth were poorly transcribed. This report demonstrated the usefulness of the ORFeome for the construction of a PCR product microarray for analysis of global gene expression in *Brucella* and may be applied to other microorganisms as well.

Rossetti et al. (2009) found that *B. melitensis* cells grown in the late-log growth phase are more invasive in HeLa (a representative epithelial cell line) cells compared to the brucellae grown to the mid-log or stationary growth phase. To identify candidate pathogen genes involved in invasion of epithelial cells, cDNA microarrays were used to characterize genome-wide transcript changes of *B. melitensis* genes in late-log growth phase (the most invasive culture) compared to the stationary growth phase (the least invasive culture). At the late logarithmic growth phase, virulent *B. melitensis* is more invasive in HeLa epithelial cells than the mid-logarithmic or stationary growth phases. Compared to the stationary growth phase, 414 up- and 40 down-regulated genes were identified in late logarithmic growth phase. The majority of up-regulated genes in the late-log phase cultures were associated with growth, including DNA replication, transcription, translation, intermediate metabolism, energy production and conversion, membrane transport, and biogenesis of the cell envelope and outer membrane. Down-regulated genes were distributed among several functional categories (Rossetti et al., 2009).

The two-component BvrR/BvrS system is essential for *B. abortus* virulence. To determine the genes regulated by BvrR/BvrS, Viadas et al. (2010) performed a whole-genome microarray

[1]http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi

Table 1 | Reviewed omics studies in *Brucella* pathogenesis and host immunity.

***Brucella* spp.**	**Host cells and spp.**	**Temporal**	**Reference**
COMPARATIVE *BRUCELLA* GENOMICS ANALYSIS BASED ON SEQUENCE BIOINFORMATICS ANALYSIS			
B. abortus, B. melitensis, B. suis	–	No	Ratushna et al. (2006)
6 classical *Brucella* species	–	No	Whatmore et al. (2007)
***BRUCELLA* GENOME ANALYSIS USING WET-LAB *BRUCELLA* GENOMIC MICROARRAY**			
B. melitensis	–	No	Rajashekara et al. (2004)
***BRUCELLA* TRANSCRIPTOMIC PROFILES USING DNA MICROARRAY**			
B. melitensis	–	No	Viadas et al. (2009)
B. melitensis	–	Yes	Rossetti et al. (2009)
WT and *bvrR B. abortus* mutant	–	No	Viadas et al. (2010)
WT and *vjbR B. melitensis* mutant	–	No	Weeks et al. (2010)
WT and *blxR B. melitensis* mutant	–	No	Rambow-Larsen et al. (2008)
***BRUCELLA* PROTEOMIC PROFILES USING PROTEOMICS MICROARRAY**			
B. abortus S2308 and S19	In mouse macrophages	Yes	Lamontagne et al. (2009)
B. suis	In mouse macrophages	No	Al Dahouk et al. (2008)
B. melitensis	In HeLa cells	Yes	Rossetti et al. (2011)
B. melitensis	In human sera	No	Liang et al. (2011)
B. melitensis	In goat and human sera	No	Liang et al. (2010)
***BRUCELLA*-INFECTED HOST TRANSCRIPTOMIC PROFILES USING DNA MICROARRAY**			
B. abortus	Mouse macrophages	No	Eskra et al. (2003)
B. melitensis	Mouse macrophages	Yes	He et al. (2006)
B. melitensis, B. neotomae, B. ovis	Mouse macrophages	No	Covert et al. (2009)
B. suis	Mouse macrophages	Yes	Chen et al. (2011)
B. abortus	Cattle macrophages	No	Rossetti et al. (2010)
B. abortus	Bovine trophoblastic cells	No	Carvalho Neta et al. (2008)
B. abortus, B. melitensis, virB mutants	Mouse spleens	No	Roux et al. (2007)
B. suis biovar 2	Eurasian wild boar spleens	No	Galindo et al. (2010)
Rough *B. ovis* strain PA	Ram buffy coat	Yes	Galindo et al. (2009a)
B. melitensis strain Rev 1	Ram buffy coat	Yes	Galindo et al. (2009b)

analysis using *B. abortus* RNAs obtained from wild type and *bvrR* mutant cells grown *in vitro* under the same conditions. Among 127 differentially expressed genes, 83 were up-regulated and 44 were down-regulated in the *bvrR* mutant. Many genes involved in cell envelope or outer membrane biogenesis, including the outer membrane proteins (OMPs; Omp25a and Omp25d), lipoproteins, stress response proteins, chaperones, flagellar genes, ABC transport protein, and genes for lipopolysaccharide (LPS) and fatty acid biosynthesis, were differentially expressed. Ten genes related to carbon metabolism (e.g., *pckA* and *fumB*) were up-regulated in the *bvrR* mutant. Three denitrification genes (*nirK*, *norC*, and *nosZ*) were also regulated. The two-component system also affects seven transcriptional regulators including VjbR, ExoR, and OmpR. Therefore, the *Brucella* BvrR/BvrS system modulates cell envelope biogenesis, controls the carbon and nitrogen metabolism, and interact with other regulators to ensure the survival of *Brucella* in an extracellular environment as well as an intracellular niche (Viadas et al., 2010).

The quorum sensing (QS) communication system regulates gene expression in response to population density and often regulates virulence determinants as well. QS typically follows production of an auto inducer such as acyl-homoserine lactone (AHL). Among the proteobacteria, the AHL signal is synthesized by *luxI* and interacts with the transcriptional regulator LuxR. Deletion of *Brucella vjbR*, a LuxR-like transcriptional regulator, greatly attenuates intracellular survival of *B. melitensis*. To better define the role of VjbR and QS in *Brucella* virulence and survival, Weeks et al. (2010) used microarrays to analyze gene expression profiles of *Brucella* under the control of VjbR and an AHL signal (*N*-dodecanoyl homoserine lactone, C_{12}-HSL). Specifically, wild type *B. melitensis* and isogenic ΔvjbR transcriptomes were grown in the presence and absence of exogenous C_{12}-HSL. A comparison of VjbR and C_{12}-HSL transcriptomes identified shared regulation of 127 genes. Of these genes, all but three genes were inversely regulated. These results suggest that C_{12}-HSL functions via VjbR to reverse gene expression. In the absence of VjbR and in the presence of C_{12}-HSL, 48 genes were up-regulated at the stationary growth phase. The differentially regulated genes included adhesins, proteases, antibiotic and toxin resistance genes, stress survival aids, transporters, membrane biogenesis genes, amino acid metabolism and transport, transcriptional regulators, energy production genes, and *fliF* and *virB* operons. Many of the differentially regulated genes have been identified as virulence factors in other bacterial pathogens. Therefore, it can be concluded that VjbR and C_{12}-HSL contribute to virulence and survival by regulating expression of virulence mechanisms (Weeks et al., 2010).

In addition to VjbR, the first LuxR-type regulatory protein identified in *Brucella*, Rambow-Larsen et al. (2008) identified a second LuxR-type regulatory protein (BlxR) in *Brucella*. Microarray analysis of a *blxR* mutant suggests that BlxR regulates the expression of genes encoding the T4SS and flagella. These results were confirmed by experimental evidence by deletion of *blxR* in *B. melitensis*. Both BlxR and VjbR are positively auto-regulated and cross-regulate the expression of each other (Rambow-Larsen et al., 2008).

ANALYSIS OF *BRUCELLA* GENE EXPRESSION PROFILES INSIDE HOST CELLS

The virulence of *Brucella* relies heavily on their ability to survive and replicate within the vacuolar phagocytic compartments of macrophages (Baldwin and Winter, 1994; Roop et al., 2009). After phagocytosis by macrophages, the brucellae reside within a vacuole that interacts with early endosomes. These *Brucella*-containing vacuoles (BCVs) avoid further interactions with the endocytic pathway and interact with endoplasmic reticulum (ER). After sustained interaction and fusion with the ER, mature BCVs become replicative compartments (i.e., replicative phagosomes) with ER-like properties. This late maturation event (for the biogenesis of an ER-derived replicative organelle) requires a functional T4SS (Celli et al., 2003). Virulent brucellae successfully fuse with ER cysternae and survive and multiply. However, attenuated brucellae fail to fuse with ER and are destroyed inside of the host phagolysosomes.

To investigate physiological adaptations of virulent *Brucella* in its intracellular lifecycle, Lamontagne et al. (2009) infected murine macrophages with virulent *B. abortus* 2308 or attenuated *B. abortus* vaccine strain 19 and then compared the proteomes of intracellular *Brucella* recovered at 3, 20, and 44 h after macrophage infections. In total, 190 *Brucella* proteins were differentially expressed in the time course of infections. Ninety *Brucella* proteins were uniquely differentially expressed by strain 2308. Thirty proteins were only differentially expressed by strain 19. The remaining 70 proteins were differentially expressed by both strains. In virulent strain 2308, carbohydrate based carbon utilization and protein synthesis processes were initially reduced when the cells switched to alternative energy sources and low oxygen tension respiration. In the later stages of strain 2308 infection, the expressions of proteins related to key metabolic processes, protein synthesis, iron acquisition, and transport were significantly up-regulated, and its cell envelope actively modified. In contrast, strain 19 adjusted its metabolic profile to a lower degree in the early stage of infection. In the later stage of infection, strain 19 was unable to revert to pre-infection protein expression levels in key processes (Lamontagne et al., 2009).

Al Dahouk et al. (2008) used a 2-D DIGE approach to characterize the intramacrophagic proteome of *B. suis* at alate stage of *in vitro* infection. Compared to extracellularly grown, stationary-phase bacteria, the concentrations of 168 proteins were altered. The majority of the 44 proteins differentially regulated at the late stage of infection participated in bacterial metabolism. Of these, 40% were down-regulated. These results indicate that intramacrophagic *B. suis* has an adaptive response in terms of quantitative reduction of processes involving energy, protein, and nucleic acid metabolism.

Brucella infects hosts primarily by adhering and penetrating mucosal epithelium surfaces. Similar to the kinetics profile of *Brucella* inside macrophages, virulent *Brucella* have an initial adaption period followed by a replicative phase inside epithelial cells. Using cDNA microarray analysis, Rossetti et al. (2011) characterized the transcriptional profile of the intracellular pathogen *B. melitensis* at 4 h (adaptation period) and at 12 h (replicative phase) following infection of HeLa cells. The study found that 161 and 115 *Brucella* genes were differentially expressed at 4 and 12 h, respectively, post infection. Most of the genes expressed were involved in pathogen growth and metabolism. At the adaptation period, 126 (78% of 161) genes were down-regulated. At the replicative phase, 86 (75% of 115) genes were up-regulated.

MACROPHAGE IMMUNE RESPONSES TO *BRUCELLA* INFECTIONS BASED ON OMICS GENE EXPRESSION DATA ANALYSIS

The above section reviewed two proteomics and one transcriptomics studies concerning profiling gene expression patterns of different *Brucella* strain inside infected macrophages (Al Dahouk et al., 2008; Lamontagne et al., 2009). DNA microarray analysis has been used frequently to analyze transcriptomic gene expression profiles in murine macrophages infected with virulent *Brucella* strains (Eskra et al., 2003; He et al., 2006). Five studies on this topic have been reported and summarized below.

Using Affymetrix murine U74A gene microarrays, Eskra et al. (2003) found that over 140 genes, of the >6000 genes, were reproducibly differentially transcribed in RAW264.7 macrophages infected with *B. abortus* for 4 h. Initially, an increase in the transcription of a number of proinflammatory cytokines and chemokines, such as TNF-α, IL-1α, and IL-1β, was observed. However, transcription of receptors and cytokines associated with antigen presentation, e.g., MHC class II and IL-12p40, were not found at 4 h post infection. Virulent *B. melitensis* also inhibited transcription of various host genes involved in apoptosis and intracellular vesicular trafficking. It appears that *Brucella* utilizes specific mechanisms to inhibit many cell pathways (Eskra et al., 2003).

Covert et al. (2009) subsequently demonstrated that the infections with *B. melitensis*, *B. neotomae*, and *B. ovis* bacteria for 4 h elicit common and distinctive defense transcriptional responses of RAW 264.7 macrophages. Although few *B. melitensis* and *B. neotomae* cells enter macrophages, *B. ovis* cells are readily ingested by macrophages. Macrophages infected with these different *Brucella* species demonstrated common changes in gene expression compared to uninfected macrophages. Compared to uninfected macrophages, macrophage infections with all three *Brucella* species induced increased transcript levels of 72 genes including chemokines and defense response genes (e.g., IL-1β, MIP-1α, Fas, and TNF). Meanwhile, decreased transcript levels of 68 genes, such as genes associated with vesicular trafficking (e.g., Rab3d) and response to external stimulus (e.g., IL-17a), were identified in macrophages infected with all three *Brucella* species. Genes with altered transcript levels of *Brucella*-infected macrophages may correlate with *Brucella* species-specific host defenses and intracellular

survival strategies. *B. melitensis*, but not *B. neotomae* or *B. ovis*, is pathogenic to human. Correspondingly, the infection with *B. melitensis*, but the other two *Brucella* spp., induced decreased gene expression in growth arrest (Gas2), immunoglobulin receptor (Fc gamma RI), and chemokine receptor (Cxcr4) genes (Covert et al., 2009).

He et al. (2006) analyzed the time course response of J774.A1 macrophages during infection with virulent *B. melitensis* strain 16 M using Affymetrix mouse 430 2.0 array containing more than 39,000 genes. Transcriptions of 243 up-regulated and 1053 down-regulated genes were identified at 4 h post infection compared to uninfected macrophages. However, compared to uninfected macrophages, only 12 genes were found up- or down-regulated after 24 h, and no genes were found differentially regulated at 48 h post infection. Although many pro-apoptosis genes were up-regulated, it is noteworthy that the caspase cascade pathways were not activated. These results suggest that some upstream component(s) that induces caspase activation is suppressed. Interestingly, caspase-2, a caspase that regulates the release of cytochrome *c* from the mitochondria, was down-regulated. Furthermore, 106 mitochondria-associated genes were down-regulated while only 4 mitochondria-associated genes were up-regulated at 4 h post infection (He et al., 2006). It seems that *B. melitensis* 16 M may prevent apoptosis in macrophages by suppressing mitochondrial gene expression involved in cytochrome *c* release, reactive oxygen species (ROS) production, and mitochondrial membrane permeability, thereby preventing activation of caspase cascades. Prevention of apoptosis in macrophages by *B. melitensis* strain 16 M ensures extensive replication after the initial killing stage. Such inhibition may contribute to the ability of *Brucella* spp. to persist chronically in the reticuloendothelial system of infected humans and animals. Many of the hypotheses generated from the microarray analyses were later confirmed by other studies (Chen and He, 2009; Chen et al., 2011). For example, wet-lab experiments from the same group found that smooth *B. abortus* strain 2308 prevents mitochondrial permeability and the release of cytochrome *c* from mitochondria. Smooth virulent *Brucella* strains that contain intact LPS are capable of inhibiting programmed cell death in infected human and mouse macrophages (Gross et al., 2000; Tolomeo et al., 2003; He et al., 2006). Rough attenuated *Brucella* strains, which lack O-antigen or produce extremely low levels of the antigen, cannot survive inside macrophages and indeed induce programmed cell death (Fernandez-Prada et al., 2003; Rittig et al., 2003; Pei and Ficht, 2004). The author's laboratory found that rough and live attenuated *B. abortus* strains RB51 (the current cattle vaccine) and RA1 induced a caspase-2-mediated apoptotic and necrotic macrophage cell death (Chen and He, 2009). An inhibition of caspase-2 prevents cytochrome *c* release and almost completely inhibited cell death induced by these rough strains.

Brucella suis primarily infects pigs and is pathogenic to humans. Our studies reveal that smooth virulent *B. suis* strain 1330 (S1330) prevents programmed cell death of infected macrophages. However, rough attenuated *B. suis* strain VTRS1 (a vaccine candidate) induces a high level of macrophage cell death. Like *B. abortus* vaccine strain RB51, VTRS1 has a *Brucella wboA* gene mutation, which results in the deficiency of LPS O-antigen as well as the rough phenotype (Winter et al., 1996). An Affymetrix microarray study was conducted to analyze temporal transcriptional responses of murine macrophage-like J774.A1 cells infected with S1330 or VTRS1, 17,685 probe sets were significantly up- or down-regulated depending on *Brucella* strain, time, and the interaction between the strain and time (Chen et al., 2011). A miniTUBA dynamic Bayesian network analysis predicted that VTRS1-induced macrophage cell death was mediated by a proinflammatory gene TNF-α, an NF-κB pathway gene IκB-α, and caspase-2. Compared to S1330, VTRS1-induced a dramatically higher level of proinflammatory response as indicated by increased transcriptions of 40 proinflammatory genes. Increased protein level production of TNF-α and IL-1β were detected in the supernatants in VTRS1-infected macrophage cell culture. Inhibition and knockout mouse studies further confirmed that VTRS1 induces a proinflammatory, caspase-2- and NF-κB-mediated macrophage cell death. Interestingly, caspase-1 does not play any obvious role in the VTRS1-induced macrophage cell death in studies utilizing a caspase-1 inhibitor (Chen et al., 2011). This novel caspase-2-mediated proinflammatory cell death differs from apoptosis (which is not proinflammatory), and differs from classical caspase-1-mediated pyroptosis. The details of the mechanism for the cell death pathway and the biological relevance of this pathway in *Brucella* pathogenesis and protective *Brucella* immunity are currently under active investigations.

Using a cDNA microarray technology, Rossetti et al. (2010) compared the early transcriptome of *B. abortus*-infected monocyte-derived macrophages (MDMs) from cattle naturally resistant (R) or susceptible (S) to brucellosis. The MDMs isolated from peripheral blood were infected with virulent *B. abortus* strain 2308 for 24 h. Their study identified slightly increased genome activation in R MDMs and a down-regulated transcriptome in S MDMs. Specifically, compared to uninfected cells, *Brucella* infection induced 46 up- and 195 down-regulated genes in S MDMs at 12 h post infection. In R MDMs, 31 genes were up- and 25 genes were down-regulated at 12 h postinfection. R MDMs had the ability to induce a type 1 immune response against *B. abortus* infection, including up-regulation of CCL4 and reduced expression of EBF1. This ability was impaired in S cells, as demonstrated by decreased expression of HSPA14, TCIRG1, and C1QBP genes. Several inflammation-associated host genes, such as IL-1A, CCL2, and CCL5, were up-regulated in infected S MDMs. These differences may explain the different resistances of MDMs to virulent *Brucella* infection.

HOST EPITHELIAL CELL RESPONSES TO *BRUCELLA* INFECTIONS BASED ON OMICS GENE EXPRESSION DATA ANALYSIS

Brucella abortus induces acute placentitis and abortion in infected animals, key events for transmission of the disease. To better understand the intricate interaction between *B. abortus* and trophoblastic cells, Carvalho Neta et al. (2008) evaluated the profile of gene expression by bovine trophoblastic cells during infection with *B. abortus*. Microarray analysis was performed after explants of chorioallantoic membranes were infected with *B. abortus* strain 2308 for 4 h. Expression of proinflammatory genes by trophoblastic cells was suppressed at 4 h after inoculation. A significant up-regulation of CXC chemokines [CXCL6 (GCP-2) and CXCL8

(interleukin 8)] was observed at 12 (but not at 6 h) after inoculation. Therefore, in trophoblastic cells infected with virulent *B. abortus*, the expression of proinflammatory mediators was suppressed during the early stages of infection. This was followed by a delayed and mild expression of proinflammatory chemokines. A similar profile of chemokine expression, including up-regulation of CXCL6 and CXCL8, was found in the placentomes of experimentally infected cows. The kinetic trophoblastic response is likely to contribute to the pathogenesis of *B. abortus*-induced placentitis (Carvalho Neta et al., 2008).

INNATE HOST SPLENOCYTE RESPONSES TO *BRUCELLA* INFECTIONS BASED ON DNA MICROARRAY ANALYSES

Soon after *Brucella* cells invade a host, infectious brucellae migrate to the spleen and liver. In spleen and liver, the course of *Brucella* infection encompasses four phases. The early preimmune infection phase is characterized by logarithmic *Brucella* growth and an accumulation of bacteria in the liver and spleen. The second bacteriostatic phase is typically accompanied with the onset of a delayed type hypersensitivity to *Brucella* antigens and granuloma formation. In the third immune effector phase, up to 90% of the bacteria may be destroyed. This phase is typically followed by a phase of obviously impaired eradication of bacteria (phase IV; Hort et al., 2003).

Spleen is most frequently used for analysis of innate and adaptive immune responses to *Brucella* infections. To identify host responses specifically regulated by the *Brucella* T4SS, Roux et al. (2007) used Affymetrix mouse 430 2.0 arrays to compare early transcriptional responses of mouse splenocytes to infection with *B. abortus*, *B. melitensis*, and *B. abortus virB* mutants defective in the T4SS. The largest number of differentially expressed genes occurred in the categories of inflammation and immunity. Galindo et al. (2010) studied gene expression changes in spleens of the wildlife reservoir species Eurasian wild boar (*Sus scrofa*), which is naturally infected with *B. suis* biovar 2. *B. suis* biovars (bv.) 2 is frequently isolated from wild boar and hares and largely restricted to Europe. This study identified 633 up-regulated genes and 1373 down-regulated genes in infected wild boar. *B. suis* bv. 2 infection induced up-regulation of genes in cell maturation, migration, and/or proliferation in infected animals. The down-regulated genes are associated with impaired activity of several important cellular metabolic pathways including metabolism, cytoskeleton organization and biogenesis, stress, apoptosis, immune response and lysosomal function, and vesicle-mediated transport. These gene expression profiles facilitate intracellular multiplication and the development of chronic infections.

ANALYSIS OF HOST BLOOD CELL IMMUNE RESPONSES TO *BRUCELLA* VACCINATION AND CHALLENGE USING MICROARRAY TECHNOLOGY

Brucella ovis causes ovine brucellosis, characterized by infertility in rams, abortion in ewes, and increased perinatal mortality in lambs. Galindo et al. (2009a) characterized differential transcriptomics gene expression in buffy coat samples of rams experimentally infected with *B. ovis* strain PA by microarray hybridization and real-time RT-PCR. The buffy coat, the fraction of an anticoagulated blood sample after density gradient centrifugation, contains most of the white blood cells and platelets. Of the 600 ruminant inflammatory and immune response genes, 20 and 14 genes in the buffy coat samples were significantly regulated, with an expression fold change >1.75 with a P-value < 0.05, at 15 and 60 days post-challenge (dpc), respectively. Specifically, in infected rams at 15 dpc, 16 were up-regulated, and 4 were down-regulated. At 60 dpc, 11 and 3 genes, respectively, were up- and down-regulated in infected rams. Four genes, desmoglein, ENaC-alpha, IL18BP, and MIF, were up-regulated at both 15 and 60 dpc. The inflammatory and innate immune pathways were activated in infected animals. The infection of *B. ovis* up-regulated phagocytosis-associated genes and down-regulated genes related to protective host defense. These responses facilitate the chronicity of *B. ovis* infection.

Omics can also be used to characterize possible correlates of protective response against *Brucella* infection. Vaccination with live attenuated *B. melitensis* Rev 1 vaccine is used to control ovine brucellosis caused by *B. ovis* in sheep. To identify possible correlates of protective response to *B. ovis* infection, Galindo et al. (2009b) used microarrays to characterize inflammatory and immune response genes differentially expressed in rams previously immunized with Rev 1 and experimentally challenged with *B. ovis*. Total RNA was isolated from buffy coat samples before vaccination (T0), 150 days after vaccination and before challenge (T1), and 60 dpc (T2). Protected and susceptible rams did not show significant differences in gene expression prior to vaccination with Rev 1 (timeT0). After vaccination, but prior to challenge (T1), the toll-like receptor 10 (TLR10) was the only gene significantly expressed at higher levels in protected rams as compared to vaccinated rams that were susceptible to *B. ovis* infection. Concomitantly, 12 proinflammatory and innate immune effectors were up-regulated in vaccinated rams that were susceptible to *B. ovis* infection. After challenge with *B. ovis* at time T2, the vaccinated and protected rams showed higher expression levels of Bcl-2-homologous antagonist/killer (Bak), annexin I (ANXI), and interleukin 6 (IL6) genes. These genes provide possible correlates of protective response to *B. ovis* infection in rams immunized with Rev 1 vaccine.

ANALYSIS OF *BRUCELLA*-SPECIFIC SEROLOGICAL ANTIBODY RESPONSES USING PROTEOMICS

Protein expression in bacteria is an important determinant in the induction of *Brucella*-specific antibodies. A systems biology approach can be used to identify antibody signatures associated with *Brucella* infections in humans and to predict serodiagnostic antigens. Using a full proteome microarray expressing 3046 cloned *B. melitensis* genes, Liang et al. (2011) identified 122 immunodominant antigens and 33 serodiagnostic antigens. The reactive antigens had enriched features in terms of membrane association and secretion as indicated by the presence of a signal peptide, a single transmembrane domain, and an outer membrane or periplasmic location. This systems biology approach facilitates the understanding of the breadth and specificity of the immune response to *B. melitensis*.

In clinical settings, the detection of agglutinating anti-LPS antibodies is the basis for current serological diagnosis of human brucellosis. To better understand the multiplicity of antibody responses that develop after *B. melitensis* infection, Liang et al.

(2010) used a protein microarray containing 1406 predicted *B. melitensis* proteins to analyze sera from experimentally infected goats and naturally infected humans from an endemic region in Peru. Eighteen antigens were differentially recognized by infected and non-infected goats. Thirteen serodiagnostic antigens were identified that differentiated human patients with acute brucellosis from syndromically similar patients. Only two of the serodiagnostic antigens overlapped between humans and goats. A number of cross-reactive antigens were found in healthy goats and healthy humans (Liang et al., 2010). This study demonstrates that an experimentally infected natural reservoir host and a naturally infected human host produce different immune responses.

BRUCELLA VACCINE TARGET PREDICTION BASED ON GENOME SEQUENCE ANALYSIS USING VAXIGN REVERSE VACCINOLOGY

Reverse vaccinology is an emerging and revolutionary vaccine development strategy that starts with the prediction of vaccine targets by bioinformatics analysis of genome sequences (Rappuoli, 2000; He et al., 2010a). Reverse vaccinology was first applied in the development of a vaccine against serogroup B *Neisseria meningitidis* (MenB; Pizza et al., 2000). The complete MenB genome was screened for genes coding for putative surface-exposed and secreted proteins. Out of ∼600 novel vaccine candidates, 350 were expressed in *Escherichia coli*; 28 were found to elicit protective immunity (Pizza et al., 2000). Reverse vaccinology has also been applied successfully to other pathogens such as *Streptococcus pneumoniae*, *Porphyromonas gingivalis*, and *Chlamydia pneumoniae* (Rappuoli, 2000).

To promote vaccine development, the author's laboratory has developed Vaxign[2], the first web-based vaccine design program based on reverse vaccinology (Xiang and He, 2009; He and Xiang, 2010; He et al., 2010b). Predicted features in the Vaxign pipeline include protein subcellular location, transmembrane helices, adhesin probability, sequence conservation among genomes of pathogenic strains, exclusion of sequences in non-pathogenic strains, exclusion of proteins shared in host spp. (e.g., human, mouse, and pigs), and epitope binding to MHC class I and class II. Currently more than 200 genomes have been pre-computed using the Vaxign pipeline. The results are available for query in the Vaxign website. Vaxign also allows dynamic vaccine target prediction based on protein sequences provided by users. A user can register for a private account in Vaxign and save predicted results for further analyses.

Based on the Vaxign reverse vaccinology approach, sequenced *Brucella* genomes have been used for predicting vaccine targets for *Brucella* spp. (Xiang and He, 2009; He and Xiang, 2010). An O-sialoglycoprotein endopeptidase was predicted to be a secreted *Brucella* protein. Among 3034 proteins in *B. abortus* strain 2308, 32 were identified as OMPs. Two of the 32 OMPs contain more than one transmembrane alpha-helixes. Twenty out of the remaining 30 proteins are predicted as adhesins or adhesin-like proteins. Fifteen of these 20 OMPs are conserved in pathogenic *B. abortus*, *B. suis*, and *B. melitensis* strains. One of the 15 proteins is homologous to a human protein. Among the final 14 proteins are two known *Brucella* protective antigens (Omp25 and Omp31-1), two flagellar hook proteins FlgE and FlgK, one porin protein Omp2b, two TonB-dependent receptor proteins. Omp2b and Omp31-1 are absent from the genome of *B. ovis*, a *Brucella* species non-pathogenic to human (He and Xiang, 2010). The feasibility of using these *Brucella* proteins for development of a safe and effective human vaccine deserves further investigation.

[2]http://www.violinet.org/vaxign

LITERATURE MINING OF *BRUCELLA* VIRULENCE FACTORS AND PATHOGENESIS NETWORK

Many virulence factors have been retrieved by literature mining of all *Brucella* publications found in PubMed. Seventy-five mutated *Brucella* genes were identified to be attenuated inside macrophages or HeLa cells, or in an *in vivo* mouse model, using a literature mining and curation system (Limix) as part of the *Brucella* Bioinformatics Portal (BBP; Xiang et al., 2006). These 75 mutated *Brucella* genes are essential for *Brucella* virulence and pathogenesis and are thus treated as *Brucella* virulence factors (Xiang et al., 2006). Based on the NCBI Clusters of Orthologous Groups (COGs; Tatusov et al., 2000), the 75 *Brucella* genes have been classified into different categories. This study confirms the well-known pathogenesis mechanisms of *Brucella* T4SS encoded by the *virB* operon (O'Callaghan et al., 1999), the BvrR–BvrS two-component regulatory system encoded by *bvrR* and *bvrS* (Sola-Landa et al., 1998), and the intact *Brucella* lipopolysaccharide (Allen et al., 1998). The curation demonstrated an important role of the transport and metabolism of various metabolites including amino acid, carbohydrate, lipid, and inorganic ions. Those *Brucella* genes participating in these events are essential for intracellular *Brucella* growth and their survival inside phagosomes of eukaryotic cells.

The updated BBP database contains 181 *Brucella* virulence factors. These are classified by the mutants' attenuated characteristics in host cells or in animals *in vivo* (**Table 2**). A new statistic COG analysis of these virulence factors confirms many of previous data mining results. Six COG categories are significantly enriched (P-value < 0.05), including: (i) Nucleotide transport and metabolism (COG category F), (ii) Cell motility (COG category N), (iii) Translation (COG category J), (iv) Carbohydrate transport and metabolism (COG category G), (v) General function prediction (COG category R), and (vi) Function unknown (COG category S; **Table 2**). Beside these groups, many other categories, such as Signal transduction mechanisms (COG category T) and Intracellular trafficking and secretion (COG category U), are also critical for *Brucella* pathogenesis. These factors may not be crucial for *Brucella* survival *in vitro*. However, their presence is critical for *Brucella* replication *in vivo*. Many *Brucella* virulent factors have no defined functions and are classified in the categories of General function prediction, Function unknown, or Not in COGs. How these factors become virulence factors deserves further investigations.

Literature mining approaches can also be used to identify genetic networks crucial for *Brucella* pathogenesis. Out of 1358 potential interactions available from more than 7000 abstracts and/or full text papers extracted from PubMed, the Limix system found 69 true positive interactions (Xiang et al., 2006). These interactions were automatically displayed using our graphic visualization program. These results allow a more comprehensive

Table 2 | *Brucella* virulence factors classified by their mutants' attenuation.

No.	Gene name	Locus tag	Attenuation	Reference (PMID)*
COG CATEGORY C: ENERGY PRODUCTION AND CONVERSION				
1	*cydB*	BMEII0759	Mice	11274104
2	*fdhA*	BMEII0378	Mice	14979322
3	*glpK*	BMEII0823	Mice, macrophages, HeLa	14979322
4	*narG*	BMEII0950	Macrophages	12438693
5	*norE*	BMEII1001	Mice	14979322
6	*pyc*	BMEI0266	Macrophages	10678941
D: CELL CYCLE CONTROL, MITOSIS, AND MEIOSIS				
7	*virB5*	BMEII0029	Macrophages	10510235
E: AMINO ACID TRANSPORT AND METABOLISM				
8	*aroC*	BMEI1506	Macrophages, HeLa, mice	11119550
9	*aspC*	BMEI0516	HeLa	12761078
10	BMEII0626	BMEII0626	Mice, macrophages, HeLa	14979322
11	BMEII0923	BMEII0923	Mice	14979322
12	*cysK*	BMEI0933	Mice, macrophages, HeLa	14979322
13	*dppA*	BMEI0433	Mice, macrophages, HeLa	14979322
14	*glnA*	BMEI0979	Macrophages	12438693
15	*gltD*	BMEII0040	Mice	10858227
16	*glyA*	BMEI1192	Macrophages	12438693
17	*hisD*	BMEI1668	Macrophages	12438693
18	*hisF*	BMEI2041	Macrophages	12438693
19	*leuA*	BMEI0451	Macrophages	12438693
20	*leuC*	BMEI0157	Macrophages	12438693
21	*lysA*	BMEI0084	Macrophages	12438693
22	*metH*	BMEI1759	Mice, macrophages, HeLa	14979322
23	*nifS*	BMEI1043	Macrophages	14979322
24	*pheB*	BMEII0136	Mice	14979322
25	*serB*	BMEI0615	Macrophages	12438693
26	*thrA*	BMEI0725	Macrophages	14979322
27	*thrC*	BMEI1450	Macrophages	14979322
F: NUCLEOTIDE TRANSPORT AND METABOLISM**				
28	*carAB*	BMEI0526	Macrophages	14979322
29	*dut*	BMEI0358	Macrophages	12438693
30	*hpt*	BMEI0082	Macrophages, HeLa	14979322
31	*ndrI*	BMEII0931	HeLa	14979322
32	*purD*	BMEI1519	Mice, macrophages	15271960
33	*purE*	BMEI0296	Mice	15271960
34	*purF*	BMEI1488	HeLa	12761078
35	*purH*	BMEI0233	Mice, macrophages	15271960
36	*purL*	BMEI1127	Macrophage, mice	15271960
37	*purM*	BMEI1240	HeLa	12761078
38	*purN*	BMEI1241	HeLa	12761078
39	*pyrB*	BMEII0670	HeLa	12761078
40	*pyrC*	BMEII0669	Macrophages, HeLa	14979322
41	*pyrD*	BMEI1611	HeLa	12761078
G: CARBOHYDRATE TRANSPORT AND METABOLISM**				
42	BMEII1045	BMEII1045	Mice, macrophages, HeLa	14979322
43	*cbbE*	BMEI1116	Macrophages, HeLa	14979322
44	*dbsA*	BMEII0300	HeLa	14979322
45	*eryC*	BMEII0428	Mice, macrophages	16177356
46	*galcD*	BMEII0485	Mice, macrophages, HeLa	14979322
47	*gluP*	BMEII1053	Mice	12414147

(Continued)

Table 2 | Continued

No.	Gene name	Locus tag	Attenuation	Reference (PMID)*
48	*gnd*	BMEII1124	Mice	12761078
49	*ilvD*	BMEI1848	Mice, macrophages	15271960
50	*malK*	BMEI1713	Macrophages	14979322
51	*manB*	BMEII0899	Mice, macrophages, HeLa	14979322
52	*mocC*	BMEII0570	Mice, HeLa	14979322
53	*ndvB*	BMEI1837	Mice, HeLa	14979322
54	*pgi*	BMEI1636	Macrophages	10678941
55	*pgm*	BMEI1886	Mice	12525425
56	*pmm*	BMEI1396	Mice, macrophages, HeLa	14979322
57	*rbsK*	BMEII0089	Macrophages	14979322
58	*ugpA*	BMEII0624	Mice	14638795
59	*xfp*	BMEII0881	Mice, macrophages, HeLa	14979322
60	*zwf*	BMEII0513	HeLa, macrophages	12761078
H: COENZYME TRANSPORT AND METABOLISM				
61	BMEI1902	BMEI1902	Mice, macrophages, HeLa	14979322
62	*caiB*	BMEI1019	Macrophages	14979322
63	*cobB*	BMEI0705	Mice	14638795
64	*hemH*	BMEII0018	Mice, macrophages, HeLa	11553564
65	*ilvC*	BMEI0624	Mice	14638795
66	*ilvI*	BMEI0617	Macrophages, HeLa	14979322
I: LIPID TRANSPORT AND METABOLISM				
67	*aidB*	BMEII0671	Macrophages	14979322
68	*bacA*	BMEI1553	Mice, macrophages	10741969
69	*uppS*	BMEI0827	Macrophages	12438693
J: TRANSLATION**				
70	*miaA*	BMEI0616	Macrophages	12438693
71	*rpsA*	BMEI1915	Macrophages	10678941
K: TRANSCRIPTION				
72	*ansC*	BMEI0357	Mice, macrophages, HeLa	14979322
73	*arsR6*	BMEI0430	Mice	16113274
74	*aspB*	BMEI0626	Macrophages	14979322
75	*deoR*	BMEII1093	Macrophages	14979322
76	*gntR*	BMEII1066	Macrophages, HeLa	14979322
77	*gntR1*	BMEII0475	Mice	16113274
78	*gntR10*	BMEII0116	Mice	16113274
79	*gntR17*	BMEI0320	Mice	16113274
80	*gntR2*	BMEI0305	Mice	16113274
81	*gntR4*	BMEI0169	Mice	16113274
82	*gntR5*	BMEI0881	Mice	16113274
83	*lysR*	BMEI0513	Macrophages, HeLa	14979322
84	*lysR12*	BMEII0390	Mice	16113274
85	*lysR13*	BMEI1913	Mice	16113274
86	*lysR18*	BMEI1573	Mice	16113274
87	*rho*	BMEI0003	HeLa	11579087
88	*RpiR*	BMEII0573	Mice	14979322
89	*rpoA*	BMEI0781	Mice	14638795
90	*vjbR*	BMEII1116	Mice, macrophages, HeLa	14979322
L: REPLICATION, RECOMBINATION, AND REPAIR				
91	*alkA*	BMEI0382	Mice, macrophages, HeLa	14979322
92	BMEI1229	BMEI1229	Mice, macrophages, HeLa	14979322
93	*mgps*	BMEI0275	Mice, macrophages, HeLa	14979322
94	*recA*	BMEI0787	Mice	8321120

(Continued)

Table 2 | Continued

No.	Gene name	Locus tag	Attenuation	Reference (PMID)*
95	*xerD*	BMEI0040	Mice, macrophages	15519045
96	*xseA*	BMEII0527	Mice	14638795
M: CELL WALL/MEMBRANE BIOGENESIS				
97	*amiC*	BMEI1056	Macrophages, HeLa	14979322
98	*galE*	BMEI1237	Macrophages, HeLa	14979322
99	*gtrB*	BMEII1101	Macrophages	14979322
100	*lpsA*	BMEI1326	Macrophages	14979322
101	*lpsB*	BMEI0509	Macrophages	14979322
102	*macA*	BMEI0359	Mice, macrophages, HeLa	14979322
103	*mtgA*	BMEI0271	Mice	15519045
104	*omp25*	BMEI1249	Mice, cattle, goats	15374004
105	*perA*	BMEI1414	Macrophages	14979322
106	*rfbD*	BMEI1413	Mice, macrophages	14979322
107	*wbdA*	BMEI0997	Macrophages	14979322
108	*wbkA*	BMEI1404	Mice	14979322
109	*wbpL*	BMEI1426	Mice, macrophages	14979322
110	*wbpW*	BMEII0900	Macrophages	14979322
111	*wbpZ*	BMEI1393	Mice, macrophages	14979322
N: CELL MOTILITY**				
112	*flgE*	BMEII0159	Mice	14979322
113	*flgI*	BMEII1084	Mice	14979322
114	*fliC*	BMEII0150	Mice	14979322
115	*motB*	BMEII0154	Mice	14979322
O: POSTTRANSLATIONAL MODIFICATION, PROTEIN TURNOVER, CHAPERONES				
116	BMEI0455	BMEI0455	Mice, macrophages, HeLa	14979322
117	*cydC*	BMEII0761	Macrophages, HeLa	14979322
118	*cydD*	BMEII0762	HeLa	12761078
119	*cysY*	BMEI1849	Macrophages, HeLa	14979322
120	*dnaK*	BMEI2002	Macrophage	11854256
121	*dsbA*	BMEI1440	Macrophages, HeLa	14979322
122	*dsbB*	BMEI0384	Mice, macrophages	14979322
123	*glnD*	BMEI1804	Macrophages, HeLa	10678941
124	*htrA*	BMEI1330	Mice, neutraphils, macrophages	8890248
125	*lon*	BMEI0876	Mice	10672180
126	*nrdH*	BMEII0932	HeLa	14979322
127	*ppiD*	BMEI0845	Mice, macrophages, HeLa	14979322
128	*tig*	BMEI1069	Mice	14638795
P: INORGANIC ION TRANSPORT AND METABOLISM				
129	BMEII0336	BMEII0336	Mice, macrophages, HeLa	14979322
130	*cysI*	BMEI1766	Mice, macrophages	14979322
131	*mgtB*	BMEII0056	Mice, macrophages, HeLa	14979322
132	*sodC*	BMEII0581	Macrophage, mice	15845493
133	*znuA*	BMEII0178	HeLa, macrophages	15472468
134	*znuC*	BMEII0177	Macrophages, HeLa	14979322
Q: SECONDARY METABOLITES BIOSYNTHESIS, TRANSPORT, AND CATABOLISM				
135	*dhbC*	BMEII0077	Pregnant goat	14979322
T: SIGNAL TRANSDUCTION MECHANISMS				
136	BMEI1448	BMEI1448	Mice, macrophages, HeLa	14979322
137	*bvrR*	BMEI2036	Mice, macrophages, HeLa	16077108
138	*bvrS*	BMEI2035	Mice, macrophages, HeLa	16077108
139	*divK*	BMEII0659	Mice	14979322
140	*feuP*	BMEI1337	Mice, macrophages	14979322

(Continued)

Table 2 | Continued

No.	Gene name	Locus tag	Attenuation	Reference (PMID)*
141	*feuQ*	BMEI1336	Mice, macrophages, HeLa	14979322
142	*ftcR*	BMEII0158	Mice	17056750
143	*glnL*	BMEI1786	Macrophages	14979322
144	*nodV*	BMEII0052	Mice, macrophages, HeLa	14979322
145	*ntrY*	BMEI0867	Mice	10678941
146	*pstP*	BMEI0190	Macrophages, HeLa	14979322
147	*spotT*	BMEI1296	Macrophages, HeLa	14979322
148	*vsrB*	BMEI1606	Mice, macrophages, HeLa	14979322
U: INTRACELLULAR TRAFFICKING AND SECRETION				
149	*flghA*	BMEII0166	Mice	14979322
150	*fliF*	BMEII0151	Mice	14638795
151	*virB3*	BMEII0027	HeLa	12761078
152	*virB2*	BMEII0026	Macrophages, mice	15322008
153	*virB8*	BMEII0032	Macrophage	10678941
V: DEFENSE MECHANISMS				
154	BMEII0318	BMEII0318	Mice, macrophages, HeLa	14979322
155	*dacF*	BMEII0350	Macrophages, HeLa	14979322
156	*exsA*	BMEI1742	Mice	14979322
R: GENERAL FUNCTION PREDICTION ONLY**				
157	*bicA*	BMEI0605	Macrophages	14979322
158	BMEI0671	BMEI0671	Macrophages	14979322
159	BMEI1443	BMEI1443	Mice, macrophages, HeLa	14979322
160	BMEI1531	BMEI1531	Mice, macrophages, HeLa	14979322
161	BMEI1859	BMEI1859	Macrophages	14979322
162	BMEII0274	BMEII0274	Macrophages	14979322
163	BMEII0935	BMEII0935	Mice, macrophages, HeLa	14979322
164	BMEII1037	BMEII1037	Mice	14979322
165	*cobW*	BMEII0308	Macrophages	12438693
166	*glt1*	BMEII0039	Mice	14979322
167	*hfq*	BMEI0872	Macrophage, mice	12730323
168	*mosC*	BMEI0267	Mice	14979322
169	*rbsC*	BMEII0701	Mice, macrophages, HeLa	14979322
170	*tldD*	BMEI1468	Mice	14979322
S: FUNCTION UNKNOWN**				
171	BMEI1809	BMEI1809	Mice, macrophages, HeLa	14979322
172	BMEII0128	BMEII0128	Mice, macrophages, HeLa	14979322
173	*pncA*	BMEI0545	Mice, macrophages, HeLa	15135535
NOT IN COGs				
174	BMEI0085	BMEI0085	Macrophages	14979322
175	BMEI1339	BMEI1339	Mice, macrophages, HeLa	14979322
176	BMEI1361	BMEI1361	Mice	14979322
177	BMEI1658	BMEI1658	Macrophages	14979322
178	BMEI1844	BMEI1844	Mice, macrophages, HeLa	14979322
179	BMEI1879	BMEI1879	Mice, macrophages, HeLa	14979322
180	*omp10*	BMEII0017	Mice	12228280
181	*omp19*	BMEI0135	Mice	12228280

**Each reference is indicated by PubMed citation ID (i.e., PMID). **Significantly enriched COG category based on Fisher's exact test (P-value < 0.05).*

investigation of *Brucella* pathogenesis and the generation of novel hypotheses (Xiang et al., 2006). For example, this study identified a possible interaction between T4SS and the BvrR–BvrS two-component regulatory system. Specifically, the secretion of the N-terminal fragment of BvrR fused to a ribosome binding site and start codon deficient chloramphenicol acetyl transferase (CAT) report gene is diminished in *virB1* and *virB10* mutants (Marchesini et al., 2004). How the T4SS regulates the BvrR/BvrS

system remains unclear. However, Martinez-Nunez et al. (2010) recently found that BvrR/BvrS regulates the expression of the T4SS VirB in *B. abortus*.

As described below, biomedical ontologies can be used to dramatically improve *Brucella* literature mining.

ONTOLOGY-BASED ANALYSIS OF *BRUCELLA* PATHOGENESIS, HOST IMMUNITY, AND VACCINE TARGETS

A biomedical ontology is a consensus-based, controlled vocabulary of terms and relations, with associated definitions that are logically formulated in such a way as to promote automated reasoning (Xiang et al., 2010). Biomedical ontologies structure and interlink knowledge and data from complex biomedical domains in such a fashion as to permit shared understanding of a specific domain among different resources.

Extensive brucellosis research has resulted in a large number of publications encompassing various medical topics ranging from basic *Brucella* genetic study to vaccine clinical trials. To support data exchange and reasoning, a Brucellosis Ontology (IDOBRU)[3] has been developed (Lin et al., 2011). IDOBRU is a biomedical ontology in the brucellosis domain and is an extension ontology of the core infectious disease ontology (IDO-core; Cowell and Smith, 2010). Currently IDOBRU contains more than 1000 ontology terms covering areas such as etiology, transmission, symptoms, virulence factors, pathogenesis, prevention, and treatment. IDOBRU has been used to model different aspects of brucellosis, including host infection and zoonotic disease transmission, symptoms, virulence factors and pathogenesis, diagnosis, intentional release, vaccine prevention, and treatment. IDOBRU is the first reported bacterial IDO that has been developed to model different disease aspects in a formal logical format (Lin et al., 2011). The ontology can serve as a knowledgebase for *Brucella* and brucellosis. IDOBRU captures the knowledge extracted from published peer-reviewed sources that cover brucellosis bench research, clinical practice, and public health. In addition, IDOBRU has stored all *Brucella* virulence factors discussed in BBP (**Table 2**).

The vaccine ontology (VO)[4] is an open-access community-supported ontology in the domain of vaccine and vaccination (He et al., 2009). VO represents various vaccines and their relations. VO has collected more than 40 curated *Brucella* vaccines or vaccine candidates that have been officially licensed or proven to provide protection in animal models. The ontology provides detailed machine-readable information for each *Brucella* vaccine, such as the vaccine type, manufacturers of licensed vaccines, and host immune responses. Fourteen protective *Brucella* antigens have been included in VO. In addition, VO has been used to integrate many other vaccine data in the VIOLIN vaccine database and analysis system[5] (Xiang et al., 2008).

IDOBRU and VO can be used to support *Brucella* and brucellosis data exchange, data integration, and automated reasoning. These two ontologies use a machine-readable Web ontology language (OWL) format and thus support OWL-based ontological reasoning. Software programs can be developed to query IDOBRU and VO and to perform statistical and reasoning analyses. One particular research area of note is the application of these ontologies to advanced literature mining. In PubMed vaccine literature indexing is poorly performed due to limited hierarchy of Medical Subject Headings (MeSH) annotation in the vaccine field. SciMiner is a literature mining system that supports literature indexing and gene name tagging (Hur et al., 2009). Our study indicates that application of VO in SciMiner will aid vaccine literature indexing and mining of vaccine–gene interaction networks. Using the abstracts of 14,947 *Brucella*-related papers, VO-SciMiner identified 140 *Brucella* genes associated with *Brucella* vaccines. These genes included known protective antigens, virulence factors, and genes closely related to *Brucella* vaccines. When a total of 67 *Brucella* vaccine terms were incorporated into the VO-based SciMiner (VO-SciMiner), the program exhibited a superior performance in retrieving *Brucella* vaccine-related papers over that obtained with a MeSH-based PubMed literature search. For example, a VO-SciMiner search of "live attenuated *Brucella* vaccine" returned 922 hits as of April 20, 2011, while a PubMed search of the same query yielded only 74 (Hur et al., 2011). VO has identified 17 live attenuated *Brucella* vaccines (Hur et al., 2011). Licensed live attenuated vaccines RB51, strain 19, and Rev. 1 have been tested in mouse and large animals. Many live attenuated *Brucella* vaccines at the research stage have recently been tested in relevant animal models. For example, microencapsulated RB51 (Arenas-Gamboa et al., 2009a) and strain 19 (Arenas-Gamboa et al., 2009b) have recently been tested in red deer. RB51 and RB51 overexpressing superoxide dismutase (*sodC*) and glycosyltransferase (*wboA*) genes has been tested in bison (Olsen et al., 2009). These studies provide support toward the development of a safe and effective vaccine for practical animal uses.

CONCLUDING REMARKS

During the past decade, systems biology, and bioinformatics approaches have widely been used for study of the mechanisms of *Brucella* pathogenesis and host protective immunity against *Brucella* infections and for support of vaccine design. This review article demonstrates that integrative experimental Omics and computational bioinformatics analyses have dramatically advanced our understanding of how different *Brucella* species infect different host species, how *Brucella* gene expressions are regulated in cell culture or inside host cells (i.e., macrophages or epithelial cells), and how host cells (macrophages, epithelial cells, splenocytes, and blood cells) respond to *Brucella* infections. Advanced literature mining provides tools to retrieve and analyze virulence factors, protective antigens, and host–*Brucella* gene interactions from thousands of *Brucella* research publications. Machine and human-readable *Brucella* Ontology and VO have provided more ways to integrate *Brucella* data with other infectious diseases and vaccine data.

One main message of the review is that systems biology and bioinformatics approaches are able to help to facilitate vaccine development and predict fundamental molecular mechanisms of host–*Brucella* interactions. With the initial high throughput experimental studies and advanced data analyses, many predictions can be made and used as novel hypotheses for further confirmation

[3]http://www.phidias.us/bbp/idobru
[4]http://www.violinet.org/vaccineontology
[5]http://www.violinet.org

by "traditional" experimental approaches. The findings from the Omics studies have opened new avenues of research. Many of these studies confirmed and expanded the results of classical approaches in the areas of *Brucella* pathogenesis and host immunity against *Brucella* infection or vaccination. From our literature data mining analysis, known *Brucella* virulence factors can be retrieved. Compared to any isolated study of *Brucella* virulence factor(s), a systematical analysis of all possible virulence factors provides a more comprehensive view of how *Brucella* survives and replicates in a hostile intracellular environment and *in vivo*. In contrast to the traditional vaccine development strategy of continuous trials after isolated hypotheses, the new strategy that starts with systems biology and bioinformatics analyses make it possible to more rationally design safe, effective, and optimized *Brucella* vaccines.

Although much progress has been made, many challenges still exist. For example, while different gene expression profiles have been discovered at different experimental conditions, how to integrate these data and make sense of the interconnected host–*Brucella* interaction mechanism remains a challenge. IDOBRU and VO may provide ontology-based platforms for obtaining a higher level of data and knowledge integration. However, currently IDOBRU and VO only provide proof-of-concept demonstrations for representing *Brucella* virulence factors and host immune responses (Lin et al., 2011). Additional efforts are required to systematically apply IDOBRU/VO and related semantic web tools to represent and analyze different levels of host–*Brucella* interaction data and knowledge. Another challenge is how to improve the translation of the knowledge learned from the systems and bioinformatics studies into the generation of new vaccines and drugs against infectious *Brucella* infections? The *Brucella* gene expression data obtained under different experimental conditions may be used to better design vaccine protein targets. The host response profiles may facilitate a deeper understanding of the protective immune response in the host. This will require diligent research and development to design new ways to make all these translational outcomes a reality.

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