

RESEARCH ARTICLE

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

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This well-written research article reports the genome-scale microarray analysis of a light-sensing histidine kinase mutant of *Brucella melitensis*. Results of the analysis indicate that the expression of 243 genes is altered and that some of these genes encode well-known virulence factors, a finding confirmed by the observed attenuated virulence of the mutant in experimentally infected C57BL/6 mice. The findings provide evidence that a light-sensing kinase is at the top (or near the top) of a regulatory hierarchy of genes involved in virulence, metabolism and stress response.

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 (QS), and type IV secretion system genes were upregulated in the Δ BM-LOV-HK strain vs. wild type. Contextually, expression results agree with other studies of transcriptional regulators involving Δ *rpoE1*, Δ *phyR*, Δ *vjbR*, and Δ *blxR* (Δ *babR*) *Brucella* strains. Additionally, deletion of BM-LOV-HK decreases virulence in mice. During C57BL/6 mouse infection, the Δ BM-LOV-HK strain had 2 logs less CFUs in the spleen 3 days postinfection, but similar levels 6 days post infection compared to wild type. Infection of IRF-1^{-/-} mice more specifically define Δ BM-LOV-HK strain attenuation with fewer bacteria in spleens and significantly increased survival of mutant vs. wild-type infected IRF-1^{-/-} mice. Upregulation of flagella, QS, and VirB genes, along with downregulation of *rpoE1* and related sigma factor, *rpoH2* (BMEI0280) suggest that BM-LOV-HK modulates both QS and general stress response regulatory components to control *Brucella* gene expression on a global level.

Key words: brucellosis; flagella; stress; 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 com-

plexity involved in resolving human infection make research on *Brucella* virulence mechanisms a priority.

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

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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 (QS), 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). *Escherichia coli* DH5 α was used as the maintenance and propagation strain for vectors. *Escherichia 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 μ g mL⁻¹. Ampicillin was used at 100 μ g mL⁻¹. See Supporting Information, Table S1 for a list of strains and vectors.

Generation of deletion strain

The Δ 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).

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 (three 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 c. 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; Kendziorowski *et al.*, 2003), where fold change calculation was Δ BM-LOV-HK/WT for positive fold change and $-1/(\Delta$ BM-LOV-HK/WT) for negative fold change.

Raw microarray data

Raw microarray data have 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. 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 iQTM 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 1×10^6 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 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 Δ BM-LOV-HK (n=12)] intraperitoneally with 1×10^6 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, b; Rajashekara et al., 2005, b). 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 Δ 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 three biological replicates comparing the Δ 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* QS 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 PhyrR, was among the groups of notably downregulated genes. These flagellar, type IV secretory system, QS, 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 3198 arrayed genes, 243 (c. 7.6%) were significantly differently expressed in the mutant vs. wild-type strain. Figure 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 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 Δ BM-LOV-HK strain are regulated by *Brucella* QS and general GSRs

Several previous studies have identified genes differentially expressed when 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 Δ BM-LOV-HK strain, we conducted an analysis of previous studies to identify QS regulated components also differentially regulated in the Δ BM-LOV-HK strain compared to *B. melitensis*. Of the 243 differentially expressed genes in the Δ 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 Δ 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 Δ 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 C57BL/6 and IRF-1^{-/-} mice infected with the Δ BM-LOV-HK strain had decreased spleen CFUs and IRF-1^{-/-} mice infected with the Δ BM-LOV-HK strain had increased survival compared to IRF-1^{-/-} mice infected with *B. melitensis*

C57BL/6 mice were infected with the Δ BM-LOV-HK strain and wild-type *B. melitensis*, and CFUs per gram of spleen tissue were determined at 3 and 6 days postinfection. At day 3, the Δ 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 Δ 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 Δ BM-LOV-HK strain, IRF-1^{-/-} mice were infected with the Δ 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., 2002a,b; Baek et al., 2004; Rajashekara et al., 2005a,b, 2006, 2008; Miyoshi et al., 2007; 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 Δ BM-LOV-HK strain. Δ 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 Δ BM-LOV-HK infected spleens than wild-type infected spleens at three and six days postinfection (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 1×10^9 CFUs at 6 days of infection, while the average bacterial load of Δ BM-LOV-HK infected spleens was 1×10^8 CFUs or less up to twelve days postinfection. These results suggest deletion of BM-LOV-HK contributes to a reduced *B. melitensis* virulence 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., 2002a,b; Baek et al., 2004; Rajashekara et al., 2005a,b, 2006, 2008; Miyoshi et al., 2007; Rambow-Larsen et al., 2008; Trant et al., 2010; Petersen et al., 2011; Arenas-Gamboa et al., 2012). Both IRF-1^{-/-} and C57BL/6

Table 1. Expression of selected genes significantly affected by BM-LOV-HK deletion.

BME#	Gene	Fold change*	LNN†	Description
General stress response				
BMEI0371	<i>rpoE1</i>	-3.8	1.00	RNA polymerase sigma-70 factor
BMEI0372	<i>phyR</i>	-2.4	1.00	Two-component response regulator
BMEI0373	-	-4.2	1.00	Hypothetical protein
BMEI0374	<i>phyK</i>	-5.9	1.00	Sensory transduction histidine kinase
BMEI0280	<i>rpoH2</i>	-3.0	1.00	RNA polymerase sigma factor
Quorum sensing				
BMEI1116	<i>vjbR</i>	2.7	1.00	Quorum sensing regulator protein
BMEI1758	<i>blxR</i>	5.3	1.00	Quorum sensing regulator protein
Type IV secretion (virB)				
BMEI0025	<i>virB1</i>	3.3	1.00	Lytic transglycosylase
BMEI0026	<i>virB2</i>	9.9	1.00	Pilus
BMEI0027	<i>virB3</i>	9.4	1.00	Channel protein
BMEI0028	<i>virB4</i>	5.4	1.00	ATPase
BMEI0029	<i>virB5</i>	7.0	1.00	Attachment mediating protein
BMEI0030	<i>virB6</i>	4.1	1.00	IM Channel protein
BMEI0031	<i>virB7</i>	2.8	1.00	Channel protein
BMEI0032	<i>virB8</i>	3.7	1.00	Channel protein
BMEI0033	<i>virB9</i>	3.4	1.00	Channel protein
BMEI0034	<i>virB10</i>	3.8	1.00	Channel protein
BMEI0035	<i>virB11</i>	4.0	1.00	ATPase
BMEI0036	<i>virB12</i>	2.8	1.00	Outer membrane protein
Flagella				
BMEI0150	<i>fliC</i>	2.3	1.00	Flagellin
BMEI0151	<i>fliF</i>	2.4	1.00	M-ring protein
BMEI0159	<i>flgE</i>	1.9	0.89	Hook protein
BMEI0160	<i>flgK</i>	1.7	0.60	Hook-associated protein (junction)
BMEI0161	<i>flgL</i>	2.0	0.98	Hook-associated protein (junction)
BMEI0162	<i>flaF</i>	1.8	0.79	Biosynthesis regulatory factor
BMEI0170	<i>flgJ</i>	1.9	0.93	Peptidoglycan hydrolyzing protein
BMEI0171	<i>flgN</i>	2.0	0.98	Type III secretion chaperone
BMEI0172	<i>fliL</i>	1.9	0.95	Basal body-associated protein
BMEI1085	<i>flgA</i>	1.8	0.88	P-ring biosynthesis protein
BMEI1086	<i>flgG</i>	2.0	0.98	Basal body rod protein
BMEI1087	<i>fliE</i>	1.8	0.83	Hook-basal body rod protein
BMEI1088	<i>flgC</i>	2.0	0.95	Basal body rod protein
BMEI1089	<i>flgB</i>	1.8	0.79	Basal body rod protein
BMEI1107	<i>flgF</i>	2.5	1.00	Basal body rod protein
BMEI1108	<i>flgF</i>	2.6	1.00	Basal body rod protein
BMEI1110	<i>fliM</i>	1.7	0.67	Motor switch protein (C-ring)
BMEI1111	-	1.9	0.94	Hypothetical protein
BMEI1113	<i>fliG</i>	1.7	0.69	Motor switch protein
BMEI1114	<i>flhB</i>	7.0	1.00	Biosynthesis protein - export apparatus
BMEI1115	-	4.9	1.00	Hypothetical protein

*Fold Change is Δ BM-LOV-HK/WT for positive fold change and $-1/(\Delta$ BM-LOV-HK/WT) for negative fold change.

†LNN value > 0.5 considered significant and calculated using EBARRAY package for Bioconductor.

mouse infections show decreased Δ 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 Δ 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 Δ vjbR and Δ blxR strains that were also differentially expressed in the Δ 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 and Tsolis, 2011). Consistent with VirB operon upregulation, mutant strain expression of QS components *vjbR* and *blxR* were also significantly increased. *VjbR* and *BlxR* are known

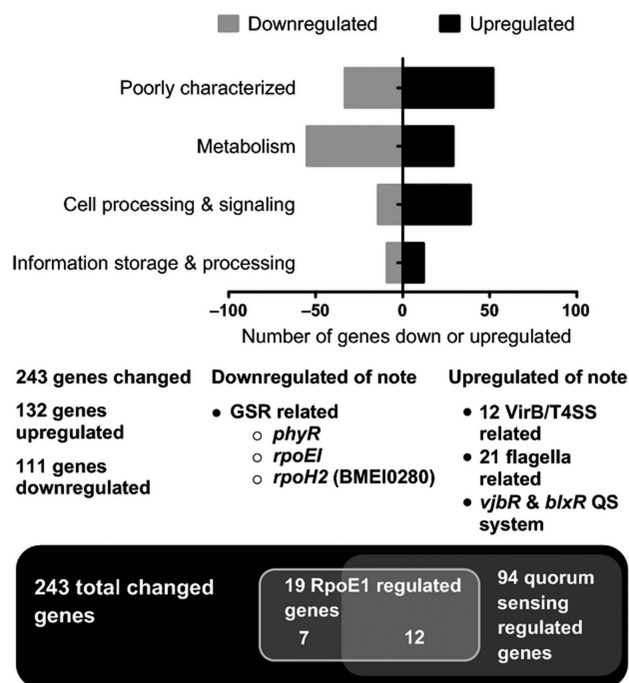


Figure 1. Differentially regulated genes in Δ BM-LOV-HK strain as compared to WT 16M strain from microarray data. One-hundred and thirty two total genes upregulated and 111 downregulated in mutant strain vs. WT. Numbers of genes are organized by COG functional category and represented by different black or grey bars. Of the 243 changed genes, 94 are known to be regulated by QS. Nineteen are regulated by RpoE1 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.

Table 2. Expression of selected genes by qRT-PCR and microarray.

BME#	Name	Fold change qRT-PCR	Fold change microarray
BMEI0280	<i>rpoH2</i>	-9.2	-3.0
BMEI0371	<i>rpoE1</i>	-8.0	-3.8
BMEI0372	<i>phyR</i>	-8.5	-2.4
BMEI0374	<i>phyK</i>	-9.2	-5.9
BMEI0884	<i>gryA</i>	-1.2	1.1
BMEI1758	<i>blxR/babR</i>	2.1	5.4
BMEI0026	<i>virB2</i>	2.7	9.9
BMEI0029	<i>virB5</i>	1.7	7.0
BMEI0035	<i>virB11</i>	1.2	4.0
BMEI0105	<i>frpB</i>	-1.6	-3.4
BMEI0150	<i>fliC</i>	2.0	2.3
BMEI0151	<i>fliF</i>	5.1	2.4
BMEI0159	<i>flgE</i>	3.2	1.9
BMEI1113	<i>fliG</i>	3.4	1.7
BMEI1116	<i>vjbR</i>	3.4	2.7

Brucella melitensis 16M number (BME#), NCBI searchable. Name: Short gene name. Fold Change is Δ BM-LOV-HK/WT for positive fold change and $-1/(\Delta$ BM-LOV-HK/WT) for negative fold change. Expression data for qRT-PCR were calculated with $\Delta\Delta C_T$ method.

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

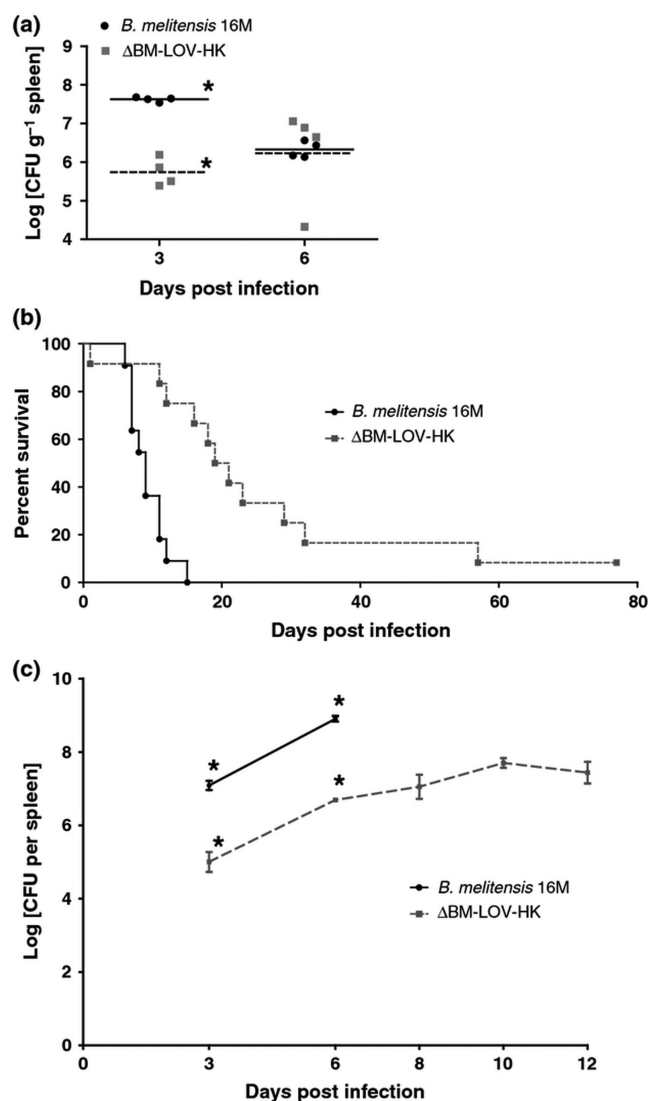


Figure 2. Decreased virulence of Δ BM-LOV-HK in mice. (a) C57BL/6 mouse spleen CFUs ($n=4$ each per time point) infected with 16M or Δ BM-LOV-HK. Δ BM-LOV-HK infected spleens contain significantly less bacteria at 3 days postinfection ($*P=0.0286$, Mann-Whitney) but not at 6 days postinfection. 16M & Δ BM-LOV-HK strains used in part (a) contain empty pBBRmcs4 plasmid. (b) Percent survival of IRF-1 $^{-/-}$ mice infected with *Brucella melitensis* 16M ($n=11$) or Δ BM-LOV-HK ($n=12$). Δ 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 Δ BM-LOV-HK. Δ BM-LOV-HK infected spleens contain significantly less bacteria at 3 and 6 days postinfection ($*P=0.0286$, Mann-Whitney) for both time points. 16M infected IRF-1 $^{-/-}$ mice begin to succumb to infection 6 days postinfection. Means shown by horizontal lines in (a) standard error of mean shown by error bars in (c) and infectious dose of 1×10^6 CFUs for a-c.

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 Δ 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 Δ BM-LOV-HK strain. This observation is con-

sistent with previous work showing a strong overlap in flagellar genes, including *fliC*, *fliF*, *flgE*, and *flaF*, being upregulated after the deletion of the RpoE1 sigma factor, which was significantly downregulated in the Δ 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, 2007; Gourion et al., 2006, 2008; Sauviac et al., 2007; Martínez-Salazar et al., 2009). Both the *rpoE1* gene and the *phyR* gene, a known GSR partner, are downregulated in the Δ BM-LOV-HK strain. Nineteen genes differentially regulated in a *B. abortus* Δ *rpoE1* strain were also differentially regulated in the Δ BM-LOV-HK strain (Kim et al., 2013). Twelve of these genes overlapped with genes differentially regulated in the above mentioned QS studies. Recently, Δ *rpoE1* and Δ *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 Δ 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. melitensis*. 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 Δ 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 Δ 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 upstream 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 Δ 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 a 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.

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 pathogen-

esis, increased initial killing of mutant bacteria, or improper trafficking within the host. Further work to determine trafficking of the Δ 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 Δ BM-LOV-HK strain and a *B. melitensis rpoE1* deletion mutant, in wild-type mice, may also be informative. Attempts to restore Δ BM-LOV-HK to wild-type virulence in mice have yielded unexpected findings of further attenuation beyond the Δ BM-LOV-HK phenotype (Fig. S1). An interpretation is that improper expression of the BM-LOV-HK gene leads to further Δ 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 Δ 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. *Brucella 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* QS and general stress systems.

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Conflict of interest statement. None declared.

SUPPLEMENTARY DATA

Supplementary material is available at Pathogens and Disease online.

Figure S1. Spleen CFUs and IRF-1^{-/-} mouse survival failed to show Δ BM-LOV-HK strain restored to WT virulence with *lac* promoter plasmid expression of BM-LOV-HK.

Table S1. Bacterial strains and vectors utilized.

Table S2. Deletion primers.

Table S3. qRT-PCR primers.

Table S4. Genes upregulated in δ BM-LOV-HK as compared to WT 16M.

Table S5. Genes downregulated in δ BM-LOV-HK as compared to WT 16M.

Table S6. Genes regulated By BM-LOV-HK, VjbR, BlxR/BabR, and RpoE1.

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