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Decreased *in vivo* virulence and altered gene expression by a *Brucella melitensis* light-sensing histidine kinase mutant

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Abstract

Brucella species utilize diverse virulence factors. Previously, *Brucella abortus* light sensing histidine kinase was identified as important for cellular infection. Here, we demonstrate that a *Brucella melitensis* LOV-HK (BM-LOV-HK) mutant strain has strikingly different gene expression than wild type. General stress response genes including the alternative sigma factor *rpoE1* and its anti-anti sigma factor *phyR* were downregulated, while flagellar, quorum sensing, and type IV secretion system genes were upregulated in the BM-LOV-HK strain versus wild type. Contextually, expression results agree with other studies of transcriptional regulators involving *rpoE1, phyR, vjbR,* and *blxR* (*babR*) *Brucella* strains. Additionally, deletion of BM-LOV-HK decreases virulence in mice. During C57BL/6 mouse infection, the BM-LOV-HK strain had 2 logs less CFUs in the spleen 3 days post infection but similar levels 6 days post infection compared to wild type. Infection of IRF-$1^{-/-}$ mice more specifically define BM-LOV-HK strain attenuation with fewer bacteria in spleens and significantly increased survival of mutant versus wild type infected IRF-$1^{-/-}$ mice. Upregulation of flagella, quorum sensing, and VirB genes, along with downregulation of *rpoE1* and related sigma factor, *rpoH2* (BMEI0280) suggest that BM-LOV-HK modulates both quorum sensing and general stress response regulatory components to control *Brucella* gene expression on a global level.

Keywords

brucellosis; flagella; secretion; stress; virulence; LOV; virB; PhyR

Introduction

Brucella spp. are zoonotic pathogens responsible for the disease brucellosis in animals and humans. Brucellosis in animals can extract a high economic cost due to spontaneous abortions and the need to cull infected herds to prevent disease spread. Although brucellosis is rarely fatal, the global distribution, high economic cost of animal infections, and the length and complexity involved in resolving human infection make research on *Brucella* virulence mechanisms a priority.

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Brucella spp. utilize few of the classic virulence factors employed by other Gram negative pathogens. Being a facultative intracellular pathogen, brucellae strike a fascinating balance being highly infective, persisting in hosts, and establishing long-term infections. Brucellae exploit a replicative niche within host phagocytic cells. Previous work revealed that the *Brucella* LOV-HK protein is important to bacterial survival in an *in vitro* macrophage model of infection (Swartz*, et al.*, 2007). An insertional gene knockout of LOV-HK in *B. abortus* (BA-LOV-HK) resulted in fewer bacteria in macrophages similar to a level observed during infections conducted in the dark (Swartz*, et al.*, 2007). LOV-HK contains a light, oxygen, and voltage (LOV) domain that binds a flavin cofactor enabling the protein to sense blue light (Swartz*, et al.*, 2007, Rinaldi*, et al.*, 2012). Light activates the enzymatic activity of LOV-HK and is necessary for full virulence of the bacterium in macrophages (Swartz*, et al.*, 2007). These results provided a clear link between LOV-HK and light-induced virulence in an *in vitro* cellular infection assay. Whether LOV-HK exerts a similar role in virulence during an *in vivo* infection is unknown.

In this report, we show deletion of *LOV-HK* (BM-LOV-HK) from *B. melitensis* altered expression of the VirB operon, flagella, quorum sensing, and general stress related genes known to be associated with *Brucella* virulence. We also demonstrate that BM-LOV-HK is necessary for full *B. melitensis* virulence in immune competent C57BL/6 and immune compromised IRF-1$^{-/-}$ mouse models of infection.

Materials and methods

Bacterial strains, vectors, and culture maintenance

Brucella melitensis strain 16M (ATCC23456) was utilized as the wild type strain for experiments, as well as for gene deletion studies. *Brucella* was propagated and maintained in brucella broth and on brucella agar (BD). *E. coli* DH5α was used as the maintenance and propagation strain for vectors. *E. coli* was propagated and maintained in Luria-Bertani broth and on Luria-Bertani agar. A suicide vector using the p-ZErO-2 backbone containing both kan^r and amp^r was used to generate the gene deletion strain. Kanamycin was utilized in media at a final concentration of 50 μg mL^{-1}. Ampicillin was used at 100 μg mL^{-1}. See Table S1 for a list of strains and vectors.

Generation of deletion strain

The BM-LOV-HK (BMEII0679) was produced using homologous recombination as previously described (Rajashekara*, et al.*, 2006). Briefly, a suicide vector was constructed containing 1 kb immediately up and downstream of the gene of interest, and a kanamycin resistance cassette was inserted between these two flanking regions. Suicide vectors also contained an ampicillin resistant cassette for counter selection. Suicide vectors were transformed into *B. melitensis* by electroporation. Desired strains were isolated by resistance to kanamycin and sensitivity to ampicillin. Gene deletion was verified by PCR. See Table S2 for gene deletion primers. Separate growth curves using brucella broth and minimal media plus glucose showed no significant difference in growth between the BM-LOV-HK mutant and wild type (data not shown). As with wild type bacteria, colonies of the BM-LOV-HK mutant strain did not stain with crystal violet (data not shown).

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Isolation of *Brucella* RNA for microarray and qRT-PCR

Brucella RNA was isolated from log phase cultures using an adaptation of the Epicentre Master Pure RNA kit/protocol with an additional DNAse treatment using Applied Biosystems TurboDNase. Briefly, liquid cultures were grown in brucella broth (BD) and treated with 1 part stop solution per 10 parts sample (stop solution was 0.5% phenol in ethanol). Treated samples were pelleted at 13,000 g and frozen at −80 °C. During lysis, samples were incubated at 65 °C for 20 min with vortexing every 5 min. RNA was eluted in nuclease free water. A second DNase digestion was conducted using the Applied Biosystems TurboDNase protocol for rigorous digestion. RNA for microarray analysis was assayed for quality and quantity by Thermo NanoDrop and Agilent 2100 Bioanalyzer.

Brucella cDNA production, labeling, microarray hybridization

All microarray analysis (3 biological replicates per strain) was conducted according to Roche NimbleGen Arrays User's Guide for Gene Expression Analysis for 385K arrays. Briefly, cDNA was produced using the NimbleGen protocol and Invitrogen Superscript II. cDNA labeling was conducted using the Nimblegen One-Color DNA Labeling Kit and protocol. Array hybridization was conducted for ~18 h at 42 °C on a Maui Hybridization System. Washed and dried arrays were scanned on a GenePix 400B Scanner. Data were extracted and normalized from scanned array images using Roche NimbleScan Software. The Bioconductor EBarray package was used to determine significance (LNN>0.5) (Newton*, et al.*, 2001, Kendziorski*, et al.*, 2003), where fold change calculation was BM-LOV-HK/WT for positive fold change and −1/(BM-LOV-HK/WT) for negative fold change.

Raw microarray data

Raw microarray data has been deposited with the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/), series record GSE42599.

qRT-PCR of *Brucella* genes

RNA was isolated three times per bacterial strain as described above. First strand cDNA synthesis conducted with the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems. A control reaction without reverse transcriptase was conducted for each biological replicate to ensure the absence of gDNA contamination. cDNA produced was used at a final dilution of 1:1000 per qRT-PCR reaction. Final primer concentration was 400 nM per primer, per reaction. qRT-PCR was conducted for 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Samples were subjected to 95 °C for 2.5 min before cycling began. Bio-Rad iQ™ SYBR® Green Supermix chemistry was used. Values were calculated by the delta-delta C_T method and normalized to expression of the *IF-1* (BMEI1671) gene (Eskra*, et al.*, 2001). qRT-PCR primers are identified in Table S3.

Measurement of spleen bacterial colony forming units (CFUs) and mouse survival

C57BL/6 or IRF-$1^{-/-}$ mice (N=4 per strain, per time point) were infected intraperitoneally with $1x10^6$ CFUs and then housed until euthanized. Spleens were harvested and macerated with disposable tissue grinders (Fisher) in 3 mL of phosphate buffered saline (PBS). Liquid

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product was 10-fold serially diluted in PBS and replica plated to determine spleen CFUs. Plates were grown for three days before counting. Results were analyzed using the Mann-Whitney test performed with GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego CA.

IRF-1$^{-/-}$ mice were infected [*B. melitensis* 16M (N=11) and BM-LOV-HK (N=12)] intraperitoneally with 1x10^{6} CFUs and then housed until they either succumbed to *Brucella* infection or were euthanized (Matsuyama*, et al.*, 1993, Kamijo*, et al.*, 1994, Taki*, et al.*, 1997, Salkowski*, et al.*, 2000, Ko*, et al.*, 2002, Rajashekara*, et al.*, 2005). Results were analyzed using the Mantel-Cox test performed with GraphPad Prism version 5.

All animal protocols and experiments were approved and conducted in accordance with the University of Wisconsin-Madison Research Animal Care and Use Committee.

Results

Differences in global gene expression between the BM-LOV-HK strain and *B. melitensis* 16M grown in broth culture

To identify the genes directly and indirectly regulated by BM-LOV-HK, a whole genome microarray expression analysis was conducted with 3 biological replicates comparing the BM-LOV-HK mutant strain to *B. melitensis* 16M wild type. RNA was isolated from log phase broth cultures to access growing bacteria. Microarray results were verified using qRT-PCR to analyze separately isolated mRNA samples. Importantly, several virulence related groups of significantly upregulated genes including flagellar genes and the *Brucella* type IV secretion system (T4SS) VirB operon were identified. Additionally, *vjbR* and *blxR* (*babR*), two *luxR* homologues and probable *Brucella* quorum sensing regulators were also upregulated in the mutant strain compared to wild type. The *Brucella* general stress response system (GSR), regulated through the RpoE1 alternate sigma factor and the anti-anti sigma factor PhyR, was among the groups of notably downregulated genes. These flagellar, type IV secretory system, quorum sensing, and general stress response genes are known contributors to *Brucella* virulence (Rambow-Larsen*, et al.*, 2008, Uzureau*, et al.*, 2010, Weeks*, et al.*, 2010). See Table 1 for a selection of notable differentially regulated genes. Of the 3,198 arrayed genes, 243 (~7.6%) were significantly differently expressed in the mutant versus wild type strain. Fig. 1 provides a graphical overview of important genes, while expression data for all significantly differentially expressed genes are presented in Tables S4 and S5 with fold changes and Log Normal Normal Values (LNN >0.5 significant). Besides COGs of poorly characterized and genes of unknown function, the largest group of differentially regulated genes were those involved in metabolism. Other categories of genes that were up or downregulated include information storage and processing, and cellular processes and signaling.

A selection of 15 genes was chosen for expression analysis by qRT-PCR to verify the microarray expression findings. Three biological replicates for each strain were isolated from log phase cultures and analyzed, and these RNA isolations were separate from isolations used to generate microarray expression data. Gene expression was normalized to

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the expression of the *IF-1* (BMEI1671) gene. Trends for gene expression were consistent with microarray data for selected genes, Table 2.

Many genes differentially expressed in the BM-LOV-HK strain are regulated by *Brucella* quorum sensing and general stress response systems

Several previous studies have identified genes differentially expressed when quorum sensing (QS) regulator genes *vjbR* and *blxR* (*babR*) are separately deleted from parent strains (Rambow-Larsen*, et al.*, 2008, Uzureau*, et al.*, 2010, Weeks*, et al.*, 2010). Because expression of both *vjbR* and *blxR* was upregulated in the BM-LOV-HK strain, we conducted an analysis of previous studies to identify QS regulated components also differentially regulated in the BM-LOV-HK strain compared to *B. melitensis*. Of the 243 differentially expressed genes in the BM-LOV-HK strain, 94 of these genes overlapped with genes differentially regulated by at least one QS regulator deletion. Additionally, the downregulation of several GSR regulatory components in the BM-LOV-HK strain led us to expand our analysis to include genes differentially regulated in a recent study comparing gene expression in a *B. abortus rpoE1* deletion mutant to wild type under oxidative stress (Kim*, et al.*, 2013). Nineteen genes were differentially regulated in both the BM-LOV-HK strain and the *B. abortus rpoE1* deletion strain. Of those 19 overlapping genes, 12 are also differentially regulated in QS studies, Fig. 1. An expanded analysis including data specific to individual genes is available in Table S6.

Both C567BL/6 and IRF-1$^{-/-}$ mice infected with the BM-LOV-HK strain had decreased spleen CFUs and IRF-1$^{-/-}$ mice infected with the BM-LOV-HK strain had increased survival compared to IRF-1$^{-/-}$ mice infected with *B. melitensis*

C57BL/6 mice were infected with the BM-LOV-HK strain and wild type *B. melitensis,* and CFUs per gram of spleen tissue were determined at 3 and 6 days post infection. At day 3 the BM-LOV-HK strain possessed decreased CFUs compared to wild type; however, by day 6, CFUs for mutant and wild type bacteria were similar (Fig 2A). These results suggest a delay in growth or greater initial killing for the BM-LOV-HK strain during initial C57BL/6 mouse infection. By day 6, mutant and wild type CFUs were similar, therefore, to better investigate *in vivo* attenuation of the BM-LOV-HK strain, IRF-1$^{-/-}$ mice were infected with the BM-LOV-HK and wild type *B. melitensis* strains.

The IRF-1$^{-/-}$ mouse provides an informative *in vivo* model for *Brucella* infection because this mouse can distinguish various levels of mutant *Brucella* strain attenuation. The ability to kill IRF-1$^{-/-}$ mice has been shown to be a strong indicator of virulence for *Brucella* mutants, and some highly attenuated bacterial mutants do not readily kill IRF-1$^{-/-}$ mice (Matsuyama*, et al.*, 1993, Ko*, et al.*, 2002, Ko*, et al.*, 2002, Baek*, et al.*, 2004, Rajashekara*, et al.*, 2005, Rajashekara*, et al.*, 2005, Rajashekara*, et al.*, 2006, Miyoshi*, et al.*, 2007, Rajashekara*, et al.*, 2008, Rambow-Larsen*, et al.*, 2008, Trant*, et al.*, 2010, Petersen*, et al.*, 2011, Arenas-Gamboa*, et al.*, 2012). To determine the importance of BM-LOV-HK to *in vivo* virulence, survival of IRF-1$^{-/-}$ mice was determined after infection with either wild type *B. melitensis* or the BM-LOV-HK strain. BM-LOV-HK infected IRF-1$^{-/-}$ mice survived significantly longer than wild type *Brucella* infected mice (Fig. 2B), and significantly fewer CFUs were recovered from BM-LOV-HK infected spleens than wild

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type infected spleens at three and six days post infection (Fig. 2C). Wild type *Brucella* infected IRF-1$^{-/-}$ mice began to succumb 6 days after infection (Fig 2B). Wild type infected mice reached an average bacterial spleen load of 1x10^9 CFUs at 6 days of infection, while the average bacterial load of BM-LOV-HK infected spleens was 1x10^8 CFUs or less up to twelve days post infection. These results suggest deletion of BM-LOV-HK contributes to a reduced *B. melitensis* virulent phenotype but does not prevent eventual death of IRF-1$^{-/-}$ mice as do some *B. melitensis* gene deletions (Matsuyama*, et al.*, 1993, Ko*, et al.*, 2002, Ko*, et al.*, 2002, Baek*, et al.*, 2004, Rajashekara*, et al.*, 2005, Rajashekara*, et al.*, 2005, Rajashekara*, et al.*, 2006, Miyoshi*, et al.*, 2007, Rajashekara*, et al.*, 2008, Rambow-Larsen*, et al.*, 2008, Trant*, et al.*, 2010, Petersen*, et al.*, 2011, Arenas-Gamboa*, et al.*, 2012). Both IRF-1$^{-/-}$ and C57BL/6 mouse infections show decreased BM-LOV-HK strain virulence compared to wild type. These results suggest BM-LOV-HK is necessary for full *B. melitensis in vivo* virulence.

Discussion

The deletion of BM-LOV-HK demonstrates that this light sensing kinase affects expression of vital *Brucella* virulence genes that may alter murine infection. Sensing of environmental signals is essential for bacterial survival. However, the extent to which a light sensing kinase may exert an effect on gene expression in a facultative intracellular pathogen had been unclear. A genome scale microarray expression comparison between the BM-LOV-HK strain and *B. melitensis* 16M identified 243 genes directly or indirectly regulated by BM-LOV-HK expression, and many of these genes are involved in control and regulation of virulence and general stress response. Of specific interest was upregulation of the T4SS/VirB operon and QS system. Our analysis of previous studies identified 94 differentially expressed genes in *vjbR* and *blxR* strains that were also differentially expressed in the BM-LOV-HK strain. The direct signaling relationship between BM-LOV-HK and the QS and T4SS remains to be identified, but *virB* genes and a functional T4SS are essential for full *Brucella* virulence and proper trafficking within cells (Hong*, et al.*, 2000, Comerci*, et al.*, 2001, Boschiroli*, et al.*, 2002, Watarai*, et al.*, 2002, Rouot*, et al.*, 2003, Zygmunt*, et al.*, 2006, Roux*, et al.*, 2007, Nijskens*, et al.*, 2008, Starr*, et al.*, 2008, Paixão*, et al.*, 2009, de Jong & Tsolis, 2011). Consistent with VirB operon upregulation, mutant strain expression of quorum sensing (QS) components *vjbR* and *blxR* were also significantly increased. VjbR and BlxR are known regulators of VirB expression and an increase in VjbR would increase VirB expression (Delrue*, et al.*, 2005, Arocena*, et al.*, 2010). There is debate as to the role of BlxR expression on VirB. BlxR could be acting as a repressor, activator, or both depending on circumstances (Rambow-Larsen*, et al.*, 2008, Uzureau*, et al.*, 2010, Caswell*, et al.*, 2012). In this case, both QS regulators and the T4SS are upregulated. Abnormal expression of *Brucella* QS and T4SS components may cause unknown pleiotropic effects, such as deleterious secretion of bacterial proteins (de Jong*, et al.*, 2008, den Hartigh*, et al.*, 2008). Two proteins, VceA and BPE123, are transported by the *Brucella* T4SS and are differentially regulated in the BM-LOV-HK strain (de Jong*, et al.*, 2008, Marchesini*, et al.*, 2011). VceA was downregulated, while BPE123 was upregulated and is encoded near flagella genes. A large collection of flagellar genes were also upregulated in the BM-LOV-HK strain. This observation is consistent with previous work showing a strong overlap in

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flagellar genes, including *fliC, fliF, flgE,* and *flaF*, being upregulated after the deletion of the RpoE1 sigma factor, which was significantly downregulated in the BM-LOV-HK strain (Ferooz*, et al.*, 2011). Also, regulation of expression of flagellar genes can be controlled by an additional RpoH sigma factor and the two-component regulator FtcR, and neither of these genes were differentially regulated in our array (Delory*, et al.*, 2006, Leonard*, et al.*, 2007). Although the presence of certain flagellar genes is important for *Brucella* virulence, the exact mechanisms by which flagellar genes contribute to *Brucella* virulence are unknown.

In addition to regulating flagellar genes, the RpoE1 sigma factor has homologues in other bacteria that regulate genes responsible for bacterial general stress response (GSR) to stressors such as heat, salt concentration, and nutrient limitation (Alvarez-Martinez*, et al.*, 2006, Gourion*, et al.*, 2006, Alvarez-Martinez*, et al.*, 2007, Sauviac*, et al.*, 2007, Gourion*, et al.*, 2008, Martínez-Salazar*, et al.*, 2009). Both the *rpoE1* gene and the *phyR* gene, a known GSR partner, are downregulated in the BM-LOV-HK strain. Nineteen genes differentially regulated in a *B. abortus rpoE1* strain were also differentially regulated in the BM-LOV-HK strain (Kim*, et al.*, 2013). Twelve of these genes overlapped with genes differentially regulated in the above mentioned QS studies. Recently, *rpoE1* and *phyR B. abortus* mutants were reported as more sensitive to oxidative stress than wild type *B. abortus* (Kim*, et al.*, 2013). Also, *Brucella rpoE1* mutants have decreased virulence in mouse models of infection, but this attenuation is much later than what we have currently demonstrated for BM-LOV-HK strain infections (Delory*, et al.*, 2006, Kim*, et al.*, 2013). We had separately deleted *phyR* and a neighboring kinase we term *phyK* (BMEI0374) from *B. melitnsis*. Consistent with Kim et al., 2013, we noted no growth difference (in culture or RAW 264.7 macrophages) or difference in survival in IRF-1$^{-/-}$ mice for either mutant strain compared to wild type *B. melitensis* (data not shown). Clearly, biochemical interaction analyses, stress condition testing, and animal experiments to directly compare persistence of the BM-LOV-HK strain and a *B. melitensis rpoE1* deletion mutant, in wild type mice, are warranted for future studies. More than a third of all differentially regulated genes in the BM-LOV-HK strain appear to be controlled at a level downstream from BM-LOV-HK by QS, GSR, or in tandem by both systems. Together, these analyses reveal two possible arms of BM-LOV-HK signaling, QS and GSR. Our results suggest BM-LOV-HK may act up-stream to modulate both QS and GSR regulatory components to control *Brucella* gene expression on a global level.

Data are emerging on direct signaling partners for BM-LOV-HK. Others have indicated LOV-HK binds and phosphorylates PhyR *in vitro* (Rinaldi*, et al.*, 2012) providing a direct link between LOV-HK and the RpoE1 sigma factor. This link could explain many of the changes in gene expression observed in our BM-LOV-HK strain, specifically with stress response and flagellar genes. Further, at least two uncharacterized kinases surround the *phyR* operon. One of these kinases, PhyK, shares significant homology to BM-LOV-HK but does not have an LOV domain and likely reacts to other environmental signals (not light). A picture of regulation is developing in which BM-LOV-HK may activate the RpoE1 pathway under certain stimuli, while another kinase such as PhyK may subsume that role or even modulate PhyR phosphorylation at a later point in growth.

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Taken together, both C57BL/6 and IRF-1$^{-/-}$ mouse infection results point to a possible important role for BM-LOV-HK during the early stage of mouse infection. The delayed death and decreased spleen CFUs in mutant infected IRF-1$^{-/-}$ mice may be explained by impaired intracellular replication or pathogenesis, increased initial killing of mutant bacteria, or improper trafficking within the host. Further work to determine trafficking of the BM-LOV-HK strain within mice may shed light on whether the mutant is abrogated in dissemination. Future animal experiments to directly compare persistence of the BM-LOV-HK strain and a *B. melitensis rpoE1* deletion mutant, in wild type mice, may also be informative. Attempts to restore BM-LOV-HK to wild type virulence in mice have yielded unexpected findings of further attenuation beyond the BM-LOV-HK phenotype (Fig. S1). An interpretation is that improper expression of the *BM-LOV-HK* gene leads to further BM-LOV-HK strain attenuation compared to wild type *B. melitensis* as suggested by Fig. S1A and B. Others have shown that improper expression of *virB* components including the overexpression of VirB5 can lead to *B. suis* 1330 attenuation (Sprynski*, et al.*, 2012). Also, as discussed above, the expression of the VirB operon is significantly altered in the BM-LOV-HK strain compared to wild type.

Results have shown that BM-LOV-HK can sense and be phosphorylated in the presence of a blue light signal (Swartz*, et al.*, 2007). Future work remains to identify and confirm the direct BM-LOV-HK interaction partners and network as well as the importance of extra organismal light activation of BM-LOV-HK for *in vivo Brucella* virulence. Is a light sensing kinase at the apex of the regulatory hierarchy for expression of these genes? Is extra organismal blue light the only activation signal to which BM-LOV-HK responds, or could there be an internal host signal that activates the protein? In the present study we have shown that BM-LOV-HK is important for *B. melitensis* virulence in an animal model of infection. Deletion of BM-LOV-HK decreases virulence of *B. melitensis* during C57BL/6 and IRF-1$^{-/-}$ mouse infections. *B. melitensis* gene expression was altered by deletion of *BM-LOV-HK*. Our results suggest that BM-LOV-HK directly or indirectly regulates members of the *Brucella* quorum sensing and general stress systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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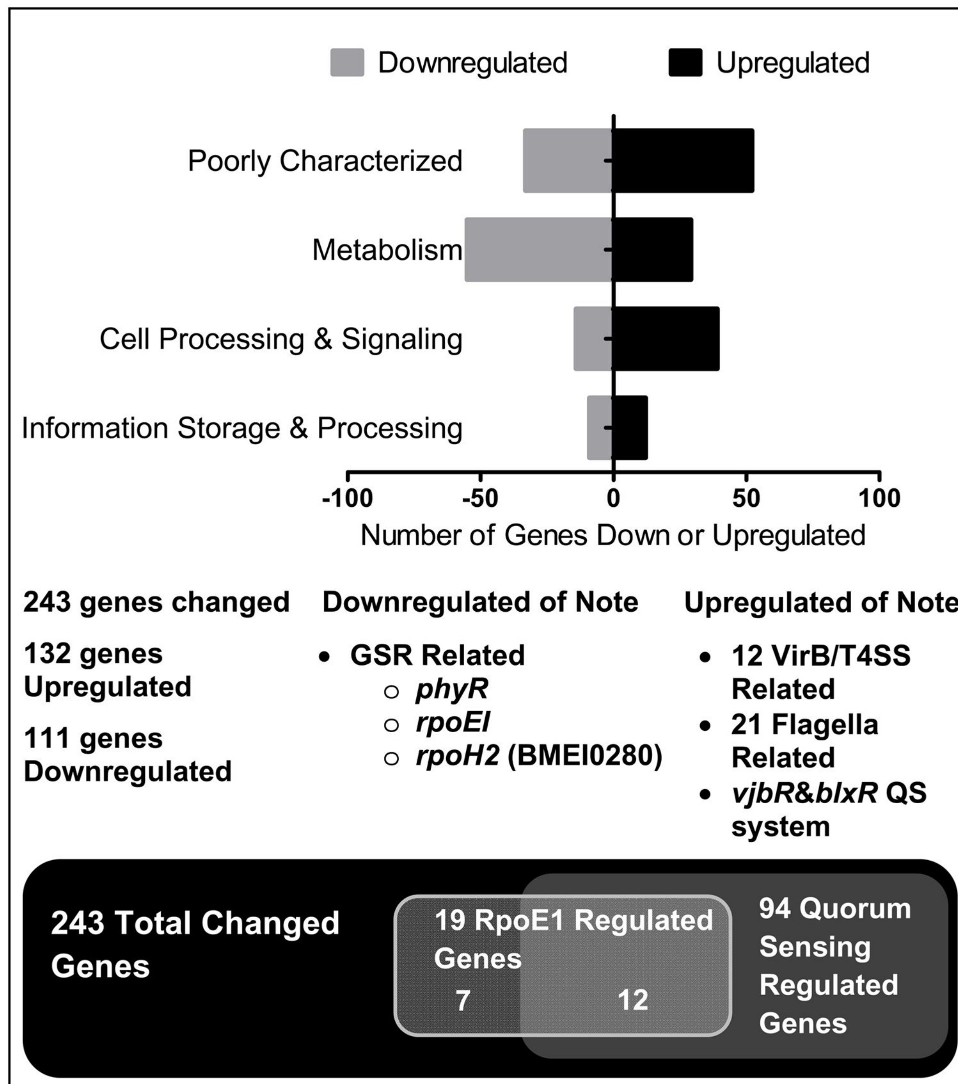

Figure 1. Differentially regulated genes in BM-LOV-HK strain as compared to WT 16M strain from microarray data

132 total genes upregulated & 111 downregulated in mutant strain vs. WT. Numbers of genes are organized by COG functional category & represented by different black or grey bars. Of the 243 changed genes, 94 are known to be regulated by QS. 19 are regulated by RpoEI and 12 of the 19 are also regulated by QS. See Table S6 for additional detail. T4SS is type IV secretion. QS is quorum sensing. GSR is general stress response.

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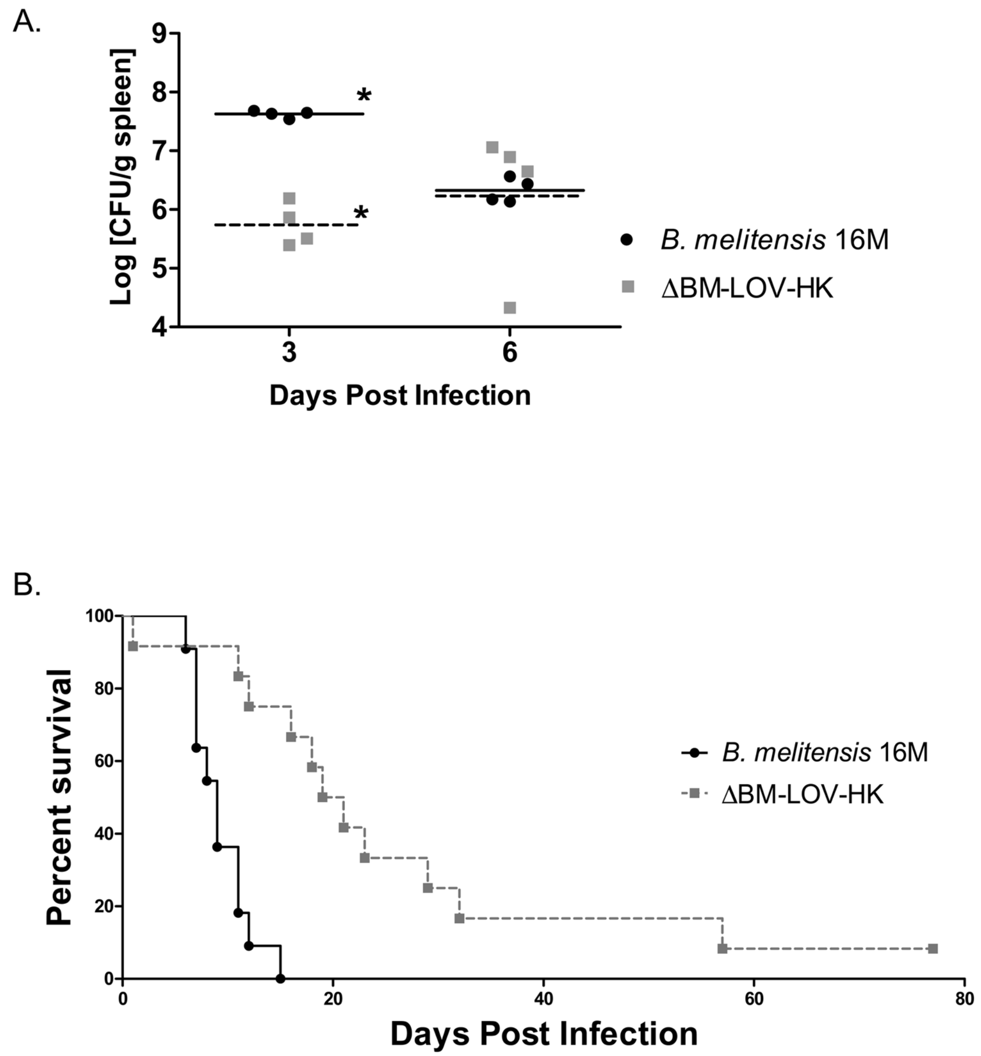

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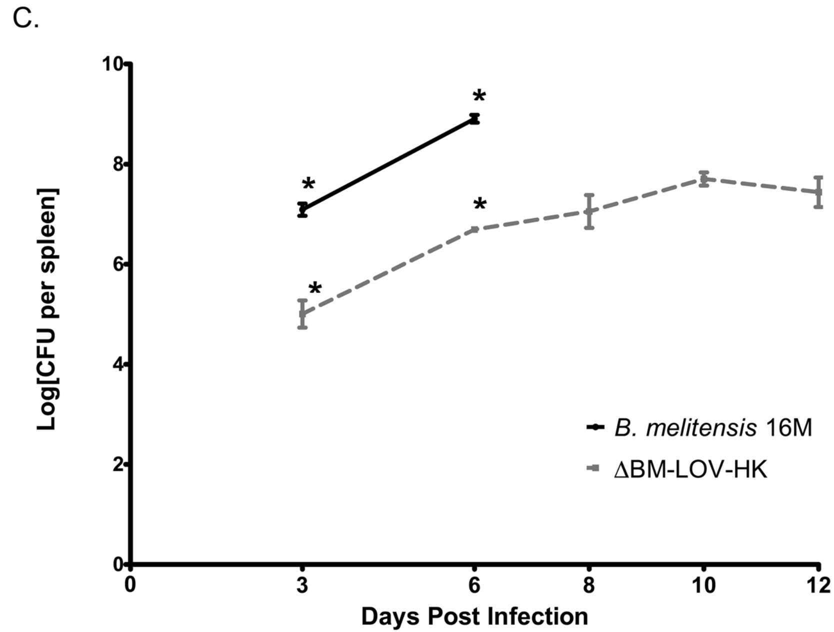

Figure 2. Decreased virulence of BM-LOV-HK in mice

A) C57BL/6 Mouse Spleen CFUs (N=4 each per time point) infected with 16M or BM-LOV-HK. BM-LOV-HK infected spleens contain significantly less bacteria at 3 days post infection (*P=0.0286, Mann-Whitney) but not at 6 days post infection. 16M & BM-LOV-HK strains used in part A contain empty pBBRmcs4 plasmid. B) Percent survival of IRF-1$^{-/-}$ mice infected with *B. melitensis* 16M (N=11) or BM-LOV-HK (N=12). BM-LOV-HK infected mice survive significantly longer than 16M infected mice (P=0.0001, Mantel-Cox). C) IRF-1$^{-/-}$ Mouse Spleen CFUs (N=4 each per time point) infected with 16M or BM-LOV-HK. BM-LOV-HK infected spleens contain significantly less bacteria at 3 & 6 days post infection (*P=0.0286, Mann-Whitney) for both time points. 16M infected IRF-1$^{-/-}$ mice begin to succumb to infection 6 days post infection. Means shown by horizontal lines in A, standard error of mean shown by error bars in C, & infectious dose of 1x10^{6} CFUs for A-C.

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Table 1

Expression of selected genes significantly affected by BM-LOV-HK deletion

BME#	Gene	Fold Change[a]	LNN[b]	Description
General Stress Response				
BMEI0371 factor	rpoE1	−3.8	1.00	RNA polymerase sigma-70
BMEI0372 regulator	phyR	−2.4	1.00	two-component response
BMEI0373	-	−4.2	1.00	hypothetical protein
BMEI0374 kinase	phyK	−5.9	1.00	Sensory transduction histidine
BMEI0280	rpoH2	−3.0	1.00	RNA polymerase sigma factor
Quorum Sensing				
BMEII1116 protein	vjbR	2.7	1.00	Quorum sensing regulator
BMEI1758 protein	blxR	5.3	1.00	Quorum sensing regulator
Type IV Secretion(virB)				
BMEII0025	virB1	3.3	1.00	Lytic transglycosylase
BMEII0026	virB2	9.9	1.00	Pilus
BMEII0027	virB3	9.4	1.00	Channel protein
BMEII0028	virB4	5.4	1.00	ATPase
BMEII0029	virB5	7.0	1.00	Attachment mediating protein
BMEII0030	virB6	4.1	1.00	IM Channel protein
BMEII0031	virB7	2.8	1.00	Channel protein
BMEII0032	virB8	3.7	1.00	Channel protein
BMEII0033	virB9	3.4	1.00	Channel protein
BMEII0034	virB10	3.8	1.00	Channel protein
BMEII0035	virB11	4.0	1.00	ATPase
BMEII0036	virB12	2.8	1.00	Outer membrane protein
Flagella				
BMEII0150	fliC	2.3	1.00	Flagellin
BMEII0151	fliF	2.4	1.00	M-ring protein
BMEII0159	flgE	1.9	0.89	Hook protein
BMEII0160 (junction)	flgK	1.7	0.60	Hook-associated protein
BMEII0161 (junction)	flgL	2.0	0.98	Hook-associated protein
BMEII0162	flaF	1.8	0.79	Biosynthesis regulatory factor
BMEII0170 protein	flgJ	1.9	0.93	Peptidoglycan hydrolyzing
BMEII0171	flgN	2.0	0.98	Type III secrtion chaperone
BMEII0172	fliL	1.9	0.95	Basal body-associated protein
BMEII1085	flgA	1.8	0.88	P-ring biosythesis protein
BMEII1086	flgG	2.0	0.98	Basal body rod protein
BMEII1087	fliE	1.8	0.83	Hook-basal body rod protein
BMEII1088	flgC	2.0	0.95	Basal body rod protein
BMEII1089	flgB	1.8	0.79	Basal body rod protein
BMEII1107	flgF	2.5	1.00	Basal body rod protein
BMEII1108	flgF	2.6	1.00	Basal body rod protein

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BME#	Gene	Fold Change[a]	LNN[b]	Description
BMEII1110	fliM	1.7	0.67	Motor switch protein (C-ring)
BMEII1111	-	1.9	0.94	Hypothetical protein
BMEII1113	fliG	1.7	0.69	Motor switch protein
BMEII1114 apparatus	flhB	7.0	1.00	Biosynthesis protein - export
BMEII1115	-	4.9	1.00	Hypothetical protein

[a]Fold Change is BM-LOV-HK/WT for positive fold change & −1/(BM-LOV-HK/WT) for negative fold change.

[b]LNN (Lognormal-normal) value >0.5 considered significant and calculated using EBarrays package for Bioconductor.

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Table 2

Expression of selected genes by qRT-PCR & microarray

BME#	Name	Fold change qRT-PCR	Fold change microarray
BMEI0280	rpoH2	−9.2	−3.0
BMEI0371	rpoE1	−8.0	−3.8
BMEI0372	phyR	−8.5	−2.4
BMEI0374	phyK	−9.2	−5.9
BMEI0884	gryA	−1.2	1.1
BMEI1758	blxR/babR	2.1	5.4
BMEII0026	virB2	2.7	9.9
BMEII0029	virB5	1.7	7.0
BMEII0035	virB11	1.2	4.0
BMEII0105	frpB	−1.6	−3.4
BMEII0150	fliC	2.0	2.3
BMEII0151	fliF	5.1	2.4
BMEII0159	flgE	3.2	1.9
BMEII1113	fliG	3.4	1.7
BMEII1116	vjbR	3.4	2.7

B. melitensis 16M number (BME#), NCBI searchable. Name: Short gene name. Fold Change is BM-LOV-HK/WT for positive fold change & −1/(BM-LOV-HK/WT) for negative fold change.

Expression data for qRT-PCR was calculated with C_T method.