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Brucella: functional genomics and host–pathogen interactions

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Abstract

Brucellosis is a zoonotic disease caused by a number of *Brucella* species and is characterized by chronic macrophage infection. However, genes that may contribute to intracellular survival of the *Brucella* species are not well studied. This review presents, first, genomic islands that are present or absent in various *Brucella* species that may help establish *Brucella* infection and survival strategies. Second, the alteration in macrophage transcription by *Brucella* to permit its long-term survival within this hostile intracellular environment. A large number of macrophage gene transcripts are altered following *Brucella* infection indicating that *Brucella* is not a silent invader of host cells. Macrophage transcript levels associated with inflammation, apoptosis, signal transduction and vesicular intracellular trafficking are altered during *Brucella* infection, and likely contribute to intracellular survival of *Brucella*. Lastly, the host–pathogen interaction events associated with *Brucella* infection in living mice visualized in real-time using biophotonic imaging. Mice are often used to evaluate *Brucella* infections; however, *Brucella* dissemination and pathogenesis is poorly understood in mice. Biophotonic imaging of *Brucella* infections revealed sites of bacterial localization similar to human infections and different patterns of infection by attenuated or virulent *Brucella*.

Keywords: *Brucella*, genome, genomic islands, microarray of macrophages, biophotonic imaging

Introduction

Brucellosis is a zoonotic disease endemic in many areas of the world and is characterized by chronic infections, abortion and sterility (Corbel, 1997). In humans, brucellosis is a systemic, febrile illness resulting in osteoarthritis, endocarditis and several neurological disorders (Young, 1995; Corbel, 1997). Brucellosis is caused by many species belonging to the genus *Brucella* that are aerobic facultative intracellular bacteria. *Brucella* species are closely related to intracellular symbionts and pathogens of plants and animals and are classified as α2-proteobacteria based on rRNA sequence comparison (Moreno *et al*., 1990).

Although the specific mechanisms of intracellular survival by *Brucella* are not clearly understood, bacteria often alter normal host function to avoid immune detection. Successful strategies for intracellular survival of *Brucella* include the ability to survive in acidified membrane-bound vesicles (Porte *et al*., 1999; Kohler *et al*., 2002; Boigegrain *et al*., 2004), alteration of macrophage apoptosis (Gross, *et al*., 2000; Fernandez-Prada *et al*., 2003; Eskra *et al*., 2003), prevention of phagosome-lysosome fusion (Porte *et al*., 2003; Bellaire *et al*., 2005), the expression of a type IV secretory system (Boschiroli *et al*., 2002a, b) and insertion of *Brucella* lipopolysaccharide (LPS) into host membranes (Lapaque *et al*., 2006). *Brucella* long-term intracellular survival strategies as well as host innate and adaptive defense mechanisms comprise the host–pathogen relationship. Defining the interaction between a host cell and

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Brucella is crucial to understanding the infectious process.

The genus *Brucella* consists of at least six species, designated on the basis of host preference, antigenic and biochemical characteristics as *Brucella melitensis* (goats and sheep), *Brucella abortus* (cattle), *Brucella suis* (pigs), *Brucella canis* (dogs), *Brucella ovis* (sheep) and *Brucella neotomae* (wood rats) (Corbel, 1988). *B. abortus*, *B. melitensis* and *B. suis* can all infect humans with similar serious disease consequences (Corbel, 1997). Recently, *Brucella* spp. have been isolated from marine mammals and are infectious for humans (Sohn *et al.*, 2003). *B. melitensis*, originally isolated as a pathogen of goats and sheep, is highly pathogenic and a frequent cause of human brucellosis. In contrast, human infections with *B. ovis* and *B. neotomae* have not been reported, and human infection with *B. canis* is rare. Although *Brucella* species exhibit host preference and virulence restrictions, they are genetically highly related and therefore have been proposed to be included under one species *B. melitensis* (Verger *et al.*, 1985). Why *Brucella* has a preferential disposition for different animal species is unclear but a greater understanding of the genomes of various *Brucella* species may provide clues. In this review, we will examine comparative genomics of *Brucella*, innate immune responses of macrophages following infection by *Brucella* spp. and lastly the pathogenesis of *Brucella* dissemination in tissues.

Identification of genomic islands (GIs) in *Brucella* using comparative genomics

Sequencing genomes of large numbers of intracellular pathogens and endosymbionts has provided information on similar and unique processes used by these pathogens to establish their intracellular niches and to enhance transmission from host to host. These intracellular pathogens all contain several megabases of DNA, yet the findings reveal there are multiple solutions to intracellular survival as well as preferential selectivity for specific hosts. A comparison of the closely related *Brucella* species indicates that 98% of the DNA among the *Brucella* species are identical (Verger *et al.*, 1987), there is an average of >94% identity at the nucleotide level among *B. melitensis*, *B. abortus* and *B. suis* (Chain *et al.*, 2005) and minimal changes have occurred within a given species worldwide (Michaux-Charachon *et al.*, 1997). Differences among *Brucella* species may involve deletions of large segments of DNA, as well as recombination/transposition/inversion events of DNA regions within the genomes (Chain *et al.*, 2005). Although the analysis of *Brucella* species is still in its genomic infancy, there has been limited analysis of different *Brucella* biovars within a species to determine what constitutes a *Brucella* biovar at the genomic level. The evolutionary pressures that maintain consistency in a given *Brucella* species leading to development of biovars are an intriguing aspect of this pathogen.

Comparative genomic analysis of various *Brucella* species revealed only 42 unique genes in *B. suis* and 32 in *B. melitensis* (Paulsen *et al.*, 2002). The sequenced *Brucella* genomes reveal fundamental similarities between animal and plant pathogens and symbionts. In *Brucella*, the preference to persist within a protected intracellular niche may prevent the genetic exchange among the brucellae, minimizing the differences among *Brucella* biovars and species (Paulsen *et al.*, 2002). The lack of plasmids or lysogenic phage within the brucellae further supports minimal exchange of genetic contents among the brucellae. *Brucella* may have assembled its genome by intracellular adaptation and reductive loss of non-essential genes from an ancestral free-living bacterium with a larger genome (Boussau *et al.*, 2004; Nilsson *et al.*, 2005; Sallstrom and Andersson, 2005).

Microarray analysis using a *B. melitensis* oligonucleotide array revealed extensive similarities among *Brucella* species in their gene content (Rajashekara *et al.*, 2004). Only 217 open reading frames (ORFs) were absent either completely or partially compared to *B. melitensis* strain 16M, as illustrated in Fig. 1. We also discovered that >3110 ORFs of the 3198 ORFs were present in any given *Brucella* species, suggesting that genomes of *Brucella* species are similar and implying that a relatively small number of genetic changes may be responsible for differences in host preference and virulence among *Brucella* species. Whether certain genes are functional in a given *Brucella* species or are regulated differently may explain host preference and potential mechanisms of virulence for different *Brucella* species. Many of the ORFs present in *B. melitensis*, but absent from other *Brucella* species, are clustered in GIs. The GI variation among the different *Brucella* species are as follows.

The *B. ovis* genome is missing five GIs that may have resulted from more active insertional sequences in this species compared to other *Brucella* spp. *B. ovis* has ~30 IS6501 copies compared to other *Brucella* species that have 4–10 copies. ORFs in GI-1 encode five hypothetical proteins, a phage-related DNA-binding protein, resolvase and a hypothetical recombinase. The presence of a phage-related DNA-binding protein and proteins involved in DNA recombination suggests that this region might have been acquired through a horizontal gene transfer event. ORFs in GI-2 and GI-5 encode factors that might be involved in *Brucella* virulence. GI-2 contains ORFs for LPS biosynthesis and GI-5 contains ORFs encoding peptide ABC-type transporters such as Dpp, Opp, and Pot systems whose homologs in other bacteria are important for root colonization, intracellular survival, attachment to host cell, and virulence (Matthysse *et al.*, 1996; Borezee *et al.*, 2000; Kuiper *et al.*, 2001). ORFs in GI-7 and -9 encode two transcriptional regulators, an antioxidant stress-related protein, enzymes for energy production, and polypeptide deformylase (PDF),

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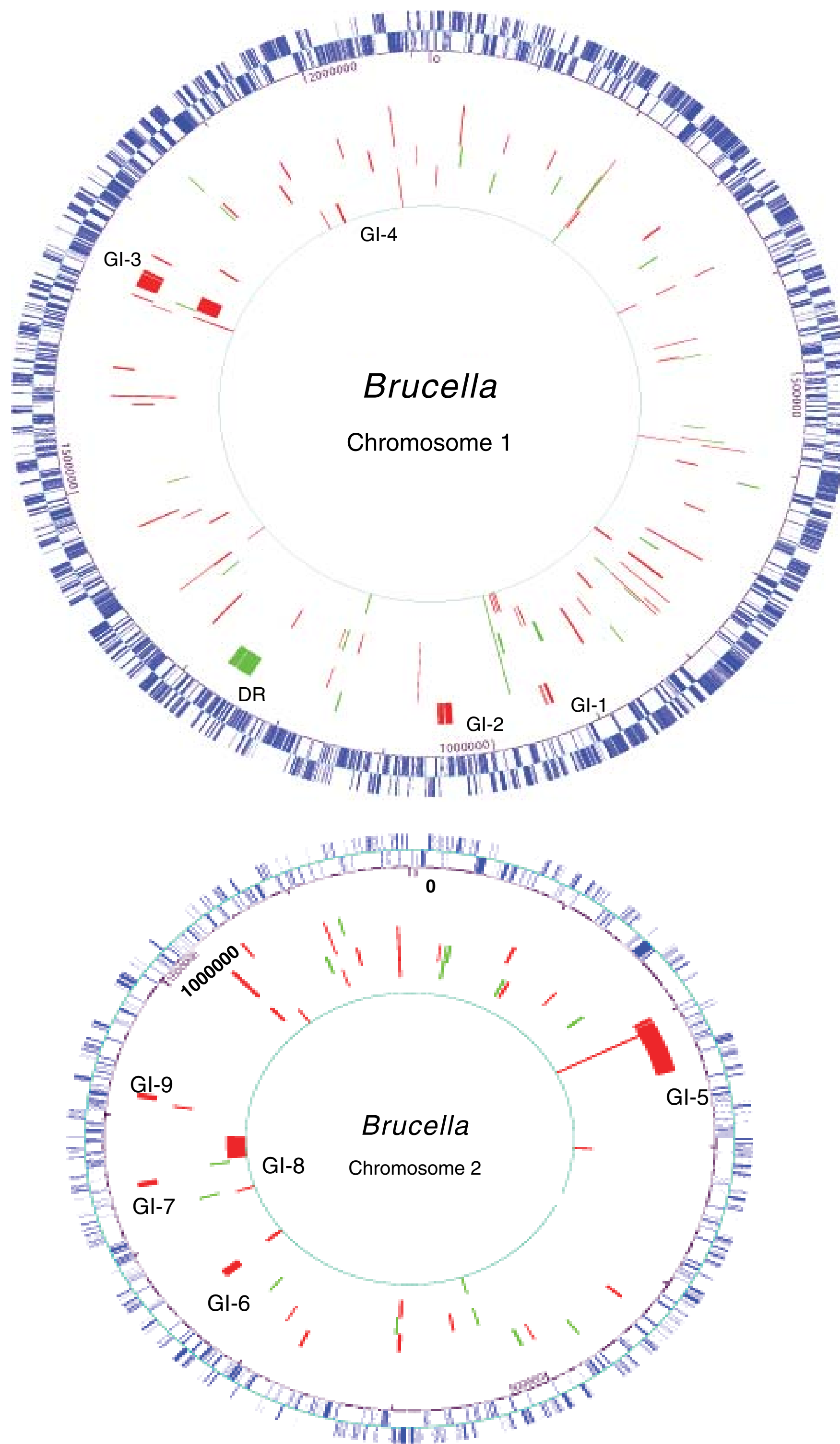

Fig. 1. Comparison of five *Brucella* species genomes to *B. melitensis* 16M by microarray. 16M arrays were hybridized with labeled genomic DNA (gDNA) from *Brucella* species (two or three hybridizations from gDNA labeled in separate reactions for each species). Deletions or duplication were determined by comparing the ratio of average probe signal for each ORF to signal for *B. melitensis* 16M. Red, predicted deletions; and green, predicted duplication. The order of strains in the (outside to inside) *B. ovis*, *B. canis*, *B. neotomae*, *B. melitensis* 710, *B. suis* and *B. abortus*. Regions where >3 contiguous ORFs have a log2 ratio significantly below the mean are designated as putative 16M GIs. One region of contiguous ORFs with log2 ratio significantly above the mean is indicated as a duplicated region (DR) (Rajashekara *et al.*, 2004).

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an essential bacterial metalloenzyme responsible for the removal of the N-formyl group from the N-terminal methionine of nascent polypeptides.

B. neotomae is a non-pathogen of humans yet lacks only 17 genes that are present in virulent *B. melitensis* including GI-6 (~7.5 kb). Transposases comprise seven of the missing genes, while five genes are annotated as a thiamine transport permease, alcohol dehydrogenase, a hemagglutinin and two transcriptional regulators. In *B. neotomae*, denitrification genes are altered, and in fact, complementation of the deleted region in *B. neotomae* by using *nirK*, *nirV* and *nnrA* from *B. melitensis* restored the ability of *B. neotomae* to reduce nitrite and decreased *B. neotomae* virulence (Baek *et al.*, 2004). Interestingly, *B. neotomae* replicates in macrophage cultures similar to wild-type *B. melitensis*. A careful assessment of why *B. neotomae* is non-pathogenic has not been performed.

In *B. canis* and *B. suis* there were 38 and 39 ORFs, respectively, that were either partially or completely absent. Interestingly, in both species an identical DNA region of approximately 21 kb (GI-3) was missing from chromosome I. In *B. abortus* 40 ORFs were absent including two clusters (GI-4, ~4.5 kb chromosome I; GI-8, ~25 kb chromosome II) (Rajashekara *et al.*, 2004).

In addition to our comparative genomic analysis using microarray, a recent comparison of genomes of three sequenced *Brucella* species, *B. abortus* strains 2308 and 9–941, *B. suis*, and *B. melitensis*, revealed that the pseudogenes, deletions and insertions in these genomes support previous studies that *B. abortus* and *B. melitensis* share a common ancestor that diverged from *B. suis* (Chain *et al.*, 2005). The comparison revealed that all three *Brucella* species have lost the genes involved in the metabolism of glycogen. The only glycogen-related gene conserved in the brucellae is the one required for virulence (Ugalde *et al.*, 2003). In most organisms, the utilization of α-D-galactose to glucose-1-phosphate is performed by four enzymes; however, in brucellae, the only enzyme present is UDP-galactose 4-epimerase. In *B. melitensis*, four genes are annotated as UDP-galactose 4-epimerase but their sequences are not similar. An ORF encoding cyclic β-1,-2-glucan synthetase, an important virulence determinant (Arellano-Reynoso *et al.*, 2005), is strictly conserved in the three *Brucella* species and is required for controlling intracellular trafficking (Arellano-Reynoso *et al.*, 2005). A unique characteristic of the *Brucella* cell envelope is the presence of phosphatidylcholine (PC), one of the major membrane-forming phospholipids, and a feature typical of eukaryotic membranes. The genomes of brucellae encode orthologs of the enzymes responsible for methylating phosphatidylethanolamine and for producing PC. A recent study on the composition of *B. abortus* 2308 phospholipids revealed that synthesis of PC requires the presence of choline, and that PC synthesis occurs exclusively via the PC synthase pathway. To promote pathogenesis, *Brucella* depend on choline from the host cell to form PC. (Comerci *et al.*, 2006).

Of serious concern in *Brucella* research is the identification of genetic factors that permit these species to multiply within the host and cause disease. Well-characterized virulence factors of many pathogenic bacteria such as cytolysins, capsules, exotoxins, secreted proteases, pili and/or fimbriae, flagella, phage-encoded toxins and virulence plasmids are absent in *Brucella* (Delvecchio *et al.*, 2002; Moreno and Moriyon, 2002). Brucellae do not possess classic virulence factors but appear to utilize a type IV secretion system, flagella or non-classical virulence factors. Pathogenic bacteria often use secretion systems to deliver signals into host eukaryotic cells. *Brucella* species examined have a complete type IV secretion system. This key virulence determinant is highly conserved at the DNA level among three *Brucella* species whose genomes have been sequenced. In contrast, the flagellar system in various brucellae appear to vary in functional ORFs. Brucellae are non-motile bacteria; however, their genomes contain a considerable number of class II and class III flagellar genes (Delrue *et al.*, 2005). The organization of these genes is the same in the three species; however, based on sequence information many of these genes appear as pseudogenes, suggesting that the lack of motility in brucellae results from inactivation of several flagellar genes and the absence of chemotactic mechanisms (Chain *et al.*, 2005). However, the recent work demonstrating a flagellar-like structure by electron microscopy (Delrue *et al.*, 2005) now requires a biological role for this identified structure since *Brucella* are non-motile.

In conclusion, comparative *Brucella* genomics has identified a set of genes, often clustered in GIs, that are missing from *Brucella* species that are not pathogenic to humans. Findings from these studies now facilitate future investigations to understand the relevance of these genes or gene clusters in host adaptation and restriction of virulence of certain *Brucella* species to humans. Furthermore, GIs present in *Brucella* are likely acquired as most islands have lesser G+C content (Rajashekara *et al.*, 2004), and many islands have large numbers of ORFs encoding proteins with unknown functions. The GIs missing in *B. ovis* are present in other *Brucella* species that are pathogenic to humans. However, *B. neotomae*, a species that is not pathogenic to humans and domestic animals, also possesses these islands. Thus, identifying why *B. neotomae* is not pathogenic to humans would be interesting. In addition, *B. canis* and *B. suis*, although differing in virulence to humans, are genetically very similar. *B. canis* lacks only three genes present in *B. suis*: a polysaccharide deacetylase, and the α and β chains for protocatechuate 3,4 deoxygenase. These findings imply that, in addition to the loss or gain of genetic content in *Brucella* species, mechanisms involving gene inactivation or altered expression of virulence traits may contribute to differences in host range and virulence of *Brucella*

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species for humans. However, it is possible that genetic contents unique to each *Brucella* species may contribute to host preference and virulence restriction differences. Future studies are necessary to understand the relevance of these GIs to the host adaptation and virulence of *Brucella* species.

Host-response to *Brucella* infection

Brucella infections are typically chronic in nature suggesting an ongoing interaction between host and pathogen. To promote long-term intracellular survival, *Brucella* minimize activating host inflammatory mechanisms. For example, *Brucella* LPS has 100- to 1000-fold less ability to activate proinflammatory TNFα or IL-1 cytokines compared to similar concentrations of *Esherichia coli* LPS (Jarvis *et al*., 2002). For intracellular bacteria such as *Brucella*, survival and replication within phagocytic cells with minimal signaling of host defenses is the key to pathogenesis. Animals and humans infected with *Brucella* rarely die.

During macrophage infection by *Brucella*, examining changes in transcript levels from macrophages exposed to *Brucella* may reveal early host responses to this facultative intracellular bacterium. A murine microarray was used to evaluate macrophage genes that were differentially transcribed in a 4 h infection by *B. abortus* (Eskra *et al*., 2003). Proinflammatory cytokine and chemokine transcripts were up-regulated and serve as evidence of an antibacterial response by host cells. Therefore, *Brucella* is not a silent invader of the macrophage. In contrast, however, transcripts of macrophage genes involved in cell cycling, apoptosis and intracellular vesicular trafficking were decreased. The last group of genes may permit bacterial intracellular survival in macrophages by redirecting *Brucella*-containing vesicles away from lysosomal contact and promoting docking to the endoplasmic reticulum membrane. A number of genes whose proteins are involved in intracellular trafficking are down-regulated 4 h following macrophage infection. Rab proteins are key regulators of membrane trafficking and function in tethering and vesicle movement. Several Rab transcripts are down-regulated, suggesting an alteration of vesicle transport from endosomes to the *trans*-Golgi. The modulation of genes involved in intracellular trafficking may increase survival of *Brucella* within macrophages by redirecting vesicular movement and preventing phagosome–lysosome fusion. Table 1 illustrates a number of macrophage gene transcripts increased by *Brucella* infection, while Table 2 presents macrophage gene transcripts decreased by *Brucella* infection.

Macrophage function depends on multiple signaling pathways that control the decision to proliferate, differentiate or initiate apoptosis. Disruption of these pathways by *Brucella* can lead to alterations that permit pathogen survival and eventual dissemination of *Brucella* to other host cells. The up-regulation of inflammation-associated genes, though less evident with *Brucella* than other pathogens, may serve to clear this pathogen. Alternatively, the influx of macrophages to the site of infection may provide additional macrophages for *Brucella* to infect and provide a vehicle for bacterial dissemination.

Apoptosis plays a significant role in regulating the pathogenesis of infection. If viable *Brucella* are released to the extracellular environment, antibodies may bind the bacteria, leading to complement activation or enhanced phagocytosis and killing of *Brucella*. To survive in a protected intracellular niche, *Brucella* may block apoptosis (Fernandez-Prada, 2000). Several enzymes in the apoptotic pathways are down-regulated in *Brucella* infection at 4 h (Eskra *et al*., 2003). Preventing apoptosis early in infection would permit intracellular *Brucella* to successfully establish a replicative niche. Pro-survival genes are up-regulated in *B. abortus* (Eskra *et al*., 2003) and *B. suis* infections (Gross *et al*., 2000) and genes encoding three proapoptotic proteins are down-regulated. Thus, early in macrophage infections *Brucella* appear to present an organized strategy to enhance its intracellular survival. However, what *Brucella* products are responsible for altering host defense mechanisms need to be identified. Knowing the *Brucella* proteins that alter macrophage function, therapeutic approaches using chemical genomic strategies can be used to disrupt the *Brucella* protein activity.

In summary, determining the macrophage genes that are transcribed during the early stages of *Brucella* infection can provide hypotheses regarding the molecular pathogenesis of brucellosis. Increase in a number of transcripts for proinflammatory cytokines was evident, similar to findings with human macrophages activated by several Gram-negative and -positive bacteria (Nau *et al*., 2002) and may constitute a general host recruitment of antibacterial defenses. However, *Brucella* may subvert newly arriving macrophages for additional intracellular infection. *Brucella* infection also led to transcript inhibition of host genes involved in apoptosis, cell-cycling and intracellular vesicular trafficking mechanisms. Selective targeting of host genes suggests that *Brucella* possess specific mechanisms to alter host gene expression. Future studies are needed to examine individual *Brucella* proteins and particular host pathways that would ensure a bacterial advantage for intracellular survival. Understanding pathogen-specific manipulations of host pathways has practical applications in designing vaccines and therapies that engage the innate immune system in a targeted fashion.

Brucella dissemination and pathogenesis in animals

Understanding the molecular basis of microbial diseases has increased dramatically in recent years. New

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Table 1. Macrophage gene transcription induced by *B. abortus* infection

Gene Symbol	Protein	Fold change
Inflammation and chemokines		
IL-1b	Interleukin-1β	188.58
MIP-2	Macrophage inflammatory protein 2	102.88
Csf3	Granulocyte colony-stimulating factor	20.55
Scya7	Small inducible cytokine A7	8.07
Scyb10	Macrophage interferon inducible protein 10 (IP-10)	6.42
MIP-1β	Macrophage inflammatory protein 1β	7.95
TNFα	Tumor necrosis factor α	57.98
IL-1rn	IL-1 receptor antagonist IL-1rn	8.35
IL-13rα2	Interleukin 13 receptor α2	6.97
Apoptosis		
Naf1	A20-binding inhibitor of NF-κB activation	3.65
Fas	Fas antigen	5.02
Gadd45b	Myeloid differentiation primary response gene	17.15
Ltβ	Lymphotoxin-β	6.78
Tnfaip3	Tumor necrosis factor induced protein 3	5.87
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	8.05
Adhesion		
Cd44	CD44	2.95
ICAM1	Intracellular adhesion molecule 1	3.77
Stress		
Sqstml	Oxidative stress-induced protein	3.58
MnSOD	Manganese superoxide dismutase (MnSOD) gene	2.43
GLRX	Glutaredoxin	3.95
Signal transduction		
Jak2	Janus kinase 2	2.13
Stk10	Serine/threonine kinase 10	3.03
Rgs16	Retinally abundant regulator of G-protein signaling	3.83
Regulatory		
SOCS3	Suppressor of cytokine signaling-3	55.72
Traf1	Tnf receptor-associated factor 1	36.30

Total RNA from uninfected and *B. abortus* S2308-infected RAW 264.7 (multiplicity of infection of 100) was extracted at 4 h after infection. The labeled cRNA from each RNA sample was prepared and hybridized to murine U74A gene chip (Affymatrix) as described (Eskra *et al.*, 2003). The sum of six difference calls from the intergroup comparisons of two uninfected and three *Brucella*-infected arrays (2×3) was calculated. A sum of greater than or equal to 8 was the cutoff for increase determination (Eskra *et al.*, 2003).

techniques, for example microarrays described in the previous section, have been developed that allow most bacterial pathogens to be studied at the molecular and cellular levels. Many pathogens share common mechanisms to subvert or survive within the host, but each species has also evolved a repertoire of unique approaches to exploit host processes. Sensitive imaging systems now permit following bioluminescence production or green fluorescent protein expression from bacteria in a living animal in real-time. The application of genetic techniques to identify genes essential for virulence in an animal in combination with real-time monitoring of bioluminescent bacteria permits identifying bacterial genes relevant to bacterial dissemination and virulence.

Acute human brucellosis is characterized by localization of bacteria in various tissues leading to chronic disease with serious clinical manifestations, such as orchitis, osteoarthritis, spondylitis, endocarditis as well as neurological disorders (Young, 1995; Corbel, 1997). Following infection, *Brucella* enter regional lymph nodes, resulting in bacteremia that facilitates dissemination into various tissues. *Brucella* infection in mice parallels that of human infection. Therefore, mice are used routinely to understand *Brucella* pathogenesis. However, the infection process can vary within individual mice, even in inbred strains of mice. Current studies of brucellosis in mice routinely examine the number of colony-forming units (CFU) in livers and spleens and kinetic studies require large numbers of mice, often representing weeks of infection. Determining the kinetics of disease progression within the same living host would reduce the number of animals required, prevent animal-to-animal variability and enhance understanding of the pathogenic process. Importantly, sequential analyses of the entire body during infection can provide a more comprehensive understanding of the overall disease process. Also, real-time sequential analysis of mutant bacteria may identify the contribution of bacterial genes to tissue localization and dissemination.

Bacterial luciferases, unlike eukaryotic luciferase systems, express biosynthetic enzymes for substrate synthesis within the engineered bacteria producing light

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Table 2. Macrophage gene transcription decreased by *B. abortus* infection

Gene symbol	Protein	Fold change
Cell membrane		
Fcgr1	Fc receptor, IgG, high affinity I	10.2
Ly86	MD-1	1.6
Apoptosis		
Bnip3	E1B 19K/Bcl-2-binding protein homolog (Nip3)	2.4
Bad	BAD protein	2.6
Siva	Proapoptotic protein (Siva) gene	2.7
Nix	NIX (Nix) mRNA, nuclear gene encoding mitochondrial protein	2.4
Birc5	TIAP mouse homolog of inhibitor of apoptosis	2.1
Caspase 3	Caspase 3, apoptosis-related cysteine protease	5.4
Intracellular trafficking		
Rab3d	GTP-binding protein (Rab3D) mRNA	4.3
Rab9	SID 99 mRNA for small GTP-binding protein	3.0
Syngr1	Synaptogyrin 1b	2.7
Gsn	Gelsolin	1.7
Siat8d	*N*-glycan α2,8-sialyltransferase	2.9
Kif4	Kinesin heavy chain member 4	1.8
Kif11	Kinesin-related mitotic motor protein	2.2
Adhesion		
Kit1	Mouse mast cell growth factor (MGF)	4.1
Cell cycle, differentiation and proliferation		
Chk2	Protein kinase Chk2 (Chk2)	2.9
Cdc6	Cdc6-related protein	4.4
Vegfb	VEGF-related factor mvrf186 precursor mRNA	1.9
Rasa3	GTPase-activating protein GAPIII	3.8
BRCA1	Breast cancer 1	6.1
Rb11	Retinoblastoma-like 1 (p107)	3.4
Wig1	p53-inducible zinc finger protein (Wig-1)	2.3
Spa1	Signal-induced proliferation associated gene 1	3.2
Agpt2	Angiopoietin 2	2.5
Ptpro	Protein tyrosine phosphatase, receptor type, O	3.0
Transcription		
Mefc2	Myocyte enhancer factor 2C	3.1
Tieg	Transcription factor GIF mRNA	2.1
E2F-1	E2F transcription factor 1	3.7
Stress		
Gpx4	Glutathione peroxidase 4	1.6
Transport		
Kcnaβ2	K^+ channel β2 subunit mRNA	2.3
Regulatory		
Rnf13	RING zinc finger protein (Rzf)	1.9
Crip	Cysteine-rich intestinal protein	1.8
Sh3bp2	SH3-binding protein 3BP2	2.4

Total RNA from uninfected and *B. abortus* S2308-infected RAW 264.7 (multiplicity of infection of 100) was extracted at 4 h after infection. The labeled cRNA from each RNA sample was prepared and hybridized to murine U74A gene chip (Affymatrix) as described (Eskra *et al.*, 2003). The sum of six difference calls from the intergroup comparisons of two uninfected and three *Brucella* infected arrays (2×3) was calculated. A sum of less than or equal to −8 was the cutoff for decrease determination (Eskra *et al.*, 2003).

without an exogenous substrate. Unlike green fluorescence protein, bioluminescence depends on the metabolic integrity of the bacteria and therefore serves as an ideal means to visualize the localization of virulent and attenuated *Brucella* strains. Furthermore, luciferase of *Photorhabdus luminescence* has a short half-life of several seconds, ensuring that the events occurring reflect processes in real time and are not artefacts of the accumulated signals. Therefore, monitoring bioluminescent *Brucella* infection in mice reflects the dynamic process of host–pathogen interaction. Bioluminescent imaging can be highly useful in analyzing sublethal infections that are often problematic because of animal-to-animal variation and unpredictable bacterial location.

The dynamics of *B. melitensis* infection have recently been evaluated in real time in mice using a bioluminescent marker inserted in the *Brucella* chromosome to monitor temporal infection (Rajashekara *et al.*, 2005). bioluminescent *B. melitensis* (GR023) is very similar to its parental wild-type 16M in its growth characteristics and

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in vitro (macrophage) and *in vivo* (mice) growth and persistence. Significant to *Brucella* pathogenesis, this bioluminescent approach revealed the following: first, the previously unknown localization of *Brucella* in salivary glands of mice that may be important to establish infection particularly in humans where infection occurs commonly through ingestion. The salivary gland may contain factor(s) that preferentially promote localization of *Brucella* or *Brucella*-containing macrophages. Since *Brucella* are non-motile, how *Brucella* rapidly traffic to this tissue location following an intraperitoneal injection is a particularly intriguing issue. Second, preferential replication of *Brucella* in murine testes may reflect the similar ability of the bacterium to localize to the testes in humans (Malik, 1997). Again, the mechanism(s) for the preferential localization of *Brucella* to this tissue site is unknown. Third, the localization of *Brucella* to vertebral joints in the tails of mice may provide a model to understand pathogenesis of chronic brucellosis in humans where it localizes to the vertebral joints. Musculoskeletal involvement is one of the most frequent complications of human brucellosis affecting the spine at any level (Lulu *et al.*, 1988). In bone tissue *Brucella* may reside in osteoclasts, which are derived from macrophage lineage. The bacteria are not present in synovial fluid and analysis of synovial fluid for a cellular profile, glucose and protein content is not particularly helpful in the diagnosis (Khateeb *et al.*, 1990). Fourth, bioluminescent analysis of *Brucella*-infected mice has revealed patterns of bacterial growth and clearance in certain tissues that are difficult to observe using the conventional method of determining CFU. Interestingly, tissue distribution in the mouse as observed by biophotonic imaging parallels the description of *Brucella* infection in humans (Madkour, 2001). As the infection disseminates to various organs throughout the body, the process can be monitored in real time. Fifth, biophotonic imaging of attenuated *Brucella* has identified the contribution of certain bacterial genes in tissue-specific replication. For example, genes encoding LPS products are important for *Brucella* to readily disseminate. *Brucella* that lack complete native LPS fail to disseminate as readily as wild-type bacteria. Therefore, detecting bioluminescent *Brucella* in living mice provides a novel visual representation of the disease process that likely reflects human infection and accelerates our understanding of *Brucella* pathogenesis. Thus, biophotonic imaging provides stronger evidence for parallel *Brucella* infections between mice and humans than previously appreciated.

In addition to tracking bacterial dissemination in an animal, bioluminescence has revealed novel sites of bacterial colonization especially in chronic and asymptomatic infections, providing insight into potential mechanisms of bacterial persistence and spread. Infection of interferon regulatory factor-1$^{-/-}$ or C57BL/6 mice with bioluminescent virulent *B. melitensis* strain GR023 resulted in strong bioluminescence in the salivary gland and testicles as early as 2 days post inoculation, even prior to evidence of bacteremia, suggesting that *Brucella* efficiently and preferentially colonize these organs. Colonization of the salivary gland is particularly intriguing since human infection often results from ingestion of contaminated dairy products. Previously, *Brucella* have been shown to colonize the parotid lymph nodes of calves after conjunctival exposure (Meador *et al.*, 1988). *Brucella* may utilize this special niche near the site of ingestion to establish infection but the advantages of this tissue contributing to the adaptation of *Brucella* to the host are unknown.

The contribution of specific *B. melitensis* genes to bacterial dissemination and persistence *in vivo* in real time has not been studied previously. As examples, two *Brucella* genes that influence dissemination or persistence will be discussed. First, the VirB system, consisting of 12 VirB proteins, is important *in vitro* for *Brucella* to establish a replicative niche in macrophages (O'Callaghan *et al.*, 1999). Consistent with previous findings (Rouot *et al.*, 2003; den Hartigh *et al.*, 2004), we have observed strong bioluminescence with a *virB4* mutant when grown in broth in log as well as stationary phase. The bioluminescent *virB4* mutant disseminates to various tissues following intraperitoneal injection as seen with the virulent *B. melitensis* strain GR023 (Rajashekara *et al.*, 2005). However, in mice the *virB4* mutant was unable to persist as long as strain GR023. These findings indicate that mutation in the *virB4* gene does not interfere with initial spread; however, bacterial replication and persistence are affected. The *virB4* mutant was also attenuated in *in vitro* macrophages similar to other VirB mutants (O'Callaghan *et al.*, 1999; Delrue *et al.*, 2001; den Hartigh *et al.*, 2004). Although *virB* mutants are defective in intracellular replication, mutant bacteria can be isolated from spleens of infected BALB/c mice even after 8 weeks post inoculation (den Hartigh *et al.*, 2004) and up to 5 weeks in C57BL/6 mice (results not shown), suggesting that other factors may contribute to *Brucella* persistence in mice. Since macrophages serve as a protective niche for the intracellular survival of *Brucella*, can *virB* mutant *Brucella* survive for extended periods in macrophages without acquiring endoplasmic reticular membranes? The *in vitro* data of others suggest that *Brucella virB* mutants cannot survive long-term in macrophages *in vitro* (Delrue *et al.*, 2001; den Hartigh *et al.*, 2004). However, *in vivo virB* mutants can survive in spleens for many days (Rajashekara *et al.*, 2006). Alternatively, *virB* mutants or wild-type *Brucella* likely survive in other cell types *in vivo*. Collectively, the data on *virB Brucella* mutants suggest that dissemination of these mutants readily occurs in the host but the mechanism of trafficking requires further study.

A second *Brucella* mutant that has an altered dissemination pattern compared to virulent *Brucella* is the *galE* mutant. Unlike *virB4*, a *galE* mutant was defective in replication as well as dissemination (Fig. 2) in interferon

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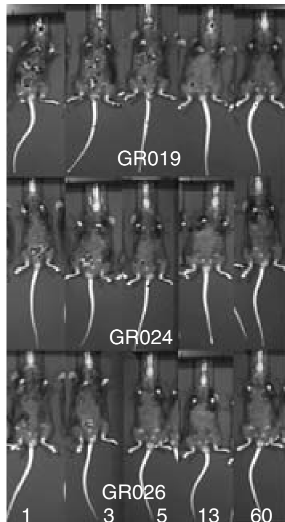

Fig. 2. Real-time analysis of attenuated bioluminescent *B. melitensis* strains in C57BL/6 mice. Mice were infected with 5×10^7 CFU of *B. melitensis* strains GR019 (*virB4*), GR024 (*galE*) and GR026 (*bmeI1090-91*) and imaged daily with a 10 min exposure. Numbers below the panels indicate the number of days post infection, and images representing the same day post infection from different groups are shown. The bioluminescent image of the same single mouse from each group is shown. The rainbow scale represents approximate photon counts (Rajashekara *et al.*, 2005).

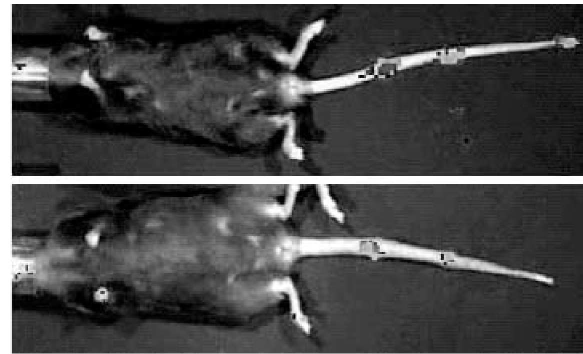

Fig. 3. Wild-type mice infected with bioluminescent *B. melitensis galE* for 2–3 months. Note localized bioluminescence in regions corresponding to joints. These bioluminescent joints were swollen and may represent joint lesion observed in humans infected with chronic brucellosis.

regulatory factor-1$^{-/-}$and wild-type C57BL/6 mice. Mice infected with the *galE* mutant had bioluminescence localized in the intervertebral region of the tail during the later stage of infection (Fig. 3), a feature not observed with *virB4* mutant. Even though bioluminescent signals reappeared, mice were asymptomatic and healthy. This skeletal localization is suggestive of chronic infection as reported for humans (Madkour, 2001). The reappearance and growth of *Brucella* at an atypical site suggests the adaptive immune system can no longer control the infection. It is likely that *Brucella* are intracellular at this atypical location to minimize an adaptive immune response. Our preliminary studies suggest that the *galE* mutant may have defective LPS. It is possible that the ability of *Brucella* to disseminate systemically in the host may depend on the intact LPS as we have a similar observation with another LPS defective bioluminescent mutant (results not shown).

In summary, this review highlights recent findings of the host–pathogen relationship between *Brucella* and mice. First, comparative genome analysis among various *Brucella* species revealed GIs selectively missing from *Brucella* species compared to *B. melitensis*. These GIs, or their absence, likely contribute to the unique properties of each *Brucella* species for adaptation to its host. Future studies of deleting particular islands from virulent *Brucella* followed by identifying an alteration in phenotype would be important to characterize the contribution of genes within each island. Current data suggest that deletion of GI-2 from virulent *Brucella* produces an attenuated mutant with an altered dissemination pattern. Second, *Brucella* can induce long-term intracellular survival with minimal activation of host inflammatory mechanisms such as proinflammatory TNFα or IL-1 cytokines. Successful intracellular survival of *Brucella* would require minimal signaling of host defenses as a key mechanism to prolonging its intracellular survival. Animals and humans infected with *Brucella* rarely die, the hallmark of a successfully adapted pathogen. Third, bioluminescent *B. melitensis* in infected interferon regulatory factor-1$^{-/-}$ and C57BL/6 mice has provided a visual progression of infection. Interestingly, *Brucella* localized to novel tissue sites in mice, e.g., salivary gland, and re-emerged as a chronic infection in osteoarticular joints. Furthermore, bioluminescent imaging of *Brucella* mutants in infected mice rapidly identified attenuation and revealed differences in the tissue distribution, thus offering insight into the contribution of *Brucella* genes in tissue-specific localization.

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