IDENTIFICATION OF GENETIC LOCI AND TRANSCRIPTIONAL NETWORKS THAT CONFER SURVIVAL AND VIRULENCE OF *BRUCELLA MELITENSIS*

A Dissertation

by

JENNI NICHOLE WEEKS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2008

Major Subject: Genetics

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ABSTRACT

Identification of Genetic Loci and Transcriptional Networks that Confer Survival and Virulence of *Brucella melitensis*. (August 2008)

Jenni Nichole Weeks, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Thomas A. Ficht

Brucella melitensis is the etiological agent of brucellosis, a zoonotic disease characterized by abortions in ruminant animals and a chronic debilitating disease in humans. Despite genome sequencing, little is known about the genetic elements behind *Brucella*'s ability to survive and cause disease. Regulatory networks provide the ability to adapt to changing environments by initiating expression from specific regulons to provide adjustments to metabolism and mechanisms that enhance survival. Little detail is known about transcriptional networks that exist in *Brucella*, but are of great interest because they could provide information about genetic loci that contribute to virulence and intracellular survival.

Transposon mutagenesis identified gene loci that are indispensable for the intracellular replication of *B. melitensis,* including virulence genes, metabolic defects, and transcriptional regulators. Two transcriptional regulators of interest were identified, MucR and VjbR. VjbR is a LuxR homologue and is associated

with the regulation of virulence genes in a density dependent manner in a number of bacterial pathogens, and is consistent with VjbR regulation of virulence genes in *B. melitensis*. Microarray analysis of Δ*vjbR* and a potential activating signal C_{12}-HSL revealed that both regulate numerous putative virulence genes, including adhesins, proteases, protein secretion/translocation components, potential effector proteins, lipoproteins, a hemolysin and stress survival aids. This analysis also revealed that C_{12}-HSL is not an activating signal of VjbR, but instead acts to suppress VjbR activity.

MucR is a transcriptional regulator shown to regulate exopolysaccharide synthesis in the closely related Rhizobiales. Microarray analysis of a Δ*mucR* mutant in *B. melitensis* suggested that MucR contributes to the regulation of nitrogen metabolism and iron sequestering/storage. MucR was also found to regulate genes involved in stress response, regulating several proteases that may contribute to enhanced survival and virulence of the organism.

This work identified approximately 1,000 genetic loci that may be important to the survival of *B. melitensis*, revealing potential virulence genes and metabolic defects. Interruption of the VjbR regulon could be a potential chemotherapeutic target for the treatment of brucellosis. Furthermore, this work describes the functions of two gene deletions that are being evaluated as novel attenuated vaccines.

DEDICATION

….To my Mom and family; without their love, encouragement and support none of this would have been possible.

….To my loving boyfriend and best friend, Chris Whittemore, for his patience, support and encouragement.

….To Dr. Don Davis, for always being a great friend, looking after me, and making sure I never went hungry or thirsty.

….To my favorite furry animals, Tango and Holly, for always making me smile.

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TABLE OF CONTENTS

CHAPTER Page

CHAPTER Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

TABLE Page

CHAPTER I

INTRODUCTION: *BRUCELLA* SPP. AND BRUCELLOSIS

THE ORGANISM

Brucella spp. are aerobic, non-motile, Gram negative, facultative intracellular coccobacilli. *Brucella* spp. are classified phylogenetically within the α-2 subgroup of Proteobacteria, family Rhizobiaceae. *Brucella* spp. and biovars are distinguished by pathogenicity, host preference, and growth characteristics; including the necessity for carbon dioxide, growth on dyes, H_2S production, lysis by phage and agglutination by monospecific sera (38, 150).

In most species, the genome consist of two circular chromosomes that are approximately 2.1 and 1.5 Mb., with approximately 3197 predicted open reading frames between the two chromosomes (98). *B. suis*'s genome organization varies between the biovars and can be either a single 3.3 Mb. chromosome as found in biovar 3, or divided between two smaller chromosomes as found in biovars 1,2, and 4 (109). Based on the clusters of replication genes, chromosome I resembles a classic bacteria chromosome while chromosome II posses a cluster of plasmid-like replication genes (109).

Comparison of the distribution of functional categories of genes between

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the two chromosomes shows remarkable asymmetry, with chromosome I enriched for the majority of core metabolic machinery for processes such as transcription, translation, and protein synthesis (109). Chromosome II largely represents auxiliary pathways for utilization of specific substrates, enriched with many genes involved in membrane transport, energy metabolism and regulation (109). Chromosome II is likely derived from a megaplasmid that was captured by an ancestral *Brucella*, as has been suggested for *Vibrio cholerae,* and is essential and not predicted to be disposable (109).

HISTORICAL PERSPECTIVE

A type of fever characterized by regular remissions has long been recognized and described along the Mediterranean basin since the time of Hippocrates in 450 B.C. In 1887 Sir David Bruce, a physician with the British Royal Army Medical Corps, was the first to isolate *Brucella* from the spleen of a fatally infected soldier in Malta (89). He called the bacteria *Micrococcus melitensis* and went on to complete Koch's postulates by culturing the bacteria, inoculating monkeys and re-isolating the bacteria from the liver and spleen of an animal that died from the infection (89).

In 1895, a Danish veterinarian pathologist and bacteriologist described a different causative organism in cattle and called it *Bacillus abortus*. In 1897, Wright and Smith detected antibodies to *M. melitensis* in human and animal

sera, demonstrating the zoonotic potential of the disease (157). A few years later in 1905, Themistocles Zammit, a Maltese physician working with the Mediterranean Fever Commission isolated the organism from the milk and urine of goats (89). This finding was crucial to the understanding and control of the disease because it was believed that the disease was vector-borne (104). In 1914, a *Brucella* species was isolated in the United States from an aborted pig fetus and was named *B. suis (104)*.

ANIMAL BRUCELLOSIS

Brucella spp. are the etiological agents of brucellosis, a highly contagious disease causing abortion and sterility in a broad range of domestic and wild animals (1). There are eight different species of *Brucella*, divided primarily by host preference, although animals can be susceptible to infection by more than one species. *B. melitensis* primarily infects goats and sheep, *B. abortus* infects cattle, *B. suis* infects swine, *B. ovis* infects sheep, *B. canis* infects dogs, *B. neotomae* infects wood rats, and *B. cetaceae* and *B. pinnepedieae* infect marine mammals.

Acute disease is characterized by infection of the reproductive tissues of the host, typically resulting in abortion of the fetus (39). Colonization of the placenta and fetus occurs rapidly, while the factors that control this tissue tropism are unknown (39). Acute infections are characterized by uncontrolled

growth of the bacteria resulting in placental necrosis and abortion or birth of weakened calves (39).

Chronic brucellosis in animals is defined as persistence of the organism without serious signs of illness and is distinct from the acute stage of infection (39). In ruminants, chronic infections usually follow the acute infection, after an animal aborts. During the chronic infection, the mammary glands and lymph nodes of the host are usually colonized with the bacteria and may be excreted in the udder and chronically shed in the milk (39).

HUMAN BRUCELLOSIS

Brucella spp. are the etiological agents of human brucellosis, a chronic infectious disease characterized in humans as undulant fever (151). Three species of *Brucella* are commonly associated with human infections; *B. melitensis, B. abortus* and *B. suis*. *B. melitensis* is often the most virulent to humans, followed by *B. suis* and *B. abortus.*

Human brucellosis is often times also referred to as Bang's disease, Mediterranean fever, Malta fever and undulant fever. Human brucellosis is rarely fatal, but can be severely debilitating and disabling. Brucellosis presents a myriad of symptoms; most commonly including fever, arthritis, osteomyelitis, spondylitis, dementia and in rare cases meningitis or endocarditis. The

incubation period is usually one to three weeks, although in rare instances the onset of symptoms can occur several months after exposure.

Prophylactics for the disease can vary, and clinical management of the disease is of particular concern because of the high failure of initial treatment and relapse rates. The World Health Organization recommended treatment for adults is rifampicin, 600 to 900 mg, and doxycycline, 100 mg twice daily, for a minimum of six weeks. Alternatively, intramuscular streptomycin with an oral tetracycline, trimethoprim-sulfamethoxazole triple regimens and gentamycin have also been reported to treat with the same or improved efficiency as the recommended treatment (89). Relapses are often less severe than the initial disease and can occur in approximately 10 % of treated patients and are often treated by repeating the antibiotic regimen (89).

TRANSMISSION

Brucellosis is a zoonotic disease that is almost invariably transmitted from animal to humans through consumption of contaminated dairy products. In addition to ingestion, transmission can also occur through inhalation or through soft membranes, such as the conjunctiva or skin abrasions (43). Additionally, brucellosis is one of the most common laboratory-transmitted infections through accidental ingestion, inhalation and mucosal or skin contact and is a major health hazard in clinical and research laboratories (89). Human to human

transmission is not likely to occur and is considered very rare, but has been suspected in a few case studies (6, 90, 101, 107).

PATHOGENESIS

Brucella spp. virulence relies on the ability to enter phagocytic and non-phagocytic cells, control the host's intracellular trafficking to avoid lysosomal degradation and replicate in a *Brucella*-containing vacuole, or brucellosome without restricting host cell functions or inducing programmed death (13, 15, 52, 74, 114). Among resident cells in a host, the macrophage cell is a major target in infected mammals (51). Upon lipid raft-mediated entry, *Brucella* interacts with early endosomes, which mature into acidic intermediate vacuoles but avoid fusion with late endosomes and phagolysosomes (14, 114, 153). The early brucellosome continues to mature by interacting with the endoplasmic reticulum (ER) in a manner that leads to fusion between these two organelles and generates a vacuole permissive for bacterial replication (14).

Lipopolysaccharide (LPS) and cyclic β-1,2-glucan appear to be important for early survival and in delaying fusion of the early brucellosome with lysosomes (14). The *virB* encoded type IV secretion system (TIVSS) has been shown to be important for interaction and fusion with the ER and maintaining the mature brucellosome (14). TIVSS deficient mutants are unable to sustain

interactions with the ER and the vacuoles ultimately fuse with lysosomes and are degraded (14).

CHAPTER II

IDENTIFICATION OF GENETIC LOCI REQUIRED FOR THE INTRACELLULAR SURVIVAL IN SIGNATURE TAGGED MUTAGENESIS SCREENING

INTRODUCTION

Random mutagenesis of bacterial genomes is a useful tool to identify genetic elements that are essential for survival and virulence. Transposon mutagenesis has been shown to be successful in *B. abortus*, *B. suis* and *B. melitensis*, identifying genes required for invasion and survival in macrophages, non-phagocytic cells, and mice (36, 62, 72, 74, 83, 84). With completion of the annotated *B. melitensis* genome sequence, it was noted that the bacterium lacked many classical virulence genes, such as exotoxins, cytolysins, secreted proteases, toxins, capsules, virulence plasmids, and exoenzymes (119). To date, only a few virulence factors required by *B. melitensis* have been identified and characterized, including the TIVSS, flagellin, cyclic β-1,2 glucan and LPS (4, 14, 25, 68). The previous mutagenesis screens conducted in *Brucella* spp. were relatively small and far from saturating the genome, limiting the genetic loci identified but demonstrating the usefulness of the technique.

Brucella spp. are facultative intracellular pathogens that encounter a number of different environments before reaching the appropriate intracellular

replicative niche: the brucellosome (74). The use of transcriptional regulators to sense the environment and coordinate a transcriptional response could be a great advantage for the survival of a pathogen by regulating a subset of genes that confer survival at that point in time. *Brucella* spp. must be able to survive conditions ranging from the extracellular, open environment to an intracellular environment in professional phagocytic cells, thus allowing for the establishment and maintenance of chronic infections. It has been shown that *Brucella* spp. actively re-direct the endosomal trafficking pathway, avoid lysosomal fusion and create a unique replicative niche, suggesting that a specific transcriptional response is employed by the bacterium at each step.

Identification of transcriptional regulators and the respective virulons of *Brucella* spp. will further the understanding of genetic loci required for survival and virulence and provide further insights into the transcriptional networks necessary for intracellular survival. Additionally, this work is expected to lead to the discovery of novel chemotherapeutic targets for treatment and identify potential gene targets for the development of attenuated novel strains for vaccine development.

In order to identify transcriptional regulators and genetic loci required for the intracellular survival of *B. melitensis*, a collection of signature tagged transposon mutants were generated and screened for attenuation in the mouse and macrophage models of infection. This study differs from previous

transposon mutagenesis studies in two ways. The first difference is in the number of mutants generated, increased to saturate the genome and identify additional genetic loci required for survival. The second difference was in the utilization of two different models to identify attenuated mutants, an *in vitro* and *in vivo* model of infection. This project was completed by the contribution of several members of the laboratory and the work described here was completed by the effort of one individual: therefore it represents only a portion of the combined data. From this work, genes coding for seven transcriptional regulators were identified to have attenuated phenotypes, as well as virulence genes and metabolic defects. VjbR, a LuxR homologue, identified in this screen is a transcriptional regulator that is of special interest because the mutant is highly attenuated and has been linked to the regulation of virulence determinants in a number of other bacteria (154).

MATERIALS AND METHODS

Bacterial strains and plasmids for cloning. *Escherichia coli* cultures were routinely cultured on Luria-Bertani agar or broth (LB) and incubated at 37°C. LB media was supplemented when appropriate with kanamycin (100 mg l^{-1}), ampicillin (100 mg l^{-1}), carbenicillin (100 mg l^{-1}) or chloramphenicol (30 mg l^{-1}). *B. melitensis* 16M bv.1 was obtained from ATCC and re-isolated from an aborted goat fetus by this lab. *B. melitensis* 16M was routinely cultured

on tryptic soy agar or broth (TSA or TSB) and incubated at 37°C. All transposon mutants were cultured in TSA or TSB supplemented with kanamycin (100 mg l^{-1}) and incubated under the same conditions as wild type. J774A.1 murine macrophage-like cells were obtained from ATCC and grown in T-75 flasks with Dulbecco's modified Eagle's medium, hepes modification (DMEM), supplemented with 1X MEM non-essential amino acids (Sigma, St. Louis, MO), 0.37 % (w/v) sodium bicarbonate and 10 % (v/v) heat-inactivated fetal bovine serum. J774A.1 macrophage-like cells were grown and incubated at 37°C in an atmosphere containing 5 % (v/v) CO_2. All work with live *B. melitensis* was performed in a biosafety level 3 laboratory at Texas A&M University College Station, as per CDC approved standard operating procedures.

Gentamycin protection assay. J774A.1 cells were seeded in 24 well plates at 2.5 x 10^5 cells $well^{-1}$ in modified DMEM and incubated for 24 hours before infection. J774A.1 cell monolayers were infected with *B. melitensis* 16M or transposon mutants in individual wells at a MOI of 20. The infection was synchronized by centrifugation at 200 x g for 5 minutes. Following a 20-minute incubation, monolayers in wells for the first time point were washed three times in peptone saline (1 % (w/v) Bacto-Peptone™ (Difco Laboratories, Becton, Dickinson and Company, Franklin Lakes, NJ) and 0.5 % (w/v) NaCl) and then incubated in modified DMEM supplemented with gentamycin (40 μg ml^{-1}) for one hour. Immediately following the wash, macrophage cells were then lysed in 0.5

% Tween-20 and serial dilutions were plated to determine bacterial colony forming units (CFU) at time zero. The remaining infected monolayers were incubated for 48 hours in modified DMEM supplemented with gentamycin (40 μg ml^{-1}). After 48 hours, the macrophage cells were washed and lysed as described for time zero and bacterial titers were determined. The survival of each mutant was examined in at least 3 independent assays.

Inverse PCR. *B. melitensis* genomic DNA was extracted using the Wizard Genomic DNA Prep kit (Promega, Madison, WI). Ten μg of genomic DNA was digested with *Rsa*I (Roche, Indianapolis, IN) by overnight digestion at 37°C. The enzyme was then heat inactivated by incubation at 65°C for 15 minutes and the DNA was precipitated with 2.5 volumes cold absolute ethanol containing 0.1 volumes of 3M sodium acetate (pH 5.4). The DNA was pelleted by centrifugation at 10,000 x g for 5 minutes and the pellet was subsequently washed in 80 % (v/v) cold ethanol and air dried for 15 minutes. The DNA pellet was directly resuspended in 1X ligase buffer containing 3 units of T4 DNA ligase (Promega, Madison, WI) and incubated at 20°C for 4 hours. Inverse PCR was performed using 0.2 volumes of the ligated DNA, primers SIGN-10 (gccgaacttgtgtataagagtcag) and SIGN-11 (aaaggtagcgttgccaatg) at 0.5 μM each, 1x PCR buffer with 0.2 mM $MgCl_2$, 200 μM each dNTPs, and 2 units Faststart Taq Polymerase (Roche, Indianapolis, IN). The inverse PCR amplified the ligated DNA fragment containing the transposon and flanking genomic DNA.

PCR products were electrophoresed through a 0.8 % (w/v) agarose gel, extracted and purified using a Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA). Purified PCR Products were submitted for sequencing (GTL, Texas A&M University) using primers SIGN-10 and SIGN-11. The sequence results were aligned to the sequenced *B. melitensis* genome using MacVector v8.0 (MacVector, Inc., Cary, NC) to identify the interrupted gene locus. Once a gene locus was identified, the DOE Joint Genome Institute, Integrated Microbial Genomes web page (http://imgweb.jgi-psf.org/cgi-bin/w/main.cgi) was utilized to determine the gene product identity, putative function and to organize the genes into their respective cluster of orthologous genes (COG's).

Acriflavine agglutination. To test for the loss of O-antigen, acrivflavine agglutination phenotypes were tested for in transposon mutants. Bacterial cells were grown to saturation for 48 hours with agitation in TSB. Approximately 1 x 10^{10} cells were mixed in a 1:1 (v/v) ratio with 0.1 % (wt/vol) of an aqueous solution of acriflavine and examined for agglutination.

RESULTS

Initial screening of signature tagged mutants. Previously, *B. melitensis* 16M was mutagenized using a pool of signature tagged mini-Tn*5*::Km2 transposons and screened using two models (J. Pei and C. Turse, data unpublished). In the J774A.1 macrophage-like *in vitro* model, *B. melitensis*

antibodies were used to label internalized bacteria in macrophage cells. Mutants were manually screened by fluorescence microscopy, selecting strains that appeared to be deficient for intracellular replication. Secondly, mutants were screened in the mouse model. Mice were infected with pools of mutants (46-47 mutants/pool) and the mutant recovery was determined by Southern blot, using probes that hybridized to a unique DNA tag (located within the transposon) for each mutant in a pool. Mutants with reduced hybridization signals in at least 2 of 3 mice were considered attenuated for *in vivo* replication. Mutants that were not able to survive in the either model were examined further, quantifying the degree of attenuation and sequenced to identify the interrupted gene loci.

Identification of interrupted genetic loci attenuated for survival. Seventy-eight mutants identified in primary screens were sequenced to identify the interrupted gene locus. The putative gene products of these mutated genes are presented in Table 1, organized by clusters of orthologous genes (COGs). A very diverse set of mutants was identified using this method. Mutants defective in metabolic functions, including energy production and carbohydrate metabolism and transport, as well as housekeeping functions, including the β subunit of DNA polymerase III, DNA helicase, 50S ribosomal protein L9 and ribonucleases were identified. Among these seventy-eight mutants, there were five mutants that could not be genetically identified, either from the inability to

TABLE 1. Identity of interrupted genetic loci by transposon mutagenesis and verified for attenuation in J774A.1 macrophage-like cells.

STM Mutant	Gene Product	BME Locus Tag	ΔCFU at 0H[a]	ΔCFU at 48H[a]	% Uptake±SD[b]
Amino Acid Transport and Metabolism					
9E3	Transporter, Drug/Metabolite Family	I 0187	0.12	0.54*	302±4.20
2G6	Cysteine Synthase A	I 0101	-0.01	0.40*	135±0.64
9C1	Branched Chain Amino Acid ABC Transporter	II 0344	0.05	0.43*	110±0.63
3H3[c]	Glutamate Synthase NADPH	II 0039	0.21	1.59***	284±4.45
18F3[c]	5-Methyltetrahydrofolate-Homocysteine Methyltransferase	I 1759	0.38	1.36*	119±1.00
6H4[c]	Glutamate Synthase	II 0040	0.52**	1.81*	36±0.06
23C3	Cysteine Synthase A	I 0101	0.00	0.97*	65±0.11
25H2	Para-Aminobenzoate Synthase Component I	II 0013	-0.37	1.26**	168±2.20
20E5	N-Acyl-L-Amino Acid Amidohydrolase	I 0033	.01	0.80*	131±1.01
Carbohydrate Transport and Metabolism					
10E2	Ribose 5-Phospate Isomerase	II 0424	0.11	0.59**	122±0.73
17G5[c]	Glucose/Galactose Transporter, GluP	II 1053	1.08**	1.39*	9±0.06
Cell Motility					
16F1	Flagellar Motor Switch Protein, FliM	II 1110	0.31	0.37*	100±0.65
Cell Wall, Membrane, and Envelope Biogenesis					
9C4[c]	Mannosyltransferase	I 0997	-0.82**	1.50***	32407±315
8A6	Mandelate Racemase	I 1707	0.14	0.75*	102±0.99
2C3	Apolipoprotein N-Acetyltransferase	I 1972	0.49	0.71*	73±0.52
3A1	Glucosamine-1-Phosphate Acetyltransferase	II 0684	0.14	0.38*	85±0.61
8C3	Mandelate Racemase	I 1707	0.31	0.90**	67±0.49
Coenzyme Transport and Metabolism					
4B3	Molybdopterin (MPT) Converting Factor	I 1254	0.40*	1.22**	71±0.41
Energy Production and Conversion					
5B5[c]	Transport ATP-Binding Protein, CydC	II 0761	0.26	1.91*	218±2.81
10C5	Pyruvate Dehydrogenase E1 Component, β Subunit	I 0855	0.46	1.07*	192±3.22
10D1	L-Lactate Permease	I 1233	-0.25	0.79***	215±2.3
5B2	Pyruvate Dehydrogenase E1 Component, β Subunit	I 0855	0.67*	3.68*	119±0.16
8C6[c]	Pyruvate Carboxylase	I 0266	0.14	0.88	112±0.88
8E3	L-Lactate Permease	I 1233	0.23	0.41**	109±0.84
10B3	Rhodocoxin Reductase	II 0557	0.64***	0.28***	578±6.28
10H4	L-Lactate Permease	I 1232	0.03	0.25	141±0.77
17B3	Cytochrome D Ubiquinol Oxidase Subunit I	II 0760	0.44	1.36*	108±1.46
General Function Prediction or Unknown					
6B6	Hypothetical Protein	I 0316	0.26	0.40***	92±0.20
8D2	31 kDa Immunogenic Protein Precursor	I 0796	0.01	0.83*	108±0.67
9E5	26 kDa Periplasmic Immunogenic Protein Precursor, Omp28	I 0536	0.02	0.90*	115±0.26
10B1	Phenazine Biosynthesis Protein, PhzF	I 0630	0.88	0.62*	209±2.61
29H6	Hypothetical Cytosolic Protein	I 1279	0.51	2.74	47±0.36
10C2	Hypothetical Phage Protein	I 1344	-1.08	0.99**	3945±38.44
6E1	Putative Membrane-Associated Alkaline Phosphatase	I 0385	-0.13	2.74**	199±0.03
30A4	NorQ Protein	II 0997	-0.11	0.89**	163±1.73
27C6	Hypothetical Cytosolic Protein	I 1277	-0.03	0.38	121±1.30
Inorganic ion transport and metabolism					
9B4[c]	Superoxide Dismutase Precursor, SodC	II 0581	-0.05	0.20	232±2.86
Intracellular Trafficking, Secretion, and Vesicular Transport					
2E2[c]	ATPase, Type IV Secretory Pathway, VirB4	II 0028	0.42	1.65**	113±0.6
7F1[c]	ATPase, Type IV Secretory Pathway, VirB4	II 0028	-0.05	2.27***	123±0.41

TABLE 1--*Continued*

STM Mutant	Gene Product	BME Locus Tag	ΔCFU at 0H[a]	ΔCFU at 48H[a]	% Uptake±SD[b]
19E6[c]	ATPase, Type IV Secretory Pathway, VirB4	II 0028	0.34	3.04**	71±0.37
30H1[c]	Type IV Secretory Pathway, VirB6	II 0030	0.01	2.21*	98±0.95
2C1[c]	Type IV Secretory Pathway, VirB8	II 0032	0.23	1.82**	68±0.09
23F3[c]	Type IV Secretory Pathway, VirB8	II 0032	-0.07*	2.27***	109±0.09
11F2[c]	Type IV Secretory Pathway, VirB9	II 0033	0.23	1.41**	102±0.51
12H3[c]	Type IV Secretory Pathway, VirB10	II 0034	0.21	1.60**	131±0.37
	Lipid Transport and Metabolism				
9A2	Acyl CoA Dehydrogenase	II 0492	0.11	0.47*	176±1.96
	Nucleotide Transport and Metabolism				
2D1[c]	Dihydroorotase	I 1281	0.01	0.44**	122±0.39
17E1	Dihydroorotase	II 0069	0.65	0.82***	47±0.18
10F2[c]	Dihydroorotate Dehydrogenase	I 1611	0.27	0.34*	303±3.46
22A5[c]	Amidophosphoribosyltransferase5	I 1488	0.46	2.62***	63±0.22
20G2[c]	Phosphoribosylformylglycinamidine Synthase I	I 1124	0.04	1.37*	89±0.98
	Replication, Recombination, and Repair				
4B2	DNA Polymerse III--β Chain	I 1942	0.88	1.17**	81±0.78
9H6	Transposase	II 0716	-0.06	0.47	435±5.5
30A1[c]	Tyrosine Recombinase, XerD	I 0040	-0.01	1.21**	323±4.4
	Secondary Metabolites Biosynthesis and Catabolism				
10D3	Acetylspermidine Deacetylase	I 1504	0.35	0.66*	666±3.44
10F3	Acetylspermidine Deacetylase	I 1504	-0.22	0.52*	200±0.74
	Signal Transduction Mechanisms				
20D2	Response Regulator Protein, PleD	II 0660	-1.46	1.64*	4690±0.61
8H5	Sensory Transduction/Histidine Kinase	II 0679	0.40	1.36*	51±0.24
10E5[c]	Transcriptional Regulatory Protein, HydG	II 0011	0.36	1.76**	169±0.65
	Transcription				
1F1	Transcriptional Regulator, LysR Family	I 1913	0.11	0.12	96±0.43
19C6	Transcriptional Regulator, LuxR Family, VjbR	II 1116	0.21	2.51**	109±0.46
3E1	Transcriptional Regulator, LacI-Family	I 1385	-0.24	0.29*	174±0.93
17B2	Ribose Operon Repressor, LacI Family	II 0312	0.09	0.72**	84±0.76
4C2[c]	Heat-Inducible Transcription Repressor, HrcA	I 1776	0.07	0.32*	95±0.62
	Translation, Ribosomal Structure and Biogenesis				
4C1	Ribonuclease PH	I 1775	0.14	0.59*	99±0.51
10B5	Ribonuclease PH	I 1777	-0.28	1.16**	173±0.90
16H1[c]	ATP-Dependent DNA Helicase	I 0275	0.11	1.21**	78±0.28
10B6	Polypeptide Deformylase	II 0812	0.31	0.50*	806±5.18
16F6	50S Ribosomal Protein L9	I 1483	0.31	1.36*	63±0.24
	Unsequenced				
15C6	-	-	0.37	1.02*	33±0.08
16E1	-	-	0.31	0.37*	100±0.65
21H1	-	-	-0.33	0.81*	222±2.54
21H4	-	-	-0.44	1.92*	186±1.62
22B2	-	-	-0.19	2.22**	121±0.38

[a] The ΔCFU was calculated by subtracting the log CFU of the mutant from the log CFU of wild-type at either immediately after uptake or after 48 hours. A two-tailed Student's *t*-test was applied to determine statistical significance. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. [b] The percent uptake is the ratio of the number of internalized mutant bacteria in the macrophage cells normalized to the respective innoculum and compared to the number of the wild type bacteria internalized by the macrophage cells normalized to the respective innoculum. [c] The mutant was identified in an independent signature tagged mutagenesis study (26, 158).

obtain an inverse PCR product or the sequence results did not align with the sequenced genome.

In addition to metabolic genes, genetic loci associated with virulence and stress responses were also identified, including the *virB* operon, which encodes the TIVSS and is potentially responsible for transporting unidentified effector protein(s) into the host cell. A gene encoding for superoxide dismutase (SodC) is important for the neutralization of the oxidizing radical superoxide (O_2^-) to O_2 and hydrogen peroxide (H_2O_2), was also identified in this screen.

Furthermore, genes encoding for seven transcriptional regulators were identified to be important for the survival of *B. melitensis*. These transcriptional regulators include a LacI ribose repressor and members of the LysR, LuxR and HrcA protein families. Signal transduction systems were also identified as attenuated in this screen, including the two-component response regulators PleD and HydG. These transcriptional regulators may be involved in the regulation of virulence genes and/or may be important in responding to the changing environment from extracellular to intracellular.

Quantification of the attenuation in transposon mutants identified in primary screens. The 78 genes identified in the primary screen were verified for attenuation in the J774A.1 macrophage-like model. The uptake of the mutants was examined by comparing the number of cells recovered early after infection to the innoculum. The uptake of each mutant was normalized to the

uptake of the wild type organism and is presented in Table 1 as the percent uptake compared to wild type. The recovery of wild type and mutant bacterial strains early after infection and at 48 hours post infection was also examined and presented in Table 1 as the log CFU difference between the mutant and wild type recovered from infected J774A.1 macrophage-like cells. Varying degrees of uptake and attenuation were observed for these mutants, with some showing a large defect in survival and some surviving comparably to wild type organism after 48 hours of infection.

Some of the most highly attenuated mutants identified in this screen included interruptions in the following genes: Pyruvate dehydrogenase E1 component β subunit, mutant 5B2 with 3.68 logs less bacteria recovered than wild type; a hypothetical cytosolic protein, mutant 29H6 with 2.74 logs less bacteria recovered; mutant 22A5, amidophosphoribosyltransferase with 2.62 logs less bacteria recovered than wild type; and mutant 19C6, a transcriptional regulator from the LuxR family with 2.51 logs less bacteria recovered. These genes, along with the other genetic loci identified in Table 1, represent metabolic pathways and structures that are indispensable for the intracellular lifestyle and provide insight into the environment encountered within the macrophage.

A few of the mutants identified as attenuated in the primary screening (mouse and macrophage models) were not found to be highly attenuated after 48 hours of infection in the secondary verification using the J774A.1

macrophage-like cell infection model. This could be due to several factors, including the difference in models. Mutants may be highly attenuated *in vivo*, but when examined *in vitro* in macrophage-like cells were not as highly attenuated, suggesting the gene products are important for survival in the entire organism and may be dispensable for replication in macrophage cells. The mouse model of infection will impose many factors and environments not present in the *in vitro* macrophage infection, including an immune response elicited against the bacteria and the presence of different host cells besides the macrophage to reside in. Additionally, the mouse model screening consisted of pools of bacteria. Possibly, some of the mutants may have been unable to survive due to the competition imposed by other mutant strains present, which is removed in the second verification screening in macrophage-like cells.

The majority of mutants survived similarly to wild type early in the infection, suggesting that many of the mutants enter the macrophage-like cells at the same rate, and potentially through the same mechanism as the wild type organism. However, there were six mutants that showed a deficiency early in infection. To determine if these deficiencies shortly after uptake were due to reduced bacterial uptake, the number of bacteria recovered at time zero was compared to the number of bacteria in the inoculum. This ratio was than compared to the uptake ratio of the wild type bacteria and expressed as the percentage of cells internalized by the J774A.1 macrophage-like cells as

compared to wild type. Three mutants were found to be deficient in uptake when compared to wild type bacteria; 6H4, 17G5 and 17E1, with only 36 %, 9 % and 47 % (respectively) internalized by the J774A.1 macrophage-like cells. The reduced uptake explains the reduction in the bacterial number recovered early in infection.

The remaining 3 mutants, 4B2, 5B2 and 10B1, were found to enter the macrophage cells in numbers similar to wild type; 81 %, 119 %, 209 % (respectively), suggesting that the interrupted gene locus may be important for survival immediately after uptake by the macrophages. The interrupted gene loci include the β subunit of DNA polymerase III (4B2), pyruvate dehydrogenase E1 component β subunit (5B2) and phenazine biosynthesis protein (10B1). The identification of these interrupted genes does not provide an immediate explanation as to why these mutants are attenuated for early survival since they were not attenuated for growth *in vitro* cultures, unless these genes are not required when grown in rich culture media and may indicate metabolic pathways required in the host cell early in the course of infection. In a nutrient-limiting environment, these metabolic pathways or metabolites may be crucial for survival even early in the infection.

In addition to mutants with uptake and early survival defects, mutants with significantly enhanced uptake and early survival capabilities were identified. This includes mutants 9C4, with an interruption in a gene locus coding for

mannosyltransferase; 10C2, a hypothetical phage protein; and mutant 20D2, a two component response regulator PleD. The observed uptakes were 324, 39 and 47 (respectively) times greater then the uptake of wild type bacteria by the macrophages. Acriflavine agglutination revealed that one of these mutants, 9C4, displayed a phenotype characteristic of mutants with an altered LPS. Sequencing of the interrupted gene locus identified that 9C4 was a mutant with an interruption in the mannosyltransferase gene, known to contribute to the production of LPS. LPS is important for entry into macrophage cells via lipid rafts, and *Brucella* spp. with an altered LPS and lacking O-antigen (rough mutants) have been found to have an enhanced invasiveness, likely through an alternate mode of entry (110, 115). Mutants 10C2 and 20D2 do not appear to have rough phenotypes by this examination and the interrupted genetic loci are not expected to be involved in LPS biosynthesis, suggesting that these mutants are more invasive to macrophages than wild type but are not able to display any enhanced survival capabilities. Uptake of these mutants may be through an alternative route and the bacterium is not able to re-direct the phagosome to develop a replicative niche and instead fuses to lysosomes.

DISCUSSION

Signature tagged transposon mutagenesis (STM) is a powerful technique, first used in *Salmonella enterica* serovar Typhimurium, and has proven useful for

identifying genes required for survival and virulence in a number of other bacteria, including *Brucella* spp. (42, 59, 62, 72, 83, 84, 132, 158). In this study, transposon mutants found to be attenuated for survival in initial screens using two different models were verified and sequenced to identify the interrupted gene loci. Seventy-eight transposon mutants were confirmed to have attenuated phenotypes. Sequencing of the gene interruptions in the attenuated mutants revealed interruptions in genes contributing to metabolic and housekeeping functions, virulence, stress response and transcriptional regulators. Twenty-two genetic loci, or approximately 28 % of the genes identified in this study, were identified in independent STM studies conducted in *B. suis, B. abortus* or *B. melitensis* and screened in a variety of models, including the two used in this study as well as HeLa cells (26, 158). These genetic loci, noted in Table 1, suggest that these genes are important among the different *Brucella* spp. and in the different models of infection used to measure the attenuated phenotypes.

Three mutants identified in this study were found to able to enter macrophages at a rate comparable to wild type but were deficient for survival following their uptake. It is possible these mutants entered through an alternative route compared to wild type and were not as successful at diverting the early phagosome to a more favorable brucellosome, resulting in a lower recovery of bacteria early after infection. There was not a growth defect observed for *in vitro* culture growth and may suggest that these

pathways/metabolites are not required in rich media but may be essential for intracellular survival. The phenazine biosynthesis protein is interesting because phenazine has been shown to be capable of oxidative activity, but the degree of this activity is dependent on the combination and variety of functional groups and has not been characterized in *Brucella* (118). Phenazines produced by *Pseudomonas aeruginosa* have been shown to be able to interact with oxygen and form reactive oxygen species, which can insult the host cell's internal redox balance in human airway epithelial cells (118). This disruption leads to an increase in secretion by the cells, thus contributing to the generation of sputum (the nutritional and physical substrate for *P. aeruginosa* in the lungs of individuals with cystic fibrosis). Phenazine production contributes to the virulence and survival of *P. aeruginosa* and may also contribute to the virulence and survival of *B. melitensis* by also disrupting the redox balance of its resident host cell.

Through this screening, gene products that have been shown to be important for *Brucella* spp. infection and survival were identified. These genes encode for gene products including the type IV secretion system (TIVSS), totaling 8 mutants in 4 loci; flagella motor switch, FliM; O antigen and LPS biosynthesis; superoxide dismutase SodC; a LuxR family transcriptional regulator, VjbR; and cytochrome *bd* oxidase (25, 35, 49, 80, 135).

The TIVSS is required for replication and survival in the host (11). Trafficking studies in macrophage cells have shown that the TIVSS is essential for the maintenance of the brucellosome by sustaining interactions with the endoplasmic reticulum (14). It is proposed that the TIVSS secretes effector protein(s) into the host cell to recruit components necessary for *Brucella* spp. to maintain an intracellular replicative niche; however, no effector proteins have been identified as yet (11).

Brucella spp. are described as being non-motile, yet contain three gene clusters containing flagellar structural genes, with many necessary components either missing or truncated (27). Transmission electron microscopy (TEM) showed that *B. melitensis* appears to produce a flagellar structure, that is sheathed by an outer cellular membrane (44). Survival studies in mice showed that flagella genes *flgI, fliF, fliC, flhA, motB* or *flgE* are required for the establishment of a chronic infection, attenuated for survival at 4 weeks post infection when compared to wild type infections (44). Currently, the precise role of flagella components in the survival and replication of *Brucella* spp. has not been defined. It is plausible that they could also function as a secretion system, based on the ancestral relationship between the type III secretion systems and flagella (47). Clustering of the type III secretion system components of plant and animal pathogens found that the plant TIIISS components are more closely related to components of the flagellar machinery than their counterparts in

animal pathogens (47). This is interesting and noteworthy because *Brucella* spp. is an animal pathogen, but is phylogenetically classified with plant pathogens.

Brucella spp. synthesize an unconventional LPS that possess properties such as low endotoxicity, high resistance to macrophage degradation and protection against host defenses (40, 41, 97). One gene identified in this screen, coding for mannosyltransferase, is involved in the synthesis of LPS and displayed a rough phenotype based on acriflavine agglutination. Additional mutants were identified in this screen that may also affect the lipid composition of the cellular membrane, including a gene encoding for an apolipoprotein, which transfers fatty acid groups to membrane lipoproteins and glucosamine-1-phosphate acetyltransferase, an important precursor of lipid synthesis. This screening also identified genes encoding for additional membrane associated protein; including a 31 kDa immunogenic protein, a membrane associated alkaline phosphatase, a drug metabolite exporter and a sensory transduction histidine kinase. Although these membrane components have not been defined in *Brucella*, these gene products could be important for membrane structure and integrity, as well as interacting or interrupting signaling cascades in the host cell or for signal transduction in the bacterial cell.

A gene responsible for the production of superoxide dismutase was identified in this mutagenesis screen and has been previously found to be

important for the survival of *B. abortus* (49). Superoxide dismutase is able to catalyze the dismutation of superoxide (O_2^-) to hydrogen peroxide (H_2O_2), protecting the cell from free radical damage. Hydrogen peroxide is also a reactive oxygen species (ROS) and is usually further neutralized to water and oxygen gas spontaneously or by the aid of an additional scavenging enzyme. In *Brucella* spp., superoxide dismutase could function to protect the cells from oxidative killing *in vivo* and confer resistance to the respiratory burst from host-derived macrophages. Additionally, superoxide dismutase can offer protection against superoxide radicals produced by metabolic pathways such as aerobic respiration (49).

The ability to sense and react to one's environment through the modulation of gene expression is an essential survival component of bacterial pathogens. Gene interruptions found in gene products involved in signaling were found to be attenuated in this screen, including signal transduction proteins and two component response regulators and transcriptional regulators. To date, the precise role of these signal transduction pathways have not been studied, but it would be interesting to establish the environmental cues and transcriptional networks of these signal transduction components. Such information would provide insight into the environments that *Brucella* spp. encounter and the genetic responses to adapt to the environment.

The transposon mutagenesis screen revealed four transcriptional regulators that were found to be attenuated for survival, including members from the LuxR, LysR, LacI and HrcA protein families. Members of the LysR family have been found to regulate genes associated with virulence and symbiosis in other organisms (12, 56, 141). A targeted mutagenesis screen examined 20 LysR transcriptional regulators in *B. melitensis* found that only three of the mutations were attenuated for survival in the models examined, including the gene locus identified in this study (55). Additional studies are needed to determine the precise role of this LysR transcriptional regulator and network that confers survival of the organism.

The LuxR family of transcriptional regulators are well known for their role in the regulation of virulence genes and have been described in a number of species, with *P. aeruginosa* serving as one of the model organisms (5, 32, 155). The LuxR family of transcriptional regulators control gene expression in a density dependent fashion by responding to a chemical signal indicative of the population present, a process commonly referred to as quorum sensing. Although the role of quorum sensing in *B. melitensis* is not fully understood, recent research has shown that VjbR, the LuxR homologue identified here, is involved in the expression of the TIVSS and flagella operons and regulates modifications of the cell surface, resulting in a clumping phenotype in *vjbR* deletion mutants (25, 147).

HrcA is a negative regulator, shown to be involved in the repression of *dnaK* and *groESL* in *Bacillus subtilis* (65). In *Helicobacter pylori,* HrcA has also been found to function as a repressor of molecular chaperones *groESL*, *hrcA-grpE-dnaK* and *chpA-hspR-orf* operons, as well as an indirect, positive influence on the expression of flagellar assembly genes (126). HrcA and other specialized repressors actively repress the chaperones expression and become inactivated during stressful conditions, leading to the de-repression and expression of the molecular chaperones: a mechanism used by a subclass of Gram-negative bacteria and exclusively by Gram-positive bacteria (126). In *B. melitensis*, HrcA involvement in the stress response has not been examined. To date, only a few factors have been shown to be involved in *Brucella* stress responses, including a sigma factor RpoH in *B. melitensis* and host factor I (HF-1) for stationary phase stress resistance in *B. abortus* (23, 125). It would be interesting to examine the potential role of HrcA in the regulation of the flagella operons, which have been shown to be important for the intracellular survival of *B. melitensis*.

In this study, genetic loci necessary for the intracellular survival of *B. melitensis* were identified by signature tagged transposon mutagenesis. This study utilized two widely accepted models for *Brucella* infection, the *in vivo* mouse model and *in vitro* J774A.1 macrophage-like cells infection model. Of the seventy-eight mutants identified here, 24 gene interruptions were independently found to be attenuated in either a different *Brucella* species or in a different

infection model. This provides further confirmation of the importance of these genetic loci in survival across species and infection models. Additionally, recent research has independently confirmed the function or involvement of some gene loci identified here in the survival of *Brucella* spp., thus confirming our methodology and approach using the mouse and macrophage infection models to identify genes required for the intercellular replication of *B. melitensis*. Although some of genetic loci identified in this study have been examined in *Brucella* spp., there are still numerous genes that either have similar counterparts in other organisms but have not been examined in *Brucella* spp., or have only a general or unknown function, leaving their contribution in the intracellular replication and virulence of *B. melitensis* speculative or unknown and of great interest.

CHAPTER III

BRUCELLA MELITENSIS QUORUM SENSING REGULON: CONTRIBUTIONS OF THE *N*-DODECANOYL HOMOSERINE LACTONE SIGNALING MOLECULE AND A LUXR HOMOLOGUE VJBR

INTRODUCTION

Quorum sensing (QS) is a communication system in bacteria used to coordinate population behavior. Autoinducers are small diffusible molecules that are produced by the bacterial cells and which accumulate in the environment. When a quorum of bacteria is present, the autoinducer interacts with its respective transcriptional regulator and coordinates activation or repression of target genes. Density dependent gene regulation was first described in luminescence genes in *Vibrio harveyi* and *V. fisheri* (102). Chemical communication, or quorum sensing, is a coordinated population social trait that has been compared to pheromone production in many social animals (31).

Quorum sensing has been shown to control virulence gene expression, acyl-homoserine lactone (AHL) production, secretion systems, cell division, and biofilm formation (3, 21, 45, 64, 137, 141, 156). Modulation of host inflammatory responses and cross-communication between different bacterial species by the AHL chemical signal has also been documented (85, 94). In *P. aeruginosa*, LuxR homologues regulate production of elastase, proteases, exotoxin A, and

the type II secretion system (146). In *Agrobacterium tumefaciens*, the LuxR homologue TraA controls Ti plasmid conjugation in the presence of octopine, a plant pheromone (46). LuxR homologues have also been found to regulate virulence genes in *Aeromonas* spp., *Yersinia pseudotuberculosis*, and *Burkholderia cepacia* (7, 66, 142).

P. aeruginosa has three QS regulatory systems that have been shown to be hierarchically arranged, with an AHL and non-AHL signal serving as the mediator between the two AHL systems. The LasR-autoinducer complex activates expression of the second quorum sensing system RhlR by inducing expression of *rhlR* (105). The RhlR-autoinducer complex activates genes also regulated by the LasR-autoinducer complex as well as a second class of specific target genes. Additionally, it has been shown that the LasR-dependent autoinducer, (*N*-(3-oxododecanoyl)-homoserine lactone, prevents binding of the RhlI-dependent autoinducer, *N*-(butryl)-homoserine lactone, to its regulator RhlR (111). It is assumed that this second level of control placed on the RhlI/RhlR autoinduction by the LasI/LasR system ensures that these two systems initiate their cascades sequentially and in the proper order (95).

The transcriptional regulators, part of the LuxR family, contain two domains, the N-terminal autoinducer binding domain and the C-terminal helix-turn-helix DNA binding domain (17, 18, 131, 138). One of these *luxR*-like genes found in *Brucella melitensis*, *vjbR*, has been previously shown to positively

regulate the type IV secretion system and flagellar genes, both shown to contribute to virulence and survival of *B. melitensis* (11, 25, 82).

The LuxI family of proteins are responsible for production of AHL signals in Gram negative bacteria. Data mining using known LuxI homologues has failed to identify any candidate genes involved in the synthesis of AHL molecules within the *B. melitensis* genome. Two AHL signaling molecules produced by *B. melitensis* have been identified: *N*-dodecanoyl homoserine lactone (C_{12}-HSL), which has been confirmed, and a second molecule, potentially *N*-(3-oxododecanoyl)-homoserine lactone, 3-oxo-C_{12}-HSL, was identified but not confirmed by mass spectrometry (144). It has been shown that two of the LuxR homologues, VjbR and BabR (locus ID BMEI 1758), are both able to interact with C_{12}-HSL (79, 147). Reports focusing on specific operons (*virB* and *flgE*) indicate that the addition of exogenous C_{12}-HSL acts as a repressor of gene expression (25). The results reported here extend those observations and suggest that C_{12}-HSL may act as a more global repressor of gene expression when interacting with VjbR.

It has been shown that the TIVSS (*virB* operon) and VjbR are necessary to sustain interactions with the endoplasmic reticulum and to maintain the brucellosome, essential for intracellular bacterial replication (14, 25, 74). We hypothesize that quorum sensing regulates genes necessary to establish and sustain the brucellosome and will promote replication of the bacterium. In the

present study, custom *B. melitensis* 70-mer oligonucleotide microarrays were utilized to identify quorum sensing regulated genes. By comparing transcript expression in wild type, a VjbR knockout and wild type treated with exogenous C_{12}-HSL, a number of genes not previously described as quorum sensing regulated in *B. melitensis* were identified. These include gene in numerous metabolic pathways as well as putative virulence genes, including adhesins, proteases, lipoproteins, outer membrane proteins, secretion systems and putative effectors. Additionally, the results confirmed genes previously found to be regulated by quorum sensing, validating the microarray approach.

MATERIALS AND METHODS

Bacteria, macrophage strains and growth conditions. *E. coli* DH5α™-T1^R competent cells (Table 2) were used for cloning and routinely grown on Luria-Bertani (LB, Difco Laboratories, Becton, Dickinson and Company, Franklin Lakes, NJ) overnight at 37°C with or without supplemental kanamycin (100 mg l^{-1}) or carbenicillin (100 mg l^{-1}). *B. melitensis* 16M was routinely grown on tryptic soy agar or broth (TSA or TSB, Difco Laboratories, Becton, Dickinson and Company, Franklin Lakes, NJ), Brucella Broth (BB, Becton, Dickinson and Company, Franklin Lakes, NJ) or Sucrose Broth (1 % (w/v) Bacto-Tryptone (Difco Laboratories, Becton, Dickinson and Company, Franklin Lakes, NJ), 0.5 % (w/v) Bacto-Yeast extract (Difco Laboratories, Becton

TABLE 2. Bacterial strains, plasmids and primers used in this study.

Bacterial Strains	Description or Genotype	Reference
16M	*Brucella melitensis* bv.1 strain obtained from ATCC and re-isolated from an aborted goat fetus by this lab	
β2155	*E. coli thrB1004 pro thi strA hsdS lacZΔM15* (F' *lacZΔM15 lacI*^{q} *traD36 proA*$^+$ *proB*$^+$) *ΔdapA::erm* (Erm^r) *pir*::RP4 [::*kan* (Km^r) from SM10	(22)
JLD271	*E. coli* K12 Δ*lac*X74 *sdi*A271::Cam	(86)
DH5α^{TM}-T1^R	F-ϕ80*lacZ*ΔM15Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_{k-},m_{k+}) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A	Invitrogen
BL21Gold(DE3)	*E. coli B F- dcm+ Hte ompT hsdS(r_B- m_B-) gal λ (DE3) endA Tet^r*	Stratagene
Plasmids	**Description of Genotype**	**Reference**
pBa1143	pFuse containing *virB2, virB3* and part of *virB4*	(9)
pBluescript KSII$^+$	f1 + origin, Ap^R, lacZ', P lac promoter, pUC origin	Stratagene
pKD4	FLP/FRT, Km^R	(60)
pEX18Ap	*sacB*, Ap^R	(128)
pMR10Kan	Km^R, NCBI Accession number AJ606312	Bourniquel, A.A. (unpublished)
pET11a	Ap^R, *T7/lacO* promoter, RBS binding site, T7 terminator	Stratagene
pAL101	*rhlR*$^+$ *rhlI*::*uxCDABE*; Tet^r p15A origin	(86)
pAL102	*rhlI*::*luxCDABE*; Ter^r p15A origin	(86)
pAL105	*lasR*$^+$*lasI*::*luxCDABE*; Tet^r p15A origin	(86)
pAL106	*lasI*::*luxCDABE*; Tet^r p15A origin	(86)
Primer Name	**Sequence (5' to 3') and 5' Linker**	**Product**
TAF577	agtgaggcgcgccattcgtcatgcgacttccgg (*Asc*I)	BMEI1582 5' Prod. Rev
TAF578	gctctagacatcaaggaagtgcggcgtc (*Xba*I)	BMEI1582 5' Prod. For
TAF579	gctctagagcaggaagaagatggtgccg (*Xba*I)	BMEI1582 3' Prod. Rev
TAF580	cgaatggcgcgcctcactttagggcggttccg (*Asc*I)	BMEI1582 3' Prod. For
TAF583	ttttcggcgcgcccttgccccaccttctccg (*Asc*I)	BMEI1751 5' Prod. Rev
TAF584	gctctagaggtccgcaaacccacaaaatc (*Xba*I)	BMEI1751 5' Prod. For
TAF581	gctctagagcacagtttgcggggtgac (*Xba*I)	BMEI1751 3' Prod. Rev
TAF582	gcaagggcgcgccgaaaattgaagaaatatttcag (*Asc*I)	BMEI1751 3' Prod. For
TAF565	cagagggcgcgcccccagtagcgtttc (*Asc*I)	BMEI1758 5' Prod. Rev
TAF566	gctctagagcgatgtggaggctgtggagcg (*Xba*I)	BMEI1758 5' Prod. For
TAF567	gctctagagcgcttgacgacggtatcattcgc (*Xba*I)	BMEI1758 3' Prod. Rev
TAF568	ctgggggcgcgccctctgacctttttccagtgtcc (*Asc*I)	BMEI1758 3' Prod. For
TAF561	cagacggcgcgccactcattggaaatatccttggtga (*Asc*I)	BMEII1116 5' Prod. Rev
TAF562	gctctagagcgattacctcctccatcgccatt (*Xba*I)	BMEII1116 5' Prod. For
TAF563	gctctagagcgtcttcgaggatgtacaattggc (*Xba*I)	BMEII1116 3' Prod. Rev
TAF563	tgagtggcgcgccgtctgatcaacatggtcg (*Asc*I)	BMEII1116 3' Prod. For
TAF569	tattgggcgcgccattcattgtatcgccctctg (*Asc*I)	BMEII0853 5' Prod. Rev
TAF570	gctctagagcattggtccagtccccctgtctc (*Xba*I)	BMEII0853 5' Prod. For
TAF571	gctctagagccaaaagcattggcgaccctg (*Xba*I)	BMEII0853 3' Prod. Rev
TAF572	tgaatggcgcgcccaatagcttgatcaggccc (*Asc*I)	BMEII0853 3' Prod. For
TAF588	aatctagagcggaaggtttccgcgcgac (*Xba*I)	BMEII1116 Comp For
TAF589	aatctagaggtttcatcactgaggatatttcc (*Xba*I)	BMEII1116 Comp Rev
TAF204	ggcgcgccacgtcttgagcgattgtgtagg (*Asc*I)	*nptII* For
TAF205	ggcgcgccggacaacaagccagggatgtaac (*Asc*I)	*nptII* Rev
TAF644	ggatcctcacaccgccatcgacagcg (*Bam*HI)	*P. aeruginosa rhlI* For
TAF645	ggatccatcgaattgctctctgaatcgc (*Bam*HI)	*P. aeruginosa rhlI* Rev
TAF646	ggatccatcgtacaaattggtcggcgcgaagagttcg (*Bam*HI)	*P. aeruginosa lasI* For
TAF647	ggatcctcatgaaaccgcccttcgctgttccacc (*Bam*HI)	*P. aeruginosa lasI* Rev
TAF648	ggatccttgcatccggcttgcgcgcgtcttatcc (*Bam*HI)	BMEI1213 For
TAF649	ggatcctcagattgcagcaaaacctctttcg (*Bam*HI)	BMEI1213 Rev

TABLE 2--*Continued*

Primer Name	Sequence (5' to 3') and 5' Linker	Product
TAF650	ggatccgtgccatggcgtggcggaggc (*Bam*HI)	BMEI0701 For
TAF651	ggatccttaaacttttgagcgcgccgcgtggc (*Bam*HI)	BMEI0701 Rev
TAF652	ggatccgtgtctttatcgcaaacgcgacttctggc (*Bam*HI)	BMEI1768 For
TAF653	ggatcctcaggctgcgacgggaacaggtttcaggg (*Bam*HI)	BMEI1768 Rev
TAF654	ggatccgtggacgccgcttttgaaaccgatgc (*Bam*HI)	BMEI1289 For
TAF655	ggatccttattctaaaactggcaaggcttcg (*Bam*HI)	BMEI1289 Rev
TAF656	ggtaccttagacgccgcgtctgttgcccc (*Bam*HI)	BMEI0093 For
TAF657	ggatccatgaaacccggccccgaaatccg (*Bam*HI)	BMEI0093 Rev
TAF658	ggatccatggctgcgacttatagaaaagacg (*Bam*HI)	BMEI1869 For
TAF659	ggatccctactttgcctgtgccctgatgattttcagcg (*Bam*HI)	BMEI1869 Rev
TAF660	ggatccatggaacggcgcatgatcccc (*Bam*HI)	BMEI0712 For
TAF661	ggatccttatttatccggcttttgaccataccagcg (*Bam*HI)	BMEI0712 Rev
TAF662	ggatccgtgagtcgcatggttgatcaaacg (*Bam*HI)	BMEI0852 For
TAF663	ggatcctcaccgccgtgcagtcactccg (*Bam*HI)	BMEI0852 Rev
TAF664	ggatccatgctccgccgccgaaatacgagc (*Bam*HI)	BMEI1093 For
TAF665	ggatccctaaaccgccaacgtgactgttaccg (*Bam*HI)	BMEI1093 Rev
TAF666	ggatccttgcttgaagggaacgcacgg (*Bam*HI)	BMEI2003 For
TAF667	ggatcctcacacagtagcgctcaccc (*Bam*HI)	BMEI2003 Rev
TAF668	atgacggatccatgggtgaattcgcggtcgtg (*Bam*HI)	BMEI0032 For
TAF669	atgacggatcctcacccgtcatttggccagttc (*Bam*HI)	BMEI0032 Rev
TAF670	atgacggatccatggcagaacagaaatcgagc (*Bam*HI)	BMEI1956 For
TAF671	atgacggatccttattgcgcgctgcagtccttggacaggc (*Bam*HI)	BMEI1956 Rev
TAF672	ggatccatgatcgatgaaaatcatccc (*Bam*HI)	BMEII1969 For
TAF673	ggatcctcagcgccggataaaagtcagataagcg (*Bam*HI)	BMEI1969 Rev
TAF674	ggatccttgcccactggcaaattgcgc (*Bam*HI)	BMEIII0678 For
TAF675	ggatccttatttcgtctgtcgggg (*Bam*HI)	BMEII0678 Rev
TAF676	ggatccatggcgcagcatcatcatctgaaggtgatcg (*Bam*HI)	BMEII0828 For
TAF677	ggatccttagcgctcctccctaacgagtgccgg (*Bam*HI)	BMEII0828 Rev

Dickinson and Company, Franklin Lakes, NJ) and 6 % (w/v) sucrose). J774A.1 murine macrophage-like cells were obtained from ATCC and grown in T-75 flasks in Dulbecco's modified Eagle's medium, hepes modification (DMEM), supplemented with 1X MEM non-essential amino acids (Sigma, St. Louis, Mo), 0.37 % (w/v) sodium bicarbonate and 10 % (v/v) heat-inactivated fetal bovine serum. All work with live *B. melitensis* was performed in a biosafety level 3 laboratory at Texas A&M University College Station, as per CDC approved standard operating procedures.

Generation of gene replacement and deletion mutants. LuxR-like proteins were identified in *B. melitensis* using NCBI BLAST protein homology searches (http://www.ncbi.nlm.nih.gov/) and PFAM domain inquiry's (http://www.sanger.ac.uk/) using known LuxR proteins. Creation of gene replacement and deletion mutations were previously described by this laboratory and were followed for the creation of *luxR* gene replacement and deletion mutations in *B. melitensis* 16M (69). Briefly, regions flanking 5 *luxR* homologues (BMEI 1582, 1751, 1758, and BMEII 0853 and 1116) were amplified by PCR. The first PCR amplified the regions upstream and downstream of each gene using the 5' forward and reverse primers and 3' forward and reverse primers, primer sequences listed in Table 2. The PCR products from the first step were purified and used as the template in a subsequent overlapping PCR using the 5' forward and 3' reverse primers, linking the upstream and downstream regions of

homology with an *Asc*I site engineered in-between the upstream and downstream regions. The resulting PCR product was digested with *Xba*I (Roche, Indianapolis, IN), purified and cloned into pBluescript II KS$^+$ (Table 2, Stratagene, La Jolla, CA). *NptII* was amplified from plasmid pKD4 with primers TAF204 and TAF205 (Table 2) and was cloned in-between the flanking regions of homology at the engineered *Asc*I site. The resulting plasmids were electroporated separately into *B. melitensis* 16M. Allelic exchange resulted in the replacement of the *luxR* gene with *nptII* and created a gene replacement mutation.

The overlapping PCR product was also cloned into a second plasmid, pEX18Ap (Table 2), containing a counter-selectable marker, *sacB*. This plasmid was electroporated into the gene replacement mutant and antibiotic resistance to carbenicillin was used to select for plasmid integration. Removal of the integrated plasmid and *nptII* gene by recombination was done so by utilizing the counter-selectable *sacB* marker by selecting for the ability to grow in sucrose broth. Gene deletion mutations were screened for by the ability to grow in the presence of sucrose and antibiotic susceptibility to both kanamycin (gene replacement mutation) and carbenicillin (from pEX18Ap). For BMEII 1116, the 16M1116::Tn*5*::Km strain (19C6) was used in place of gene replacement mutant and the 16MΔ1116 gene deletion mutation was created as described above.

For complementation of BMEII 1116, the gene locus was amplified by PCR primers TAF588 and TAF589 (Table 2) and cloned into pMR10-Kan (Table 2) at *Xba*I sites. The plasmid was electroporated into the 16MΔ1116 gene deletion mutant and selected for by kanamycin resistance.

Screening of *luxR* mutants for attenuation. The *luxR* gene replacement and deletion mutants were screened for attenuation in J774A.1 macrophage-like cells. J774A.1 cells were seeded in 24 well plates at a density of $2.5x10^5$ CFU $well^{-1}$ and allowed to rest at 37°C in an atmosphere containing 5 % (v/v) CO_2 for 24 hours in supplemented DMEM. J774A.1 cells were infected with *B. melitensis* 16M or mutant strains in individual wells at an MOI of 20. To synchronize the infection, the monolayers were centrifuged for 5 min at 200 x g and incubated at 37°C in an atmosphere containing 5 % (v/v) CO_2 for 20 minutes. The infected monolayers for the first time point were washed three times in Peptone Saline (1 % (w/v) Bacto-Peptone (Difco Laboratories, Becton, Dickinson and Company, Franklin Lakes, NJ) and 0.5 % (w/v) NaCl) and then lysed in 0.5 % Tween-20 and serial dilutions were plated to determine bacterial CFU, defining time zero. The remaining infected monolayers were incubated for 48 hours at 37°C in an atmosphere containing 5 % (v/v) CO_2 in modified DMEM supplemented with gentamycin (40 μg ml^{-1}). After 48 hours, the macrophage cells were washed and lysed as described at time zero and bacterial titers determined.

RNA collection. Bacterial cultures were inoculated 1:1000 from a 48 hour starter culture grown in Brucella Broth at 37°C with agitation. Cultures for the AHL experiments were grown with the addition of exogenous *N*-dodecanoylhomoserine lactone (C_{12}-HSL, Sigma, St. Louis, Mo) added at inoculation at a concentration of 50 ng ml^{-1}. Cell density was estimated by spectrophotometry at an optical density of 600 nm. Total RNA at a mid-exponential growth phase was extracted when an OD_{600}=0.4 was reached, and at an early stationary growth phase when an OD_{600}=1.5 was reached. The RNA extraction was performed using the hot acidic phenol extraction method adapted from a previously described protocol developed for *E. coli* (124). Three biological replicates were collected for each time point. At harvest time, 14 % (v/v) of an ethanol solution containing 5 % (v/v) acidic water saturated phenol (Ultrapure, Invitrogen, Carlsbad, CA) was added to the bacterial culture and immediately centrifuged at 10900 x g for 3 minutes. The supernatant was removed and the bacterial pellet was either frozen in liquid nitrogen and stored at -80°C or processed immediately. RNA was collected by re-suspending the bacterial pellets in 0.5 mg ml^{-1} lysozyme (Amresco, Solon, OH) dissolved in TE, followed by the addition of 0.97 % (v/v) SDS. The cells were then incubated at 64°C for 2 minutes. Next, 0.5 M sodium acetate (pH 5.2) was added, mixed gently by inversion and an equal volume of acidic water saturated phenol (Ultrapure, Invitrogen, Carlsbad, CA) was added and mixed well by inversion.

The bacteria-phenol mix was incubated at 64°C for 6 minutes and mixed by inversion several times every 40 seconds. The bacteria-phenol mix was chilled on ice for 30 minutes and the layers were separated by centrifugation at 28900 x g for 13 minutes. The aqueous layer was removed and placed in a clean tube. An equal volume of chloroform (Sigma, St. Louis, MO) was added to the aqueous layer and mixed well by inversion. The layers were separated by centrifugation at 20700 x g for 7 minutes. This aqueous layer was placed into a clean tube and total RNA was precipitated by adding an equal volume of cold isopropanol and 0.1 volumes of 3M sodium acetate (pH 5.4). RNA was precipitated for one hour at -80°C and then pelleted by centrifugation at 19000 x g for 30 minutes, washed with 80 % (v/v) ethanol, centrifuged for 5 minutes at 19000 x g, and the resulting pellets were air-dried for 20 minutes. The dried pellets were resuspended in RNAse-free water, and subjected to DNAseI treatment.

The DNA was degraded and removed by incubation with 5X DNAseI buffer (50 mM $MgCl_2$, 50 mM Tris-Cl pH 7.5, 5 mM EDTA, 5 mM dithiothreitol (DTT)), 10 units of DNAseI (Qiagen, Valencia, CA) and 10 units of RNAse Inhibitor (Roche, Indianapolis, IN). The reaction was incubated at 37°C for one hour. Following the DNAseI digestion, the total RNA was further purified using the HighPure RNA isolation kit (Roche, Indianapolis, IN), following manufacturer's instructions and included the optional (second) DNAseI

incubation. The RNA was eluted with the provided elution buffer, precipitated with 3 volumes of cold 95 % (v/v) ethanol and 0.1 volumes 3 M sodium acetate (pH 5.2) and washed with 80 % (v/v) ethanol. The pellet was air dried for 15 minutes and then resuspended in The RNA Storage Solution (Ambion, Austin, TX). RNA integrity, purity and concentration was measured using a 2100 bioanalyzer (Agilent, Santa Clara CA), electrophoresis using 0.8 % (w/v) agarose gel and the Nanodrop® ND-1000 (Nanodrop, Wilmington, DE).

RNA and DNA labeling for microarrays. *B. melitensis* 16M genomic DNA was extracted using a Wizard genomic DNA extraction kit (Promega, Madison, WI), labeled by direct incorporation with Cy-5 and served as a common reference for each array. Genomic DNA was processed into cDNA using components of the BioPrime® Plus Array CGH Indirect Genomic Labeling System (Invitrogen, Carlsbad, CA) with the exception of the supplied dNTPs. The reaction consisted of 1.5 μg of genomic DNA, 1x random primers, 0.12mM dATP, 0.12mM dTTP, 0.12mM dGTP, 60μM dCTP (PCR grade, Invitrogen, Carlsbad, CA), 40μM dCTP-Cy5 (Amersham, Piscataway, NJ) and 40 units Exo-Klenow Fragment. Samples were incubated overnight at 37°C. The reaction was stopped by adding 45mM EDTA pH 8.0 and purified of unincorporated dye using PCR purification columns (Qiagen, Valencia, CA) following the manufacturer's instructions and eluted in 0.1X of the supplied elution buffer. The

fluorescence and concentration was measured using a Nanodrop® ND-1000 (Nanodrop, Wilmington, DE).

Total RNA extracted from *B. melitensis* 16M was transcribed into cDNA incorporating 5-(3-aminoallyl)-dUTP (aha-dUTP) for indirect labeling with Alexa Fluor 555 (Invitrogen, Carlsbad, CA), a Cy3-like fluorescein. The cDNA synthesis combined 10 μg of total RNA, 6 μg random primers (Invitrogen, Carlsbad, CA), 1X first strand buffer (Invitrogen, Carlsbad, CA), 10 mM DTT, 0.5 mM each dATP, dTTP and dGTP, 0.3 mM dTTP (PCR grade, Invitrogen, Carlsbad, CA), 0.2 mM aha-dUTP (Invitrogen, Carlsbad, CA), 400 units SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and 20 units protector RNAse inhibitor (Roche, Indianapolis, IN). The reaction was incubated at 23°C for one minute and then at 46°C overnight. The reaction was then treated with 0.4 M NaOH and incubated at 65°C for 15 minutes to stop the reaction and remove residual RNA.

The pH was brought back to neutral with the addition of 0.2M HCl, then the cDNA was purified to remove unincorporated aha-dUTP and free amines using PCR Purification columns (Qiagen, Valencia, CA) with the modified wash (5 mM KPO_4 (pH 8.0) and 80 % (v/v) ethanol) and elution buffers (4 mM KPO_4 (pH 8.5)). The manufacturer's instructions were modified to include 2 washes with the substituted phosphate wash buffer, and the elution was carried out in 2

steps: 50 μl each elution (yielding 100 μl total) with a one minute incubation of the elution buffer in the column at room temperature before centrifugation.

Alexa-Fluor 555 (Invitrogen, Carlsbad, CA) was coupled to the aha-dUTP incorporated into the cDNA following the coupling procedure outlined in the BioPrime® Plus Array CGH Indirect Genomic Labeling System (Invitrogen, Carlsbad, CA) and purified using PCR purification columns (Qiagen, Valencia, CA) following manufacturer's instructions. Concentration and labeling efficiency of the labeled cDNA was examined using the Nanodrop® ND-1000 (Nanodrop, Wilmington, DE). Labeled RNA samples were dried completely (Eppendorf Vacufuge™, Westbury, NY) and resuspended in ddH_2O immediately before hybridization to the microarrays.

Microarray construction. Unique 70-mer oligonucleotides (Sigma, St. Louis, MO) representing 3,227 ORFs of *B. melitensis* 16M and unique sequences from *B. abortus* and *B. suis* were suspended in 3x SSC (Ambion, Austin, TX) at 40 μM. The oligonucleotides were spotted in quadruplicate onto ultraGAP glass slides (Corning, Corning, NY) by a custom built robotic arrayer (Magna Arrayer) assembled at Dr. Stephen A. Johnston's lab at the University of Texas Southwestern Medical Center (Dallas, TX). The printed slides were steamed, UV cross-linked, and stored in a desiccator until use.

Microarray prehybridization, hybridization and washing. Printed slides were submerged in 0.2 % SDS (Ambion, Austin, TX) for 2 minutes and

washed three times in containers with ample ddH_2O. The microarray slides were placed in a prehybridization solution (5X SSC (Ambion, Austin, TX), 0.1 % SDS (Ambion, Austin, TX), and 1 % (w/v) BSA (Sigma, St. Louis, MO)) pre-warmed to 45°C for a minimum of 45 minutes. Following prehybridization, the slides were washed five times in containers containing ample ddH_2O, submerged once in isopropanol, and then immediately dried by centrifugation at 207 x g for 2 minutes at room temperature in a Jouan CR422 table top centrifuge.

The resuspended labeled RNA was combined with 1 μg of labeled genomic DNA from *B. melitensis* 16M to a total volume of 35 μl. The sample was heated at 95°C for 5 minutes and held at 45°C until hybridization to the microarray slide. The labeled cDNA was combined with 1X hybridization buffer (25 % (v/v) formamide (Sigma, St. Louis, Mo), 1X SSC (Ambion, Austin, TX) and 0.1 % (v/v) SDS (Ambion, Austin, TX)) and applied to the microarray with a 22 x 60 mm LifterSlip (Erie Scientific Company, Portsmouth, NH). The microarray was incubated in a water bath for approximately 21 hours at 42°C in a sealed hybridization chamber (Corning, Corning, NY).

After hybridization, microarray slides were washed three times with agitation at room temperature. The first wash buffer (2X SSC (Ambion, Austin, TX) and 0.2 % (v/v) SDS (Ambion, Austin, TX)) was pre-heated to 42°C and the microarray was washed for 10 minutes, followed by a 5 minute wash in 2X SSC

(Ambion, Austin, TX). The microarray was subsequently washed a third time in 0.2X SSC (Ambion, Austin, TX) for 5 minutes. Immediately after the last wash, the arrays were dried by centrifugation for 2 minutes at 207 x g at room temperature in a Jouan CR422 tabletop centrifuge.

Microarray data acquisition and analysis. All slides were scanned using a commercial laser scanner GenePix 4100A (Molecular Devices, Sunnyvale, CA) and Genepix 6.1 Pro software. The auto-PMT (photomultiplier tube) function was used to determine the optimal gain for each laser channel with each slide. Spots that were flagged as bad or absent were removed from the analysis and background values were subtracted. Raw Cy-3 signals were normalized against the mean of the baseline Cy-5 gDNA reference. M vs. A plots with a LOESS fit were plotted using Prism 4 (data not shown, GraphPad Software, La Jolla, CA) to verify that the normalization procedure improved the trend of the data. Before normalization, the mean of the log values centered around 1 or 2 and after normalization the M vs. A plots show that the mean is centered at zero, confirming that the normalization procedure reduced the technical variations between the microarrays. GeneSifter (VizX Labs, Seattle, WA) was used to perform global mean normalization on the signal values already normalized to the reference channel. Genes were retained if their signals were altered by at least a 1.5-fold change between conditions and a *p* value of 0.05 or less was obtained applying a Student's *t*-test. To further filter

data based on biological relevance, individual pairwise comparisons were also performed on signal values after global mean normalization using Spotfire DecisionSite 9.0 (Spotfire, Inc., Somerville, MA). Any fold-change observed between test conditions were expected to be at least 50 % greater in magnitude (e.g., 1.5-fold greater) than the fold-change observed between any two biological replicate samples. Genes that did not meet the criteria were removed from the analysis.

For the samples extracted during the exponential growth phase, the analysis was modified to account for variation revealed by scatter plots fitted with a trend line using the normalized signal values in the wild type samples. In addition to the analysis described above, a separate comparison was performed that included only the two similar wild type samples. The resulting fold-change values were expected to be greater than 50 % of the value obtained for wild type 1 versus wild type 2. A Student's *t*-test was performed, retaining genes that had a *p* value of less than 0.05. Genes that passed the conditions in either analysis method were considered statistically significant.

A second, independent analysis of the microarray data was performed by Seralogix, Inc. (Austin, TX) using a z-score ranking method to determine significantly altered genes. Additionally, Serologix applied their dynamic Bayesian network analysis to determine the top scoring pathways in our model.

Quantitative real-time PCR. Taqman® universal probes and primer pairs were selected using Roche's Universal Probe Library and probefinder software (www.universalprobelibrary.com) and are shown in Table 3. Total RNA was reverse transcribed to cDNA using the Transcriptor First Strand cDNA synthesis kit (Roche, Indianapolis, IN) following manufacturer's instructions using 2 μg of total RNA and random primers. PCR reactions were set up with 1X TaqMan® universal PCR master mix, no AmpErase® UNG (Applied Biosystems, Foster City, CA), 200 nM of each primer and 100 nM of probe. Quantitative real-time PCR was performed in triplicate for each sample within a plate and repeated 3 independent times using the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Gene expression was normalized to 16s rRNA, and is shown relative to 16M wild type using the $\Delta\Delta C_t$ method (Applied Biosystems Prism 7700 User Bulletin #2). To verify reaction efficiencies, the absolute value of the slope resulting from the log input amount versus ΔC_t was less than 0.1 for all comparisons, validating the use of the $\Delta\Delta C_t$ calculations (data not shown).

Screen for a putative AHL synthase. Fifteen *B. melitensis* gene loci (BMEI 0032, 0093, 0701, 0712, 0852, 1093, 1213, 1289, 1768, 1869, 1956, 1969, 2003 and BMEII 0678 and 0828) as well as *lasI* and *rhlI* from *P. aeruginosa* were cloned into pET-11a (Table 2, Stratgene, La Jolla, CA) expression vector utilizing the *Bam*HI sites, and transformed by heat-shock into

TABLE 3. Primers and Taqman® universal probes used for quantitative real-time PCR.

Gene Loci BME	Forward Primer	Reverse Primer	Universal Probe*
I 0047	agatcaaggcgcgctataaa	acatcacggaatcgctcct	22
I 0148--1	catcgaccgttcgcaact	tcggtttggaaaaactcgtc	154
I 0148--2	tttttccaaaccgacctgat	ctcaccacgccataggattt	58
I 0155	aacctatggctggttcttcg	tttgcggagcacaaaataga	70
I 0414	gaattgttccgcaatcagg	cgggtataacgcgtgagc	104
I 0561	gaagatgcttatggcgttcc	cgccatagtcggtttcaag	137
I 0831	ggaaggcaagaaaaacgtca	agagattcggtgcaaagca	70
I 0984	gacgcatgaggaggtcca	cttttggcgaggatgaagtc	70
I 1470	atcgaccagtcgcaaagg	gcgagattgtagcttccag	153
I 1621	ccggatatcatggcgatt	cgccataggtcgttccat	70
I 1758	cgaaatcctgcgttggac	ttgaggattgtcccgatgat	155
I 1878	tccgttgcttacgacgtg	gaacccgtatagcccatgc	83
II 0025	catccatcatcgcagtcg	ttgcgggttgtacggttt	83
II 0151	ccggttatgagctgttcgac	cacgggtaatctcctgcataa	45
II 0659	catgaagggcgatgagga	gatcggcttcgagatataggc	83
II 0753	agatcaagctgccgctca	aattggcaacgaaggtcatc	121
II 0838	gcatgtgcagttttcctatcg	cagggtcagaaatgtcacca	45
II 1069	gaccggtgagaacggtgat	ttgaatgcgagcccagat	138
II 1116	ttgcgggttgtacggttt	caaggaattgcgtacggtct	39

*Universal probe refers to the Taqman® probe number utilized from the Roche Universal probe library

E. coli BL21-Gold(DE3) cells (Table 1, Stragene, La Jolla, CA). The primer sets used to amplify the 15 genes, as well as *lasI* and *rhlI* are shown in Table 1. The *E. coli* clones with the candidate *luxI* genes were cross streaked on LB agar supplemented with 2 mM IPTG with *E. coli* JLD271 + pAL105 (Table 2) and JLD271 + pAL106 (Table 2) for detection of C_{12}-HSL production, and *E. coli* JLD271 + pAL101 (Table 2) and JLD271 + pAL102 (Table 2) for detection of C_4-HSL production. Luminescence was detected with the FluorChem Imaging System (Alpha-Innotech, San Leandro, CA).

RESULTS

Screening for attenuation of Δ*luxR* mutants in J774A.1 macrophage-like cells. A *luxR*-like gene, *vjbR*, was identified in a mutagenesis screen conducted by this laboratory and others (data not published, (26). Six additional LuxR homologues were identified in *B. melitensis* by BLAST protein homology searches and PFAM domain inquiries of the autoinducer binding domain and LuxR DNA binding domain. Two of these LuxR homologues (BMEI 1758 and BMEII 1116) contained the two domains typical of quorum sensing LuxR proteins, illustrated in Fig. 1. The remaining five LuxR homologues (BMEI 1582, 1607, 1752, and BMEII 0051 and 0853) were found to only contain significant amino acid similarity to the C-terminal LuxR DNA binding domain. The N-terminal region of these proteins do not contain the autoinducer binding domain,

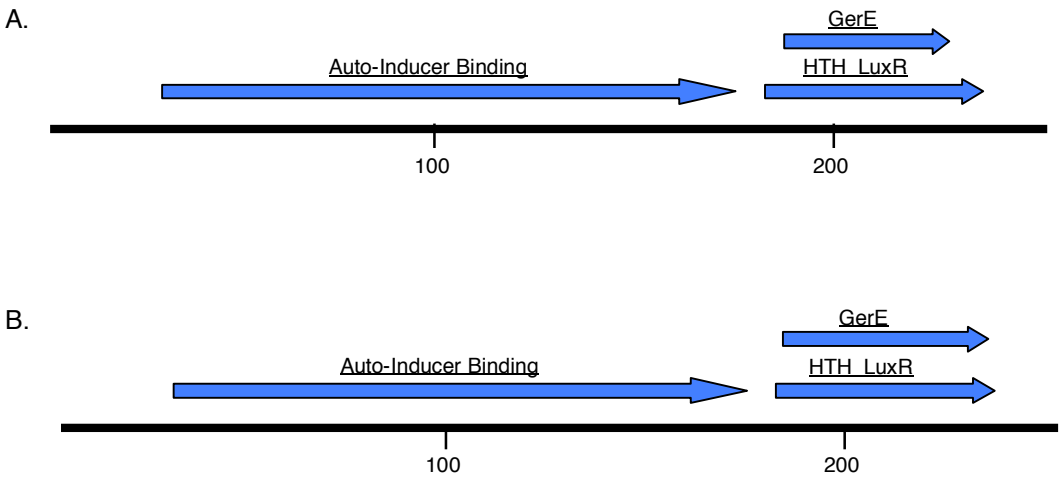


Fig. 1. Domains found in the LuxR-like proteins in the *B. melitensis* genome. Conserved domains found in proteins (A) BMEII 1116 VjbR and (B) BMEI 1758 BabR. The auto-inducer binding domain (pfam03472) binds autoinducer molecules. The GerE domain (pfam00196) and HTH_LuxR domain (smart00421) is conserved among LuxR proteins and represents the DNA binding domain.

but has conserved amino acid similarity to response receiver domains found in two-component response regulators, as illustrated in Fig. 2. While the activation domain of these genes is not usually associated with quorum sensing, LuxR proteins are members of the FixJ-NarL superfamily which is part of a larger protein family that includes two-component response regulators that contain response receiver domains found in these additional *luxR*-like genes (70). Comparison of the amino acid similarity between all seven *B. melitensis* LuxR-like proteins, TraR from *A. tumefaciens* and LasR from *P. aeruginosa* found that the similarities ranged from 27-57 % (Table 4), which was higher than expected since it was reported that LuxR proteins overall, from end to end, share 18-23 % amino acid similarity (45). For these reasons the seven *luxR*-like genes were considered for this study.

Gene replacement and deletion mutations were created for five of the seven *luxR*-like homologues in *B. melitensis* 16M. Figure 3 illustrates the genomic organization for gene locus BMEI 1582 in wild type and in the gene replacement mutant, where BMEI 1582 is replaced with a kanamyacin cassette, as well as restriction enzyme sites used for Southern blot verification of the mutations. PCR amplification and Southern Blot probing, as demonstrated in Fig. 4, confirmed two potential gene replacement mutants for BMEI 1582. For the PCR amplification, primers TAF579 and TAF579 were used and genomic DNA from wild type and the gene replacement mutants served as the templates.

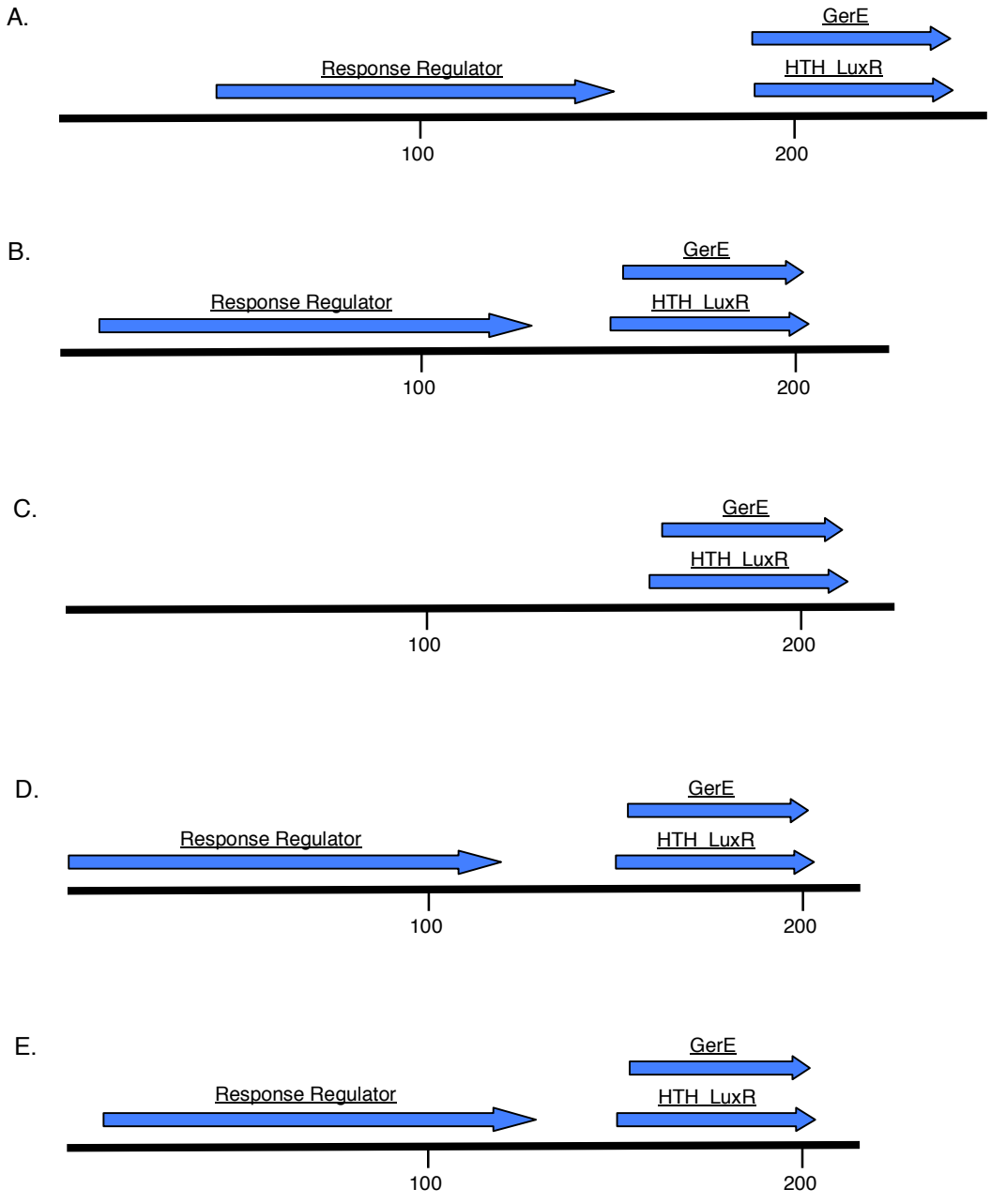


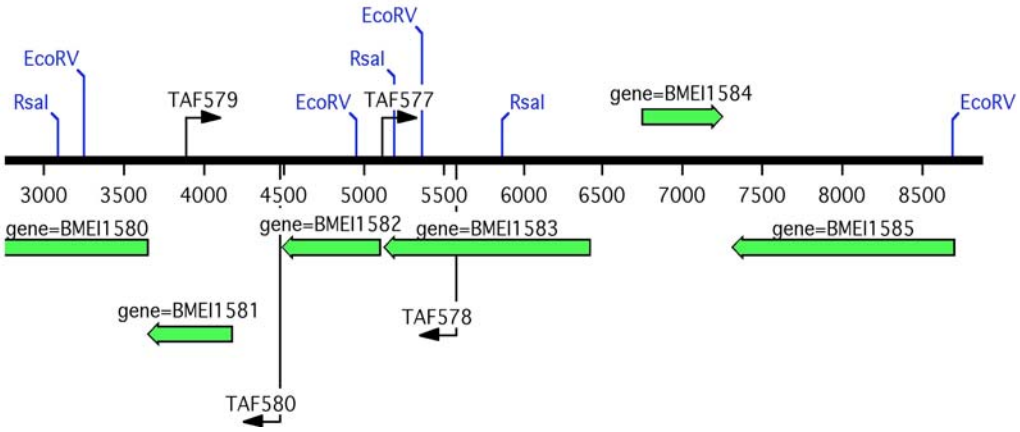
Fig. 2. Domains found in proteins containing the LuxR-like DNA binding domain in the *B. melitensis* genome. Conserved protein domains found in (A) BMEII 0853, (B) BMEI 1582, (C) BMEI 1751, (D) BMEI 1607 and (E) BMEII 0051 are demonstrated. The response regulator domain (pfam0072) is responsible for receiving the signal from the sensor partner in bacterial two-component systems. The GerE domain (pfam00196) and HTH_LuxR (smart00421) domain are conserved among LuxR proteins and represent the DNA binding domain.

TABLE 4. Amino acid similarities from ClustalW alignments of LuxR-like proteins.

BME	I 1582	I 1751	I 1758	II 0853	II 0051	I 1607	TraR	LasR
I 1116	30.1	32.2	37.7	34.9	27.2	28.8	32.7	39.5
I 1582	-	40.3	29.5	35.3	39.8	46.6	29	36.5
I 1751	-	-	34	57.3	38.1	41.2	32.4	34.1
I 1758	-	-	-	31.2	31.7	30.6	33.7	42.7
II 0853	-	-	-	-	38	36.5	30.1	31.8
II 0051	-	-	-	-	-	45.3	33.1	29.3
I 1607	-	-	-	-	-	-	32.4	31.4
TraR	-	-	-	-	-	-	-	40.7

BME numbers represent the locus designation of the LuxR-like genes in *B. melitensis*. TraR is a LuxR homologue from *A. tumefaciens* and LasR is a LuxR homologue from *P. aeruginosa*.

A.



B.

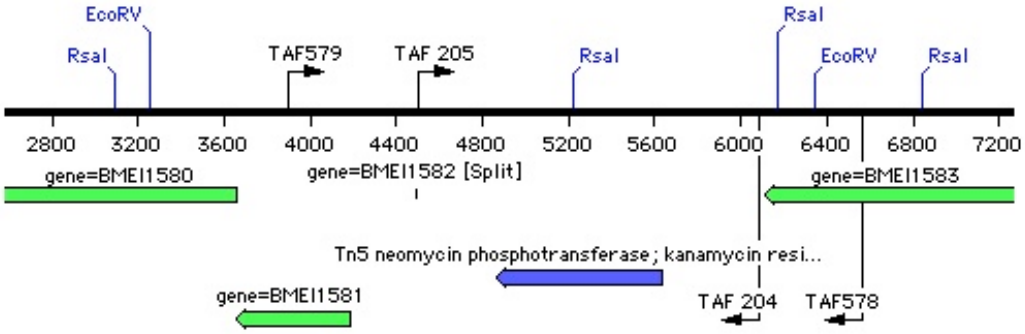


Fig. 3. Genomic organization of *B. melitensis* gene locus BMEI 1582. (A) 16M wild type genomic organization of BMEI 1582, showing primers used to amplify the flanking regions and restriction enzyme sites used for Southern blot verification. (B) Genome map of 16M BMEI 1582::Km gene replacement mutation; showing primers TAF204 and TAF205, which are part of the kanamycin cassette used for the gene replacement mutation. Restriction enzyme sites shown were used for Southern blot verification.

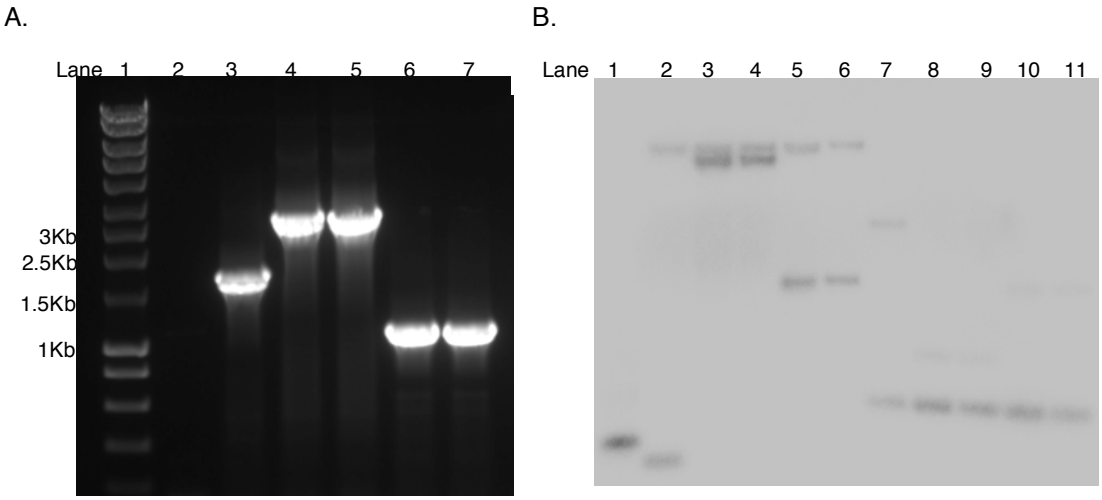


FIG. 4. PCR and Southern blot verification of gene replacement and deletion mutations of gene locus BMEI 1582. (A) PCR amplification products electrophoresed on a 0.8 % agarose gel using genomic DNA as the template and primer TAF579 and TAF578. Lanes: 1, Hyperladder I (Bioline) molecular weight marker; 2, PCR, no template; 3, 16M wild type (1697 bp); 4, 16M I1582::Kan clone A (2674 bp) ; 5, 16M I1582::Kan clone B (2674 bp); 6, 16MΔI1582 clone A (1073 bp); 7, 16MΔI1582 clone B (1073 bp). (B) Southern Blot verification of gene replacement and gene deletion mutations using P^{32} labeled 5' PCR product, forward primer TAF577 and reverse primer TAF578, as the probe. Lanes: 1, 5'1582 PCR Product (471 bp); 2, *Eco*RV digested 16M (3320 and 410 bp); 3, *Eco*RV digested 16M I1582::Kan clone A (3320 and 3084 bp); 4, *Eco*RV digested 16M I1582::Kan clone B (3320 and 3084 bp); 5, *Eco*RV digested 16MΔI1582 clone A (3320 and 1483 bp); 6, *Eco*RV digested 16MΔI1582 clone B (3320 and 1483 bp); 7, *Rsa*I digested 16M wild type (2100 and 670 bp); 8, *Rsa*I digested 16M I1582::Kan clone A (950 and 670 bp); 9, *Rsa*I digested 16M I1582::Kan clone B (950 and 670 bp); 10, *Rsa*I digested 16MΔI1582 clone A (1476 and 670 bp); 11, *Rsa*I digested 16MΔI1582 clone B (1476 and 670 bp).

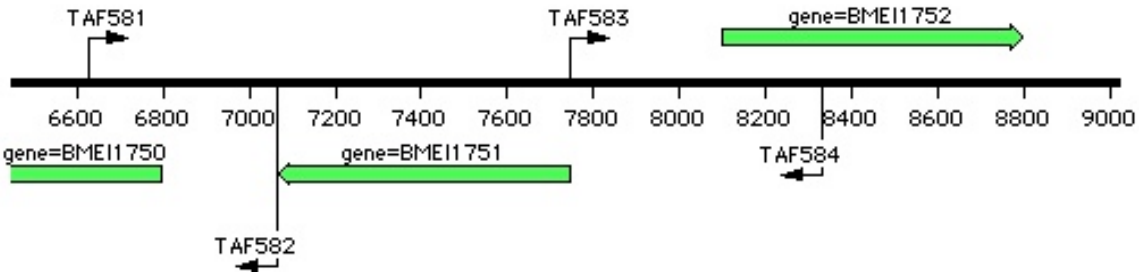
A PCR amplification product of 1697 bp was predicted and obtained from the wild type organism and a PCR product of 2674 bp from the potential gene replacement mutants was predicted and obtained (Fig. 4A, lanes 3, 4 and 5), confirming the mutation. For the Southern blot analysis, genomic DNA from wild type and the two potential gene replacement mutants were digested with *Eco*RV or *Rsa*I and probed with a ^{32}P labeled DNA fragment upstream of gene locus BMEI 1582. The labeled probe was predicted to bind to DNA fragments from the wild type organism of 3320 bp and 410 bp for the *Eco*RV digest and 2100 bp and 670 bp for the *Rsa*I digest. The labeled probe bound the predicted DNA fragments for wild type organism for both the *Eco*RV digest (Fig. 4B, lane 2) and for the *Rsa*I digest (Fig. 4B, lane 7). For the gene replacement mutations, the labeled probe was predicted to bind DNA fragments of 3320 bp and 3084 bp for the *Eco*RV digest and 950 bp and 670 bp for the *Rsa*I digested fragments. As expected, the labeled probe bound DNA fragments of the expected size for both restriction enzyme digests (Fig. 4B, lanes 3 and 4 for the *Eco*RV digest and lanes 8 and 9 for the *Rsa*I digest), confirming the two gene replacement mutants.

A gene deletion mutation was generated from the gene replacement mutant, and two potential gene deletion mutants were screened and verified by PCR and Southern blot. For the PCR verification, genomic DNA from the potential gene deletion mutants and wild type organism was amplified with

primers TAF579 and TAF578. Amplification products of 1073 bp were predicted and obtained, verifying that both potential mutants contained the correct deletion (Fig. 4A, lanes 6 and 7). For the Southern Blot verification, the labeled probe was predicted to bind DNA fragments of 3320 bp and 1483 bp for *Eco*RV digests and 1476 bp and 670 bp from the *Rsa*I digests of the genomic DNA from the potential gene deletion mutants. The probe was bound to the predicted DNA fragments, demonstrated in Fig. 4B, lanes 5 and 6 for the *Eco*RV digest and Lanes 10 and 11 for the *Rsa*I digested genomic DNA, verifying the gene deletion mutations.

The genomic organization for gene locus BMEI 1751 in *B. melitensis* 16M (wild type) and the gene replacement mutant is illustrated in Fig. 5, where BMEI 1751 is replaced with a kanamyacin cassette by allelic exchange. PCR amplification verified gene replacement and gene deletion mutations of BMEI 1751, shown in Fig. 6. PCR amplification using primers TAF581 and TAF584 were expected to produce a product of 1706 bp for the wild type organism and 2632 bp fragment from the gene replacement mutant. Amplification of the wild type organism produced a product the predicted size (Fig. 6, lane 3). Gene replacement mutants A and B do not appear to contain the intended mutation, as the PCR amplification product was the same size as wild type, suggesting these two mutants were spontaneously resistant for kanamycin or the allelic exchange occurred elsewhere in the genome (Fig. 6, lanes 4 and 5). The gene

A.



B.

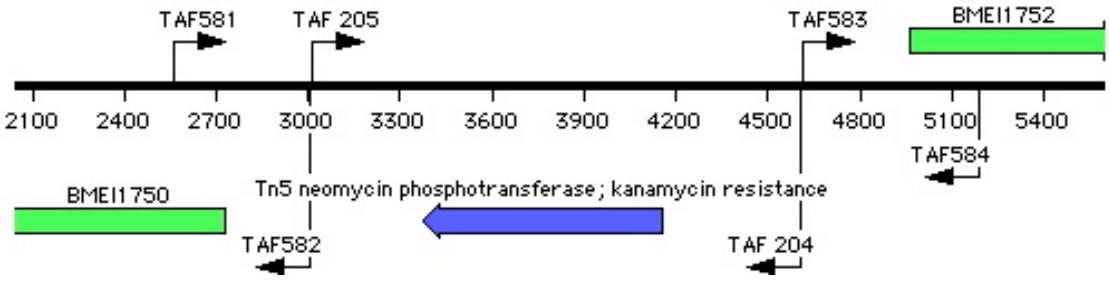


Fig. 5. Genomic organization of *B. melitensis* gene locus BMEI 1751. (A) 16M wild type genomic organization of BMEI 1751, showing primers used to amplify the flanking regions. (B) Genome map of 16M I1751::Km gene replacement mutation, showing primers TAF204 and TAF205, which are part of the kanamycin cassette used for the gene replacement mutation.

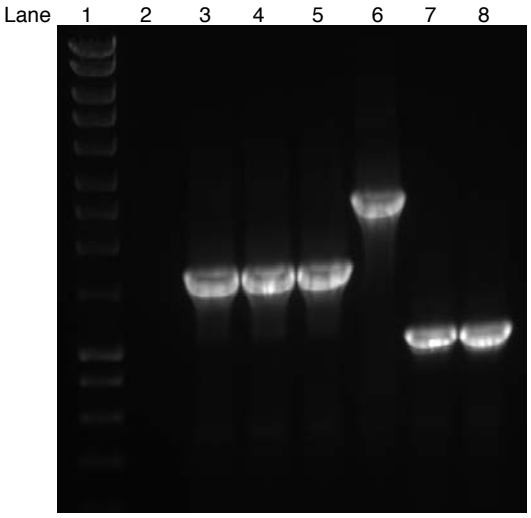
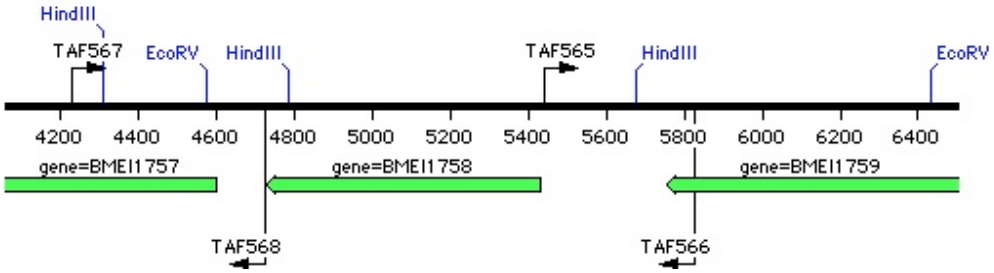


Fig. 6. PCR verification of gene replacement and deletion mutations of gene locus BMEI 1751. PCR amplification products electrophoresed on a 0.8 % agarose gel using genomic DNA as the template and primers TAF581 and TAF584. Lanes: 1, HyperladderI (Bioline); 2, PCR without template; 3, 16M wild type (1706 bp); 4, 16M I1751::Km clone A (1706 bp); 5, 16M I1751::Km clone B (1706 bp); 6, 16M 1751::Km clone C (2632 bp); 7, 16MΔI1751 clone A (1031 bp); 8, 16MΔI1751 clone B (1031 bp).

replacement mutant C appears to contain the correct gene replacement mutation and a PCR amplification of the predicted 2632 bp was obtained (Fig. 6, lane 6) and was subsequently used for the production of the gene deletion mutants. Two potential gene deletion mutants were screened by PCR amplification. Both of these mutants contained the correct deletion, with the PCR amplification products matching the predicted 1031 bp, also shown in Fig. 6, lanes 7 and 8.

The genomic organization for *B. melitensis* BMEI 1758 wild type and a gene replacement mutant is illustrated in Fig. 7, where gene locus BMEI 1758 is replaced with a kanamyacin cassette by allelic exchange. Fig. 8 demonstrates the PCR and Southern blot verification for the potential gene replacement and gene deletion mutations. For the verification of the gene replacement mutant, PCR amplification using primers TAF567 and TAF566 was predicted to produce DNA fragments of 1601 bp for the wild type organism and 2489 bp for the gene replacement mutants. The correct PCR products were obtained, shown in Fig. 8A, lane 4 for the wild type organism and lane 5 for the gene replacement mutant. This mutation was also verified by Southern blot, probing with a ^{32}P labeled DNA fragment located downstream of gene locus BMEI 1758. Genomic DNA from the wild type organism and the potential gene replacement mutants were digested with *Eco*RV or *Hind*III. The probe was predicted to bind to DNA fragments from the wild type organism of 2310 bp and 1856 bp from the *Eco*RV digest and 5387 bp and 474 bp from the *Hind*III digested DNA. The probe

A.



B.

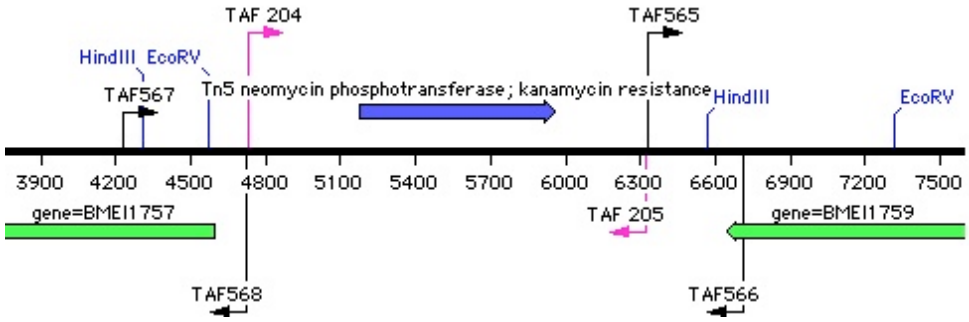


Fig. 7. Genomic organization of *B. melitensis* gene locus BMEI 1758. (A) 16M wild type genomic organization of BMEI 1758, showing primers used to amplify the flanking regions and restriction enzyme sites used for Southern blot verification. (B) Genome map of 16M BMEI 1758::Km gene replacement mutation, showing primers TAF204 and TAF205, which are part of the kanamycin cassette used for the gene replacement mutation. Restriction enzyme sites shown were used for Southern blot verification.

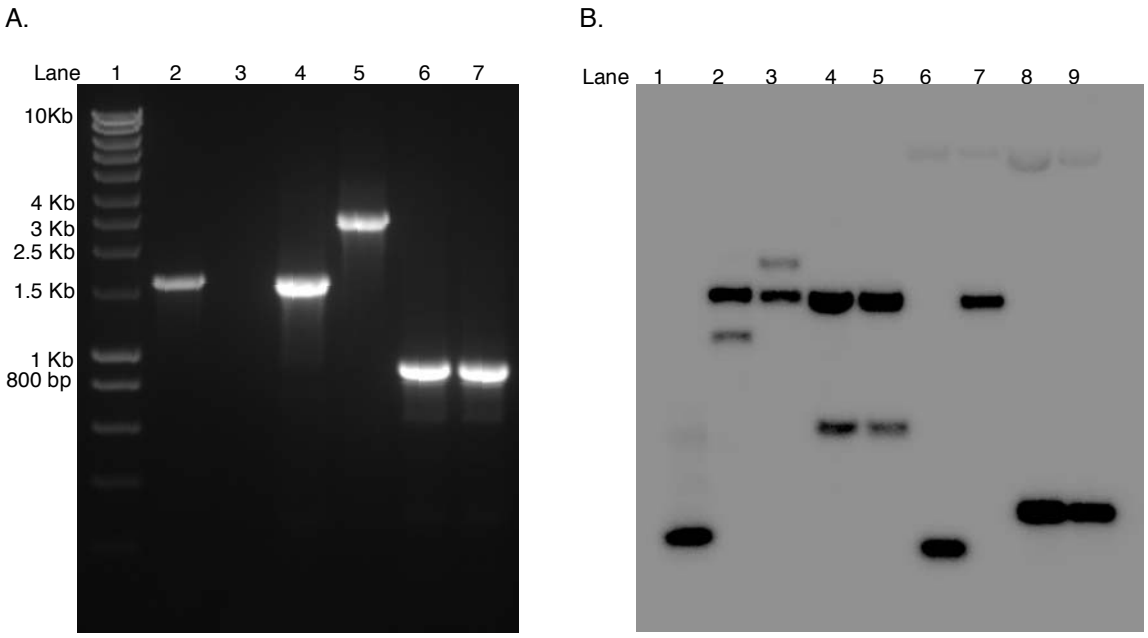


Fig. 8. PCR and Southern blot verification of gene replacement and deletion mutations of gene locus BMEI 1758. (A) PCR amplification products electrophoresed on a 0.8 % agarose gel using genomic DNA as the template and primers TAF567 and TAF566. Lanes: 1, HyperladderI (Bioline); 2, Kanamycin cassette PCR with forward primer TAF204 and reverse primer TAF205 (1600 bp); 3, PCR without template; 4, 16M wild type (1601 bp); 5, 16M I1758::Km (2489 bp); 6, 16MΔI1758 clone A (891 bp); 7, 16MΔI1758 clone B (891 bp). (B) Southern Blot verification of gene replacement and deletion mutants using P^{32} labeled 3' PCR product, forward primer TAF567 and reverse primer TAF568, as the probe. Lanes: 1, 3' PCR product (502bp); 2, *Eco*RV digested 16M wild type (2310 and 1856 bp); 3, *Eco*RV digested 16M I1758::Km (2744 and 2310 bp); 4, *Eco*RV digested 16M ΔI1758 clone A (2310 and 1143 bp); 5, *Eco*RV digested 16M ΔI1758 clone B (2310 and 1143 bp); 6, *Hind*III digested 16M wild type (5387 and 474 bp); 7, *Hind*III digested 16M I1758::Km (5387 and 2256 bp); 8, *Hind*III digested 16MΔI1758 clone A (5387 and 655 bp); 9, *Hind*III digested 16ΔI1758 clone B (5387 and 655 bp).

bound to the predicted DNA fragment sizes, demonstrated in Fig. 8B, lanes 2 and 6. For the gene replacement mutant, genomic DNA was also digested with *Eco*RV or *Hind*III, and the probe was predicted to bind DNA fragments of 2744 and 2310 bp for the *Eco*RV digest and DNA fragments of 5387 and 2256 bp for the *Hind*III digest. The labeled probe was found to bind DNA fragments corresponding to the predicted sizes, shown in Fig. 8, lanes 3 and 7. The probe was found to have a very low binding affinity for the 5387 bp DNA fragment from the *Hind*III digest. The restriction enzyme site is close to the end of the labeled region, and it is likely there were not enough nucleotides present in the digested fragment to interact and form an association with the probe that was strong enough to withstand the washing procedure to remove background signal.

A deletion mutation was produced from the gene replacement mutant and verified by PCR amplification and Southern blot. Two potential gene deletion mutants were examined for verification. For the PCR amplification, products were predicted to be 891 bp and were obtained from both potential mutants (Fig. 8A, lanes 6 and 7). The mutations were also verified by Southern blot analysis, probed with a labeled DNA fragment from the downstream region of BMEI 1758. Genomic DNA was digested with either the restriction enzyme *Eco*RV or *Hind*III. The probed was predicted to bind DNA fragments of 2310 bp and 1143 bp for the *Eco*RV digest and fragments of sizes 5387 bp and 655 bp for the *Hind*III digest. The probe was found to bind the correct sizes of DNA fragments, shown

in Fig. 8B, lanes 4 and 5 for the *Eco*RV digest and lanes 8 and 9 for the *Hind*III digest, verifying the gene deletion mutation.

The genomic organization for *B. melitensis* gene loci BMEII 1116 in the wild type and the transposon mutant is illustrated in Fig. 9. Southern blot and PCR amplification were used to verify both the transposon and gene deletion mutations, demonstrated in Fig. 10. The PCR amplification product from the transposon mutant was found to be 4486 bp and the wild type product was 2150 bp, as predicted (Fig. 10A, lane 6 and lane 5, respectively). For additional confirmation, a Southern blot using a ^{32}P labeled probe from the downstream region of the gene locus BMEII 1116, with genomic DNA from the wild type and transposon mutant digested with *Hind*III or *Rsa*I. The probe was predicted to bind DNA fragments of 1107 bp for the *Hind*III digest and 743 bp for the *Rsa*I digest, shown in Fig. 10B, lanes 2 and 3 (respectively) for the wild type organism. For the transposon mutant, the labeled probe was predicted to bind DNA fragments of 1107 bp for the *Hind*III digested genomic DNA and 743 bp for the *Rsa*I digested DNA. The results are shown in Fig. 10, lanes 4 and 5, confirming the insertion of the transposon were predicted.

Two gene deletion mutants were selected for PCR amplification to verify the deletion of both the transposon and the gene locus BMEII 1116. The amplification product of the predicted 1350 bp was obtained (Fig. 10A, lane 2

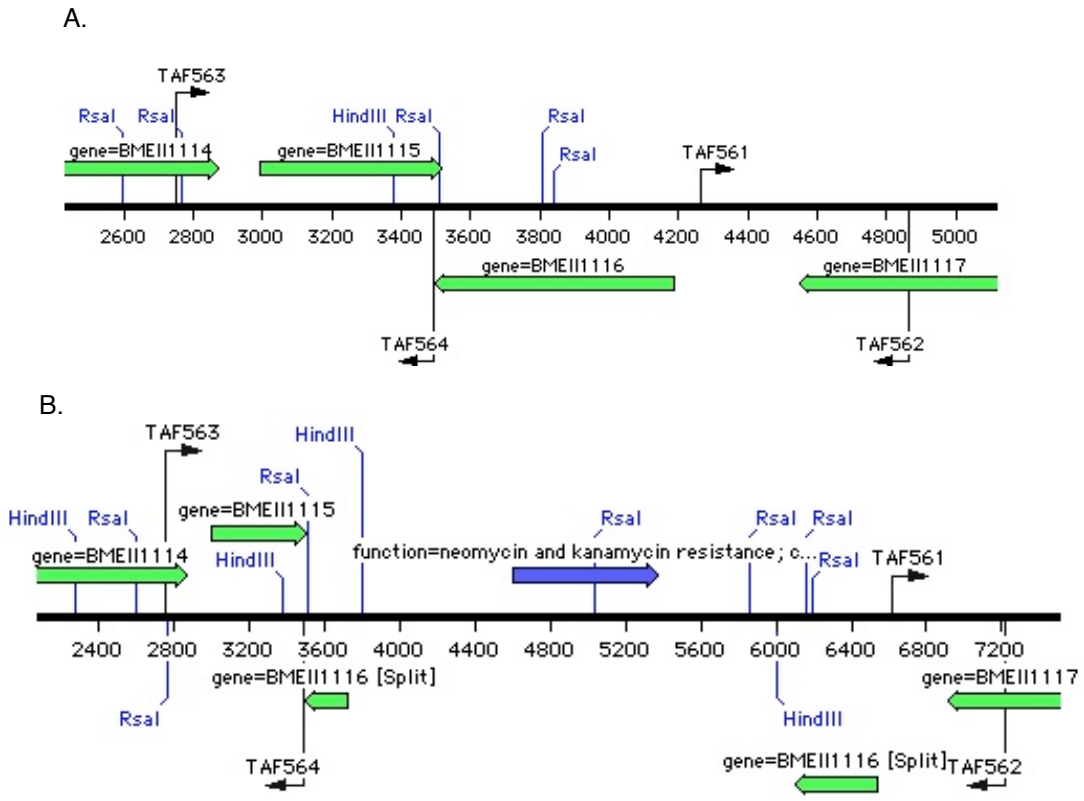


Fig. 9. Genomic organization of *B. melitensis* gene locus BMEII 1116. (A) 16M wild type genomic organization of BMEII 1116, showing primers used to amplify the flanking regions and restriction enzyme sites used for Southern blot verification. (B) Genome map of 16M II1116::tn5Km transposon mutant used for the gene deletion mutation. Restriction enzyme sites shown were used for Southern blot verification.

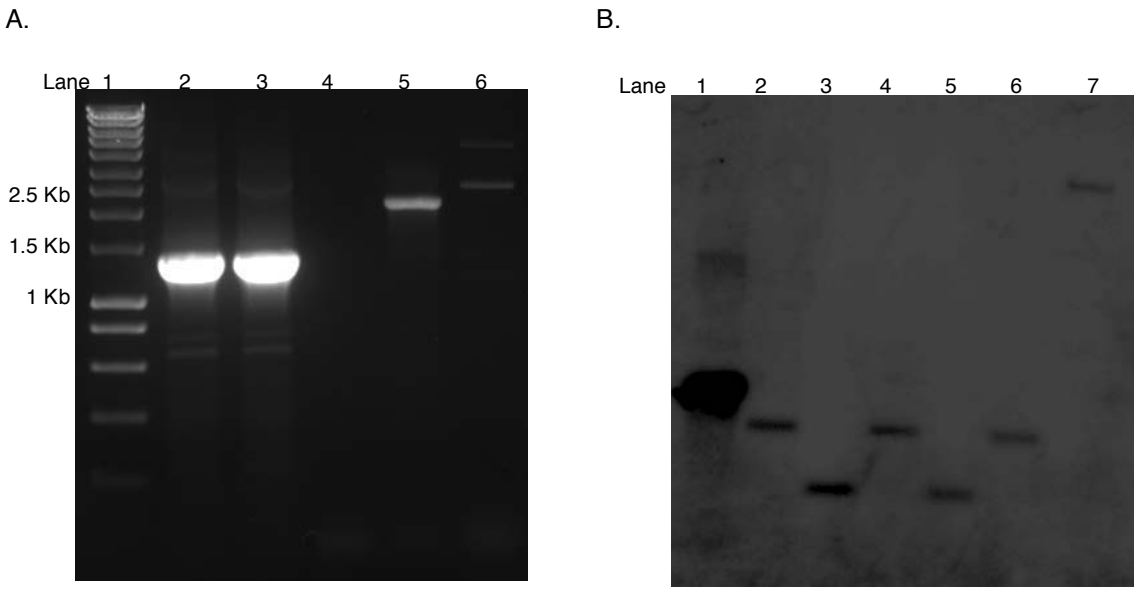


Fig. 10. PCR and Southern blot verification of transposon and gene deletion mutations of gene locus BMEII 1116. (A) PCR amplification products electrophoresed on a 0.8 % agarose gel using genomic DNA as the template and primers TAF563 and TAF562. Lanes: 1, HyperladderI (Bioline); 2, 16MΔII1116 clone A (1350 bp); 3, 16MΔII1116 clone B (1350 bp); 4, PCR without template; 5, 16M wild type (2150 bp); 6, 16M II1116::tn5Km (4486 bp). (B) Southern blot verification of transposon and deletion mutations using P^{32} labeled 3' PCR product, forward primer TAF563 and reverse primer TAF564, as the probe. Lanes: 1, PCR product, using forward primer TAF563 and reverse primerTAF562 (1354 bp); 2, *Hind*III digested 16M wild type (1107 bp); 3, *Rsa*I digested 16M wild type (743 bp); 4, *Hind*III 16M II1116::tn5Km (1107 bp); 5, *Rsa*I digested 16M II1116::tn5Km (743 bp); 6, *Hind*III digested 16MΔII1116 (1107 bp); 7, *Rsa*I digested 16MΔII1116 (4376 bp).

and 3), confirming the loss of the transposon and gene locus BMEII 1116. To further verify the mutation, a Southern blot with genomic DNA from the potential mutants was digest with *Rsa*I and *Hind*III, and probed with a labeled DNA fragment downstream of BMEII 1116. For the *Hind*III digest, the labeled probe was predicted to bind to a DNA fragment of 1107 bp. For the *Rsa*I digest, the probe was predicted to bind a DNA fragment corresponding to 4376 bp. The Southern blot confirmed the deletion, with the labeled probe binding DNA fragments of the predicted sizes (Fig. 10B, lanes 5 and 6).

To provide further confirmation the BMEII 1116 gene deletion mutant contains the correct mutation for downstream applications, the PCR product obtained using primers TAF562 and TAF563 (Table 2) using the deletion mutant genomic DNA and wild type as the template were sequenced. The sequence data was aligned to the published *B. melitensis* genome 16M (GenBank database, accession nos. AE008917 and AE008918) and is shown in Fig. 11, providing additional confirmation that 16MΔII1116 contains the anticipated deletion mutation (28).

The genomic organization for *B. melitensis* 16M BMEII 0853 in 16M wild type and in the gene replacement mutant, where BMEII 0853 is replaced with a kanamyacin cassette, is shown in Fig. 12. PCR and Southern blots confirmed the two gene replacement mutations, shown in Fig. 13. PCR amplification using

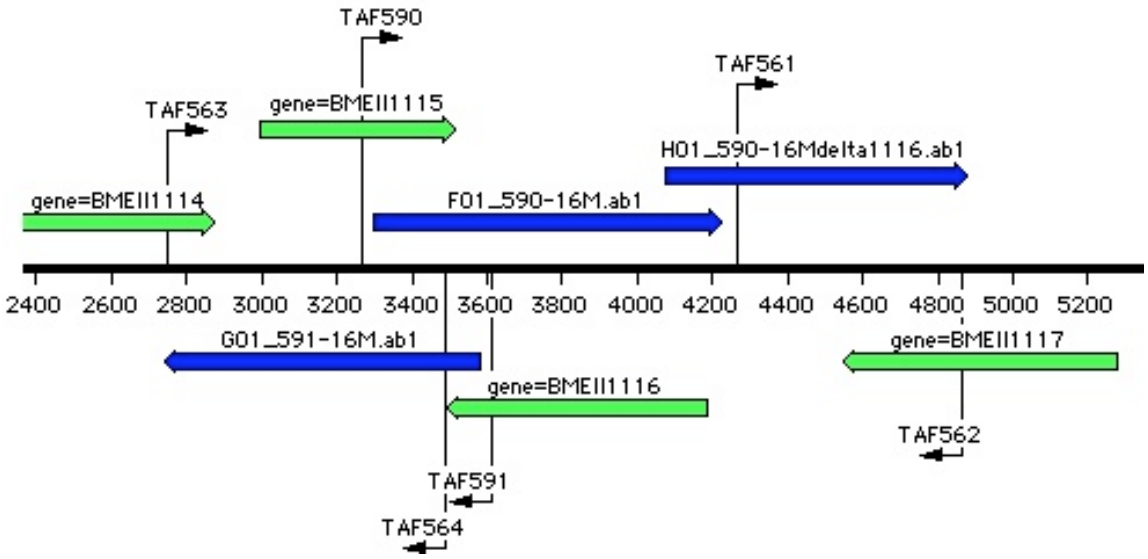


Fig. 11. Sequencing alignment of *B. melitensis* 16M wild type and 16M Δ1116 mutant. Both strains were sequenced using primers TAF590 and TAF591. The sequencing of 16M Δ1116 with TAF591 is a negative control because the primer binds within the deleted region; thus no sequence was obtained. ABI sequence files were aligned to the *B. melitensis* wild type genomic sequence using MacVector 8.1.2 (MacVector) and are shown in blue. G01_591-16M and F01_590-16M are the sequence alignments for 16M wild type using primers TAF591 and TAF590 (respectively). H01_590-16Mdelta1116 is the sequence alignment from the 16M Δ1116 mutant sequenced with primer TAF590.

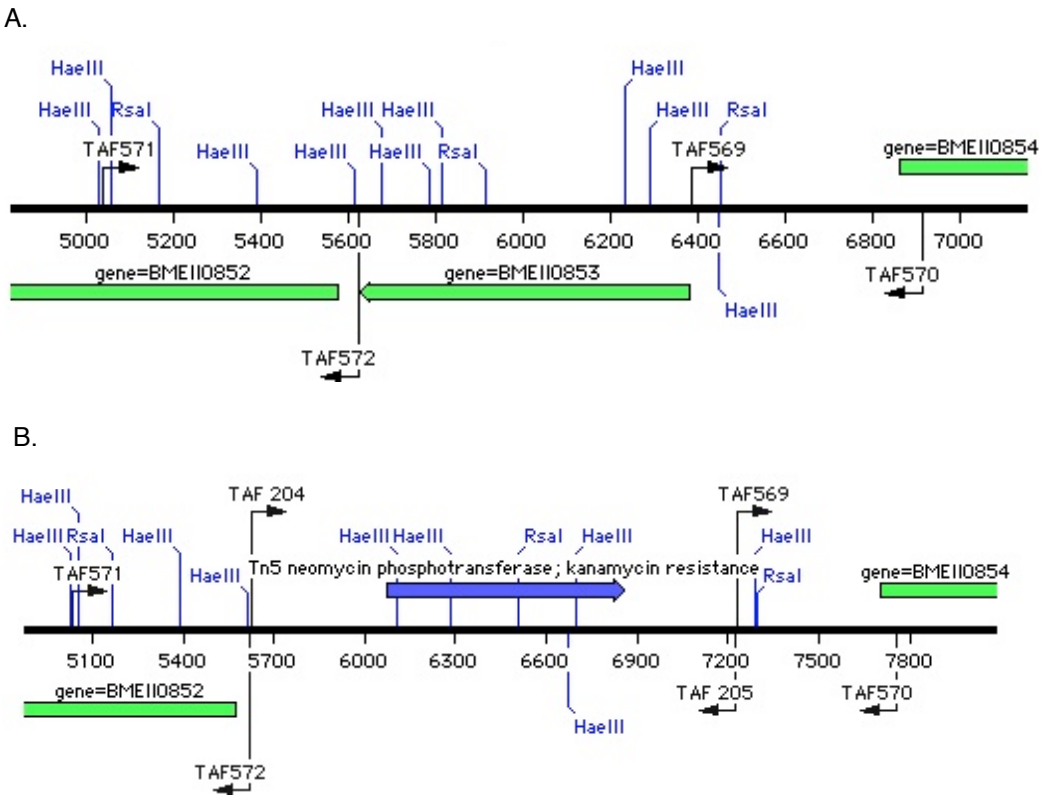


Fig. 12. Genomic organization of *B. melitensis* gene locus BMEII 0853. (A) 16M wild type genomic organization of BMEII 0853, showing primers used to amplify the flanking regions and restriction enzyme sites used for Southern blot. (B) Genome map of 16M BMEII 0853::Km gene replacement mutation, showing primers TAF204 and TAF205, which are part of the kanamycin cassette used for the gene replacement. Restriction enzyme sites are also shown were used for Southern blot verification.

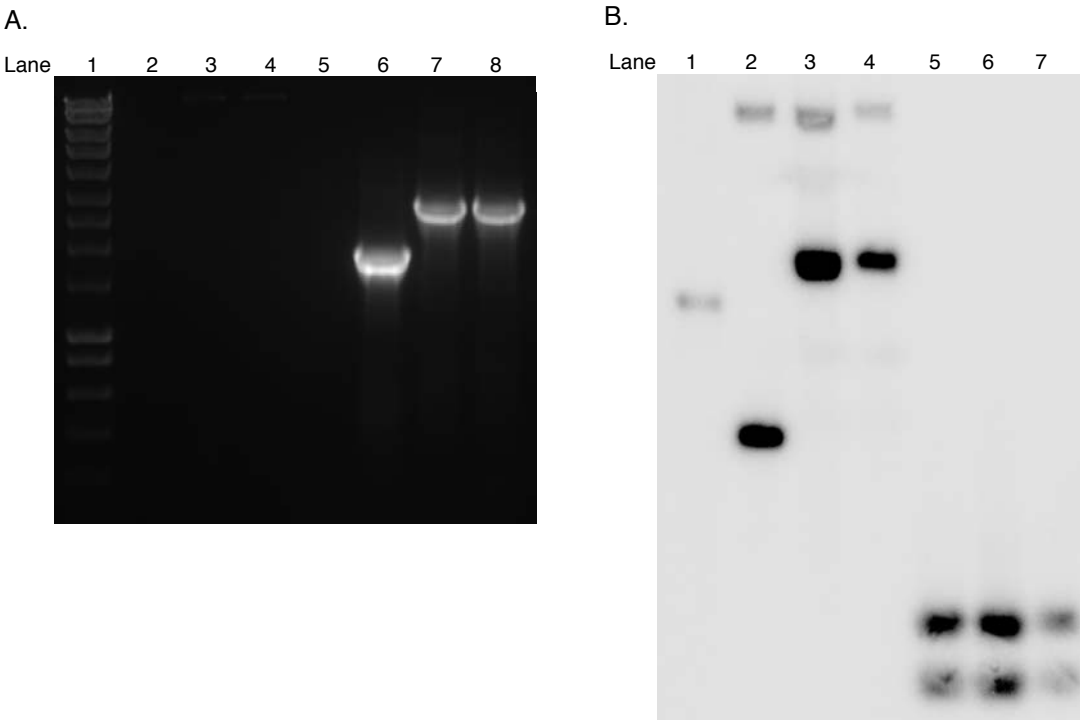


Fig. 13. PCR and Southern blot verification of the gene replacement mutation of gene locus BMEII 0853. (A) PCR amplification products electrophoresed on a 0.8 % agarose gel using genomic DNA as the template and primers TAF571 and TAF570. Lanes: 1, HyperladderI (Bioline); 2-5, blank; 6, 16M wild type (1880 bp); 7, 16M II0853::Km clone A (2723 bp); 8, 16M II0853::Km clone B (2723 bp). (B) Southern blot verification of gene replacement mutation using P^{32} labeled 3' PCR product, forward primer TAF571 and reverse primer TAF57, as the probe. Lanes: 1, PCR product amplified with forward primer TAF571 and reverse primer TAF570 (1125 bp); 2, *Rsa*I digest 16M wild type, (2153 and 749 bp); 3, *Rsa*I digest 16M II0853::Km clone A (2153 and 1339 bp); 4, *Rsa*I digest 16M II0853::Km clone B (2153 and 1339 bp); 5, *Hae*III digest 16M wild type (333 and 222 bp); 6, *Hae*III digest 16M II0853::Km clone A (333 and 222 bp); 7, *Hae*III digest 16M II0853::Km clone B (333 and 222 bp).

primers TAF570 and TAF571 was predicted to produce a product of 1880 bp for wild type and 2723 bp for the gene replacement mutation. The PCR products obtained were of the predicted sizes for both wild type and the gene replacement mutants (Fig. 13A, lanes 6, 7 and 8). To further confirm the gene replacement mutations, a Southern blot with a ^{32}P labeled probe from the downstream region of BMEII 0853 was used to verify binding to DNA fragments of the predicted sizes. Genomic DNA from the wild type and gene replacement mutants was digested with the restriction enzymes *Rsa*I or *Hae*III. The probe was predicted to bind wild type *Rsa*I digested DNA fragments of 2153 bp and 749 bp and was confirmed to do so (Fig. 13B, lane 2). The probe was predicted to bind DNA fragments of 333 bp and 222 bp from wild type DNA digested by *Hae*III and was confirmed to bind the predicted DNA fragments (Fig. 13B, lane 5). For the two gene deletion mutants that were being examined, the labeled probe was predicted to bind DNA fragments corresponding to 2153 bp and 1339 bp for the *Rsa*I digest and DNA fragments of 333 bp and 222 bp for the *Hae*III digest. The Southern blot confirmed the probe was bound to DNA fragments of the predicted sizes for both of the mutants being examined (Fig. 13B, lanes 3, 4, 6 and 7).

Numerous attempts to delete the kanamycin gene and produce a gene deletion mutation for BMEII 0853 were unsuccessful. Many of the gene deletion candidates screened were sucrose resistant and antibiotic sensitive for both kanamycin and carbencillin, however, the PCR amplification did not produce

products of the predicted size (data not shown), suggesting that the homologous recombination was not recombining in the desired location.

Attenuation for the gene replacement and gene deletion mutants were measured in a gentamycin protection assay with J774A.1 macrophage-like cells. The results of bacterial CFU recovery from infected macrophages are shown in Fig. 14. Four out of the five LuxR homologues examined do not appear to be required for replication or survival in the macrophage cell. As previously reported, mutations in BMEII 1116, *vjbR*, were found to be highly attenuated in this model, recovering 1.5 logs fewer bacteria than wild type from the infected macrophages 48 hours after infection (25). This result is similar to the transposon mutant 19C6, identified in a bank of random transposon mutants conducted in this laboratory and reported elsewhere (26).

To confirm that the attenuation observed is due to mutation of Δ*vjbR*, the deletion mutant was complemented with VjbR expressed in trans. Survival of the VjbR complemented strain was restored, only slightly more attenuated when compared to wild type strain. Although expression of VjbR in trans did not completely restore survival to wild type levels, the defect was reduced when compared to the deletion and transposon mutant and could be due to a problem resulting from gene copy number or regulation problems due to gene expression in trans. Additionally, the results shown here confirm previous findings that

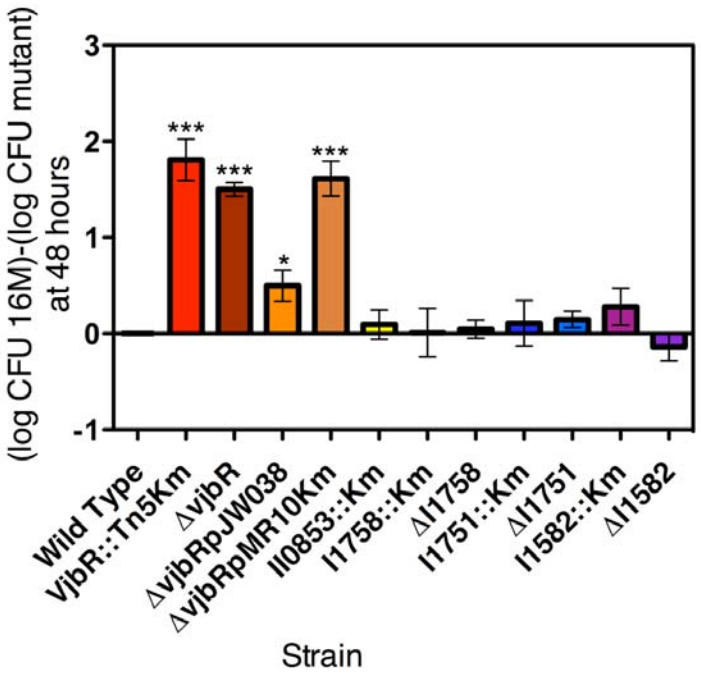


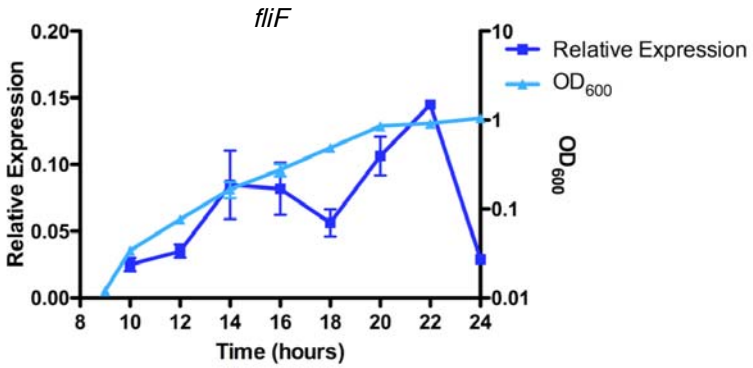
Fig. 14. Attenuation of *luxR*-like gene replacement and deletion mutants in J774A.1 murine macrophage-like cells. The attenuation was measured as the log difference between the CFU recovery of the mutant from the CFU of wild type recovered from infected macrophages at 48 hours post infection. Shown are the averaged CFU recovery from at least 3 experiments, with each sample performed in triplicate. The error bars represent the SEM. Mutants were compared to wild type using a Student's two tailed *t*-test, the resulting *p* values are as follows: *, $P<.0.05$; ***, $P<0.001$. The strains are as follows, in order left to right: wild type, *B. melitensis* 16M wild type; VjbR::tn5Km, 16M II1116::tn5Km transposon mutant; ΔvjbR, 16MΔII1116 deletion mutant; ΔvjbRpJW038,16M ΔII1116 complemented mutant; ΔvjbRpMR10Km, 16M ΔII1116 with empty cloning vector; II0853::Km, 16M II0853::Km gene replacement mutant; I1758::Km, 16M I1758::Km gene replacement mutant; Δ1758, 16M ΔI1758 gene deletion mutant; I1751::Km, 16M I1751::Km gene replacement mutant; ΔI1751, 16M ΔI1751 gene deletion mutant; I1582::Km, 16M I1582::Km gene replacement mutant; ΔI1582, 16M ΔI1582 gene deletion mutant.

interruption or deletion of BMEI1758 does not significantly attenuate *B. melitensis* intracellular survival in the J774A.1 macrophage-like model (79).

Microarray analysis reveals quorum sensing is a global regulator of gene expression and is dependent on the condition and growth phase examined. To identify genes regulated by the quorum sensing factors VjbR and C_{12}-HSL, microarray analysis was used to compare transcripts in wild-type *B. melitensis* 16M with transcripts in either an isogenic Δ*vjbR* deletion mutant or wild-type 16M supplemented with exogenous C_{12}-HSL at the time of inoculation. Timepoints for isolating RNA were selected based on observed increases in the expression of *fliF* and *virB*, two genes previously found to be regulated by VjbR. *FliF* and *virB2* gene expression was examined over time using quantitative real time PCR and is demonstrated in Fig. 15. *FliF* and *virB* expression were found to increase at different stages of growth. *FliF* expression was found to increase during the beginning of the exponential growth phase (OD_{600}=0.40) and *virB* expression increased later, at the early stages of the stationary growth phase (OD_{600}=1.5). Total RNA was collected from three independent cultures for each experiment and are referred to as biological replicates.

Identification of significantly altered transcripts was obtained using two different approaches to analyze the microarrays, as described in the Material and Methods section. Scatter plot analysis of biological replicate samples were

A.



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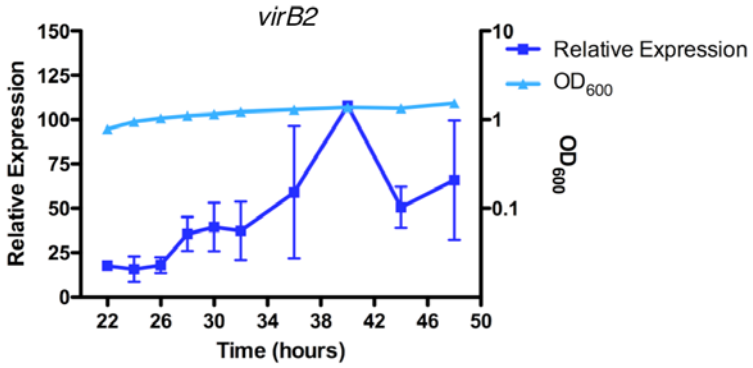
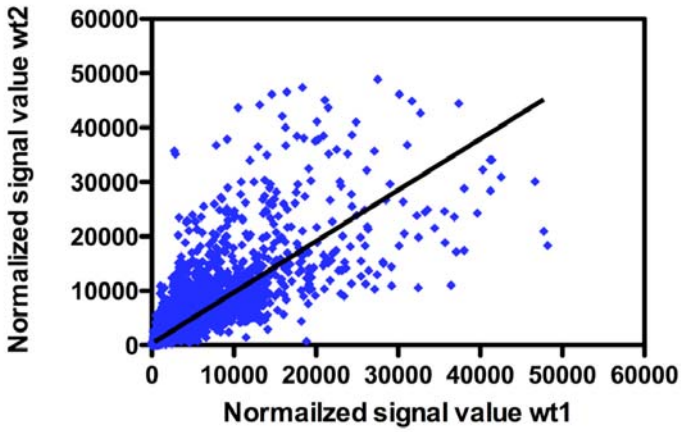


Fig. 15. Quantitative real-time PCR of *fliF* and *virB2* transcript expression over time. Expression units are calculated using the $\Delta\Delta C_t$ calculation and are normalized to 16S ribosomal RNA. (A) Expression of *fliF* over time and (B) expression of *virB2* over time in wild type 16M. The averaged values are from at least two independent samples and the error bars represent the SEM.

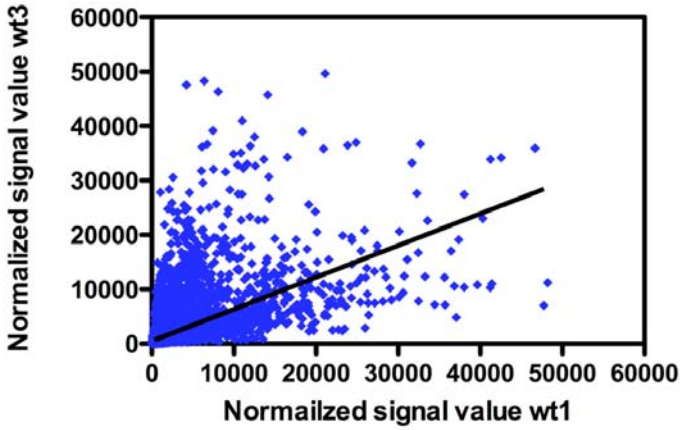
completed to compare the variability among the samples, using normalized signals values fitted with a trend line and are shown in Fig. 16 for the exponential growth phase wild type samples and Fig. 17 for the stationary growth phase wild type samples.

At the exponential growth phase time point, it was noted that the retained gene list resulting from the use of all three wild type replicates did not include *fliF*, a target gene known to be regulated by VjbR and was the basis for this time point analysis (25). Further examination of the raw signal data revealed several differences in wild type replicate sample number 3. First, it was noted that this particular microarray contained a high number of flagged genes, 100 total compared to 40 and 27 flags for wild type biological replicates 1 and 2 (respectively). The higher incident of flags suggests that this microarray had a technical issue not observed with the other 2 microarrays and likely had a higher level of background or interference. Next, scatter plots with the biological replicates using normalized signal values and fitted with a trend line were compared and shown in Fig. 16. Microarrays compared to the wild type replicate number 3 produced low R^2 values, 0.34 when compared to wild type replicate 1 and 0.39 when compared to wild type replicate 2. For comparison, wild type replicates 1 and 2 were found to have a R^2 value of 0.66. The analysis at the exponential growth phase was modified to exclude wild type replicate 3 as described in the materials and methods section of this paper because of the lack

A.



B.



C.

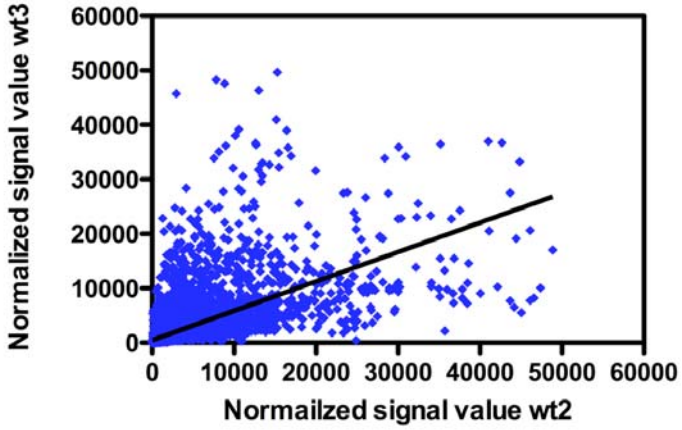


Fig. 16. Scatter plots of microarray normalized signal values fitted with a trend line for the wild type biological replicates at the exponential growth phase. (A) Biological replicates 1 and 2 with an R^2 value of 0.6510, (B) biological replicates 1 and 3 with an R^2 value of 0.3409 and (C) biological replicates 2 and 3 with an R^2 value of 0.3936.

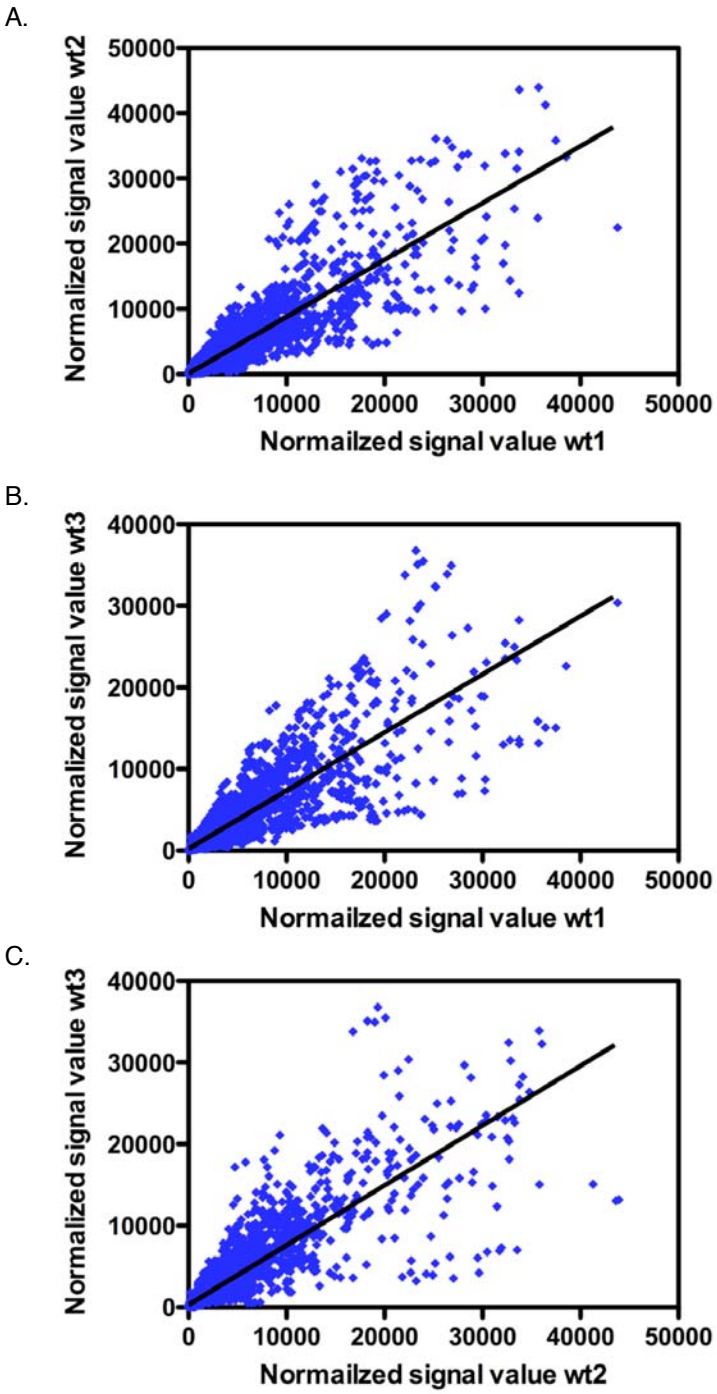
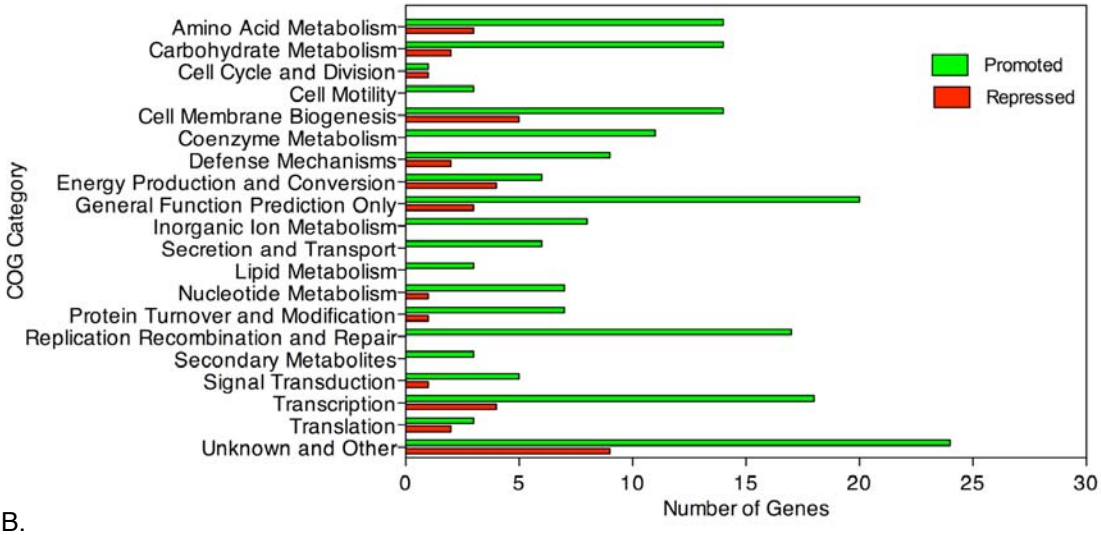


Fig. 17. Scatter plots of microarray normalized signal values fitted with a trend line for the wild type biological replicates at the stationary growth phase. (A) Biological replicates 1 and 2 with an R^2 value of 0.7921, (B) biological replicates 1 and 3 with an R^2 value of 0.7600 and (C) biological replicates 2 and 3 with an R^2 value of 0.7696.

of confidence in the data contributed by this microarray. Inclusion of this microarray would have likely introduced spurious results and skewed the data. A Student's *t*-test using fewer biological replicates was applied to increased the confidence in the data by increasing the stringency of the statistical test that was applied at this time point to offset the use of fewer biological repeats.

Gene products have been categorized by clusters of orthologous genes (COGs) using the DOE Joint Genome Institute Integrated Microbial Genomics website (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi?page=findGenes) and are summarized in Fig. 18 for VjbR and Fig. 19 for C_{12}-HSL, divided by growth phase and further divided by positive or negative regulation. To determine if any COG category is over or under represented by either condition examined, the number of genes regulated by VjbR or C_{12}-HSL were compared to the total number of genes in the genome that belong to that COG category (Fig. 20). This approach revealed that defense mechanisms, intracellular trafficking and secretion and nucleotide transport and metabolism were all found to be over-represented within the VjbR regulon. Conversely, lipid transport and metabolism, amino acid transport and metabolism and energy production and conversion were all highly under-represented within the VjbR regulon. Similar examination of the C_{12}-HSL regulated genes reveals that cell division, defense mechanisms and intracellular trafficking and secretion COGs categories are highly over-represented; while

A.



B.

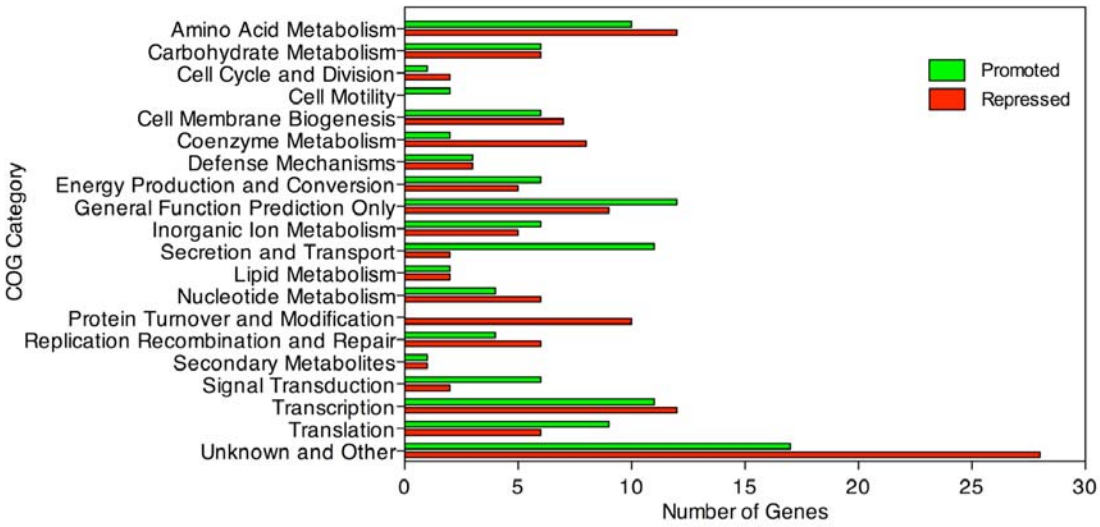
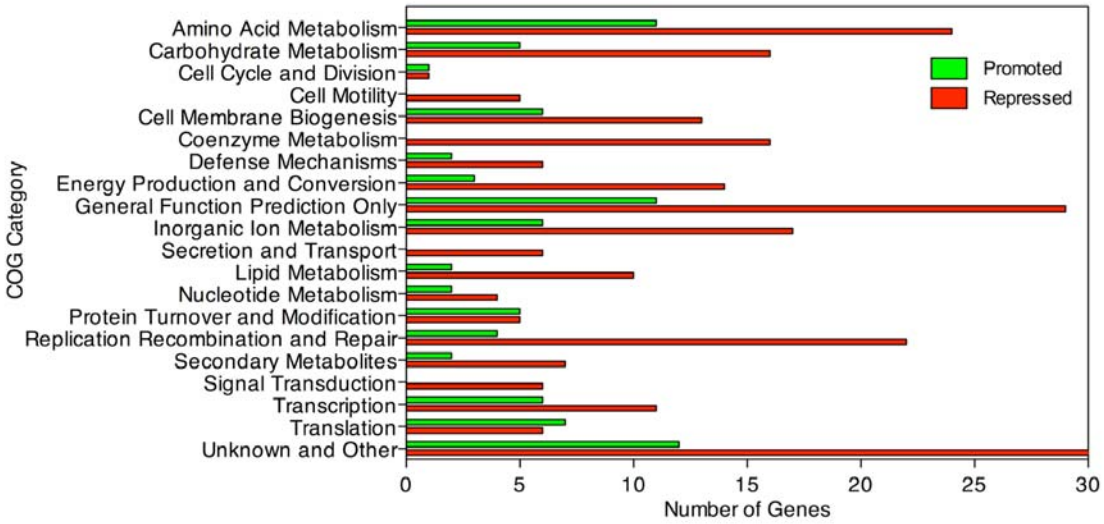


Fig. 18. Number of genes in each COG functional category found to be regulated by VjbR. (A) Demonstrates genes positively and negatively regulated by VjbR at the exponential growth phase and (B) at the stationary growth phase.

A.



B.

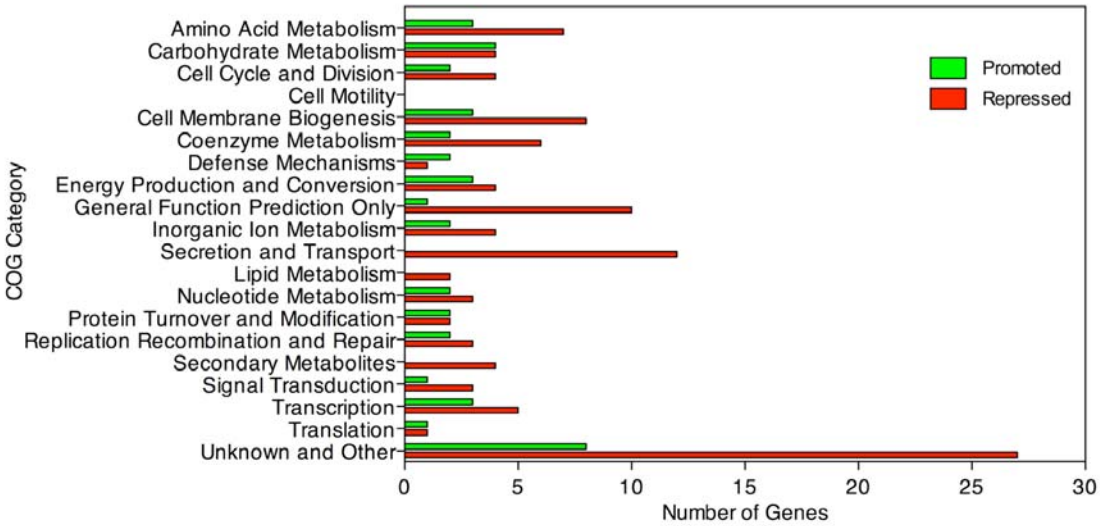


Fig. 19. Number of genes in each COG functional category found to be regulated by C_{12}-HSL. (A) Demonstrates genes positively and negatively regulated by C_{12}-HSL at the exponential growth phase and (B) at the stationary growth phase.

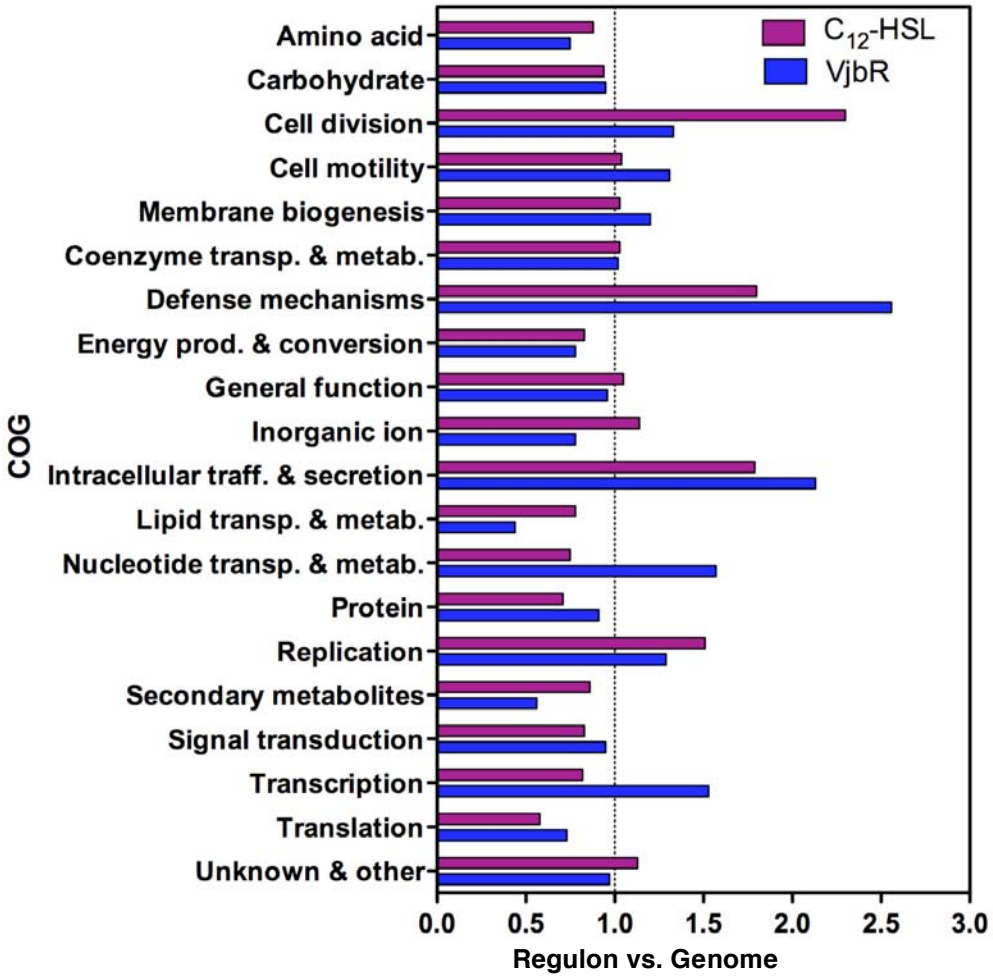


Fig. 20. COG functional categories over and under represented by VjbR and C_{12}-HSL. Values were calculated by the comparing the ratio of genes in the regulon to the ratio of the total number of genes in each COGs present in the *B. melitensis* genome.

posttranslational modification, protein turnover and chaperones, nucleotide transport and metabolism and lipid transport and metabolism were found to be under-represented.

As an alternative approach to the microarray analysis, Seralogix (Ausin, TX) trained a dynamic Bayesian model, applying any prior biological knowledge of pathways and gene ontologies, as well the condition of time to establish any pathways that are different from the control (wild type). One limitation of this model is that it is only able to measure the degree of perturbation, but does not indicate if the pathway is positively or negatively influenced. The top scoring effected pathways for VjbR and C_{12}-HSL are presented in Tables 5 and 6 (respectively). The top scoring pathway for both conditions are ABC transporters, which is interesting because examination of the altered gene lists shows a high number of transporters, but when classified by COG, they fall into many different categories, depending upon the substrate that is being transported. This analysis highlights many of the metabolic functions affected by either VjbR or C_{12}-HSL. In both conditions, many amino acid pathways are altered, as well as fatty acid metabolism, cellular respiration and energy pathways including glycolysis, pyruvate metabolism and acetyl CoA biosynthesis.

Overall, 499 genes were found to be regulated by VjbR and/or C_{12}-HSL at the exponential growth phase and 334 genes regulated by VjbR and/or C_{12}-HSL

TABLE 5. Altered pathways from the deletion of VjbR compared to the control wild type.

Rank	Score	VjbR Top Scoring Pathways
1	2.364	ABC transporters
2	1.698	Histidine metabolism
3	1.619	Butanoate metabolism
4	1.603	Aminoacyl-tRNA biosynthesis
5	1.572	Two componenent system
6	1.536	Purine metabolism
7	1.504	Valine, leucine and isoleucine biosynthesis
8	1.492	Aminophosphonate metabolism
9	1.485	Nicotinate and nicotinamide metabolism
10	1.457	Flagellar assembly
11	1.378	Pyruvate metabolism
12	1.352	Tyrosine metabolism
13	1.317	Urea cycle and metabolism of amino groups
14	1.308	Lysine biosynthesis
15	1.298	Fatty acid metabolism
16	1.249	Taurine and hypotaurine metabolism
17	1.248	Ascorbate and aldarate metabolism
18	1.219	Inositol phosphate metabolism
19	1.218	Glycine, serine and threonine metabolism
20	1.2	Glutamate metabolism
21	1.191	1- and 2-Methylnaphthalene degradation
22	1.177	Bile acid biosynthesis
23	1.176	Glycerophospholipid metabolism
24	1.173	Alanine and aspartate metabolism
25	1.161	Pantothenate and CoA biosynthesis
26	1.155	Glyoxylate and dicarboxylate metabolism
27	1.151	Phenylalanine metabolism
28	1.149	D-Alanine metabolism
29	1.147	β-Alanine metabolism
30	1.123	Benzoate degradation via hydroxylation
31	1.112	Valine, leucine and isoleucine degradation
32	1.099	Biotin metabolism
33	1.073	Riboflavin metabolism
34	1.068	Metabolism of xenobiotics by cytochrome P450
35	1.053	Cyanoamino acid metabolism
36	1.045	Fatty acid elongation in mitochondria
37	1.041	C5-Branched dibasic acid metabolism
38	1.037	Glycolysis / Gluconeogenesis
39	1.015	Nitrobenzene degradation
40	1.004	Oxidative phosphorylation

The score is a normalized log likelihood measure of how different the experiment data is from the control data used to train a Bayesian model.

TABLE 6. Altered pathways from the addition of C_{12}-HSL compared to the control wild type.

Rank	Score	C_{12}-HSL Top Scoring Pathways
1	2.473	ABC transporters
2	1.898	Pyruvate metabolism
3	1.896	Valine, leucine and isoleucine biosynthesis
4	1.886	Histidine metabolism
5	1.879	Tyrosine metabolism
6	1.788	Porphyrin and chlorophyll metabolism
7	1.768	Glycerophospholipid metabolism
8	1.743	Phenylalanine metabolism
9	1.704	Glycine, serine and threonine metabolism
10	1.691	Glyoxylate and dicarboxylate metabolism
11	1.669	Oxidative phosphorylation
12	1.669	Purine metabolism
13	1.651	Glycolysis / Gluconeogenesis
14	1.642	Pyrimidine metabolism
15	1.57	Pentose phosphate pathway
16	1.558	Aminophosphonate metabolism
17	1.537	Nitrobenzene degradation
18	1.513	Benzoate degradation via hydroxylation
19	1.497	Folate biosynthesis
20	1.489	Galactose metabolism
21	1.489	Riboflavin metabolism
22	1.469	Lysine biosynthesis
23	1.45	Glutamate metabolism
24	1.446	Metabolism of xenobiotics by cytochrome P450
25	1.435	Butanoate metabolism
26	1.413	Urea cycle and metabolism of amino groups
27	1.41	Lysine degradation
28	1.399	Styrene degradation
29	1.398	Starch and sucrose metabolism
30	1.393	Glycerolipid metabolism
31	1.373	Taurine and hypotaurine metabolism
32	1.368	Alanine and aspartate metabolism
33	1.339	Aminoacyl-tRNA biosynthesis
34	1.335	Pantothenate and CoA biosynthesis
35	1.331	Nitrogen metabolism
36	1.309	Fluorene degradation
37	1.308	Bile acid biosynthesis
38	1.292	Sulfur metabolism
39	1.291	Nucleotide sugars metabolism
40	1.29	Carbon fixation

The score is a normalized log likelihood measure of how different the experiment data is from the control data used to train a Bayesian model.

at the stationary growth phase. Venn diagrams illustrate the relationships of these gene numbers by growth phase and then sub-divided by condition as shown in Fig. 21A. Conversely, the genes numbers are also categorized by condition and then sub-divided by growth phase as shown in Fig. 21B. This data suggests that VjbR is an activator of gene expression at the exponential growth phase and exerts an equal affect on gene regulation at the stationary growth phase. Examination of the C_{12}-HSL data suggests that it is a repressor of gene expression at both growth phases, but exerts a larger impact on gene regulation during the exponential growth phase.

VjbR and C_{12}-HSL regulate a common set of genes. In many organisms, LuxR transcriptional regulators and the AHL signal affect gene transcription simultaneously. To examine the relationship between VjbR and C_{12}-HSL gene regulation, the significantly altered genes from the VjbR regulon were compared to the significantly altered gene list for the C_{12}-HSL regulon. Genes that are co-regulated by both conditions are presented in Table 7. Seventy-six genes representing 15 % of total genes regulated at the exponential growth phase and 58 genes representing 17 % of the total genes regulated at the stationary phase were co-regulated by VjbR and C_{12}-HSL.

Among the genes co-regulated by both conditions, the impact on gene expression was inversely correlated between the two conditions in all but three genes, strongly suggesting that C_{12}-HSL is an antagonist of VjbR activity when

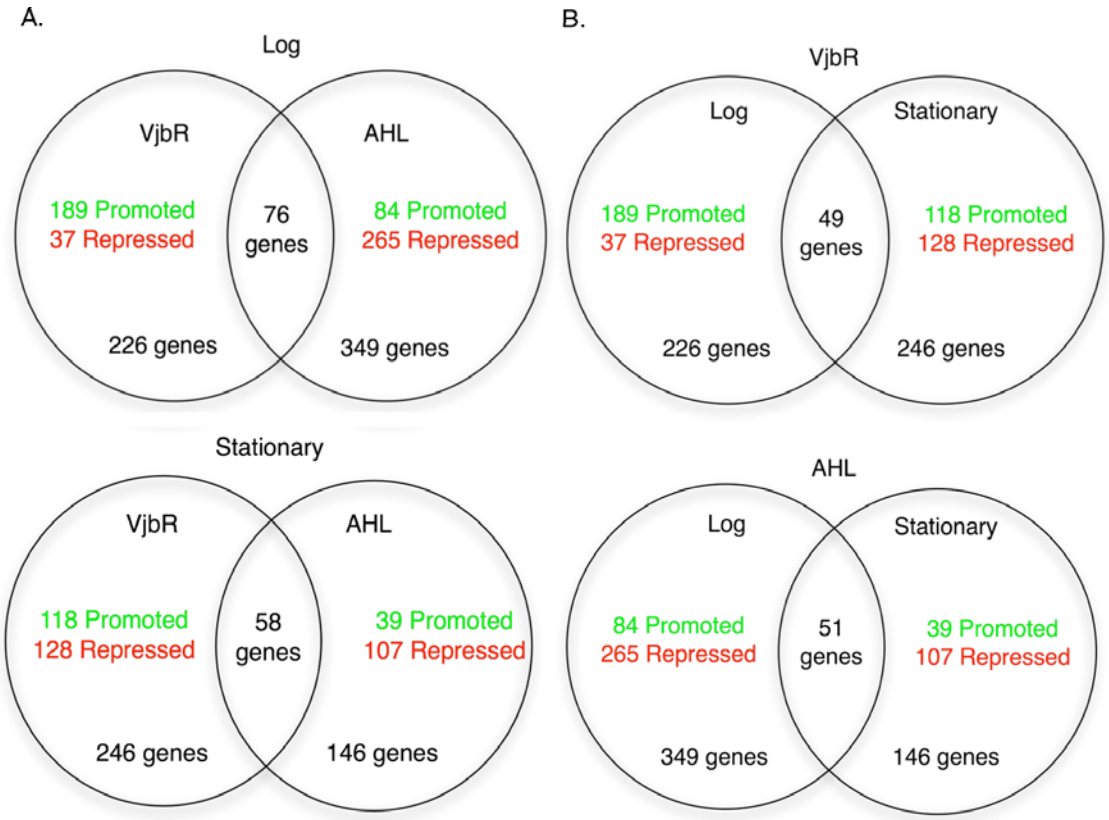


Fig. 21. Venn diagrams demonstrating the relationship between genes regulated over time and by the experimental conditions. (A) Total number of genes regulated at the log and stationary growth phase further divided by conditions, showing total numbers of genes promoted and repressed by each condition individually and total number of genes found to be shared by the two conditions at each growth phase. (B) Total number of genes regulated by each condition then further sub-divided by the growth phases; demonstrating the total number of positively and negatively regulated genes at each time point as well as the total number pf genes found to be regulated at both growth phases by each condition.

TABLE 7. Genes altered by VjbR and C_{12}-HSL at exponential or stationary growth phase.

BME Loci	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Condition	Growth Phase
	Amino Acid Transport and Metabolism				
I 0114	ABC-Type Transporter Asparagine-Binding	1.7	1.5	VjbR	Log
	Periplasmic Protein	2.4	1.7	AHL	
I 0843	Indole-3-Glycerol Phosphate Synthase	2.0	1.6	VjbR	Log
		2.1	1.4	AHL	
II 0484	ABC-Type Transporter Spermidine/	-2.2	-2.4	VjbR	Log
	Putrescine Integral Membrane Protein	-2.4	-2.5	AHL	
I 0208	γ-Glutamyl Phosphate Reductase	-2.1	-1.6	VjbR	Stat
		-2.2	-1.8	AHL	
I 0263	ABC-Type Transporter Leucine, Isoleucine,	2.1	2.1	VjbR	Stat
	Valine, Threonine and	2.0	2.1	AHL	
I 0451	2-Isopropylmalate Synthase	1.9	1.9	VjbR	Stat
		2.4	2.3	AHL	
II 0909	Glutamate, γ-Aminobutyrate Antiporter	-	-2.1	VjbR	Stat
		-	-1.7	AHL	
II 1133	Ornithine Decarboxylase	-2.3	-2.5	VjbR	Stat
		-1.8	-1.7	AHL	
	Carbohydrate Transport and Metabolism				
I 0070	Aquaporin Z	-2.0	-1.7	VjbR	Log
		-2.1	-2.3	AHL	
I 0267	Membrane Protein, MosC	2.0	1.3	VjbR	Log
		2.3	1.6	AHL	
I 1087	β-Hexosaminidase A	-1.8	-1.3	VjbR	Log
		-2.2	-2.1	AHL	
II 0048	Extragenic Suppressor Protein, SuhB	-3.2	-3.1	VjbR	Log
		-2.7	-2.6	AHL	
I 0070	Aquaporin Z	1.7	1.9	VjbR	Stat
		1.6	1.8	AHL	
	Cell Cycle Control, Cell Division and Chromosome Partitioning				
I 1174	Chromosome Partitioning, ParA Family	1.8	1.9	VjbR	Stat
		1.7	1.8	AHL	
	Cell Motility				
II 0151	Flagellar M-Ring Protein, FliF	-2.4	-2.0	VjbR	Log
		-2.3	-1.9	AHL	
	Cell Wall, Membrane and Envelope Biogenesis				
I 0795	Glutamate Racemase	2.1	1.5	VjbR	Log
		1.8	1.2	AHL	
I 1037	Glycosyltransferase	1.7	1.5	VjbR	Log
		2.2	1.6	AHL	
II 0157	Soluble Lytic Murein Transglycosylase	-1.8	-1.9	VjbR	Log
		-1.9	-2.2	AHL	
II 0730	UDP-Glucose 4-Epimerase	-2.1	-2.1	VjbR	Log
		-2.3	-2.5	AHL	
II 0830	dTDP-4-Dehydrorhamnose 3,5-Epimerase,	3.0	-2.5	VjbR	Log
	dTDP-4-Dehydrorhamnose Reductase	-1.6	-2.5	AHL	
II 0838	Succinoglycan Biosynthesis Transport	-1.9	-2.0	VjbR	Log
	Protein, ExoT	-5.0	-3.5	AHL	

TABLE 7—*Continued*

BME Loci	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Condition	Growth Phase
I 0499	Soluble Lytic Murein Transglycosylase	2.1	1.8	VjbR	Stat
		2.1	1.6	AHL	
I 0561	Membrane-Bound Lytic Murein	-2.2	-1.8	VjbR	Stat
	Transglycosylase B	-1.7	-1.6	AHL	
I 1037	Glycosyltransferase	2.5	2.8	VjbR	Stat
		1.9	2.0	AHL	
II 0036	OMP, OprF, VirB12	-1.9	-1.5	VjbR	Stat
		-1.8	-1.6	AHL	
	Coenzyme Transport and Metabolism				
I 0286	Putative Nucleotide-Binding Protein	-2.0	-1.5	VjbR	Log
		-2.9	-2.0	AHL	
I 0315	5-Formyltetrahydrofolate Cyclo-Ligase	-2.0	-2.0	VjbR	Log
		-1.8	-2.2	AHL	
I 0701	Precorrin-4 C11-Methyltransferase	-1.7	-1.4	VjbR	Log
		-2.1	-1.7	AHL	
I 0954	2-Amino-4-Hydroxy-6- Hydroxy-	-2.2	-1.9	VjbR	Log
	Methyldihydropteridine Pyrophosphokinase	-1.6	-2.3	AHL	
I 1517	Pyridoxamine 5'-Phosphate Oxidase	-2.0	-2.1	VjbR	Log
		-1.8	-1.7	AHL	
I 1768	Uroporphyrin-III C-Methyltransferase /	-2.1	-2.0	VjbR	Log
	Precorrin-2 Oxidase / Ferrochelatase	-2.3	-3.7	AHL	
I 2039	Pantothenate Kinase	-2.0	-2.2	VjbR	Log
		-1.7	-2.1	AHL	
II 0957	VdcC Protein	-2.4	-1.6	VjbR	Log
		-2.5	-1.4	AHL	
I 0221	Pyridoxine Kinase	1.6	1.6	VjbR	Stat
		1.7	1.5	AHL	
I 0842	Molybdenum Cofactor Biosynthesis Protein	1.7	1.7	VjbR	Stat
	C	1.6	1.5	AHL	
I 1768	Uroporphyrin-III C-Methyltransferase /	1.6	1.8	VjbR	Stat
	Precorrin-2 Oxidase / Ferrochelatase	-2.3	-2.0	AHL	
	Defense Mechanisms				
I 0654	ABC-Type Multi-Drug Transporter ATP	-1.7	-1.6	VjbR	Log
	Binding Protein	-1.9	-2.2	AHL	
I 0655	ABC-Type Multi-Drug Transporter ATP	-1.8	-	VjbR	Log
	Binding Protein	-2.3	-2.2	AHL	
I 0945	6-Aminohexanoate-Dimer Hydrolase	-2.5	-2.2	VjbR	Log
		-2.2	-2.2	AHL	
II 0258	Bacitracin Resistance Protein, BacA	1.9	1.1	VjbR	Log
		-2.6	-2.0	AHL	
	Energy Production and Conversion				
I 2037	Phosphoenolpyruvate Carboxykinase	-1.8	-1.8	VjbR	Log
		-1.6	-1.6	AHL	
II 0394	Glycerol Trinitrate Reductase	-2.0	-1.7	VjbR	Log
		-2.4	-2.4	AHL	
I 0017	Alkanal Monooxygenase α-Chain	2.0	2.0	VjbR	Stat
		2.0	2.1	AHL	
II 0786	NADH Dehydrogenase	-1.8	-1.5	VjbR	Stat
		-1.7	-1.6	AHL	

TABLE 7—*Continued*

BME Loci	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Condition	Growth Phase
	General Function Prediction Only				
I 0273	GlcG Protein	-2.9	-2.4	VjbR	Log
		-2.3	-2.3	AHL	
I 0282	Zinc Metalloprotease	-1.8	1.0	VjbR	Log
		-1.9	-1.4	AHL	
I 1143	Metal Dependent Hydrolase	-1.6	-2.0	VjbR	Log
		2.0	-2.1	AHL	
I 1531	Tetratricopeptide Repeat Family Protein	-1.7	-2.4	VjbR	Log
		-1.8	-2.9	AHL	
I 2003	Trans-1,2-Dihydrobenzene-1,2-Diol Dehydrogenase, D-Xylose 1-Dehydrogenase	-2.5	-1.8	VjbR	Log
		-2.0	-2.5	AHL	
II 0149	Extracellular Serine Protease	-3.8	-2.6	VjbR	Log
		-2.0	-1.6	AHL	
II 0211	Penicillin Acylase	-2.0	-1.7	VjbR	Log
		-2.7	-2.7	AHL	
II 1060	2,5-Diketo-D-Gluconic Acid Reductase	1.7	1.4	VjbR	Log
		1.7	1.2	AHL	
I 0187	DME Family Transporter	-1.8	-1.7	VjbR	Stat
		-2.3	-2.1	AHL	
I 0739	Integral Membrane Protein, Rhomboid Family	-2.0	-1.8	VjbR	Stat
		-1.8	-1.8	AHL	
I 1304	Porin, F Precusor	-1.8	-1.6	VjbR	Stat
		-2.0	-2.0	AHL	
I 1499	Pirin	-1.7	-1.7	VjbR	Stat
		-1.9	-2.1	AHL	
I 1554	Transporter	-2.6	-2.0	VjbR	Stat
		-2.2	-1.7	AHL	
	Inorganic Ion Transport and Metabolism				
I 0317	Integral Membrane Protein, TerC	-1.8	-1.9	VjbR	Log
		-1.5	-1.9	AHL	
I 0659	Metal Chelate Transport System	-1.8	-	VjbR	Log
		-2.0	-	AHL	
I 0992	Arsenate Reductase	-2.3	-1.6	VjbR	Log
		-2.2	-1.6	AHL	
I 1954	ABC Transporter Substrate Binding Protein	-1.7	-2.2	VjbR	Log
		-1.5	-1.7	AHL	
II 0005	ABC-Type Transporter Molybdate-Binding Protein	-2.8	-2.5	VjbR	Log
		-2.3	-2.4	AHL	
I 1739	ABC-Type Nitrate/Sulfonate/Bicarbonate Transporter	-1.8	-1.6	VjbR	Stat
		-2.2	-1.9	AHL	
I 1954	ABC-Type Metal Ion Transport System Substrate Binding Protein	2.0	2.0	VjbR	Stat
		2.1	2.0	AHL	
II 0798	ABC-Type Nitrate Transport System, ATP Binding Protein, NtrC	-2.2	-2.0	VjbR	Stat
		-2.1	-2.0	AHL	
	Intracellular Trafficking, Secretion and Vesicular Transport				
I 1077	Hypothetical Exported Protein, YajC	-1.7	-1.3	VjbR	Log
		-2.1	-2.0	AHL	
II 0025	Attachment Mediating Protein, VirB1	-2.1	-2.3	VjbR	Log
		-2.1	-1.6	AHL	
II 0025	Attachment Mediating Protein, VirB1	-2.7	-2.4	VjbR	Stat
		-2.2	-2.2	AHL	

TABLE 7—*Continued*

BME Loci	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Condition	Growth Phase
II 0026	Attachment Mediating Protein, VirB2	-4.8	-3.7	VjbR	Stat
		-3.9	-3.3	AHL	
II 0027	Channel Protein, VirB3	-4.3	-3.5	VjbR	Stat
		-3.4	-3.0	AHL	
II 0029	Attachment Mediating Protein, VirB5	-6.2	-5.1	VjbR	Stat
		-4.9	-4.1	AHL	
II 0030	Channel Protein, VirB6	-3.1	-2.4	VjbR	Stat
		-2.4	-2.1	AHL	
II 0032	Channel Protein, VirB8	-3.9	-2.7	VjbR	Stat
		-3.0	-2.2	AHL	
II 0033	Channel Protein, VirB9	-2.0	-1.5	VjbR	Stat
		-2.1	-1.7	AHL	
II 0034	Channel Protein, VirB10	-2.2	-1.7	VjbR	Stat
		-2.0	-1.8	AHL	
	Lipid Transport and Metabolism				
I 1289	4'-Phosphopantetheinyl Transferase	-1.7	-1.2	VjbR	Log
		-1.9	-1.6	AHL	
I 1922	Acetoacetyl-CoA Synthase	-1.6	-1.3	VjbR	Log
		-1.7	-1.8	AHL	
II 1103	Phosphatidylglycero-Phosphatase B	-2.5	-2.1	VjbR	Stat
		-2.3	-2.1	AHL	
	Nucleotide Transport and Metabolism				
I 1430	Ureidoglycolate Hydrolase	-1.7	-1.7	VjbR	Log
		-1.6	-1.7	AHL	
II 0088	Nucleoside Hydrolase	-3.2	-2.5	VjbR	Log
		-2.3	-2.7	AHL	
I 1090	Deoxyguanosinetriphosphate	2.0	2.1	VjbR	Stat
	Triphosphohydrolase-Like Protein	1.6	1.7	AHL	
I 1430	Ureidoglycolate Hydrolase	-1.9	-1.7	VjbR	Stat
		-2.7	-2.3	AHL	
	Posttranslational Modification, Protein Turnover and Chaperones				
I 1852	ABC-Type Transporter Heme Exporter	-2.0	-1.6	VjbR	Log
	Protein B	-1.9	-1.8	AHL	
I 0887	Peptidyl-Prolyl Cis-Trans Isomerase	1.7	1.7	VjbR	Stat
		1.6	1.6	AHL	
I 1804	PII Uridylyl-Transferase	-	2.3	VjbR	Stat
		-	-2.4	AHL	
	Replication, Recombination and Repair				
I 0040	Tyrosine Recombinase	-1.8	-1.6	VjbR	Log
		-1.7	-1.9	AHL	
I 0902	Recombinase	-2.3	-1.9	VjbR	Log
		-2.3	-2.4	AHL	
I 1002	Transposase	-1.8	-1.4	VjbR	Log
		-1.8	-1.6	AHL	
I 1053	Transposase	-2.9	-1.6	VjbR	Log
		-5.2	-3.2	AHL	
I 1664	Hypothetical Cytosolic Protein	-2.3	-2.0	VjbR	Log
		-1.9	-1.8	AHL	
II 0183	Transposase	-3.3	-3.7	VjbR	Log
		-3.3	-3.0	AHL	

TABLE 7—*Continued*

BME Loci	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Condition	Growth Phase
II 0227	Transposase	-2.0	-2.0	VjbR	Log
		-1.8	-1.9	AHL	
II 0445	Transposase	-2.9	-2.3	VjbR	Log
		-2.6	-2.4	AHL	
I 1664	Recombinase, XerD	2.7	2.3	VjbR	Stat
		1.9	1.8	AHL	
	Secondary Metabolites Biosynthesis, Transport and Catabolism				
II 0580	Probable Blue-Copper Protein, YacK	-2.8	-2.5	VjbR	Log
		-1.8	-1.7	AHL	
II 0879	Putative Cytochrome P450, YjiB	-1.7	-1.8	VjbR	Log
		-2.1	-2.6	AHL	
II 0062	Probable Carbonyl Reductase [NADPH]	-1.9	-	VjbR	Stat
		-1.6	-1.5	AHL	
	Signal Transduction Mechanisms				
I 0370	Sensory Transduction Histidine Kinase	-2.2	-1.2	VjbR	Log
		-2.5	-1.7	AHL	
I 0950	Phosphohistidine Phosphatase Protein, SixA	-3.3	-2.1	VjbR	Log
		-3.4	-2.6	AHL	
II 0051	Nodulation Protein W	-1.9	-1.9	VjbR	Log
		-2.7	-2.8	AHL	
II 0245	Universal Stress Protein Family, UspA	-2.0	-1.6	VjbR	Log
		-1.8	-1.6	AHL	
I 2034	Sensor Protein, ChvG	-2.1	-1.8	VjbR	Stat
		-1.6	-1.5	AHL	
II 0245	Universal Stress Protein Family, UspA	-2.9	-2.1	VjbR	Stat
		-3.0	-2.0	AHL	
II 0854	CRP Family Transcriptional Regulator	-1.7	-1.2	VjbR	Stat
		-2.0	-1.3	AHL	
	Transcription				
I 0896	LysR Family Transcriptional Regulatory Protein	-1.6	-1.1	VjbR	Log
		-2.2	-1.9	AHL	
I 1098	AsnC Family Transcriptional Regulator	-1.7	-1.7	VjbR	Log
		-1.6	-2.3	AHL	
I 1631	TetR Family Transcriptional Regulator	-1.7	-2.0	VjbR	Log
		-2.0	-2.1	AHL	
I 1700	Predicted Transcriptional Regulator	2.2	1.7	VjbR	Log
		3.3	2.4	AHL	
I 1776	Heat-Inducible Transcription Repressor, HrcA	-1.6	-1.2	VjbR	Log
		-1.8	-1.6	AHL	
II 0219	IclR Family Transcriptional Regulator	-3.5	-2.8	VjbR	Log
		-6.4	-5.2	AHL	
II 1098	AraC Family Transcriptional Regulator	-1.9	-1.7	VjbR	Log
		-2.8	-2.8	AHL	
I 0447	Leucine-Responsive Regulatory Protein	-2.6	-2.1	VjbR	Stat
		-1.8	-1.7	AHL	
I 1641	TetR Family Transcriptional Regulator	-1.8	-1.6	VjbR	Stat
		-2.0	-1.6	AHL	
II 1098	AraC Family Transcriptional Regulator	1.8	2.0	VjbR	Stat
		-	1.5	AHL	

TABLE 7—*Continued*

BME Loci	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Condition	Growth Phase
	Translation, Ribosomal Structure and Biogenesis				
I 0480	Peptidyl-tRNA Hydrolase	2.3	-1.9	VjbR	Log
		2.7	2.1	AHL	
II 1056	Histidyl-tRNA Synthetase	-1.9	-1.6	VjbR	Stat
		-1.7	-1.6	AHL	
	Unknown and Other				
I 0400	Hypothetical Protein	1.9	1.5	VjbR	Log
		2.7	1.9	AHL	
I 1000	Hypothetical Protein	-3.1	-3.4	VjbR	Log
		-2.6	-2.9	AHL	
I 1425	Hypothetical Protein	1.5	1.1	VjbR	Log
		1.6	1.2	AHL	
I 1680	Hypothetical Protein	-2.2	-2.1	VjbR	Log
		-2.0	-2.3	AHL	
I 1685	Hypothetical Protein	-2.0	-1.4	VjbR	Log
		-2.3	-2.2	AHL	
II 0057	Hypothetical Protein	-	-2.0	VjbR	Log
		-1.9	-1.8	AHL	
I 0154	Hypothetical Membrane Spanning Protein	1.9	1.8	VjbR	Stat
		2.0	2.0	AHL	
I 0366	Hypothetical Protein	1.7	1.9	VjbR	Stat
		1.7	1.7	AHL	
I 0368	Hypothetical Protein	1.7	1.9	VjbR	Stat
		1.7	1.8	AHL	
I 0431	Hypothetical Protein	-1.7	-1.5	VjbR	Stat
		1.6	-1.5	AHL	
I 0515	Hypothetical Protein	1.6	1.9	VjbR	Stat
		2.0	1.9	AHL	
I 0620	Hypothetical Protein	1.6	1.9	VjbR	Stat
		1.8	1.9	AHL	
I 0798	Hypothetical Protein	1.9	2.1	VjbR	Stat
		2.0	1.9	AHL	
I 1524	Hypothetical Protein	-1.6	-1.3	VjbR	Stat
		-1.9	-1.6	AHL	
I 1694	Hypothetical Protein	-2.2	-1.9	VjbR	Stat
		-2.7	-2.3	AHL	
I 2044	Hypothetical Membrane Spanning Protein	1.8	1.6	VjbR	Stat
		-2.0	1.8	AHL	
II 0231	Hypothetical Protein	1.9	1.9	VjbR	Stat
		1.6	1.6	AHL	
II 0516	Hypothetical Protein, Predicted Membrane Protein	-3.0	-2.6	VjbR	Stat
		-2.4	-2.3	AHL	

*Wild type (WT) replicates 1&2 for exponential growth phase and replicates 1,2&3 for the stationary growth phase were used for the Student's *t*-test analysis. Fold change (FC) values are the log ratio of normalized signal values. FC values shown in grey did not pass the statistical test in that column. Abbreviations are as follows: FC, Fold Change; OMP, Outer Membrane Protein; DME, Drug/Metabolite Exporter.

added exogenously. Although there is some indirect evidence that VjbR and C_{12}-HSL interact through the response domain of VjbR, this data suggests that only a very small portion of the genes found to be regulated by either condition in the individual analysis were shared between the two (147).

Putative and known virulence factors were found to be co-regulated by VjbR and C_{12}-HSL, including proteases (BMEI 0282 and 0739 and BMEII 0149), flagellar m-ring protein FliF (BMEII 0151) and the type IV secretion system (*virB*, BMEII 0025-0036). Additional genes of interest include: ParA, involved in cell division; several soluble lytic murein transglycolases, which have been implicated in cell membrane recycling during cell division/elongation; signal transduction; and transcription (123). The transcriptional regulators include family members from LysR, AsnC, TetR, HrcA, IclR, AraC and one of the LuxR-like proteins, BME II0051, which was found to be regulated by both conditions, promoted by VjbR and repressed by C_{12}-HSL.

VjbR and C_{12}-HSL regulate independent sets of genes at both an exponential and early stationary growth phase. Microarray analysis was used to determine genes regulated by VjbR or C_{12}-HSL at two different growth stages. Comparison between the microarray analyses of VjbR and C_{12}-HSL revealed that the majority of genes are dependent on only one of the conditions measured, with only a very small portion (16-18 %) of the genes were found to be co-regulated. The significantly altered genes found to be regulated by either

VjbR or C_{12}-HSL at the exponential growth phase are presented in Table 8 and the genes found to be regulated at the stationary growth phase are presented in Table 9. Commonly, it was found that the AHL signal and LuxR transcriptional regulator form a positive feedback loop that will enhance the expression of one another (130). Interestingly, it was observed that the addition of C_{12}-HSL to 16M wild type culture did not have a large impact on the induction of VjbR and was not included in the list of altered genes in the microarray analysis. Quantitative real-time PCR found VjbR to be repressed an average of 2.9-fold by C_{12}-HSL relative to wild type at the stationary growth phase (data not shown). This fold change is modest, but does indicate that C_{12}-HSL represses the expression of VjbR. At the exponential growth phase, a few trends in the gene products regulated by VjbR and C_{12}-HSL were found. Although the two conditions did not regulate the same gene locus, both conditions promoted the expression of ribosomal subunits and inversely regulated expression of DNA polymerase III subunits. An inverse relationship between the regulation of different flagella and motility genes was also observed, with VjbR promoting and C_{12}-HSL repressing gene expression. Exopolysaccharide synthesis and transport (including cyclic β-1,2-glucan) are also inversely regulated, with VjbR promoting expression of *exoF* and *ndvA* and C_{12}-HSL repressing expression of *opgC* at the exponential growth

TABLE 8. Genes altered at mid-exponential growth phase by VjbR or addition of C_{12}-HSL.

Locus	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student 's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Condition
	Amino Acid Transport and Metabolism			
l 0079	Prephenate Dehydrogenase	-1.9	-1.4	VjbR
l 0143	Threonine Efflux Protein	-1.8	-2.2	AHL
l 0256	D-AA Dehydrogenase	1.9	1.4	AHL
l 0260	ABC-Type High-Affinity Branched Chain AA Transport, BraF	2.6	1.5	AHL
l 0617	Acetolactate Synthase III Large Subunit	-1.7	-	VjbR
l 0642	Urea Transporter	-1.6	-2.1	AHL
l 0844	Anthranilate Phosphoribosyltransferase	1.9	1.3	AHL
l 0978	Nitrogen Regulatory Protein P-II	2.6	1.8	AHL
l 1022	ABC-Type Arginine, Ornithine Binding Periplasmic Protein Precursor Transporter	3.0	2.5	AHL
l 1171	N-Acetyl-γ-Glutamyl-Phosphate Reductase	3.0	1.7	AHL
l 1209	ABC-Type General L-AA Transport System, AapM	-1.8	-2.2	AHL
l 1213	Cystathionine β-Lyase	1.9	1.5	AHL
l 1217	ApeA Protein	2.0	1.8	AHL
l 1683	Zinc-Dependent Metallopeptidase	-1.8	-1.7	AHL
l 1719	Sarcosine Oxidase γ Subunit	-1.7	-1.9	AHL
l 1722	Sarcosine Oxidase β Subunit	-1.9	-2.0	AHL
l 1755	Sulfate Adenylyltransferase Subunit 2	-1.8	-1.9	AHL
l 1869	Homoserine Lactone Efflux Protein	-2.5	-2.0	AHL
l 1934	ABC-Type Transporter, Periplasmic Oligopeptide-Binding Protein Precursor	-1.8	-2.0	AHL
l 2058	Shikimate 5-Dehydrogenase	-2.0	-2.1	VjbR
II 0099	ABC-Type High-Affinity Branched Chain AA Transport ATP Binding Protein, LivG	-1.6	-	VjbR
II 0101	ABC-Type High Affinity Branched-Chain AA Transport System, LivM	-2.0	-2.1	AHL
II 0193	ABC-Type Spermidine, Putrescine Transport ATP Binding Protein, PotA	-1.9	-	VjbR
II 0199	ABC-Type Oligopeptide Transport ATP Binding Protein, OppF	-2.6	-3.0	AHL
II 0209	ABC-Type Dipeptide Transport System Permease Protein, DppB	-1.7	-2.0	AHL
II 0221	ABC-Type Dipeptide Transport System Permease Protein, DppC	-1.8	-1.9	AHL
II 0273	Acetylglutamate Kinase	2.8	1.7	AHL
II 0366	N-Formylglutamate Deformylase	-1.8	-2.0	AHL
II 0368	Imidazolonepropionase	-1.8	-2.1	VjbR
II 0396	Arginase	-1.6	-1.9	AHL
II 0441	Acetylornithine Aminotransferase	-1.7	-1.2	VjbR
II 0484	ABC-Type Transport System Spermidine/Putrescine, Integral Membrane Protein	-2.2	-2.4	VjbR
II 0548	ABC-Type Glycine Betaine, L-Proline Transport, ProV	-1.8	-1.9	AHL
II 0571	IolD Protein	-1.7	-1.8	AHL
II 0632	High-Affinity Branched Chain AA Transport System Permease Protein, LivH	-1.8	-	VjbR
II 0873	High Affinity Branched Chain AA Transport System, LivF	-1.9	-2.7	AHL
II 0908	Glutaminase	-1.8	-2.1	AHL
II 0923	Spermidine, Putrescine-Binding Periplasmic Protein of Transport System	-2.4	-2.8	AHL
	Carbohydrate Transport and Metabolism			
l 0396	Diohydroxyacetone Kinase	2.9	2.0	AHL
l 0398	Deoxyribonucleoside Regulator, Dihydroxyacetone Kinase	4.7	1.6	AHL
l 0556	α--Ketoglutarate Permease	-2.5	-2.4	AHL
l 0665	ABC-Type Sugar Transport ATP Binding Protein	-1.6	-	VjbR
l 0667	FucU Protein	-1.9	-1.7	AHL
l 0720	Sugar Fermentation Stimulation Protein	-2.1	-1.8	AHL

TABLE 8--*Continued*

Locus	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student 's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Condition
I 1385	LacI Family Transcriptional Regulator	-3.1	-3.2	AHL
I 1387	Xylose Isomerase	-1.8	-1.9	VjbR
I 1389	IolE Protein	-1.7	-1.4	VjbR
I 1773	Putative Lactoylglutathione Lyase	-1.6	-1.1	VjbR
II 0113	ABC-Type SN-G3P Transport System, UgpA	-1.9	-1.8	AHL
II 0115	ABC-Type G3P-Binding Periplasmic Protein	-3.2	-3.2	AHL
II 0139	Phosphotyrosyl Phosphatase Activator, PtpA	3.8	1.8	AHL
II 0189	L-Fuculose Phosphate Aldolase	-2.4	-1.0	VjbR
II 0362	ABC-Type Xylose Transport System, XylH	-2.4	-2.5	AHL
II 0422	Fructose-1,6-Bisphosphatase	-2.4	-2.3	VjbR
II 0476	Uronate Isomerase	-2.4	-1.4	VjbR
II 0478	D-Mannonate Oxidoreductase	-1.7	-2.0	AHL
II 0502	ABC-Type Transport System Sugar-Binding Protein	-	-2.5	VjbR
II 0596	Methylenomycin A Resistance Protein	-2.4	-2.6	AHL
II 0821	Multi-drug Resistance Protein	2.4	1.6	VjbR
II 0850	GDP-Fucose Synthetase	-2.7	-2.9	VjbR
II 0939	ThuA Protein	-1.7	-2.3	AHL
II 1096	Putative Tartrate Transporter	3.0	2.1	AHL
	Cell Cycle Control, Cell Division and Chromosome Partitioning			
I 0313	Hypothetical Cytosolic Protein, ZapA	-1.8	-1.4	VjbR
I 0342	Cell Cycle Protein, MesJ	3.2	2.6	VjbR
I 0633	Camphor Resistance Protein, CrcB	-2.5	-3.0	AHL
	Cell Motility			
II 0155	Chemotaxis MotC Protein Precursor	-1.5	-1.9	VjbR
II 0160	Flagellar Hook-Associated Protein, FlgK	-1.9	-2.1	AHL
II 0161	Flagellar Hook-Associated Protein 3	-3.3	-2.0	AHL
II 0162	FlaF Protein	-2.3	-1.8	VjbR
II 0165	Flagellar Biosynthesis Protein	-2.8	-2.8	AHL
II 0167	Flagellar Biosynthesis Protein, FlhA	-2.1	-2.5	AHL
	Cell Wall, Membrane and Envelope Biogenesis			
I 0204	Acetyltransferase	-2.8	-1.6	VjbR
I 0359	ABC-Type Efflux System, MacA	-1.9	-1.3	VjbR
I 0363	TonB Protein	-1.8	-1.5	VjbR
I 0418	Lic2B Protein	-2.0	-1.1	AHL
I 0566	Soluble Lytic Murein Transglycosylase	-2.0	-1.5	AHL
I 0682	Potassium Efflux System, KefA	-1.9	-2.2	VjbR
I 0831	UDP-3-O-[3-hydroxymyristoyl] Glucosamine N-Acyltransferase	2.6	1.9	AHL
I 0835	Lipid-A-Disaccharide Synthase	-1.7	-1.9	AHL
I 0913	Penicillin-Binding Protein 6	1.5	-	VjbR
I 1393	Mannosyltransferase C	-2.1	-1.8	AHL
I 1602	Glycosyltransferase	3.1	2.7	AHL
I 1972	Apolipoprotein N-Acyltransferase	-1.9	-2.1	AHL
I 1799	Lipoprotein Signal Peptidase	3.4	1.0	VjbR
I 1904	3-Deoxy-Manno-Octulosonate Cytidylyltransferase	-2.0	-1.9	VjbR
I 2052	Membrane-Bound Lytic Murein Transglycosylase A	-1.7	-1.2	VjbR
II 0260	GTP-Binding Protein LepA	-2.1	-1.7	AHL
II 0374	Alanine Racemase, Catabolic	-2.0	-2.3	VjbR
II 0376	Heat Resistant Agglutinin 1 Precursor	2.5	1.6	AHL
II 0839	Putative Undecaprenyl-Phosphate α-N-Acetylglucosaminyl-Transferase	-2.0	-2.2	AHL
II 0851	Exopolysaccharide Production Protein ExoF	-2.0	-2.1	VjbR
	Coenzyme Transport and Metabolism			

TABLE 8--*Continued*

Locus	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student 's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Condition
I 0177	Proporphyrinogen-III Synthetase	-1.9	-2.4	AHL
I 0703	Cobalt-Precorrin-6A Synthase, CbiD	-1.7	-1.6	VjbR
I 0707	CbiB Protein	-1.9	-2.3	AHL
I 0712	Precorrin-3B C17-Methyltransferase Protein, CbiG	-2.2	-1.7	AHL
I 0713	Precorrin-2 C20-Methyltransferase	-1.9	-1.5	AHL
I 1293	Coproporphyrinogen III Oxidase	-1.9	-2.2	AHL
	Defense Mechanisms			
I 0361	ABC-Type Antimicrobial Peptide Transporter ATP Binding and Permease Protein	-1.9	-	VjbR
I 0472	ABC-Type Transporter ATP Binding Protein	2.4	1.5	AHL
I 0656	ABC-Type Daunorubicin Resistance Membrane Protein	1.7	-	VjbR
I 0926	Multi-drug Resistance Protein A	-2.9	-2.8	VjbR
I 0984	ABC-Type β-(1->2) Glucan Export ATP Binding, Protein NdvA	-2.6	-1.6	VjbR
I 1883	Pantothenate Kinase	-1.8	-1.7	AHL
II 0319	6-Aminohexanoate-Dimer Hydrolase	2.6	1.4	AHL
II 0452	Type I Restriction-Modification Enzyme	-1.6	-2.0	AHL
	Energy Production and Conversion			
I 0911	NifU Protein	2.0	-1	VjbR
I 1016	Fumarate Hydratase Class I	-2.3	-1.4	AHL
I 1145	NADH Dehydrogenase Subunit N	-1.8	-2.1	AHL
I 1149	NADH Dehydrogenase Subunit J	-2.0	-2.4	AHL
I 1527	Glycolate Oxidase Subunit GlcD	-1.7	-1.3	VjbR
I 1559	Salicylaldehyde Dehydrogenase	2.6	1.9	AHL
I 1591	Ferredoxin-NADP Reductase	-1.6	-1.8	AHL
I 1802	NADP-Dependent Malic Enzyme	2.4	-	AHL
II 0061	2-Oxoisovalerate Dehydrogenase β Subunit	-1.9	-2.3	AHL
II 0135	5-Carboxymethyl-2-Hydroxymuconate	2.5	2.0	AHL
II 0218	Dihydrolipoamide Acetyltransferase	-2.1	-1.8	AHL
II 0225	6-Oxohexanoate Dehydrogenase	-3.8	-2.6	VjbR
II 0242	Aldehyde Dehydrogenase	-2.1	-2.5	AHL
II 0553	Alcohol Dehydrogenase	2.0	1.5	VjbR
II 0880	Acetate Kinase	1.5	1.2	VjbR
II 1064	(S)-2-Hydroxy-Acid Oxidase Chain D	-3.1	-2.4	VjbR
II 1073	Cytochrome B561	-2.3	-1.6	AHL
	General Function Prediction Only			
I 0125	Acetyltransferase	-2.1	-1.5	AHL
I 0206	GTP Binding Protein	2.6	2.0	AHL
I 0330	OpgC Protein	-1.6	-2.3	AHL
I 0709	4-Hydroxyphenylacetate 3-Monooxygenase	-2.0	-1.4	VjbR
I 0852	Methyltransferase	-2.3	-1.9	AHL
I 0920	MazG Protein	-1.8	-2.0	AHL
I 0922	Sodium, Bile Acid Cotransporter Homolog	-1.9	-2.4	AHL
I 0925	Alcohol Dehydrogenase	-	2.8	AHL
I 1034	HesB Protein	1.9	1.3	AHL
I 1102	Transporter	-2.0	-1.5	AHL
I 1305	Porin	-2.7	-1.9	AHL
I 1119	Predicted Esterase of the α/β Hydrolase Fold	-1.9	-1.4	VjbR
I 1198	RDD Family Protein	1.5	1.2	VjbR
I 1446	Phosphoglycolate Phosphatase	-1.8	-1.9	AHL
I 1470	YicC Protein	-2.3	-1.2	VjbR
I 1501	Transglycosylase Associated Protein	-1.7	-1.9	AHL
I 1502	Alkaline Phosphatase Like Protein	-1.7	-1.8	AHL
I 1597	Murein Hydrolase Exporter	-2.9	-2.3	AHL

TABLE 8--*Continued*

Locus	Gene Product	Δ*vjbR* / wt*or wt+ahl / wt* Student 's *t*-test FC	Z-score FC	Condition
I 1764	Oxidoreductase	-1.8	-1.4	VjbR
I 1820	Acetyltransferase	-3.2	-2.5	AHL
I 1822	S-Formylglutathione Hydrolase	-1.6	-1.3	VjbR
II 0234	Ubiquinone Biosynthesis Protein, AarF	2.5	1.2	AHL
II 0307	Vegetatible Incompatibility Proein HET-E-1	-2.0	-1.6	AHL
II 0347	Membrane Protein Related to Metalloendopeptidase	2.1	1.5	AHL
II 0400	Putative DNA-Binding Protein	-1.6	-1.8	AHL
II 0410	Acetylglutamate Kinase	-1.8	-1.9	AHL
II 0466	Tetratricopeptide Repeat Family Protein	3.0	1.5	AHL
II 0448	Zinc Metallopeptidase	-1.7	-1.8	AHL
II 0574	Myo-Inositol 2-Dehydrogenase	-1.8	-1.8	VjbR
II 0578	Alkyl Hydroperoxide Reductase Subunit D	-1.8	-1.4	VjbR
II 0655	Alkaline Phosphatase	2.5	1.6	AHL
II 0701	Ribose Transport System, RbsC	2.2	-	AHL
II 0831	Aminopeptidase-Like Domain	-1.9	-2.1	AHL
II 0980	Ribitol 2-dehydrogenase	-2.2	-1.9	AHL
II 1036	Zinc Protease	-2.0	-2.0	AHL
II 1070	Adhesin AidA-I	2.0	1.3	VjbR
II 1136	ABC-Type Uncharacterized Transport System	-1.8	-1.9	AHL
	Inorganic Ion Transport and Metabolism			
I 0053	Cation Transporting ATPase, PacS	2.4	2.0	AHL
I 0284	Thiamine Transport System, ThiP	-2.2	-1.7	AHL
I 0637	ABC-Type Cobalt Transport Protein, CbiQ	2.8	1.8	AHL
I 0641	ABC-Type Co^{2+} Transport System	2.1	1.7	AHL
I 0671	Integral Membrane Protein, Hemolysin	-2.3	-3.0	AHL
I 1367	Superoxide Dismutase (MN)	-2.2	-1.5	VjbR
I 1753	CysQ Protein	3.0	1.1	AHL
I 1754	Binfunctional Sulfate Adenylyltransferase Subunit 1, Adenylylsulfate Kinase Protein	2.4	1.7	AHL
II 0056	Mg^{2+} Transport, P-Type	-1.6	-2.0	AHL
II 0109	ABC-Type Aliphatic Sulfonates Binding Lipoprotein	-1.8	-1.8	AHL
II 0176	ABC-Type High-Affinity Zn Uptake System Membrane Protein, ZnuB	-2.3	-2.3	AHL
II 0201	Oligopeptide Transport System Permease Protein, OppC	-1.9	-	AHL
II 0418	Mg^{2+} Transporter Protein, MgtE	-3.4	-2.9	VjbR
II 0606	ABC-Type Ferric Anguibactin Transport System Permease Protein, FatD	-	-2.4	AHL
II 0770	Potassium Efflux System Protein, PhaA, PhaB	-2.1	-2.0	AHL
II 0897	Chloride Channel Protein, EriC	2.6	1.8	AHL
II 0964	Asparagine Synthetase B (Glutamine-Hydrolyzing)	-1.8	-2.1	AHL
	Intracellular Trafficking, Secretion and Vesicular Transport			
I 0365	Biopolymer Transport Protein, ExbB	-1.8	-1.5	VjbR
I 1141	Predicted Exported Protein	-1.6	-1.8	AHL
I 1873	Autotransporter adhesin	-2.2	-2.1	VjbR
	Lipid Transport and Metabolism			
I 0022	3-Hydroxybuturyl CoA Dehydratase	2.1	1.7	AHL
I 0552	Lysophospholipase L2	-2.5	-2.1	AHL
I 0799	Methylmalonyl-CoA Mutase	1.9	1.3	AHL
I 1196	Enoyl-CoA Hydratase	-1.7	-2.2	AHL
I 1928	Enoyl-CoA Hydratase	-1.8	-1.8	AHL
I 1977	1-Acyl-SN-G3P Acyltransferase	-2.3	-2.7	AHL
I 1252	CDP-Diacylglycerol--G3P3-Phosphatidyltransferase	-2.0	-2.4	AHL
II 0643	3-Oxoadipate CoA-Transferase Subunit A	-	-2.1	AHL

TABLE 8--*Continued*

Locus	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student 's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Condition
II 0815	Acetyl-CoA Synthetase	-1.7	-1.8	AHL
	Nucleotide Transport and Metabolism			
I 0989	Thymidylate Kinase	1.8	1.1	VjbR
I 1318	AMP Nucleosidase	2.2	1.1	AHL
I 1772	Putative Deoxyribonucleotide Triphosphate Pyrophosphatase	-1.5	-1.4	VjbR
II 0369	Atrazine Chlorohydrolase	2.1	1.7	AHL
II 0420	Thymidylate Synthase	-1.7	-1.8	VjbR
	Posttranslational Modification, Protein Turnover and Chaperones			
I 0004	Thioredoxin Domain Protein	3.1	1.4	AHL
I 0093	ExsD Protein	-1.7	-2.0	AHL
I 0611	HflC Protein, Stomatin, Prohibitin, Flotillin, HflK-C Domains	-1.7	-1.4	VjbR
I 0783	Protease DO	2.4	1.7	AHL
I 0958	Thioredoxin Reductase	-2.1	-2.3	AHL
I 1041	ABC-Type Transporter ATP Binding Protein	2.4	1.6	AHL
I 1172	Cytochrome C Oxidase Assembly Protein Cox15	2.3	1.6	AHL
I 1650	Urease Accessory Protein, UreF	-2.1	-2.4	VjbR
I 1784	Small Heat Shock Protein, HspA	-1.7	-	VjbR
I 1808	NifU-Like Protein	-2.4	-1.9	VjbR
I 1994	Mg^{2+} Chelatase Family Protein	4.2	2.9	AHL
II 0042	Heat Shock Protein A	-1.6	-1.6	VjbR
	Replication, Recombination and Repair			
I 0333	Holiday Junction DNA Helicase, RuvA	-1.7	-1.7	AHL
I 0334	Holliday Junction DNA Helicase, RuvB	-2.1	-1.2	AHL
I 0784	ATPase Protein	2.6	1.7	AHL
I 1093	Exodeoxyribonuuclease III	-2.2	-1.9	AHL
I 1163	Transposase	-2.1	-2.0	AHL
I 1223	Transposase	-2.3	-1.9	VjbR
I 1362	ATPase	-2.4	-2.4	VjbR
I 1397	Transposase	-2.1	-1.9	AHL
I 1409	Transposase	3.5	-1.1	AHL
I 1420	Transposase	-1.6	-1.9	VjbR
I 1442	A/G-Specific Adenine Glycosylase	-1.7	-1.9	AHL
I 1876	DNA Polymerase III, α Subunit	-1.9	-1.6	AHL
I 1908	DNA Polymerase III, δ and τ Subunits	-1.7	-1.3	VjbR
I 1941	RecF Protein	3.0	2.0	AHL
I 1946	Formamidopyrimidine-DNA Glycosylase	2.5	2.0	AHL
II 0184	Transposase	-1.7	-2.0	VjbR
II 0453	Transposase	-1.8	-1.9	AHL
II 0714	Transposase	-1.8	-1.9	AHL
II 0718	Transposase	-1.9	-2.0	AHL
	Secondary Metabolites Biosynthesis, Transport and Catabolism			
I 1167	Putative Aromatic Compound Catabolism Protein	3.0	2.2	AHL
I 1504	Acetylspermidine Deacetylase	-2.7	-2.5	VjbR
I 1860	Predicted ABC-type Transport System Lysophospholipase L1 Biosynthesis	-1.9	-	AHL
II 0079	Isochorismatase	2.4	1.6	AHL
II 0889	Phenylacetic Acid Degradation Protein, PaaI	-1.8	-2.2	AHL
	Signal Transduction Mechanisms			
I 0929	Diguanylate Cyclase/Phosphodiesterase Domain 1, GGDEF Domain	2.0	1.8	VjbR
II 0011	Transcriptional Regulatory Protein, HydG	-1.8	-2.1	AHL
II 0654	Diguanylate Cyclase/Phosphodiesterase Domain 1, GGDEF Domain	-3.1	-1.8	AHL

TABLE 8--*Continued*

Locus	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student 's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Condition
Transcription				
I 0446	MarR Family Transcriptional Regulator	3.4	2.3	AHL
I 0781	DNA-Directed RNA Polymerase α Subunit	3.4	2.2	AHL
I 1291	AraC Family Transcriptional Regulator	-1.6	-2.1	AHL
I 1383	AraC Family Transcriptional Regulator	-2.3	-2.5	VjbR
I 1598	LysR Family Transcriptional Regulator	-1.8	-2.0	AHL
I 1607	LuxR Family Glycerol Metabolism Activator	3.5	2.5	AHL
II 0370	GntR Family, Histidine Utilization Repressor	-1.7	-1.9	VjbR
II 0520	MarR Family Transcriptional Regulator	-2.3	-1.5	VjbR
II 0576	LysR Family Transcriptional Regulator	-1.8	-	VjbR
II 0657	Regulator of Nucleoside Diphosphate Kinase	3.5	2.6	AHL
II 0800	AraC Family Transcriptional Regulator	2.1	1.3	VjbR
II 0807	GntR Family Transcriptional Regulator	-1.5	-2.0	VjbR
II 0810	ArsR Family Transcriptional Regulator	2.5	1.4	AHL
Translation, Ribosomal Structure and Biogenesis				
I 0277	Heat Shock Protein 15	1.9	1.2	AHL
I 0327	Protein Translation Elongation Factor P (EF-P)	-2.0	-3.1	AHL
I 0428	tRNA (5-Methylaminomethyl-2-Thiouridylate)-Methyltransferase	-2.1	-2.2	AHL
I 0444	Methyltransferase	3.2	2.2	AHL
I 0747	50S Ribosomal Protein L10P	-1.8	-1.4	VjbR
I 0779	30S Ribosomal Protein S13	2.6	1.6	AHL
I 0890	Queuine tRNA-Ribosyltransferase	-1.7	-2.0	AHL
I 1089	Arginyl-tRNA Synthetase	-1.5	-1.5	VjbR
I 1103	NifR3-Like Protein	-1.9	-1.7	AHL
I 1862	2-5 RNA Ligase	-1.9	-1.6	AHL
I 1961	Polyribonucleotide Nucleotidyltransferase	1.8	1.2	AHL
II 0289	Glutamyl-tRNA(GLN) Amidotransferase Subunit A	2.0	-	AHL
II 0500	Lysyl-tRNA Synthetase	-2.0	-2.3	AHL
Unknown and Other				
I 0011	Hypothetical Protein	2.5	1.9	AHL
I 0038	Hypothetical Protein	-2.2	-2.1	AHL
I 0041	Hypothetical Protein	-1.8	-1.6	AHL
I 0055	Hypothetical Protein	3.2	2.0	AHL
I 0057	Hypothetical Membrane Spanning Protein	3.1	1.5	AHL
I 0172	Hypothetical Protein	-1.7	-1.9	VjbR
I 0194	Hypothetical Cytosolic Protein	-2.2	-1.3	AHL
I 0220	Hypothetical Protein	-2.4	-2.1	VjbR
I 0290	Hypothetical Cytosolic Protein	-1.8	-1.7	AHL
I 0308	Hypothetical Protein	-2.1	-1.7	VjbR
I 0389	Hypothetical Protein	-1.7	-2.0	AHL
I 0419	Hypothetical Protein	-1.5	-1.7	AHL
I 0443	Hypothetical Protein	2.9	1.8	AHL
I 0448	Hypothetical Protein	2.1	1.6	AHL
I 0535	Hypothetical Protein	2.2	1.6	VjbR
I 0627	Hypothetical Protein	-2.1	-2.1	AHL
I 0652	Hypothetical Protein	2.3	1.5	VjbR
I 0699	Hypothetical Protein	-1.9	-1.5	VjbR
I 0723	Hypothetical Protein	-3.0	-2.1	AHL
I 0751	Hypothetical Protein	-1.5	-1.1	VjbR

TABLE 8--*Continued*

Locus	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student 's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Condition
I 0308	Hypothetical Protein	-2.1	-1.7	VjbR
I 0389	Hypothetical Protein	-1.7	-2.0	AHL
I 0419	Hypothetical Protein	-1.5	-1.7	AHL
I 0443	Hypothetical Protein	2.9	1.8	AHL
I 0448	Hypothetical Protein	2.1	1.6	AHL
I 0535	Hypothetical Protein	2.2	1.6	VjbR
I 0627	Hypothetical Protein	-2.1	-2.1	AHL
I 0652	Hypothetical Protein	2.3	1.5	VjbR
I 0699	Hypothetical Protein	-1.9	-1.5	VjbR
I 0723	Hypothetical Protein	-3.0	-2.1	AHL
I 0751	Hypothetical Protein	-1.5	-1.1	VjbR
I 0822	Hypothetical Protein	-1.9	-1.9	AHL
I 0809	Predicted Membrane Protein	-1.6	-1.2	VjbR
I 1006	Hypothetical Cytosolic Protein	-1.8	-2.2	AHL
I 1008	Hypothetical Protein	-1.9	-2.5	AHL
I 1011	Hypothetical Protein	-2.7	-2.8	AHL
I 1013	Hypothetical Membrane Spanning Protein	-1.7	-2.0	AHL
I 1048	Hypothetical Membrane Associated Protein	-1.9	-2.4	VjbR
I 1072	Hypothetical Protein	-2.4	-2.8	VjbR
I 1095	Hypothetical Protein	-1.8	-2.3	AHL
I 1107	Hypothetical Cytosolic Protein	-1.8	-2.2	AHL
I 1135	Hypothetical Protein	-1.7	-1.3	VjbR
I 1141	Predicted Exported Protein	-1.6	-1.6	VjbR
I 1162	Hypothetical Protein	-2.7	-2.8	AHL
I 1165	Hypothetical Membrane Spanning Protein	2.5	1.9	AHL
I 1214	Hypothetical Protein	2.5	2.0	VjbR
I 1314	Hypothetical Protein	2.1	1.8	AHL
I 1356	Hypothetical Protein	1.8	1.2	AHL
I 1361	Hypothetical Cytosolic Protein	-1.9	-1.7	VjbR
I 1434	Hypothetical Protein	-2.1	-2.3	AHL
I 1595	Hypothetical Protein	-2.0	-2.1	AHL
I 1674	Hypothetical Protein	-2.0	-2.1	AHL
I 1699	Hypothetical Protein	-1.6	-2.2	VjbR
I 1703	Hypothetical Protein	-2.1	-2.5	VjbR
I 1724	Hypothetical Protein	-1.9	-1.8	AHL
I 1756	Hypothetical Protein	-2.1	-1.2	VjbR
I 1788	Hypothetical Protein	-1.9	-2.0	AHL
I 1826	Hypothetical Protein	-1.7	-1.8	VjbR
I 1842	Hypothetical Protein	-1.8	-2.2	AHL
I 1891	Hypothetical Protein	-1.7	-2.0	AHL
I 1920	Hypothetical Protein	-1.6	-1.7	VjbR
I 1929	Hypothetical Protein	-2.3	-2.6	AHL
I 2006	Hypothetical Cytosolic Protein	-2.0	-2.6	AHL
II 0118	Hypothetical Protein	-2.4	-2.5	AHL
II 0153	Hypothetical Protein	-1.6	-1.8	AHL
II 0187	Hypothetical Cytosolic Protein	-2.1	-1.8	VjbR
II 0191	Hypothetical Protein	-	-1.9	AHL
II 0330	Hypothetical Protein	-2.2	-2.5	AHL
II 0331	Hypothetical Cytosolic Protein	2.3	1.4	AHL
II 0412	Hypothetical Protein	-2.1	-2.2	AHL
II 0647	Hypothetical Protein	-2.8	-2.7	AHL
II 0693	Hypothetical Cytosolic Protein	-2.2	-2.3	AHL
II 0726	Hypothetical Protein	-2.6	-2.0	VjbR

TABLE 8--*Continued*

Locus	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student 's *t*-test FC	Z-score FC	Condition
II 0833	Hypothetical Protein	-1.7	-2.1	AHL
II 0841	Hypothetical Protein	-2.1	-2.2	AHL
II 0877	Hypothetical Protein	-2.5	-2.7	AHL

*Wild type (WT) replicates 1&2 were used for the Student's *t*-test analysis. Fold change (FC) values are the log ratio of normalized signal values. FC values shown in grey did not pass the statistical test indicated in that column. Abbreviations are as follows: FC, Fold Change; AA, Amino Acid; OMP, Outer Membrane Protein, G3P, Glycerol 3 Phosphate.

TABLE 9. Genes altered at early stationary growth phase by VjbR or C_{12}-HSL.

BME Loci	Gene Name	Δ*vjbR* / wt or wt+ahl / wt: Student's *t*-test FC	Δ*vjbR* / wt or wt+ahl / wt: Z-score FC	Condition
	Amino Acid Transport and Metabolism			
I 0207	γ-Glutamyl Kinase	1.6	1.7	VjbR
I 1378	L-Asparaginase II	1.6	1.7	VjbR
I 1621	Bifunctional N-Succinyldiaminopimelate-Aminotransferase, Aacetylornithine Transaminase Protein	1.8	1.8	AHL
I 1939	D-3-Phosphoglycerate Dehydrogenase	-1.8	-1.6	VjbR
I 2043	Imidazole Glycerol Phosphate Synthase Subunit, HisH Protein	-1.7	-1.4	VjbR
II 0038	D-Serine, D-Alanine, Glycine Transporter	-1.9	-1.7	AHL
II 0070	ABC-Type Transporter Leucine, Isoleucine, Valine, Threonin and Alanine Binding Protein	-2.7	-2.3	VjbR
II 0098	ABC-Type Transporter High-Affinity Branched Chain AA Transport ATP Binding Protein, LivF	1.6	1.5	VjbR
II 0285	ABC-Type Transporter Dipeptide Transport System Permease Protein, DppB	1.6	1.8	VjbR
II 0339	Alcohol Dehydrogenase (Acceptor)	1.6	1.7	VjbR
II 0348	4-Aminobutyrate Aminotransferase	1.6	1.5	VjbR
II 0407	Aspartate-Semialdehyde Dehydrogenase	-1.6	-1.5	VjbR
II 0567	ABC-Type Spermidine/Putrescine/Fe^{3+} Transport ATP Binding Protein, SfuC	1.8	1.9	VjbR
II 0583	ABC-Type Spermidine/Putrescine/Fe^{3+} Transport ATP Binding Protein, SfuC	-1.9	-1.7	VjbR
	Carbohydrate Transport and Metabolism			
I 0309	Phosphoglycerate Kinase	1.5	1.6	VjbR
I 1435	Polysaccharide Deacetylase	1.6	1.7	VjbR
II 0106	Xylose Transcriptional Repressor	-	1.6	VjbR
II 0181	N-Acetylglucosamine Kinase	-2.2	-1.7	VjbR
II 0301	ABC-Type Transporter Ribose /Xylose/ Arabinose/ Galactoside Transport System Permease Protein, RbsC	-2.0	-1.8	VjbR
II 0357	2-Dehydro-3-Deoxygalactonokinase	-1.7	-1.2	VjbR
II 0358	2-Dehydro-3-Deoxyphosphogalactonate Aldolase, Keto-Hydroxyglutarate-Aldolase/ Keto-Deoxy-Phosphogluconate Aldolase	-1.8	-1.5	VjbR
II 0568	Myo-Inositol-1(Or 4)-Monophosphatase	-1.8	-1.7	AHL
II 0700	Galactoside Transport System Permease Protein, MglC	-1.9	-2.2	VjbR
II 0702	ABC-Type Surface Lipoprotein, Simple Sugar Transport System Periplasmic Binding Protein	-2.8	-	AHL
II 0821	MDR Protein	-1.9	-1.5	AHL
II 0941	ABC-Type Maltose/Maltodextrin ATP Binding Transport Protein, MalK	-2.0	-1.9	VjbR
II 1092	Hydroxypyruvate Isomerase	1.8	1.8	AHL
	Cell Cycle Control, Cell Division and Chromosome Partitioning			
I 0007	Glucose Inhibited Division Protein A, GidA	-2.0	-1.6	AHL
I 0213	Metalloendopeptidase	2.0	2.2	VjbR
I 0313	Hypothetical Cytosolic Protein, ZapA	-1.7	-1.6	AHL
II 0093	Replication Protein A	-1.9	-1.7	AHL
II 0469	Integral Membrane Protein	-1.6	-	AHL
II 0470	Integral Membrane Protein, Chromosome Condensation	-2.5	-2.0	VjbR
	Cell Motility			
I 1692	Flagellar Protein, FlgJ	-1.9	-1.7	VjbR
II 1109	Chemotaxis Protein, MotA	-2.0	-1.4	VjbR

TABLE 9—*Continued*

BME Loci	Gene Name	Δ*vjbR* / wt or wt+ahl / wt: Student's *t*-test FC	Δ*vjbR* / wt or wt+ahl / wt: Z-score FC	Condition
	Cell Wall, Membrane and Envelope Biogenesis			
I 0402	31 KDa OMP Precursor	2.0	-1.7	VjbR
I 0581	UDP-N-Acetylenolpyruvoyl-Glucosamine Reductase	-2.0	-1.9	AHL
I 0830	Outer Membrane Protein, Omp89	-1.7	-1.5	VjbR
I 0991	Rare Lipoprotein A	2.5	2.7	VjbR
I 1056	N-Acetylmuramoyl-L-Alanine Amidase	-2.1	-1.9	VjbR
I 1079	Lipoprotein NlpD	-1.9	-1.8	AHL
I 1177	Putative Colanic Biosynthesis UDP-Glucose Lipid Carrier Transferase	1.5	1.5	VjbR
I 1356	Peptidoglycan Binding Protein	-1.7	-1.4	VjbR
I 1417	Perosamine Synthetase, WbkB	-2.4	-2.1	AHL
I 1858	Phosphinothricin N-Acetyltransferase	-1.6	-1.6	AHL
	Coenzyme Transport and Metabolism			
I 0700	Precorrin-3B C17-Methyltransferase Protein, CbiG	2.0	2.2	VjbR
I 0702	Precorrin-6x Reductase	-1.9	2.5	VjbR
I 0703	Cobalt-Precorrin-6A Synthase Protein, CbiD	-2.2	-1.9	AHL
I 1021	Molybdopterin-Guanine Dinucleotide Biosynthesis Protein B	-1.6	-1.3	VjbR
I 1771	Coproporphyrinogen III Oxidase	-	1.6	VjbR
I 1834	Ubiquinone/Menaquinone Biosynthesis Methyltransferase, UbiE	-2.0	-1.7	VjbR
II 0528	Glutamate-Cysteine Ligase	-2.5	-2.0	AHL
	Defense Mechanisms			
I 0240	Fusaric Acid Resistance Protein	-1.6	-1.3	VjbR
I 0356	Type 1 Capsular Polysaccharide Biosynthesis Protein J	1.6	1.6	VjbR
I 0893	Acriflavin Resistance Protein B	1.6	1.5	AHL
I 1743	ABC-Type Multidrug Transporter ATP Binding Protein	-1.8	-1.6	AHL
II 0382	Acriflavin Resistance Protein D	-1.9	-1.7	VjbR
II 0795	MDR Protein B	1.8	1.7	AHL
	Energy Production and Conversion			
I 0928	Acetate CoA-Transferase α-Subunit	-1.5	-1.3	VjbR
I 0967	NAD-Dependant Malic Enzyme, Phosphate Acetyltransferase	-1.6	-1.3	VjbR
I 1903	Cytochrome C-552	1.6	1.9	VjbR
II 0224	Formyl-Coenzyme A Transferase	-2.0	-1.7	VjbR
II 0429	Erythritol-4-Phosphate Dehydrogenase	-	1.5	VjbR
II 0952	Nitrate Reductase Δ Chain	1.6	1.7	VjbR
II 0965	Pseudoazurin	-2.0	-1.6	AHL
II 1073	Cytochrome B561	-2.0	-1.5	VjbR
	General Function Prediction Only			
I 0129	Hydroxyacylglutathione Hydrolase	1.5	1.5	VjbR
I 0506	DME Family Transporter	-	1.5	VjbR
I 0698	Transporter	1.6	1.6	AHL
I 0982	Permeases Of The DMT Superfamily Transporter	-1.9	-1.6	VjbR
I 1094	Exopolysaccharide Production Negative Regulator Precursor, TPR Repeat	2.0	2.2	VjbR
I 1239	Predicted Permease	1.9	2.1	VjbR
II 0655	Alkaline Phosphatase	-2.5	-1.7	VjbR
II 0677	DME Family Transporter	1.7	1.5	VjbR
II 0831	Hypothetical Protein, Aminopeptidase-Like Domain	-2.3	-2.5	VjbR
II 1037	Zinc Protease	-1.9	-1.8	VjbR
II 1100	Cellobiose Phosphotransferase System Protein, CelC	1.5	1.6	VjbR
II 1121	ABC-Type Fe^{3+} Transport System Permease Protein, SfuB	-2.0	-1.8	AHL

TABLE 9—*Continued*

BME Loci	Gene Name	Δ*vjbR* / wt or wt+ahl / wt: Student's *t*-test FC	Δ*vjbR* / wt or wt+ahl / wt: Z-score FC	Condition
	Inorganic Ion Transport and Metabolism			
I 0044	CBS Domain Containing Protein	1.7	1.7	VjbR
I 0622	Kup System Potassium Uptake Protein	-1.7	-1.6	VjbR
I 0641	ABC-Type Co^{2+} Transport System Hypothetical Protein	-1.9	-1.6	VjbR
I 0660	ABC-Type Cobalamin/Fe^{3+} Siderophores Transport ATP Binding Protein	1.6	1.5	VjbR
I 0995	Secretion Activator Protein	1.7	1.8	VjbR
I 1292	Fosmidomycin Resistance Protein	-2.2	-2.3	VjbR
II 0704	Bacterioferritin	-1.7	-1.5	VjbR
	Intracellular Trafficking, Secretion and Vesicular Transport			
I 0883	Multiple Antibiotic Resistance Protein	1.8	1.7	VjbR
I 1692	Flageller Protein, FlgJ	-2.2	-2.0	AHL
II 0188	Hypothetical Cytosolic Protein, Type III Effector Hrp-Dependent Outer Proteins Domain	1.6	1.8	VjbR
II 1069	Adhesin, AidA	-1.6	-1.3	VjbR
	Lipid Transport and Metabolism			
I 0827	Undecaprenyl Pyrophosphate Synthetase	-1.6	-1.3	VjbR
I 1956	3-Hydroxydecanoyl-ACP Dehydratase	1.6	1.5	VjbR
	Nucleotide Transport and Metabolism			
I 0155	Putative Allantoin Permease	-1.6	-1.2	AHL
I 0476	Adenine Phosphoribosyltransferase	1.6	1.6	VjbR
I 0608	Thymidylate Synthase	1.6	1.6	VjbR
I 1117	Adenylosuccinate Lyase	-2.0	-1.6	VjbR
I 1318	AMP Nucleosidase	1.7	1.7	VjbR
I 1611	Dihydroorotate Dehydrogenase	-1.8	-1.4	VjbR
	Posttranslational Modification, Protein Turnover and Chaperones			
I 0887	Peptidyl-Prolyl Cis-Trans Isomerase	1.7	1.7	VjbR
I 1049	Bacterioferritin Comigratory Protein	1.6	1.5	VjbR
I 1080	Protein-L-Isoaspartate O-Methyltransferase	1.5	1.5	VjbR
I 1619	Hsp33-Like Chaperonin	1.8	1.7	VjbR
I 1851	ABC-Type Heme Exporter Protein C Transport System	1.6	1.9	VjbR
I 2048	ATP-Dependent Protease ATP Binding Subunit	1.6	1.6	VjbR
	Replication, Recombination and Repair			
I 0728	Single-Stranded-DNA-DNA-Specific Exonuclease, RecJ	-1.9	-1.5	VjbR
I 0784	ATPase Protein	-1.7	-	VjbR
I 1424	Transposase	-2.3	-1.8	VjbR
I 1942	DNA Polymerase III β Subunit	-1.8	-1.4	VjbR
I 2015	Dinucleoside Polyphosphate Hydrolase	-	1.5	VjbR
	Secondary Metabolites Biosynthesis, Transport and Catabolism			
I 0032	3-OxoAcyl-(ACP) Reductase	1.7	1.8	VjbR
	Signal Transduction Mechanisms			
I 0372	Two-Component Response Regulator	1.7	1.8	AHL
I 1678	Hypothetical Protein, Sensory Transduction Protein Kinase	-2.3	-1.8	VjbR
I 1811	Acid Tolerance Regulatory Protein, ActR	1.6	1.8	VjbR
II 0659	Two Component Response Regulator	-1.9	-1.6	VjbR
II 1014	Two Component Response Regulator	1.6	1.5	VjbR
	Transcription			
I 0387	IclR Family Transcriptional Regulator	-1.9	-1.8	AHL
I 0604	TetR Family Transcriptional Regulator	-	1.5	VjbR
I 0891	TetR Family Transcriptional Regulator	1.5	1.6	VjbR
I 1291	AraC Family Transcriptional Regulator	1.7	1.7	VjbR

TABLE 9—*Continued*

BME Loci	Gene Name	Δ*vjbR* / wt or wt+ahl / wt: Student's *t*-test FC	Δ*vjbR* / wt or wt+ahl / wt: Z-score FC	Condition
I 1885	LysR Family Transcriptional Regulator	-1.6	-1.5	VjbR
II 0127	IclR Family Acetate Operon Repressor	-1.9	-1.6	VjbR
II 0345	LysR Family Transcriptional Regulator	2.0	1.5	AHL
II 0641	AraC-Type Transcriptional Regulator	-1.8	-1.4	VjbR
II 0810	ArsR Family Transcriptional Regulator	1.8	1.7	VjbR
II 0894	LysR Family Hydrogen Peroxide-Inducible Gene Activator	-1.9	-1.5	VjbR
II 0966	CRP Family Transcriptional Regulator	-	1.6	VjbR
II 1007	GntR Family Transcriptional Regulator	-	1.6	VjbR
II 1022	IclR Family Transcriptional Regulator	-2.1	-1.7	VjbR
	Translation, Ribosomal Structure and Biogenesis			
I 0322	50S Ribosomal Protein L31	1.6	1.9	VjbR
I 0429	23S Ribosomal RNA Methyltransferase	-1.9	-1.6	VjbR
I 0752	30S Ribosomal Protein S12	-	1.5	VjbR
I 0987	Methionine—tRNA Ligase	1.6	1.6	VjbR
I 1184	Small Protein A	-	1.5	VjbR
I 1203	Ribonuclease D	-2.0	-2.3	VjbR
I 1267	Dimethyladensine Transferase, KsgA	-1.7	-1.3	VjbR
I 1360	Glutamyl-tRNA(GLN) Amidotransferase Subunit A, Amidase	-2.3	1.0	VjbR
II 0002	Ribosomal Protein Serine Acetyltransferase	-2.1	-1.7	VjbR
II 0675	Glutamyl-tRNA Amidotransferase Subunit A	1.5	1.5	VjbR
II 0812	Peptide Deformylase	1.7	1.8	VjbR
II 1056	Histidyl-tRNA Synthetase	-1.9	-1.6	VjbR
	Unknown and Other			
I 0051	Hypothetical Protein	-2.1	-1.7	VjbR
I 0212	Hypothetical Protein	-2.0	-1.8	AHL
I 0304	Hypothetical Cytosolic Protein	-2.0	-1.7	AHL
I 0354	Hypothetical Membrane Spanning Protein	1.5	1.5	VjbR
I 0362	Predicted Periplasmic protein	2.0	2.2	VjbR
I 0602	Hypothetical Protein	2.0	2.1	VjbR
I 0738	Hypothetical Protein	1.8	1.8	VjbR
I 0806	Hypothetical Protein	1.7	1.8	VjbR
I 0952	Predicted Membrane Protein	1.7	1.6	VjbR
I 0993	Hypothetical Protein	1.6	1.6	VjbR
I 1242	Hypothetical Membrane Spanning Protein	1.7	1.7	AHL
I 1290	Hypothetical Membrane Spanning Protein	-2.1	-1.5	AHL
I 1358	Hypothetical Cytosolic Protein	-1.6	-1.4	VjbR
I 1572	Predicted Membrane Protein	-1.7	-1.3	VjbR
I 1647	Hypothetical Protein	-2.0	-1.8	VjbR
I 1658	Hypothetical Protein	-	1.5	VjbR
I 1684	Hypothetical Protein	-1.7	-1.8	VjbR
I 1696	Hypothetical Membrane Spanning Protein	-1.9	-1.8	AHL
I 1842	Hypothetical Protein	-1.9	-1.6	VjbR
I 1865	Hypothetical Protein	1.5	1.6	VjbR
I 1933	Hypothetical Protein	-1.9	-1.7	VjbR
II 0022	Hypothetical Protein	-2.1	-1.9	AHL
II 0153	Hypothetical Protein	-1.7	-1.4	VjbR
II 0244	Hypothetical Protein	-2.0	-1.5	AHL
II 0379	Hypothetical Protein	1.6	1.7	VjbR
II 0399	Hypothetical Protein	-1.6	-1.4	AHL
II 0503	Hypothetical Protein	-1.7	-1.5	VjbR
II 0525	Hypothetical Protein	1.8	2	VjbR

TABLE 9—*Continued*

BME Loci	Gene Name	Δ*vjbR* / wt or wt+ahl / wt: Student's *t*-test FC	Δ*vjbR* / wt or wt+ahl / wt: Z-score FC	Condition
II 0534	Hypothetical Protein	-1.8	-1.6	AHL
II 0595	Hypothetical Protein	1.6	1.8	VjbR
II 0615	Hypothetical Protein	1.5	1.7	VjbR
II 0732	Hypothetical Protein	-1.7	-1.5	AHL
II 0788	Hypothetical Protein	1.6	1.5	VjbR
II 0842	Hypothetical protein	-1.8	-1.6	VjbR
II 0918	Hypothetical Protein	-	1.6	VjbR
II 0919	Hypothetical Protein	1.6	1.7	VjbR
II 1013	Hypothetical Protein	1.6	1.8	VjbR

Fold change (FC) values are the log ratio of normalized signal values. FC values shown in grey did not pass the statistical test. Abbreviations as follows: FC, Fold Change; OMP, Outer Membrane Protein; DME, Drug/Metabolite Exporter; DMT, Drug/Metabolite Transporter; ACP, Acyl-Carrier Protein.

phase. Additionally, both conditions down regulate signal transduction systems and exert an affect on downstream transcriptional regulators; including members of MarR, AraC, LysR, GntR, ArsR and a LuxR-like transcriptional regulator (BMEI 1607).

Putative virulence factors and effector proteins were found to be regulated by VjbR at the stationary growth phase. VjbR positively regulates a protease and an adhesin protein that could be important for uptake or interaction with the brucellosome membrane. VjbR is also involved in the repression of two potential effector or secreted proteins; a secretion activating protein (BMEI 0995) and a HRP-type effector of the type III secretion system (BMEII 0188) at the stationary growth phase.

Genes regulated by quorum sensing components VjbR or C_{12}-HSL are regulated in a bi-phasic manner. A small subset of genes regulated by VjbR and/or C_{12}-HSL were found to be significantly altered at both time points examined, mid log and early stationary growth phases. A total of 101 genes were found to be regulated in a bi-phasic manner; 45 by VjbR, 47 by C_{12}-HSL and 9 genes regulated by both conditions and are presented in Table 10. The majority of the genes (75 %) regulated by VjbR at both time points were inversely related, while only 25 % of the C_{12}-HSL genes were regulated in different directions at the two time points. Microarray analysis of quorum

TABLE 10. Altered genes regulated bi-phasicly by VjbR or C_{12}-HSL.

BME Loci	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Growth Phase	Condition
	Amino Acid Transport and Metabolism				
I 0114	Asparagine-Binding Periplasmic Protein	1.7	1.5	Log	VjbR
		1.7	1.9	Stat	
I 0208	γ-Glutamyl Phosphate Reductase	-1.8	-1.8	Log	VjbR
		-2.1	-1.6	Stat	
I 0615	SerB Protein	-1.8	-1.8	Log	VjbR
		1.6	1.8	Stat	
I 1381	Choline Dehydrogenase	-	-1.6	Log	AHL
		-1.9	-1.8	Stat	
II 0205	ABC-Type Dipeptide Transport ATP Binding Protein, DppF	-1.7	-2.1	Log	VjbR
		1.6	1.5	Stat	
II 0484	ABC-Type Spermidine/Putrescine Transporter Integral Membrane Protein	-2.4	-2.5	Log	AHL
		-2.1	-1.9	Stat	
II 0517	ABC-Type Branched Chain AA Transport Permease Protein, AzlC	-1.7	-1.8	Log	VjbR
		-2.4	-2.0	Stat	
II 0602	N-Methylhydantoinase, 5-Oxoprolinase	-	-1.7	Log	AHL
		-2.1	-1.8	Stat	
	Carbohydrate Transport and Metabolism				
I 0070	Aquaporin Z	-2.0	-1.7	Log	VjbR
		1.7	1.9	Stat	
I 0070	Aquaporin Z	-2.1	-2.3	Log	AHL
		1.6	1.8	Stat	
I 0663	ABC-Type D-Ribose-Binding Periplasmic Protein Precursor Transport System	-	-1.4	Log	AHL
		-	-2.2	Stat	
II 0300	ABC-Type Ribose ATP-BindingTransport Protein, RbsA	-2.0	-1.7	Log	VjbR
		1.7	1.7	Stat	
II 0724	Endoglucanase H	-1.7	-1.8	Log	AHL
		1.6	1.7	Stat	
	Cell Cycle Control, Cell Division and Chromosome Partitioning				
I 1174	Chromosome Partitioning ATPase, ParA Family	1.8	1.8	Log	AHL
		1.7	1.8	Stat	
	Cell Wall, Membrane and Envelope Biogenesis				
I 1037	Glycosyltransferase	1.7	1.5	Log	VjbR
		2.5	2.8	Stat	
I 1037	Glycosyltransferase	2.2	1.6	Log	AHL
		1.9	2.0	Stat	
II 0157	Soluble Lytic Murein Transglycosylase	-1.9	-2.2	Log	AHL
		-2.3	-1.9	Stat	
II 0440	dTDP-Glucose 4,6-Dehydratase	-1.5	-1.6	Log	VjbR
		1.9	2.0	Stat	
II 0730	UDP-Glucose 4-Epimerase	-2.1	-2.1	Log	VjbR
		1.8	1.7	Stat	
II 0838	Succinoglycan Biosynthesis Transport Protein ExoT	-5.0	-3.5	Log	AHL
		-1.7	-1.6	Stat	
	Coenzyme Transport and Metabolism				
I 0315	5-Formyltetrahydrofolate Cyclo-Ligase	-1.8	-2.2	Log	AHL
		-1.7	-1.5	Stat	
I 1517	Pyridoxamine 5'-phosphate Oxidase	-1.8	-1.7	Log	AHL
		-2.2	-1.8	Stat	

TABLE 10—*Continued*

BME Loci	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Growth Phase	Condition
I 1768	Uroporphyrin-III C-Methyltransferase, Precorrin-2 Oxidase, Ferrochelatase	-2.3	-3.7	Log	AHL
		-2.3	-2.0	Stat	
I 1768	Uroporphyrin-III ^{1}C-Methyltransferase / Precorrin-2 Oxidase / Ferrochelatase	-2.1	-2.0	Log	VjbR
		1.6	1.8	Stat	
II 0235	Phosphopantothenoylcysteine Synthase/Decarboxylase	-1.6	-1.6	Log	VjbR
		-	1.5	Stat	
	Defense Mechanisms				
I 0654	ABC-Type Multi-Drug Transporter ATP Binding Protein	-1.7	-1.6	Log	VjbR
		1.9	2.0	Stat	
II 0473	Acriflavin Resistance Protein F	-1.9	-1.7	Log	VjbR
		-	1.8	Stat	
II 0801	ABC-Type Daunorubicin Resistance Transmembrane Protein	-1.7	-2.8	Log	VjbR
		-1.9	-1.5	Stat	
	Energy Production and Conversion				
I 0017	Alkanal Monooxygenase α-Chain	-1.5	-1.7	Log	AHL
		2.0	2.1	Stat	
I 1900	Cytochrome O Ubiquinol Oxidase Subunit I	-	-1.6	Log	AHL
		-2.0	-1.8	Stat	
I 2037	Phosphoenolpyruvate Carboxykinase	-1.6	-1.6	Log	AHL
		1.6	1.5	Stat	
II 0074	Thiosulfate Reductase Cytochrome β Subunit	2.7	2.9	Log	VjbR
		-1.9	-1.6	Stat	
II 0394	Glycerol Trinitrate Reductase	-2.0	-1.7	Log	VjbR
		2.4	1.8	Stat	
II 0974	Nitrous Oxide Reductase	-3.2	-3.1	Log	AHL
		-1.7	-1.7	Stat	
	General Function Prediction Only				
I 0394	2-Deoxy-D-Gluconate 3-Dehydrogenase	2.1	1.7	Log	AHL
		-1.9	-1.5	Stat	
I 0739	Integral Membrane Protein, Rhomboid Family	-	-1.6	Log	AHL
		-1.8	-1.8	Stat	
I 1038	Phenylacetic Acid Degradation Protein, PaaD	-1.6	-1.5	Log	VjbR
		1.7	1.8	Stat	
I 1531	Tetratricopeptide Repeat Family Protein	-1.7	-2.4	Log	VjbR
		-1.7	1.8	Stat	
I 2003	Trans-1,2-Dihydrobenzene-1,2-Diol Dehydrogenase, D-Xylose 1-Dehydrogenase	-2.5	-1.8	Log	VjbR
		2.0	2.2	Stat	
II 0149	Extracellular Serine Protease	-2.0	-1.6	Log	AHL
		-1.6	-1.7	Stat	
II 0211	Penicillin Acylase	-2.0	-1.7	Log	VjbR
		1.8	1.8	Stat	
II 0611	Integral Membrane Protein, Predicted Permease	-2.3	-1.5	Log	VjbR
		-2.4	-3.9	Stat	
II 0865	1-Carboxy-3-Chloro-3,4-Dihydroxycyclo Hexa-1,5-Diene Dehydrogenase	-	-1.9	Log	AHL
		-2.3	-2.0	Stat	
II 0866	Oxidoreductase	-1.9	-1.5	Log	VjbR
		1.8	2.0	Stat	

TABLE 10—*Continued*

BME Loci	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Growth Phase	Condition
	Inorganic Ion Transport and Metabolism				
I 1739	ABC-Type Nitrate, Sulfonate, Bicarbonate Transporter	-2.0	-1.6	Log	AHL
	Integral Membrane Protein	-2.2	-1.9	Stat	
I 1954	ABC Transporter Substrate Binding Protein	-1.7	-2.2	Log	VjbR
		2.0	2.0	Stat	
I 1954	ABC-Type Metal Ion Transport System Transporter Substrate	-1.5	-1.7	Log	AHL
	Binding Protein	2.1	2.0	Stat	
II 0097	Cation Transport, P-Type	-	-1.7	Log	AHL
		-1.8	-1.6	Stat	
	Intracellular Trafficking, Secretion and Vesicular Transport				
II 0025	Attachment Mediating Protein, VirB1	-2.1	-2.3	Log	VjbR
		-2.7	-2.4	Stat	
II 0025	Attachment Mediating Protein, VirB1	-2.1	-1.6	Log	AHL
		-2.2	-2.2	Stat	
II 0026	Attachment Mediating Protein, VirB2	-2.3	-1.9	Log	AHL
		-3.9	-3.3	Stat	
II 0029	Attachment Mediating Protein, VirB5	-1.8	-2.1	Log	VjbR
		-6.2	-5.1	Stat	
II 0034	Channel Protein, VirB10	-1.5	-1.5	Log	AHL
		-2.0	-1.8	Stat	
	Lipid Transport and Metabolism				
II 0646	Acetyl-CoA Acetyltransferase	-1.7	-1.7	Log	VjbR
		1.6	1.6	Stat	
II 1103	Phosphatidylglycero-Phosphatase B	-	-1.7	Log	AHL
		-2.3	-2.1	Stat	
	Nucleotide Transport and Metabolism				
I 0082	Hypoxanthine-Guanine Phosphoribosyltransferase H	-1.5	-1.5	Log	VjbR
		1.9	1.7	Stat	
I 1090	Deoxyguanosinetriphosphate Triphosphohydrolase-Like	-	-1.6	Log	AHL
	Protein	1.6	1.7	Stat	
I 1430	Ureidoglycolate Hydrolase	-1.6	-1.7	Log	AHL
		-2.7	-2.3	Stat	
I 1430	Ureidoglycolate Hydrolase	-1.7	-1.7	Log	VjbR
		-1.9	-1.7	Stat	
II 0627	Probable Adenine Deaminase	-2.1	-1.6	Log	VjbR
		-	1.6	Stat	
	Posttranslational Modification, Protein Turnover and Chaperones				
I 0887	Peptidyl-Prolyl Cis-Trans Isomerase	-1.7	-1.6	Log	AHL
		1.6	1.6	Stat	
I 1463	Cytochrome C Oxidase Assembly Protein	-1.9	-1.6	Log	VjbR
		1.7	1.9	Stat	
I 1464	Protoheme IX Farnesyltransferase	-2.1	-2.7	Log	AHL
		-2.2	-1.9	Stat	
I 1804	PII Uridylyl-Transferase	-	3.2	Log	VjbR
		-	2.3	Stat	
	Replication, Recombination and Repair				
I 0040	Tyrosine Recombinase	-1.8	-1.6	Log	VjbR
		1.6	1.5	Stat	
I 1097	Uracil-DNA Glycosylase	-2.3	-2.2	Log	AHL
		-2.0	-1.6	Stat	
I 1664	Recombinase XerD	-2.3	-2.0	Log	VjbR
		2.7	2.3	Stat	

TABLE 10—*Continued*

BME Loci	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Growth Phase	Condition
I 1664	Recombinase, XerD	-1.9	-1.8	Log	AHL
		1.9	1.8	Stat	
I 1818	ATP Dependent Helicase, HrpB	-2.0	-2.0	Log	AHL
		-1.9	-1.8	Stat	
II 0183	Transposase	-3.3	-3.0	Log	AHL
		-1.6	-1.8	Stat	
II 0445	Transposase	-2.9	-2.3	Log	VjbR
		1.5	1.5	Stat	
II 1038	Methyltransferase	-	-2.0	Log	VjbR
		-	1.7	Stat	
	Secondary Metabolites Biosynthesis, Transport and Catabolism				
I 2034	Sensor Protein, ChvG	-	-1.7	Log	AHL
		-1.6	-1.5	Stat	
II 0245	Universal Stress Protein Family	-1.8	-1.6	Log	AHL
		-3.0	-2.0	Stat	
II 0580	Probable Blue-Copper Protein, YacK	-1.8	-1.7	Log	AHL
		-2.0	-1.7	Stat	
	Signal Transduction Mechanisms				
II 0245	Universal Stress Protein Family, UspA	-2.0	-1.6	Log	VjbR
		-2.9	-2.1	Stat	
	Transcription				
I 0019	LacI Family Transcriptional Regulator	-3.4	-2.4	Log	VjbR
		1.9	1.8	Stat	
I 0305	DeoR Family Transcriptional Regulator	-1.7	-1.7	Log	VjbR
		1.9	-1.9	Stat	
I 0447	Leucine-Responsive Regulatory Protein	1.7	1.5	Log	VjbR
		-2.6	-2.1	Stat	
I 0518	Cold Shock Protein, CspA	1.7	1.5	Log	VjbR
		1.6	1.7	Stat	
I 0623	TetR Family Transcriptional Regulator	-2.4	-2.7	Log	AHL
		-2.4	-1.5	Stat	
I 0899	Phage-Related DNA Binding Protein	-2.0	-1.5	Log	VjbR
		1.6	1.6	Stat	
I 1098	AsnC Family Transcriptional Regulator	-1.7	-1.7	Log	VjbR
		-1.6	-1.6	Stat	
II 0985	LacI Family Transcriptional Regulator	-3.1	-1.9	Log	VjbR
		-3.1	-1.7	Stat	
II 1022	IclR Family Transcriptional Regulator	-1.9	-1.6	Log	AHL
		-2.1	-2.0	Stat	
II 1098	AraC Family Transcriptional Regulator	-1.9	-1.7	Log	VjbR
		1.8	2.0	Stat	
II 1098	AraC Family Transcriptional Regulator	-2.8	-2.8	Log	AHL
		-	1.5	Stat	
II 1116	LuxR Family Transcriptional Regulator, VjbR	-1.9	-2.7	Log	VjbR
		-2.1	-1.7	Stat	
	Translation, Ribosomal Structure and Biogenesis				
I 0480	Peptidyl-tRNA Hydrolase	2.7	2.1	Log	AHL
		3.4	1.9	Stat	
II 1039	tRNA Pseudouridine Synthase A	1.7	1.5	Log	VjbR
		-1.9	-1.7	Stat	
II 1072	Novel RNA Polymerase II Holoenzyme	-2.5	-1.8	Log	VjbR
		-1.8	-1.5	Stat	

TABLE 10—*Continued*

BME Loci	Gene Product	Δ*vjbR* / wt* or wt+ahl / wt* Student's *t*-test FC	Δ*vjbR* / wt* or wt+ahl / wt* Z-score FC	Growth Phase	Condition
	Unknown and Other				
I 0052	Hypothetical Protein	2.0	1.8	Log	VjbR
		-1.9	-1.6	Stat	
I 0065	Hypothetical Protein	-1.6	-1.6	Log	VjbR
		-1.9	-1.5	Stat	
I 0262	Hypothetical Protein	-1.7	-1.9	Log	AHL
		-2.6	-2.3	Stat	
I 0368	Hypothetical Protein	1.7	1.5	Log	VjbR
		1.7	1.9	Stat	
I 0373	Hypothetical Protein	2.4	2.1	Log	VjbR
		1.8	2.0	Stat	
I 0400	Hypothetical Protein	1.9	1.5	Log	VjbR
		-1.9	-1.6	Stat	
I 0431	Hypothetical Protein	-	-1.6	Log	AHL
		1.6	-1.5	Stat	
I 0534	Hypothetical Protein	-1.6	-1.5	Log	AHL
		-2.3	-2.1	Stat	
I 0651	Hypothetical Cytosolic Protein	-1.7	-2.1	Log	AHL
		-1.9	-1.8	Stat	
I 0691	Hypothetical Protein	-	-1.7	Log	AHL
		-2.0	-1.9	Stat	
I 1222	Hypothetical Protein	2.4	1.7	Log	AHL
		2.2	1.9	Stat	
I 1472	Hypothetical Protein	-	-1.6	Log	AHL
		-2.1	-1.7	Stat	
I 1660	Hypothetical Protein	-1.8	-1.8	Log	VjbR
		1.6	1.8	Stat	
I 1690	Hypothetical Protein	-	-1.7	Log	AHL
		-2.0	-1.9	Stat	
I 1694	Hypothetical Protein	-1.6	-1.7	Log	AHL
		-2.7	-2.3	Stat	
II 0231	Hypothetical Protein	-	-1.6	Log	AHL
		1.6	1.6	Stat	
II 0480	Hypothetical Protein	-1.5	-2.0	Log	AHL
		-1.7	-1.5	Stat	
II 0913	Predicted Membrane Protein	-3.2	-3.5	Log	AHL
		-2.3	-2.0	Stat	
II 1138	Hypothetical Protein	-1.7	-1.8	Log	AHL
		-2.3	-2.1	Stat	

*Wild type (WT) replicates 1 & 2 were used at the exponential growth phase for the Student's *t*-test analysis column. Fold change (FC) values are the log ratio of normalized signal values. FCs listed in grey did not pass the statistical test indicated in that column. Abbreviations as follows: FC, fold change; AA, Amino acid.

sensing regulated genes in *P. aeruginosa* also found that genes were regulated in a bi-phasic manner as observed in this study (152).

Several genes previously described to be important for infection either in *Brucella* spp. or other intracellular pathogens were found to be regulated by VjbR or C_{12}-HSL. One protein containing a tetratricopeptide (TPR) repeat domain was found to be promoted by VjbR at both growth phases. Although this protein function has not been examined in *Brucella* spp., proteins containing TPR domains have been shown to be secreted independently of the type II and type IV secretion system in *Legionella pneumophila* and are required for infection and cellular trafficking (103). The addition of C_{12}-HSL was shown to negatively regulate ChvG, a homologue of the BvrR/BvrS two component system. The BvrR/BvrS two component system has been shown to regulate outer membrane proteins in *B. abortus* and is attenuated for intracellular survival in macrophage and HeLa cells (53, 140).

C_{12}-HSL affects gene expression independent of VjbR. Only a very small proportion of genes were co-regulated by the two quorum sensing components (VjbR and C_{12}-HSL), suggesting these two components may regulate gene expression independent of one another. Additionally, there is evidence that C_{12}-HSL interacts with a second LuxR homologue, BabR (BMEI 1758) (79). To examine the activity of C_{12}-HSL on gene expression independent of VjbR, transcripts analysis comparing the VjbR deletion mutant with and

without the addition of C_{12}-HSL to the growth media was conducted using microarray technology. Transcript analysis revealed that C_{12}-HSL is able to modulate gene expression in the absence of VjbR. C_{12}-HSL was found to regulate the expression of 102 genes, with 46 genes at the mid-exponential growth phase and 56 genes at the stationary growth phase. The altered genes from both time points are presented in Table 11. At the mid-exponential growth phase, addition of C_{12}-HSL exerts an equal effect on gene expression, promoting and repressing 23 different genes. At the stationary phase, the addition of C_{12}-HSL was found to only promote the expression of 56 genes. Comparing this data to the addition C_{12}-HSL in the wild type background, 13 % of the genes were found to be regulated by C_{12}-HSL in both the presence and absence of VjbR. Independent of VjbR, C_{12}-HSL acts as a promoter of gene expression at the stationary growth phase, suggesting that C_{12}-HSL may interact and activate gene expression in a quorum sensing-like manner with a second LuxR regulatory protein. Indirect evidence has shown that C_{12}-HSL also interacts with BabR, a second LuxR homologue, and may be responsible for the gene expression profile observed in the absence of VjbR (79). Although the regulation BabR was not detected by microarray experiments, transcript comparison of

TABLE 11. Genes altered by C_{12}-HSL in the absence of VjbR.

BME Loci	Gene Product	Δ*vjbR*+ahl / Δ*vjbR* Student's *t*-test FC	Growth Phase
	Amino Acid Transport and Metabolism		
I 0110	Agmatinase	2.8	Log
I 0978	Nitrogen Regulatory Protein P-II	2.7	Log
I 1217	Aminopeptidase ApeA	3.0	Log
I 1309	Histidinol-Phosphate Aminotransferase	4.4	Log
I 1848	Dihydroxy-Acid Dehydratase	2.6	Log
II 0506	ABC-Type Oligopeptide Transport System Permease Protein OppC	-1.8	Log
II 0554	Glutamine Synthetase	-2.9	Log
I 0231	NAD-Specific Glutamate Dehydrogenase	4.0	Stat
I 0260	ABC-Type High-Affinity Branched-Chain Amino Acid Transport ATP-Binding Protein BraF	3.0	Stat
I 0414	ABC-Type Spermidine/Putrescine Transport System Permease Protein PotI	2.3	Stat
I 0706	CobC Protein	2.8	Stat
I 1638	Glutamate Synthase (NADPH) Small Chain	2.2	Stat
II 0583	ABC-Type Spermidine/Putrescine/Fe^{3+}-Transport ATP-Binding Protein SfuC	3.0	Stat
	Carbohydrate Transport and Metabolism		
II 0512	6-Phosphogluconolactonase	2.6	Log
II 0753	ABC-Type Sorbitol/Mannitol Transport Inner Membrane Protein	2.5	Log
II 0755	ABC-Type Sugar-Binding Transport System Protein	2.4	Log
II 0945	ABC-Type Maltose-Binding Periplasmic Protein Transport System	2.9	Log
I 0326	Myo-Inositol-1(Or 4)-Monophosphatase	4.1	Stat
II 0358	Keto-Hydroxyglutarate-Aldolase/Keto-Deoxy- Phosphogluconate Aldolase	2.1	Stat
II 0544	ABC-Type Sn-Glycerol-3-Phosphate Transport ATP-Binding Protein UgpC	3.7	Stat
II 0942	ABC-Type Maltose Transport System Permease Protein MalG	2.1	Stat
	Cell Motility		
I 1692	Flagellar Protein FlgJ	-2.1	Log
	Cell Wall, Membrane and Envelope Biogenesis		
I 1831	Penicillin-Binding Protein 1A	3.0	Stat
I 1878	Soluble Lytic Murein Transglycosylase	3.3	Stat
II 0384	Glucosamine-6-Phosphate Isomerase	2.8	Stat
	Coenzyme Transport and Metabolism		
II 0678	Lipoate-Protein Ligase B	2.3	Log
I 0001	Uroporphyrinogen Decarboxylase	1.6	Stat
I 0286	Putative Nucleotide-Binding Protein	2.3	Stat
I 0713	Precorrin-2 Methyltransferase	2.2	Stat
II 0130	Adenosylmethionine-8-Amino-7-Oxononanoate Aminotransferase	2.9	Stat
	Defense Mechanisms		
II 0795	Multidrug Resistance Protein B	-2.0	Log
I 0323	ABC-Type Multidrug Transport ATP-Binding Protein MsbA	3.3	Stat
	Energy Production and Conversion		
I 1149	NADH ehydrogenase subunit J	-2.1	Log
I 0137	Malate Dehydrogenase	1.8	Stat

TABLE 11--*Continued*

BME Loci	Gene Product	Δ*vjbR*+ahl / Δ*vjbR* Student's *t*-test FC	Growth Phase
I 0380	Malate Synthase G	2.6	Stat
II 0553	Alcohol Dehydrogenase	4.9	Stat
	General Function Prediction Only		
I 0006	Predicted GTPase	5.9	Log
I 0196	Protein ErfK/SrkK	2.6	Log
I 0631	Predicted Flavin-Nucleotide-Binding Protein	-1.9	Log
I 1110	Secretion Activator Protein	-1.9	Log
I 1637	CoxG Protein	-2.9	Log
I 1951	Putative Hydrolase	2.6	Log
II 0462	ATP-Dependent Helicase	-3.8	Log
II 0828	Possible S-Adenosylmethionine-Dependent Methyltransferase	-2.3	Log
II 1136	Uncharacterized ABC transporter	-2.2	Log
I 0282	Zinc Metalloprotease	3.4	Stat
I 0319	BioY Protein	2.4	Stat
I 0614	NAD(FAD)-Utilizing Dehydrogenases	1.8	Stat
I 0694	CobW Protein	2.5	Stat
I 0709	4-Hydroxyphenylacetate 3-Monooxygenase	2.7	Stat
I 1894	Gramicidin S Biosynthesis GrsT Protein	3.3	Stat
I 1969	SAM-Dependent Methyltransferase	2.3	Stat
II 0771	Hydroxyacylglutathione Hydrolase	1.9	Stat
II 0997	NorQ Protein	3.0	Stat
	Inorganic Ion Transport and Metabolism		
I 1754	Binfunctional Sulfate Adenylyltransferase Subunit 1, Adenylylsulfate Kinase Protein	3.6	Log
II 0490	ABC-Type Nickel Transport ATP-Binding Protein NikD	2.7	Stat
	Intracellular Trafficking, Secretion and Vesicular Transport		
I 0131	Signal Recognition Particle Receptor FtsY	2.1	Stat
	Nucleotide Transport and Metabolism		
I 1558	Ada Regulatory Protein / O-6-Methylguanine-Dna-Alkyltransferase	3.8	Log
I 1611	Dihydroorotate Dehydrogenase	3.2	Stat
I 0332	Endodeoxyribonuclease RuvC	2.3	Stat
I 0333	DNA Helicase RuvA	1.9	Stat
I 1801	DNA Mismatch Repair Protein MutS	1.6	Stat
I 1818	ATP-Dependent Helicase HrpB	2.2	Stat
	Posttranslational Modification, Protein Turnover and Chaperones		
I 0047	Molecular Chaperone, DNAJ Family	-3.1	Log
	Replication, Recombination and Repair		
I 0246	Primosomal Protein N	2.9	Log
I 1411	Transposase	2.6	Log
II 0183	Transposase	3.6	Log
	Secondary Metabolites Biosynthesis, Transport and Catabolism		
I 0965	ABC-Type Toluene Tolerance Protein Ttg2B	-3.6	Log
II 0079	Isochorismatase	3.1	Log
		1.8	Stat
	Signal Transduction Mechanisms		
I 2034	Sensor Protein ChvG	-2.1	Log
I 1975	PhoH Protein	1.8	Stat
	Transcription		
I 0744	Transcription Antitermination Protein NusG	-2.4	Log
I 1750	Glycerol-3-Phosphate Regulon Repressor	3.1	Log

TABLE 11--*Continued*

BME Loci	Gene Product	Δ*vjbR*+ahl / Δ*vjbR* Student's *t*-test FC	Growth Phase
I 0280	RNA Polymerase Sigma-32 Factor	2.0	Stat
II 0392	Transcription Accessory Protein (S1 RNA Binding Domain)	2.2	Stat
II 0688	Transcription-Repair Coupling Factor	2.0	Stat
	Translation, Ribosomal Structure and Biogenesis		
I 0444	Methyltransferase	3.4	Log
I 0132	Fe-S Oxidoreductase	1.8	Stat
I 0377	Ribosomal Large Subunit Pseudouridine Synthase D	3.0	Stat
I 0774	Ssu Ribosomal Protein S5P	2.2	Stat
I 1529	Glycyl-tRNA Synthetase β Chain	2.5	Stat
	Unknown and Other		
I 0142	Hypothetical Membrane Spanning Protein	-2.3	Log
I 0535	Hypothetical Protein	-3.5	Log
I 0652	Hypothetical Protein	-2.6	Log
I 1107	Hypothetical Cytosolic Protein	-1.9	Log
I 1595	Hypothetical Protein	-3.5	Log
I 1684	Hypothetical Protein	-2.7	Log
I 1844	Hypothetical Protein	3.5	Log
II 0022	Hypothetical Protein	-2.1	Log
II 0074	Hypothetical Protein	-2.9	Log
II 1050	Hypothetical Protein	2.6	Log
I 0367	Hypothetical Protein	2.0	Stat
I 0376	Hypothetical Protein	4.0	Stat
I 0484	Hypothetical Protein	1.8	Stat
I 0710	Hypothetical Protein	2.3	Stat
I 0813	Hypothetical Protein	2.8	Stat
I 1539	Hypothetical Protein	2.1	Stat
I 1783	Hypothetical Membrane Spanning Protein	2.9	Stat
II 0464	Hypothetical Membrane Associated Protein	1.9	Stat
II 0809	Hypothetical Membrane Spanning Protein	2.0	Stat
II 1025	Hypothetical Protein	2.3	Stat

Fold change (FC) values are the log ratio of normalized signal values.

using qRT-PCR revealed that BabR is induced 27-fold by the addition of C_{12}-HSL to wild type cells when compared to wild type. To see if VjbR was involved in the induction of BabR, expression was also examined in the Δ*vjbR* background. In the absence of VjbR, the addition of C_{12}-HSL induced expression of BabR 93-fold. This strongly suggests that VjbR may partially inhibit BabR expression; potentially by binding and inactivating C_{12}-HSL's inductive activity.

Verification of microarray results by quantitative real-time PCR. Transcript profiling is a great technique to survey global changes due to an altered genotype or condition. To verify a portion of the microarray data, quantitative real-time PCR (qRT-PCR) was performed to verify the fold changes of gene expression relative to wild type 16M. The extracted total RNA samples that were used for the microarray preparation were also used for the qRT-PCR experiments for consistency and because obtaining new RNA extractions was not an option at this time. Genetic loci from each microarray experiment were selected for examination based either on the interest of the particular gene locus, or by the direction and magnitude of the fold change. Results are shown in Table 12, along with the corresponding microarray data.

Five genes regulated by VjbR were selected to verify that the fold changes associated with these genes occurred in the same direction as the microarray data. Quantitative real-time PCR confirmed that the fold changes for

TABLE 12. Quantitative real-time PCR and microarray fold changes of selected genetic loci regulated by VjbR and/or C_{12}-HSL.

	Fold Change		
		Microarray	
BME Gene Loci	qRT-PCR	Student's *t*-test	Z-score
VjbR			
I 0984	-2.5	-2.6	-1.6
II 1069	-1.9	-1.6	-1.3
I 1470	-1.1	-2.3	-1.2
II 0659	-1.2	-1.9	-1.6
C_{12}-HSL			
I 0831	2.2	2.6	1.9
I 1621	-1.8	1.8	1.8
I 0155	-1.7	-1.6	-1.2
I 1758	27.5	NA	NA
VjbR & C_{12}-HSL			
II 0151	-7.9	-2.4	-2
II 0151	-3.8	-2.3	-1.9
I 0561	-1.7	-2.2	-1.8
I 0561	1.2	-1.7	-1.6
II 0025	-4.1	-3.9	-3.3
II 0025	-2.5	-4.8	-3.7
II 0838	-1.7	-5	-3.5
II 0838	1.7	-1.7	-1.6
ΔVjbR + C_{12}-HSL			
I 0414	1.0	2.3	NA
I 1878	1.2	3.3	NA
I 0047	1.2	-3.1	NA
II 0753	1.5	2.5	NA
I 1758	93.0	NA	NA

Fold change (FC) values for the microarray date are the log ratio of normalized signal values. FCs listed in grey did not pass the statistical test indicated in that column. QRT-PCR fold changes were calculated by the $\Delta\Delta C_t$ method and are shown relative to the wild type 16M bacteria.

all 5 of the selected genes were in agreement with the microarray data. Three genes from the addition of exogenous C_{12}-HSL were examined and it was observed that all but one of the fold changes associated with these genes was in the same direction as the microarray data. Four genes found to be regulated by both VjbR and C_{12}-HSL were examined in samples from both conditions. Two of the genes were found to have fold changes that agreed with the direction of the fold change in the microarray data for both of the conditions; however, for the two remaining genes only one out of the two experimental conditions agreed with the direction of the fold change obtained from the microarray data. Five genes were examined from the microarray experiments in the Δ*vjbR* background. This included BabR, a LuxR homologue that was not identified in by the microarray analysis. The quantitative real time PCR fold changes were found to be altered in the same direction in all but one of the five genes selected.

A total of nineteen genetic loci identified in the microarray analysis were verified for an alteration in gene expression. The fold changes for fifteen of these selected genes obtained by qRT-PCR analysis agree with the direction of the fold changes obtained via microarray studies. Although only a small portion of the genes were selected for verification (n=19), comparison of the microarray and qRT-PCR data demonstrated a concordant gene expression of approximately 79 % of the genes. The microarray analysis retained genes that passed the Student's *t*-test with a *p*-value of less then or equal to 0.05; allowing

for one false positive in every 20 genes due to random chance. The correlation between the microarray data and qRT-PCR fold changes reveal that the number of false positives observed is only slightly greater then the number expected, and may be skewed by the small sample size. Furthermore, the degree of correlation between these two measurements of gene expression observed here is in agreement with the verification results found in studies also using custom printed oligonucleotide microarrays and different analysis procedures (81, 143, 160).

The RNA samples used for the microarray studies were stored at -80°C and later used for the qRT-PCR in an attempt to maintain consistency; however, the data may have been complicated by the storage and potential degradation of the RNA during the microarray analysis period. Twelve RNA samples were re-analyzed using the 2100 bioanalyzer (Agilent, Santa Clara, CA), and it was found that the RNA integrity numbers obtained ranged from 8.0 to 9.6 and were comparable to the RNA integrity numbers obtained from the freshly extracted RNA, indicating the storage did not degrade the RNA and is likely not accounting for the variation.

Screening of candidate AHL synthase genes fails to identify a unique AHL synthesis gene. Fifteen candidate AHL synthesis genes were selected by the gene product's potential interaction with the immediate precursors of AHL, S-adenosyl-L-methionine (SAM) and acylated acyl carrier

protein (acyl-ACP) (108). The gene locus examined include BMEI 0032, 0093, 0701, 0712, 0852, 1093, 1213, 1289, 1768, 1869, 1956, 1969, 2003 and BMEII 0678 and 0828. An *E. coli* expression system was utilized because *B. melitensis* has been shown to produce an AiiD-like lactonase protein capable of inactivating C_{12}-HSL (J.J. Letesson, personal communication), thus complicating the testing in *B. melitensis*. The examination of the fifteen *B. melitensis* genes examined failed to produce an AHL signal that induced the sensor stain. A limitation to this screen is the assumption that the AHL signals produced by *B. melitensis* are produced by a single enzymatic step. It is possible that *B.* melitensis may possess a unique AHL synthase and AHL synthesis is carried out in several steps and requires multiple enzymes.

DISCUSSION

The goal of this work was to further the understanding of the role of quorum sensing (QS) in the virulence and survival of *B. melitensis*. VjbR is a homologue of LuxR, a transcriptional regulator shown to interact and coordinate gene expression in the bacterial population by a diffusible AHL signal. Using custom *B. melitensis* microarrays, we were able to examine the regulons controlled by VjbR and the AHL signal, C_{12}-HSL. We found that VjbR and C_{12}-HSL regulate a number of genes involved in virulence of the organism; including adhesins, proteases, lipoproteins, a hemolysin, secretion systems and putative

effectors. VjbR and C_{12}-HSL also regulates genes from a number of metabolic pathways involved in energy production, amino acid, carbohydrate, and lipid metabolism. Additionally, VjbR and C_{12}-HSL regulates genes coding for components involved with transport of many different substrates across the cell membrane. Pathway analysis revealed that VjbR and C_{12}-HSL are major regulators of ABC transporters, which was the top scoring altered pathway. The microarray analysis conducted in this study also confirmed previous findings that *fliF* and the *virB* operon are regulated by VjbR and exogenous C_{12}-HSL; validating our microarray approach for the identification of additional genes regulated by the putative QS components.

Virulence. *Brucellae* spp. contain genetic locus that contribute to survival and pathogenesis. Beyond metabolic genes that have been found to be attenuated for survival in mutagenesis screenings, several virulence genes have been described in *B. melitensis* and found to be regulated by VjbR and C_{12}-HSL (25). Such genes found in the studies described here include *fliF* and other flagellar genes and the *virB* operon, which is responsible for the Type IV Secretion System (25). In addition to confirming virulence genes known to be regulated by VjbR and C_{12}-HSL, this study identified a number of putative virulence genes regulated by VjbR and C_{12}-HSL; including three adhesins, a component of β-(1,2) glucan exporter, exopolysaccharide biosynthesis, three lipoproteins, additional protein secretion/translocation components, three

potentially secreted effector proteins and sixteen proteases and peptidases. There are also a large number of genes regulated by VjbR and C_{12}-HSL that modulate resistance to antibiotics, toxic or mutagenic compounds; including tellurium (BMEI 0671), bacitracin (BMEII 0258), multiple antibiotic resistance protein MarC (BMEI 0883), multi-drug resistance protein A (BMEI 0926), multi-drug resistance protein B (BMEII 0795), arsenate reductase (BMEI 0992) and resistance or degradation of uncharacterized toxic substances (BMEI 0992, 1641 and BMEII 0879 and 0795).

The TIVSS has been shown to be involved in the recruitment of ER-derived markers associated with the brucellosome, potentially by an unidentified effector molecule secreted by *Brucella* spp. (14). Additional genetic loci that may be involved in protein secretion or translocation were found to be regulated by VjbR or C_{12}-HSL. Genes coding for proteins that contain tetratricopeptide repeats were regulated at the both of the examined growth phases (BMEI 1531, 1094 and BMEII 0466) and have been implicated in protein translocation in *Legionella pneumophilia* (103). Three genes found to be regulated in this study may produce gene products that may be potential effector secreted proteins (BMEI 1077, 1141 and BMEII 0188) and a secretion activating protein (BMEI 0995) was also identified in this study, all of which need to be examined for their secretion potential in *Brucella*. *YajC*, BMEI 1077, was found to produce an immunogenic protein in mice vaccinated with RB51, and is shown to be a

membrane protein, forming a complex with SecDF involved in SEC-dependent protein translocation and could also involved in protein secretion in *Brucella* spp. (99, 149).

Brucella spp. are described as being non-motile, yet the genome contains three gene clusters required for flagella assembly (28). Although these gene clusters are present, it was found that many genes were truncated and crucial components are absent (P ring, L ring), including a chemotatic system (27). Transmission electron microscopy (TEM) showed that the flagella genes localize to the membrane and that the flagella components are required for the survival of the bacteria in the mouse model (44).

Currently, the precise role of the flagella components in the survival and replication of *Brucella* spp. has not been defined, but is possible that they function as a secretion system, based upon the ancestral relationship between the type III secretion system (TIIISS) and flagella (47). The basal body of flagellum biogenesis shares a high degree of homology with subunits that form the contact dependent type III secretion apparatus (159). Genome sequencing of the *B. melitensis* genome reveals that it contains all of the necessary basal body structures except for *flgA*, coding for the P ring (28). The function of the flagella genes is currently difficult to establish, but appears to have a biological function and will be interesting to continue the investigation into the role of these genes.

An alternative or second potential role for the non-functional flagella could be to subvert the host immune response in favor of the bacteria. It was found that flagella mutants are not attenuated for survival in the mouse model 1 week post-infection, but are unable to establish a chronic infection, with reduced survival at 4 weeks post-infection (44). Further investigation is needed to determine if the immunomodulatory role of flagellin in the pathogen associated molecular pattern (PAMP) via the TLR5 signaling is important for the establishment of a chronic disease in the host (57).

To date, two transcriptional regulators have been found to influence the expression of the flagellar genes, VjbR and FtrC (25, 82). One gene found to be negatively regulated at the exponential growth phase by C_{12}-HSL, *exsD* (BMEI 0093), is a negative regulator of the TIIISS in *P. aeruginosa*, preventing inappropriate expression the secretion system (92). It would be interesting to examine if this repressor may be involved in the regulation of the flagellar clusters in *Brucella* spp.

VjbR was found to positively influence the gene locus of the ATP binding protein of the cyclic β-(1,2) glucan export apparatus and exopolysaccharide (EPS) biosynthesis protein ExoF (BMEI 0984). Additionally, C_{12}-HSL was found to repress expression of *opgC* (BMEI 0330), which has been shown to add substitutions to cyclic β-(1,2) glucan (127). Cyclic β-(1,2) glucan has been shown to modulate lipid raft portions of cellular membranes, as well as

preventing the phago-lysosomal fusion and recruitment of ER markers for the brucellosome (4). This activity can modulate lipid raft organization of the early endosomes, potentially interfere with host functions and contributes to *Brucella* spp. modulation of host cellular trafficking (4). In trafficking studies with VjbR mutants, they do not appear to be deficient in subverting the trafficking from the early endosomal pathway; however, these QS components may not be the only regulatory control of such an important function and it can't be ruled out that cyclic β-(1,2) glucan isn't important for vacuole modulation or other unknown functions later in the infection (25).

Exopolysaccharides are important for biofilm formation, which is commonly regulated by quorum sensing (133). Mutations in Δ*vjbR* have been found to have a clumping phenotype and to alter expression of exopolysaccharides (147). Factors other than EPS, such as flagella, adhesions and outer membrane proteins could also be contributing to the clumping phenotype and can't be ruled out. It is interesting that quorum sensing is involved in the regulation of all of these genetic locus and the potential for biofilm formation may be interesting to pursue.

VjbR was found to regulate three three genes coding for adhesins, AidA (BMEII 1069), AidA-1 (BMEII 1070), and a cell surface protein (BMEI 1873). Within the AidA family of adhesions, the product of BMEII 1069 contains an autotransporter domain (type V secretion system), while the product of BMEII

1070 does not contain any known domains. It has been shown that adhesins that are released into the extracellular milieu can be potent biological effectors that can affect the inflammatory and apoptotic responses (2, 61, 93).

The second adhesin, coded by BMEII 1070, has been shown to be involved in the attachment of *E. coli* to enterocytes leading to colonization and is responsible for autoaggregation leading to biofilm formation *in vitro* (122). Recently, a *B. melitensis* Δ*vjbR* mutant was shown to exhibit a clumping phenotype, due to VjbR's regulation of Omp31 (147). Although Omp31 and VjbR were shown to contribute to the clumping phenotype, the contributions of other membrane structures cannot be ruled out (147). VjbR's repressive affect on this adhesin may also be contributing to the observed clumping phenotype as well and would need to be investigated.

The third adhesin, coded by BMEI 1873, is described as a cell surface protein and may function as an autotransporter protein. It contains two domains also found in invasins and haemagglutinins. Adhesins have also been shown to play a critical role in recognition of host cells in *Bordetella pertussis*, discriminating between macrophages and ciliated epithelial cells in humans (61). These adhesins could play a crucial role not only in recognition of host cells, but could also be involved the biogenesis and maintenance of the brucellosome; interacting with the interior of the brucellosome membrane.

VjbR and C_{12}-HSL influence the genetic loci expression of sixteen different proteases and peptidases (BMEI 0213, 0282, 0611, 0783, 1079, 1102, 1217, 1683, 1799, 2048 and BMEII 0149, 0347, 0448, 0831, 1036 and 1037). Three serine proteases, coded by loci BMEI 0611 (HflC), BMEII 0149 and protease DO BMEI 0783 (HtrA) were found to be regulated by VjbR and C_{12}-HSL. HtrA has been characterized in *B. abortus* and was shown to function as a stress response protease, potentially functioning as a secondary defense mechanism against oxidative stresses (112). This protein could be essential for repairing damage that occurs during trafficking from the endocytic pathway to the brucellosome.

VjbR and C_{12}-HSL influence the gene expression of five zinc metalloproteases (BMEI 1102, 1683 and BMEII 0448, 1036, 1037). Although the function has not been examined in *Brucella* spp., ZmpB and ZmpA are zinc metalloproteases in *B. cenocepacia* that have been shown to cause direct tissue damage and modulation of the host immune system by degrading type IV collagen, fibronectin, α-1 proteinase inhibitor, α_2-macroglobulin and γ-interferon (76, 77). Interestingly, ZmpA and ZmpB were both found to be regulated by the quorum sensing circuits CepIR and CciIR in *B. cenocepacia* (77, 88, 139).

Three genes that code for lipoproteins, BMEI 1079, 1217 and 1799, were found to be altered by VjbR and C_{12}-HSL. Lipoproteins have been found to be important for virulence, protein secretion and motility in *Legionella pneumophilia*

(50). Interestingly, one lipoprotein, NlpD (coded by BMEI 1079), is commonly organized in the genome with RpoS and YajC (106). *Brucella* spp. does not contain a RpoS homologue, but is found to be organized in the chromosome with YajC (BMEI 1077), a *B. abortus* immunogenic protein that is also found to be regulated by both VjbR and C_{12}-HSL (149). YajC forms a complex with SecDF and is involved in SEC-dependent protein translocation. It would be interesting to examine if NlpD is secreted in a SEC dependent manner with the assistance of YajC. (99, 149)

Outer membrane proteins (Omps) were initially identified three decades ago and have been found to be immunogenic and potentially protective antigens. The genes of three Omps were found to be regulated by VjbR and C_{12}-HSL; Omp31, BMEI 0412; Omp89, BMEI 0830; and OmpF, BMEI 1304. Omp89 was previously found to be regulated by VjbR, and the gene locus was confirmed in this study (147). It was also found that the addition of C_{12}-HSL decreased the gene expression of Omp89, mediated through VjbR's signal binding domain (147). Omp31 has been shown to be regulated by VjbR and is shown to contribute to the aggregation of Δ*vjbR* mutants in culture by exopolysaccharide (EPS) secretion (147). Omp31 has also been found to be a haemin-binding protein in *B. suis* and could also be important for the sequestering of iron from the host cell (24).

Transporters. A large number of genetic loci coding for transporters (49 genes) were found to be regulated by VjbR and C_{12}-HSL in *B. melitensis*. Among the transporters, the ABC type was highly represented, totaling just under half of the total number of transporters found. In addition to the ABC type transporters, VjbR and C_{12}-HSL regulate many other transporter genes that may be related to the virulence of the organism and increase the fitness of the bacteria within the host cell. These genes include a multidrug efflux pump (BMEII 0382), an exporter of the O-antigen (BMEII 0838), transport of amino acids (BMEI 0114, 1869, 0284 and BMEII 0099, 0632, 0517, 0101, 0873), iron (BMEI 0659), sugars (BMEI 0655 and BMEII 0821, 1096) urea (BMEI 0162), nucleotide bases (BMEI 0155, 0284), polyamines (BMEII 0193, 0484, 0567, 0583, 0923) and an uncharacterized porin (BMEI 1305).

It has been found that the number of ABC transporters coded for in a genome correlates with the physiological niches in which bacteria live and not as a function of the genome size (48). Analysis of the *B. suis* genome reveals that over two-thirds of the transporters found within the entire genome are ABC-type transporters (91 systems, 284 genes), with the majority being amino acid transporters (22 transporters) (109). This correlates with the ability of *Brucellae* spp. to survive in both extracellular and intracellular environments. While transporters are overrepresented in *Brucellae* species, a biological pathway analysis shows ABC transporters as the top scoring perturbed pathway by both

conditions examined here. *Brucella* spp. survival may be enhanced within the host by constantly surveying the surrounding environment and respond to the changing atmosphere. Changes in the regulation and thus the expression of different membrane transporters could help the bacteria survive and obtain available or necessary substrates in new environments to maintain cellular efficiency. In this case, it appears that quorum sensing may help obtain nutrients needed or that are available to support growth in the brucellosome.

AHL synthesis. To date, there has been little information available on the synthesis of C_{12}-HSL by *B. melitensis*. BLAST searches of known AHL synthesis genes against the sequenced *B. melitensis* genome failed to reveal any candidate genes. To further complicate screening of synthase genes, some bacteria also produce an AHL-lactonase that breaks down the AHL signals in stationary phase, potentially complicating the use of an AHL sensing strain for identification. A close relative of *Brucellae*, *A. tumefaciens*, was found to produce such an AHL-lactonase to degrade its own AHL signal in stationary phase (161). Taking all of this together, identifying the *luxI* synthesis gene(s) in *Brucella* spp. has remained elusive. In other quorum sensing models, a positive feedback loop is formed by the AHL synthesis. Based on that observation, it may have been possible to identity AHL synthesis genes from the gentic loci regulated by VjbR or C_{12}-HSL for AHL production, using an *E. coli* based AHL detection system. The genes selected and screened failed to identify any AHL

synthase genes in our microarray data. A potential flaw to the screening system described here is that it assumes that *Brucella* production of the AHL signals relies on a single enzyme, while it could be dependent on several steps and multiple enzymes.

Stress response. VjbR was found to regulate a number of stress response genes, including molecular chaperones and heat shock proteins. This includes three heat shock genes, Hsp33-like (BMEI 1619), heat shock protein (BMEI 2048), Hsp20 (BMEII 0042) and the gene coding for a protein with a pro-isomerase domain (pfam00160) that has been shown to accelerate protein folding. Heat shock chaperones can protect other proteins against heat-induced denaturation and aggregation. Hsp33 as been shown to be activated by exposure to oxidants, forms oxidized dimers and binds many substrates to prevent their aggregration and may function in the same manner in *Brucella* spp. (136). VjbR also activates the expression of two stress response genes; YicC (BMEI 1470), an uncharacterized stress response protein; and universal stress protein A, UspA (BMEII 0245), which is a general stress response protein that enhances survival during stress and may be involved in responding to oxidative agents and confer resistance (100).

Brucella spp. have been shown to be able to resist reactive oxygen and nitrogen species (ROS and RNS, respectively) produced by activated phagocytes (37, 67). Superoxide dismutase has been found to confer protection

against the bactericidal effects of the oxidative burst in phagocytic cells and is indispensable for survival in the host cell (49). Microarray analysis reveals that VjbR is an important regulator for the neutralization and repair of oxidative damage to the cellular components. This damage may occur in the phagosome before the re-direction of trafficking and development of the brucellosome. These components could also be important for neutralizing reactive oxidative intermediates resulting from aerobic respiration and serve to self protect.

Transcription. In this study, 42 transcriptional regulators and 14 two-component response regulators or signal transducing mechanisms genes were found to be regulated by VjbR and C_{12}-HSL. From this data, genes directly or indirectly regulated by VjbR or C_{12}-HSL can't be differentiated and further studies would need to be conducted to determine if either condition regulates a gene directly or indirectly. Interestingly, microarray studies conducted with *P. aeruginosa* also found that a high number of transcriptional regulators regulated by QS components (152).

VjbR and C_{12}-HSL were found to regulate transcriptional regulator families associated with the regulation of virulence genes in other organisms. This includes members from the LysR, TetR, IclR, AraC, DeoR, GntR, ArsR, MarR and Crp families of transcriptional regulators (16, 20, 34, 54-56, 96, 121, 129). Although many of these transcriptional regulators have not been studied in *Brucella* spp., a targeted mutagenesis study previously looked at the attenuation

in 25 of the regulators described here and found that only one transcriptional regulator (GntR family, BMEI 0305) was attenuated in the mouse model (55). This finding was surprising, but may suggest that the regulators influenced by VjbR or C_{12}-HSL have only a minimal (if any) contribution and demonstrates the importance of these quorum sensing components as global regulatory networks that are indispensable to *Brucella* spp.

The regulation of *virB* is found to be influenced not only by VjbR and C_{12}-HSL, but also several other factors; including integration host factor (IHF), LuxR like regulator BabR or BlxR (BMEI 1758), and a stringent response mediator Rsh (25, 33, 120, 134). One transcriptional regulator regulated by VjbR and C_{12}-HSL from the AraC family (BMEII 1098) has also been shown to contribute to the expression of the *virB* operon, although it was not determined if this contribution was direct or indirect (55). It is possible that VjbR and C_{12}-HSL regulation of *virB* is mediated through this AraC transcriptional regulator.

Quorum sensing. VjbR and C_{12}-HSL are both global regulators, contributing to virulence and metabolic gene regulation. Examining the relationship in respect to gene regulation between VjbR and C_{12}-HSL suggests that C_{12}-HSL is an antagonist of VjbR activity, based upon the following observations: 1) the inverse correlation in gene expression of all but three genes found to be regulated by both conditions, 2) adding exogenous C_{12}-HSL to growth media mimics the deletion of VjbR in regards to gene expression, 3) the

observed *virB* repression by the addition of C_{12}-HSL is alleviated by deleting the response receiver domain of VjbR and 4) in the absence of VjbR, C_{12}-HSL has a markedly different affect on gene expression at the stationary growth phase, only promoting gene expression (147). The promotion of gene expression could potentially occur through a second LuxR-like protein BabR, which was found to be induced 125 fold by the addition of AHL in the absence of VjbR.

Although this evidence is indirect, it may suggest that there are two dueling quorum sensing circuits (VjbR and BabR) and C_{12}-HSL may provide a level of regulation between the two systems, activating BabR gene regulation and deactivating VjbR in stationary phase. Fig. 22 demonstrates the observed activity of C_{12}-HSL and proposed model of *in vitro* quorum sensing and potential hierarchical arrangement for *B. melitensis*. The data presented in this work suggests that C_{12}-HSL induces expression of 4 *luxR*-like genes, demonstrated by microarray and/or qRT-PCR. Independent microarray analysis of VjbR and C_{12}-HSL regulatory targets suggests that C_{12}-HSL is an antagonist of VjbR activity and inhibits VjbR target gene expression. It is proposed that C_{12}-HSL induces gene expression thru BabR at the stationary growth phase, and that the regulation of the *luxR*-like transcriptional regulator BMEII 0051 is mediated by VjbR and is a downstream effect of interaction with VjbR; however these are suggestions that need to be investigated and confirmed.

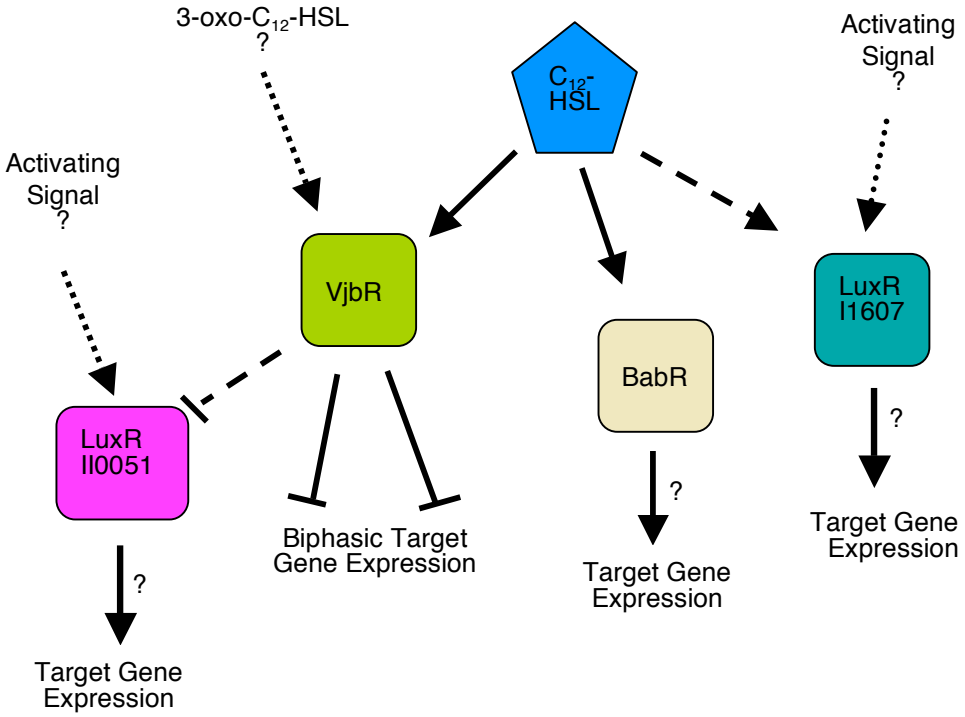


Fig. 22. Proposed model of C_{12}-HSL signaling activity and hierarchical arrangement of the quorum sensing components in *B. melitensis*. C_{12}-HSL signaling activity is demonstrated, activating expression of VjbR, BabR and LuxR BMEI 1607 and repressing the expression of BMEII 0051, potential mediated thru VjbR. It is proposed that the activation of gene expression by C_{12}-HSL occurs thru interactions with BabR. Currently, the activating signals of many of the LuxR transcriptional regulators is not known.

It has been shown that C_{12}-HSL is able to bind both LuxR transcriptional regulators VjbR and BabR, and investigation of C_{12}-HSL in a 16MΔ*vjbR* background gives a very different gene expression profile at the stationary growth phase, only activating gene expression (79, 147). This may provide some indirect evidence that this is a turning point from VjbR activity to BabR is at the stationary growth phase. It will be interesting to see if the decrease in *virB* expression observed further into stationary phase is a result of C_{12}-HSL accumulation *in vitro* (29). Continuing investigation of these putative quorum sensing components *in vitro* and *in vivo* will be very interesting, particularly to determine if these components work in a quorum sensing dependent manner in the host cell, or if they function more in a diffusion or spatial sensing context to differentiate between the intracellular and extracellular environments (58).

CHAPTER IV

CHARACTERIZATION OF THE MUCR TRANSCRIPTIONAL REGULON IN *BRUCELLA MELITENSIS*

INTRODUCTION

Bacteria have mechanisms in place to regulate the expression of a specific subset of genes that confer a growth or survival advantage in a given situation. In many cases, transcriptional regulators control the expression of many genes which, in response to an environmental or cellular signal, can bind DNA promoter regions and promote or repress gene expression as needed to adapt to the new environment.

A recent transposon mutagenesis screen using a mariner transposable element identified a novel transcriptional regulator, MucR. This transcriptional regulator is of great interest because of its high degree of attenuation of the Δ*mucR* mutant in the mouse and macrophage models of infection (3.0 and 2.0 logs less bacteria recovered, respectively) (158). The only studies investigating MucR activity are in the closely related plant pathogens *A. tumefaciens* and *Sinorhizobium meliloti* (19, 71, 87, 91).

MucR contains no common protein domains, but is related to 90 other MucR proteins (pfam05443) within phylum Proteobacteria. In *A. tumefaciens*, MucR is required for succinoglycan production and also negatively regulates

virC and *virD* operons, located within the virulence region of the Ti plasmid (19). In *S. meliloti*, MucR represses the expression of the *exp* operon that codes for the production of galactoglucan and activates expression of a second exopolysaccharide, sucinoglycan. Exopolysaccharides, particularly sucinoglycan, is important for developing the proper nodule invasion and developing a symbiotic relationship with plant nodules (71, 87, 91).

The goal of this study is to investigate the MucR transcriptional regulon in an attempt to explain the high attenuation observed in *B. melitensis* by the loss of *mucR* expression. By applying microarray technology, transcripts from wild type and a Δ*mucR* deletion mutant were analyzed at a late exponential growth phase to determine genes not equally expressed *in vitro* cell culture. These results suggest that MucR regulates exopolyaccharide biosynthesis similarly to the plant symbionts, as well as genes involved in iron sequestration and storage, nitrogen metabolism, stress response, stationary growth phase survival and several proteases that may be involved in the virulence of the organism.

MATERIALS AND METHODS

Bacteria and growth conditions. *B. melitensis* 16M was routinely grown on tryptic soy agar or broth (TSA or TSB, Difco Laboratories, Becton, Dickinson and Company, Franklin Lakes, NJ) or Brucella Broth (BB, Becton, Dickinson and Company, Franklin Lakes, NJ). All work with live *B. melitensis*

was performed in a biosafety level 3 laboratory at Texas A&M University College Station, as per CDC approved standard operating procedures.

Creation of a Δ*mucR* deletion mutant. A Δ*mucR* deletion mutation was created in *B. melitensis* wild type 16M (M. Kahl-McDonagh, unpublished). Creation of the gene replacement mutation and gene deletion mutant in two steps was performed as described in Chapter III. The following primers were used to amplify the upstream and downstream regions of MucR (BMEI 1364) for homologous recombination: 5' forward primer TAF561 (gctctagagcccatcaacaacaggacaaacgg), 5' reverse primer TAF562 (ggcggcgcgcctgttgctccgaactatgctg), 3' forward primer TAF563 (ccaggcgcgccgccgctgcgtatttcataatc) and 3' reverse primer TAF564 (gctctagagcctttgcaggttttccgtatcttt). PCR amplification using genomic DNA from potential mutants for template and forward primer TAF561 and reverse primer TAF564 verified the potential gene replacement and gene deletion mutations of Δ*mucR* (M. Kahl-McDonagh, unpublished).

RNA collection. Bacterial cultures were inoculated 1:1000 from a 48 hour starter culture grown in Brucella Broth at 37°C with agitation. Total RNA was collected at a late exponential growth phase, extracted when an optical denisty at 600 nm =0.9, measured by spectrophotometry. RNA extraction was performed using the hot acidic phenol extraction method adapted from a previously described protocol developed for *E. coli* (124). Three biological

replicates were collected. At harvest time, 14 % (v/v) of an ethanol solution containing 5 % acidic water saturated phenol (Ultrapure, Invitrogen, Carlsbad, CA) was added to the bacterial culture and immediately centrifuged at 10900 x g for 3 minutes. The supernatant was removed and the bacterial pellet was either frozen in liquid nitrogen and stored at -80°C or processed immediately. RNA was collected by re-suspending the bacterial pellets in 0.5 mg ml^{-1} lysozyme (Amresco, Solon, OH) dissolved in TE, followed by the addition of 0.97 % (v/v) SDS. The cells were than incubated at 64°C for 2 minutes. Next, 0.5 M sodium acetate (pH 5.2) was added, mixed gently by inversion and then an equal volume of acidic water saturated phenol (Ultrapure, Invitrogen, Carlsbad, CA) was added and mixed well by inversion. The bacteria-phenol mix was then incubated at 64°C for 6 minutes and mixed by inversion several times every 40 seconds. The bacteria-phenol mix was chilled on ice for 30 minutes and the layers were separated by centrifugation at 28900 x g for 13 minutes; the aqueous layer was removed and placed in a clean tube. An equal volume of chloroform (Sigma, St. Louis, MO) was added to the aqueous layer and mixed well by inversion. The layers were separated by centrifugation at 20700 x g for 7 minutes. The aqueous layer was placed into a clean tube and total RNA was precipitated by adding an equal volume of cold isopropanol and 0.1 volumes of 3M sodium acetate (pH 5.4). RNA was precipitated for one hour at -80°C and then pelleted by centrifugation at 19000 x g for 30 minutes, washed with 80 %

(v/v) ethanol, centrifuged for 5 minutes at 19000 x g, and the resulting pellet was air-dried for 20 minutes. The dried pellets were resuspended in RNAse-free water, and subjected to DNAseI treatment.

The DNA was degraded and removed by incubation with 5X DNAseI buffer (50 mM $MgCl_2$, 50 mM Tris-Cl pH 7.5, 5 mM EDTA, 5 mM dithiothreitol (DTT)), 10 units of DNAseI (Qiagen, Valencia, CA) and 10 units of RNAse Inhibitor (Roche, Indianapolis, IN). The reaction was incubated at 37°C for one hour. Following the DNAseI digestion, the RNA was further purified using the HighPure RNA Isolation kit (Roche, Indianapolis, IN), following manufacturer's instructions, including the optional (second) DNAseI incubation. The RNA was eluted with the provided elution buffer, and precipitated with 3 volumes of cold 95 % (v/v) ethanol and 0.1 volumes 3 M sodium acetate (pH 5.2); the resulting pellet was washed with 80 % (v/v) ethanol and air dried for 15 minutes. The dried pellet was resuspended in The RNA Storage Solution (Ambion, Austin, TX). RNA integrity, purity and concentration was measured using a 2100 bioanalyzer (Agilent, Santa Clara CA), electrophoresis in 0.8 % agarose and the Nanodrop® ND-1000 (Nanodrop, Wilmington, DE).

RNA and DNA labeling for microarrays. *B. melitensis* 16M genomic DNA was extracted using a Wizard genomic DNA extraction kit (Promega, Madison, WI), labeled by direct incorporation with Cy-5 and served as a common reference for each array. Genomic DNA was processed into cDNA

using components of the BioPrime® Plus Array CGH Indirect Genomic Labeling System (Invitrogen, Carlsbad, CA) with the exception of the supplied dNTPs. The reaction consisted of 1.5 μg of genomic DNA, 1x random primers, 0.12mM dATP, 0.12mM dTTP, 0.12mM dGTP, 60μM dCTP (PCR grade, Invitrogen, Carlsbad, CA), 40μM dCTP-Cy5 (Amersham, Piscataway, NJ) and 40 units Exo-Klenow Fragment. Samples were incubated overnight at 37°C. The reaction was stopped by adding 45 mM EDTA (pH 8.0) and purified of unincorporated dye using PCR purification columns (Qiagen, Valencia, CA) following the manufacturer's instructions and eluted in 0.1X of the supplied elution buffer. The fluorescence and concentration was measured using a Nanodrop® ND-1000 (Nanodrop, Wilmington, DE).

Total RNA extracted from *B. melitensis* 16M was transcribed into cDNA incorporating 5-(3-aminoallyl)-dUTP (aha-dUTP) for indirect labeling with Alexa Fluor 555 (Invitrogen, Carlsbad, CA), a Cy3-like fluorescein. The cDNA synthesis combined 10 μg of total RNA, 6 μg random primers (Invitrogen, Carlsbad, CA), 1X first strand buffer (Invitrogen, Carlsbad, CA), 10 mM DTT, 0.5 mM each dATP, dTTP and dGTP, 0.3 mM dTTP (PCR grade, Invitrogen, Carlsbad, CA), 0.2 mM aha-dUTP (Invitrogen, Carlsbad, CA), 400 units SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and 20 units Protector RNAse Inhibitor (Roche, Indianapolis, IN). The reaction was incubated at 23°C for one minute and then at 46°C overnight. The reaction was then

treated with 0.4 M NaOH and incubated at 65°C for 15 minutes to stop the reaction and remove residual RNA.

The pH was brought back to neutral with the addition of 0.2 M HCl and then the cDNA was purified to remove unincorporated aha-dUTP and free amines using PCR Purification columns (Qiagen, Valencia, CA) with modified wash (5 mM KPO_4 (pH 8.0) and 80 % (v/v) ethanol) and elution buffers (4 mM KPO_4 (pH 8.5)). The manufacturer's instructions were modified to include 2 washes with the substituted phosphate wash buffer, and the elution was carried out in 2 steps: 50 μl each elution with a one minute incubation of the elution buffer in the column at room temperature before centrifugation.

Alexa-Fluor 555 (Invitrogen, Carlsbad, CA) was coupled to the aha-dUTP incorporated into the cDNA following the coupling procedure outlined in the BioPrime® Plus Array CGH Indirect Genomic Labeling System (Invitrogen, Carlsbad, CA) and purified using PCR purification columns (Qiagen, Valencia, CA) following manufacturer's instructions. Concentration and labeling efficiency of the labeled cDNA was examined using the Nanodrop® ND-1000 (Nanodrop, Wilmington, DE). Labeled RNA samples were dried completely (Eppendorf Vacufuge™, Westbury, NY) and resuspended in ddH_2O immediately before hybridization to the microarrays.

Microarray construction. Unique 70-mer oligonucleotides (Sigma, St. Louis, MO) representing 3,227 ORFs of *B. melitensis* 16M and unique

sequences from *B. abortus* and *B. suis* were suspended in 3X SSC (Ambion, Austin, TX) at 40 μM. The oligonucleotides were spotted in quadruplicate onto ultraGAP glass slides (Corning, Corning, NY) by a custom built robotic arrayer (Magna Arrayer) assembled at Dr. Stephen A. Johnston's lab at the University of Texas Southwestern Medical Center (Dallas, TX). The printed slides were steamed, UV cross-linked and stored in a desiccator until use.

Microarray prehybridization, hybridization and washing. Printed slides were submerged in 0.2 % SDS (Ambion, Austin, TX) for 2 minutes and washed three times in containers with ample ddH_2O. The microarrays were placed in a prehybridization solution (5X SSC (Ambion, Austin, TX), 0.1 % SDS (Ambion, Austin, TX), and 1 % (w/v) BSA (Sigma, St. Louis, MO)) pre-warmed to 45°C for a minimum of 45 minutes at 45°C. Following prehybridization, the slides were washed five times in containers containing ample ddH_2O, submerged briefly in isopropanol, and then immediately dried by centrifugation at 207 x g for 2 minutes at room temperature in a Jouan CR422 table top centrifuge.

The resuspended labeled RNA was combined with 1 μg of labeled genomic DNA from *B. melitensis* 16M to a total volume of 35 μl. The sample was heated at 95°C for 5 minutes and held at 45°C until hybridization to the microarray slide. The labeled cDNA was combined with 1X hybridization buffer (25 % (v/v) formamide (Sigma, St. Louis, MO), 1X SSC (Ambion, Austin, TX) and

0.1 % (v/v) SDS (Ambion, Austin, TX)) and applied to the microarray with a 22 x 60 mm LifterSlip (Erie Scientific Company, Portsmouth, NH). The microarray was incubated in a water bath for approximately 21 hours at 42°C in a sealed hybridization chamber (Corning, Corning, NY).

After hybridization, microarray slides were washed three times with agitation at room temperature. The first wash buffer (2x SSC (Ambion, Austin, TX) and 0.2 % (v/v) SDS (Ambion, Austin, TX)) was pre-heated to 42°C and the microarray was washed for 10 minutes, followed by a 5 minute wash in 2X SSC (Ambion, Austin, TX). The microarray was subsequently washed a third time in 0.2X SSC (Ambion, Austin, TX) for 5 minutes. Immediately after the last wash, the arrays were dried by centrifugation for 2 minutes at 207 x g force at room temperature in a Jouan CR422 tabletop centrifuge.

Microarray data acquisition and analysis. All slides were scanned using a commercial laser scanner GenePix 4100A (Molecular Devices, Sunnyvale, CA) and Genepix 6.1 Pro software. The auto-PMT (photomultiplier tube) function was used to determine the optimal gain for each laser channel with each slide. Spots that were flagged as bad or absent were removed and background values were subtracted. Raw Cy-3 signals were normalized against the mean of the baseline Cy-5 gDNA reference. M vs. A plots with a LOESS fit were plotted using Prism 4 (data not shown, GraphPad Software, La Jolla, CA) to verify that the normalization procedure improved the trend of the data.

GeneSifter (VizX Labs, Seattle, WA) was used to perform global mean normalization on the signal values already normalized to the reference channel and a Student's *t*-test was performed. Genes were retained if they were altered by at least 1.5-fold between conditions and a *p* value of 0.05 or less was obtained from the Student's *t*-test. To further filter data based on biological relevance, individual pairwise comparisons were also performed on signal values after global mean normalization using Spotfire DecisionSite 9.0 (Spotfire, Inc., Somerville, MA). Any fold-change observed between test conditions were expected to be at least 50 % greater in magnitude (e.g., 1.5-fold greater) then the fold-change observed between any two biological replicate samples. Genes that did not meet the criteria were removed from the analysis.

A second, independent analysis of the microarray data was performed by Seralogix, Inc. (Austin, TX) using a z-score ranking method to determine significantly altered genes. Additionally, Serologix applied their dynamic Bayesian network analysis to determine the top scoring pathways in our model.

Quantitative real-time PCR. Taqman® universal probes and primer pairs were selected using Roche's Universal Probe Library and probefinder software (www.universalprobelibrary.com) and are located in Table 13. Total RNA was reverse transcribed to cDNA using the Transcriptor First Strand cDNA synthesis kit (Roche, Indianapolis, IN) following manufactor's instructions using 2 μg of total RNA and random primers. PCR reactions were set up with 1X

TABLE 13. Primers and Taqman® universal probes used for quantitative real-time PCR.

Gene Locus BME	Forward Primer	Reverse Primer	Universal Probe*
I 0148--1	catcgaccgttcgcaact	tcggtttggaaaaactcgtc	154
I 0148--2	tttttccaaaccgacctgat	ctcaccacgccataggattt	58
I 0058	tcatccacaacagttaatctttcttc	atgacgctatcacttccatcc	39
I 0060	ccaaccccgaaaatcaga	aatctgttggatttccctgtataaat	138
I 0430	ggcacgactgaacgctatc	aaaagactggcctcgaaaaat	136
I 0829	ggggtttgactggctgatac	caggttcaacagcccgatac	147
I 1094	gcctacaagaacgggcataa	catacatacgcgccagctt	135
I 1194	tcaagaagctggttgaaatgg	attgcccttgtcggtcag	138
I 1364	gaacccgaagaagtctgttca	tgacgcttcagcgacttg	137
I 1501	ggcagcaagattgtcaataaaa	cgccgaagaaggtgaaaata	37
I 1816	tgattctccagcgtgaacag	cttgaccaccagcgcaat	70
I 1830	cttatttcggcggtcaggt	ttggtgcggttttcaagc	57
II 0698	ggctgagggcaaatccat	caaaggcgcttcacctctt	83
II 0851	tgcaggaaaacgtaaattcca	aggcttgatttggcagca	138
II 0891	cactatactggctctttatgtgctg	gacaccgcattgcacaaa	135
II 1127	gctccaagacgacgatgg	ccataaggccgggaagaa	132

*The Universal Probe number indicates the probe utilized from the Roche universal probe library.

TaqMan® universal PCR master mix, no AmpErase® UNG (Applied Biosystems, Foster City, CA), 200 nM of each primer and 100 nM of probe. Quantitative real-time PCR was performed in triplicate for each sample within a plate and repeated 3 independent times using the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Gene expression was normalized to 16s rRNA, and is shown relative to 16M wild type using the $\Delta\Delta C_t$ method (Applied Biosystems Prism 7700 User Bulletin #2). The absolute value of the slope resulting from the log input amount versus ΔC_t was less than 0.1 for all comparisons, validating the use of the $\Delta\Delta C_t$ calculations (data not shown).

RESULTS

***MucR* expression increases at a late exponential growth phase in *Brucella melitensis*.** To determine an ideal time point for transcript analysis of the MucR regulon, expression of the transcriptional regulator MucR was examined in *B. melitensis* 16M wild type over time using quantitative real time PCR (qRT-PCR), shown in Fig. 23. Results show that *mucR* transcript levels increased 3-fold over a period of 6 hours and then began to decrease later in stationary phase. The expression of *mucR* mRNA peaked at 22 hours, when cell cultures reached an average OD_{600}=0.96, and therefore was selected as the time point for microarray analysis.

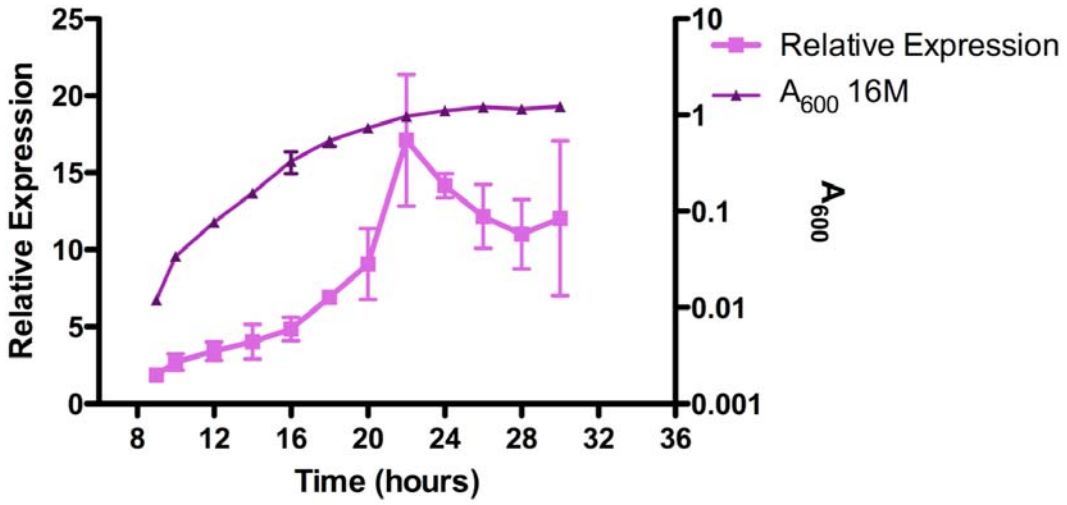


Fig. 23. Quantitative real time PCR of the *mucR* transcript expression level over time in *B. melitensis* 16M wild type. *MucR* transcript levels were normalized to 16srRNA and were calculated by the $\Delta\Delta C_t$ method. Values represent transcripts from 3 biological replicates and the error bars represent the SEM.

MucR regulates gene expression during the late exponential growth phase. Transcript analysis of the MucR regulon at the late exponential growth phase (22 hours) revealed that MucR exerts an equal effect on gene expression, regulating a total of 105 genes: promoting the expression of 53 genes and repressing the expression of 52 genes. Genes regulated by MucR at the late exponential growth phase are listed in Table 14.

Examination of the altered genes revealed that MucR promotes genes involved in nitrogen metabolism, including *nolR* (BMEI 0430), a nitroreductase (BMEII 0246), pseudozurin (BMEII 0965), two ammonium transporters (BMEI 0167 and 0645), nitrous oxide reductase *nosX* (BMEII 0967) and a nitrogen fixation protein, *fixR* (BMEI 1832). MucR was also found to decrease expression of genes that have been found to be important for an acid shock response, including chaperone *hdeA* (BMEII 0906) and *omp25* (10).

MucR regulates genes that may be involved in survival or virulence of the organism, including two extracellular serine proteases, BMEI 0058 and BMEII 0149 and a metalloprotease (BMEI 0829). MucR also regulates a gene within the HlyD protein family (BMEI 0359), which may be involved in protein, drug or toxin secretion across the cell envelope. Two genes that may be involved in the acquisition of iron (BMEII 0704 and 1121) were also found to be regulated by MucR.

TABLE 14. Genes altered by MucR at a late exponential growth phase.

BME locus	Gene Function	Δ*mucR* / wt Student's *t*-test FC	Δ*mucR* / wt Z-Score FC
	Amino Acid Transport and Metabolism		
II 0067	ABC-Type Branched-Chain AA Transport Permease Protein, LivM	4.4	4.2
II 0068	ABC-Type Branched-Chain AA Transport Permease Protein, LivH	2.5	2.3
II 0629	ABC-Type Branched-Chain AA Transport ATP-Binding Protein, LivG	-2.5	-2.4
II 0909	Glutamate/γ-Aminobutyrate Antiporter	-	-3.5
	Carbohydrate Transport and Metabolism		
I 1392	Ribose ABC-Type Transport, ATP-Binding Protein, RbsA	3.1	-
I 1636	Glucose-6-Phosphate Isomerase	3.4	3.0
II 0622	SN-Glycerol-3-Phosphate ABC-Type Transport Permease Protein, UgpE	-4.3	-2.1
	Cell Wall, Membrane and Envelope Biogenesis		
I 0359	Efflux System, HlyD Family	-2.6	-2.5
I 0402	31 KDa Outer-Membrane Immunogenic Protein Precursor	-6.9	-4.4
I 0829	Membrane Zn-Metalloprotease	-1.9	-2.0
I 1056	N-Acetylmuramoyl-L-Alanine Amidase	-2.3	-2.2
I 1426	Putative Undecaprenyl-Phosphate α-N-Acetylglucosaminyltransferase	-1.9	-1.9
I 1501	Transglycosylase Associated Protein	4.8	4.6
I 1502	Alkaline Phosphatase Like Protein	3.1	2.5
I 1830	25 kDa Outer-Membrane Immunogenic Protein Precursor	2.7	2.5
II 0844	31 kDa Outer-Membrane Immunogenic Protein Precursor	-3.1	-3.1
	Coenzyme Transport and Metabolism		
I 0209	Nicotinic Acid Mononucleotide Adenyltransferase	-3.1	-2.8
II 0967	NosX	-3.1	-1.9
	Defense Mechanisms		
I 0655	ABC-Type Multidrug Transport ATP-Binding Protein	-2.2	-1.9
I 1470	Protein YicC	-2.7	-2.5
II 0318	6-Aminohexanoate-Dimer Hydrolase, Penicillin Binding Protein	4.0	3.5
	Energy Production and Conversion		
I 1232	L-Lactate Permease	5.8	5.0
I 1773	Lactoylglutathione Lyase, Putative	-1.8	-1.8
I 1898	Cytochrome O Ubiquinol Oxidase Operon Protein, CyoD	2.3	1.9
II 0246	Nitroreductase	-2.0	-1.8
II 0867	Alcohol Dehydrogenase	-2.6	-2.2
II 0965	Pseudoazurin (Plastocyanin)	-3.8	-2.0
	General Function Prediction Only		
I 0554	Trans-Aconitate Methyltransferase	-3.4	-2.6
I 0709	4-Hydroxyphenylacetate 3-Monooxygenase	-2.7	-2.4
I 1637	CoxG protein	3.9	3.7
II 0084	Uncharacterized ABC-Type Transport System	-2.6	-2.2
II 0149	Extracellular Serine Protease	-3.2	-2.4
II 0611	Integral Membrane Protein Predicted Permease	-5.3	-3.6
II 0698	ABC-Type Sugar Transport ATP-Binding Protein	3.3	2.8
II 0831	Hypothetical Protein, Aminopeptidase-Like Domain	-4.3	-4.2
II 1121	Fe^{3+} ABC-Type Transport System Permease Protein, SfuB	2.4	2.3
	Inorganic Ion Transport and Metabolism		
I 0167	Ammonium Transporter	-2.6	-2.3
II 0704	Bacterioferritin	-7.6	-7.1

TABLE 14--*Continued*

BME locus	Gene Function	Δ*mucR* / wt Student's *t*-test FC	Z-Score FC
	Intracellular Trafficking, Secretion and Vesicular Transport		
I 0058	Extracellular Serine Protease, Type V Secretory Pathway	2.7	2.3
II 0030	Channel Protein, VirB6	1.7	1.7
	Lipid Transport and Metabolism		
I 1832	FixR Protein	-2.4	-2.1
	Nucleotide Transport and Metabolism		
II 0825	Uracil Permease	-2.2	-1.9
II 1040	Cytosine Deaminase	2.0	2.0
	Posttranslational Modification, Protein Turnover and Chaperones		
I 0645	Urease Accessory Protein, UreF	2.8	2.0
II 0891	Disulfide Bond Formation Protein B, DsbB	4.1	3.5
II 0906	HdeA	3.5	3.3
II 0932	Glutaredoxin	3.6	3.2
	Replication, Recombination and Repair		
I 1223	Transposase	2.7	2.7
I 1402	Transposase	3.9	3.3
I 1403	Transposase	3.1	2.2
I 1876	DNA Polymerase III α-Subunit	-2.4	-2.1
II 0183	Transposase	-3.2	-2.4
II 0453	Transposase	-2.3	-2.1
	RNA Processing and Modification		
I 0063	Hypothetical Membrane Spanning Protein	3.1	2.6
	Secondary Metabolites Biosynthesis, Transport and Catabolism		
I 0546	Pyrazinamidase / Nicotinamidase	-3.0	-2.5
	Signal Transduction Mechanisms		
I 1816	Sensory Transduction Protein Kinase	-2.0	-1.9
	Transcription		
I 0430	Nodulation Protein, NolR	2.6	2.4
I 0510	Leucine-Responsive Regulatory Protein	2.3	2.1
I 1098	AsnC Family	-2.6	-2.2
II 0712	Transcriptional Regulator	-3.6	-3.5
II 0721	AraC Family	-2.1	-1.9
II 1022	Transcriptional Regulator	-3.1	-2.2
	Unknown and Other		
I 0014	Hypothetical Protein	5.2	3.0
I 0030	Hypothetical Cytosolic Protein	13.1	9.9
I 0060	Hypothetical Protein	59.9	24.2
I 0061	Hypothetical Protein	19.2	13.3
I 0062	Hypothetical Protein	4.7	4.2
I 0117	Hypothetical Protein	-2.6	-2.1
I 0212	Hypothetical Protein	-3.8	-2.2
I 0495	Hypothetical Protein	3.3	3.0
I 0502	Hypothetical Membrane Spanning Protein	5.3	4.7
I 0542	Hypothetical Protein	5.1	3.2
I 0650	Hypothetical Protein	-2.1	-1.9
I 0683	Hypothetical Protein	-5.5	-2.5
I 0699	Hypothetical Protein	-2.1	-1.9
I 0994	Hypothetical Protein	-3.0	-2.0
I 1000	Hypothetical Protein	-3.1	-2.4
I 1173	Hypothetical Membrane Spanning Protein	-1.8	-1.6
I 1221	Hypothetical Cytosolic Protein	6.3	5.5

TABLE 14--*Continued*

BME locus	Gene Function	Δ*mucR* / wt Student's *t*-test FC	Δ*mucR* / wt Z-Score FC
I 1222	Hypothetical Protein	2.4	2.3
I 1224	Hypothetical Protein	59.9	43.7
I 1311	Hypothetical Protein	3.0	2.7
I 1472	Hypothetical Protein	-2.1	-2.0
I 1509	Hypothetical Protein	2.6	2.3
I 1628	Hypothetical Protein	2.2	1.4
I 1516	Hypothetical Protein	3.2	2.6
I 1703	Hypothetical Protein	2.2	2.1
I 1704	Hypothetical Protein	3.9	2.6
I 1705	Hypothetical Protein	3.2	2.0
I 0807	Hypothetical Protein	-2.1	-1.9
I 1826	Hypothetical Protein	-2.3	-2.1
I 1844	Hypothetical Protein	-3.5	-2.5
I 1896	Hypothetical Membrane Spanning Protein	7.1	6.6
I 1920	Hypothetical Protein	1.8	2.0
I 1931	Hypothetical Protein	5.1	4.8
I 1932	Hypothetical Cytosolic Protein	2.6	2.4
II 0147	Putative Integral Membrane Protein	-2.5	-2.2
II 0182	Hypothetical Protein	-2.5	-2.2
II 0187	Hypothetical Cytosolic Protein	-3.5	-2.1
II 0412	Hypothetical Protein	2.6	2.3
II 0480	Hypothetical Protein	-3.4	-2.5
II 0503	Hypothetical Protein	4.2	3.9
II 0705	Hypothetical Protein	-7.2	-6.3
II 0918	Hypothetical Protein	7.1	6.4
II 0924	Hypothetical Protein	6.5	6.1

Fold change (FC) values are the log ratio of normalized signal values. FC values listed in grey did not pass the statistical test as indicated in that column.

Genes were categorized according to their clusters of orthologous genes (COGs) using the DOE Joint Genome Institute Integrated Microbial Genomics website (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi?page=findGenes) and are summarized in Fig. 24. To determine if any COG functional category was over or under represented within the MucR regulon; the number of genes regulated by MucR were compared to the total number of genes within each COG category found in the genome (Fig. 25). Using this approach, it was found that cell membrane, defense mechanisms, replication and unknown functions are highly over represented in the gene set regulated by MucR. Additionally, it was also found that MucR had no impact on regulation of genes involved in cell division, cell motility and translation. Furthermore, amino acids, carbohydrate, coenzyme, inorganic ion, lipid metabolism, secondary metabolites and signal transduction were found to be under represented by MucR.

To understand the biological role of MucR, Seralogix, Inc. trained a dynamic Bayesian model that is able to utilize any prior biological knowledge of pathways and gene ontologies, as well the condition of time to establish any pathways that are different from the control (wild type). This model is useful because it is not limited to the list of significantly altered genes and is able to apply the expression trend observed for an entire pathway. One limitation of this model is that it is only able to measure the degree of perturbation, but does not

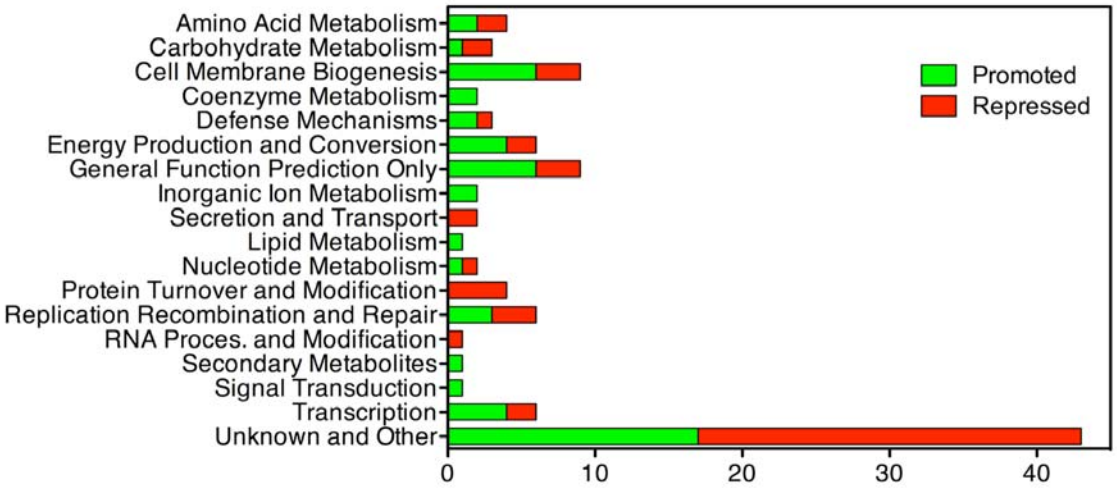


Fig. 24. Total number of promoted and repressed genes in each COG functional category found to be regulated by MucR at a late exponential growth phase.

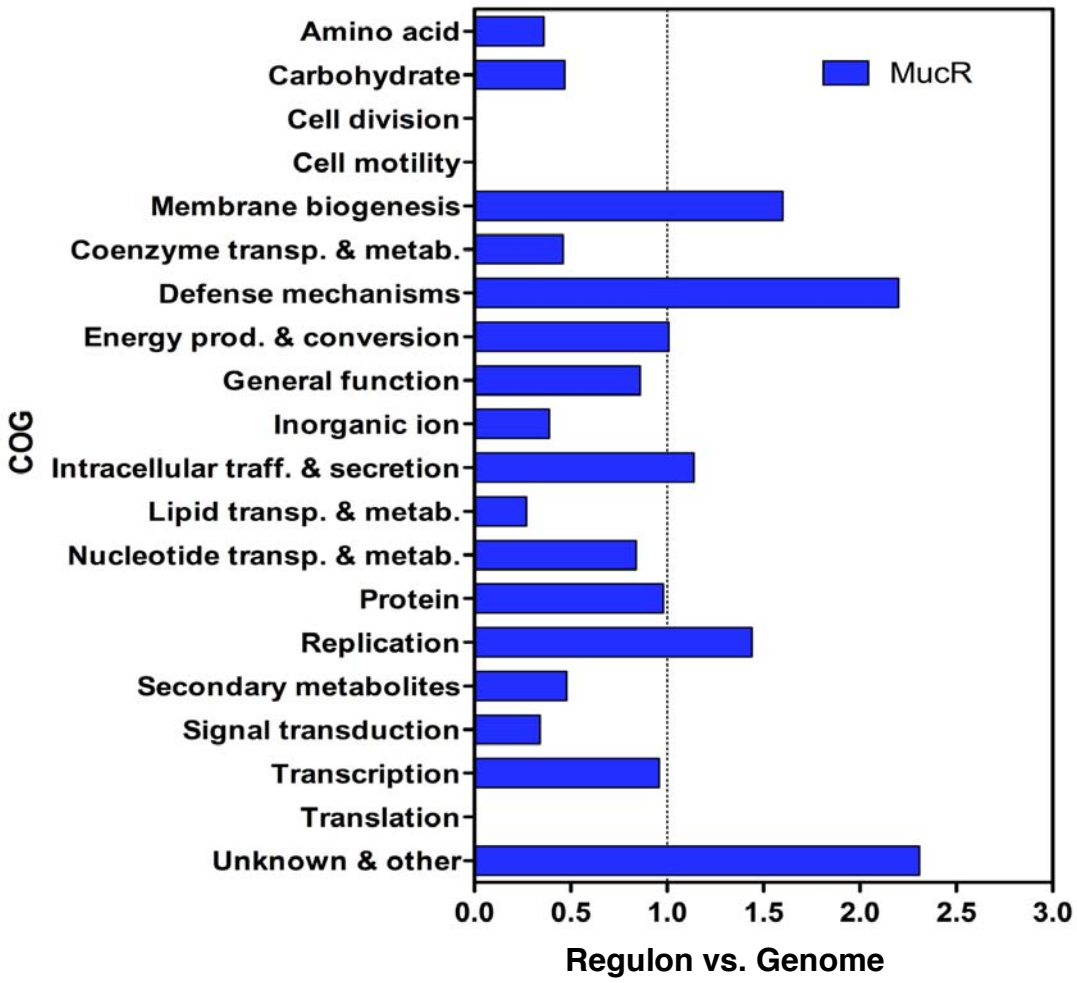


Fig. 25. Over and under representation of COG categories found to be regulated by MucR at a late exponential growth phase. The values shown are the ratio of genes regulated by MucR in each category compared to the ratio of genes present in each COG in the *B. melitensis* genome.

indicate if the pathway is positively or negatively influenced. The 40 top scoring altered pathways are listed in Table 15. ABC transporters are the top scoring altered pathway, which is interesting because they fall into many different COG categories, dependent on the substrate that is transported. ABC transporters included in the significantly altered gene list include transporters for amino acids, carbohydrates and iron.

Verification of microarray results by quantitative real-time PCR. Transcript profiling is a great technique to survey global changes due to an altered genotype or condition. To verify a portion of the microarray data, quantitative real-time PCR was performed to calculate the fold changes of selected gene loci relative to wild type 16M. Genetic loci were selected by either a gene product of interest or by the direction or magnitude of the fold change. The RNA samples that were used for the microarray preparation were also used for the quantitative real time PCR experiments for consistency and because obtaining fresh RNA extractions was not an option at this time.

Nine genes were examined for confirmation of the microarray data and are shown in Table 16. The fold changes for all but one of the genes verified by qRT-PCR were found to be altered in the same direction as the microarray results. Only a small portion of the genes was selected for verification (n=9) and

TABLE 15. Top scoring pathways found to be altered by MucR at a late exponential growth phase.

Rank	Score	MucR Top Scoring Pathway
1	3.109	ABC transporters
2	2.11	Aminoacyl-tRNA biosynthesis
3	1.887	Pyruvate metabolism
4	1.862	Tyrosine metabolism
5	1.86	Purine metabolism
6	1.841	Two componenent system
7	1.84	Pentose and glucuronate interconversions
8	1.832	Benzoate degradation via hydroxylation
9	1.831	Nicotinate and nicotinamide metabolism
10	1.775	Phenylalanine metabolism
11	1.773	Benzoate degradation via CoA ligation
12	1.769	Pyrimidine metabolism
13	1.766	Tryptophan metabolism
14	1.733	Histidine metabolism
15	1.714	Butanoate metabolism
16	1.709	Glycine, serine and threonine metabolism
17	1.705	Glycolysis / Gluconeogenesis
18	1.69	Aminophosphonate metabolism
19	1.682	Taurine and hypotaurine metabolism
20	1.669	Fatty acid metabolism
21	1.664	Valine, leucine and isoleucine degradation
22	1.629	Alkaloid biosynthesis II
23	1.623	Alkaloid biosynthesis I
24	1.611	Styrene degradation
25	1.609	Pentose phosphate pathway
26	1.605	Glycerolipid metabolism
27	1.601	Novobiocin biosynthesis
28	1.598	Biphenyl degradation
29	1.591	Glycerophospholipid metabolism
30	1.585	Limonene and pinene degradation
31	1.548	Porphyrin and chlorophyll metabolism
32	1.501	Glutamate metabolism
33	1.496	Propanoate metabolism
34	1.495	Methane metabolism
35	1.483	β-Alanine metabolism
36	1.478	Metabolism of xenobiotics by cytochrome P450
37	1.468	Arginine and proline metabolism
38	1.442	Bile acid biosynthesis
39	1.385	Urea cycle and metabolism of amino groups
40	1.384	Alanine and aspartate metabolism

The score is a normalized log likelihood measure of how different the experiment data is from the control data used to train a Bayesian model.

TABLE 16. Quantitative real-time PCR and microarray fold changes of selected gene loci regulated by MucR.

	Fold Change		
		Microarray	
BME Gene Loci	qRT-PCR	Student's *t*-test	Z-score
I 0058	8.9	2.7	2.3
I 0060	212.6	59.9	24.2
I 0430	1.1	2.6	2.4
I 0829	1.1	-1.9	-2.0
I 1364	-1720000	-	-
I 1501	3.5	4.8	4.6
I 1830	3.9	2.7	2.5
II 0698	1.5	3.3	2.8
II 0891	6.5	4.1	3.5

Fold change (FC) values for the microarray date are the log ratio of normalized signal values. FCs listed in grey did not pass the statistical test indicated in that column. QRT-PCR fold changes were calculated by the $\Delta\Delta C_t$ method and are shown relative to the wild type 16M bacteria.

could skew the correlation; however, comparison of the microarray and qRT-PCR data demonstrated a concordant gene expression of 88 % in the genes included in microarray gene list. The microarray analysis retained genes that passed the Student's *t*-test with a *p*-value of less than or equal to 0.05; allowing for one false positive in every 20 genes due to random chance. The correlation between the microarray data and qRT-PCR fold changes reveal that the number of false positives observed is only slightly greater then the number expected and may skewed slightly by the small sampling. Furthermore, this correlation is in agreement with the verification results from studies also using custom printed oligonucleotide microarrays with different analysis methods (81, 143, 160).

The list of altered genes includes genes that passed at least one of two different statistical tests. For the Student's *t*-test, only 33 of the 105 total genes passed this particular statistical test, most likely from a difference in the background subtraction that was included in this analysis. Quantitative RT-PCR examined 4 genes that did not pass the Student's *t*-test and confirmed that 3 of these were indeed altered in the same direction as the microarray data, in some cases the fold change was underestimated by the microarray. The one gene that was not altered in the same direction also deviated from the alternative microarray analysis that applied the z-score analysis.

MucR was expected to be included in the list of altered genes, but was absent from the microarray data set. To confirm that *mucR* expression was

altered between the control and Δ*mucR* deletion mutant, qRT-PCR was used to verify that a difference in the gene expression did occur. qRT-PCR confirmed a decrease in *mucR* expression by a -1.72 x 10^6 fold change, suggesting *mucR* was likely excluded from the microarray data due a technical error. The fold change of *mucR* is extremely large because the wild type sample is being compared to the mutant, which does not express *mucR*, resulting in the wild type being compared to only background levels of the PCR reaction, inflating the fold change found between these two samples.

MucR represses expression of exopolysaccharide synthesis. The microarray analysis did not provide any information as to MucR's involvement in the regulation of exopolysaccharide (EPS) biosynthesis as it does in *S. meliloti*. To determine if regulation of EPS is conserved in *B. melitensis*, four genetic loci involved in EPS production were examined by qRT-PCR; *exoF* (BMEII 0851), *exoQ* (BMEII 1127), an EPS production negative regulator precursor (BMEI 1094) and *mucS* (BMEI 1194). Expression of *exoF, exoQ* and the negative regulator was found to be repressed by MucR 6.5, 6.2 and 1.4-fold (respectively), relative to wild type. This is interesting because MucR was found to promote the expression of *exo/exs* genes in *S. meliloti* SU47, but may be responsible for repression in the EFB1 isolate (91). MucR has also been found to be necessary for the production of *mucS,* which also regulates EPS production in *S. meliloti* EFB1 (87). In *B. melitensis*, MucR was found to

promote the expression of *mucS* 1.4-fold, similar to the findings in *S. meliloti* EFB1 (91). Although this is below the microarray analysis cut-off threshold, it could still be biologically significant and is interesting because of the related function in *S. meliloti*. MucR has been shown to have a different regulatory affect on EPS production in the two different strains of *S. meliloti* examined (91). From this data, it appears that MucR gene regulation in *B. melitensis* mirrors that activity in *S. meliloti* EFB1 rather than strain SU47.

DISCUSSION

MucR is a transcriptional regulator found in some α- and Δ-Proteobacteria and one Magnetococcus. MucR has been shown to be important for exopolysaccharide production in nitrogen-fixing *S. meliloti* and regulation of the *virCD* operon and exopolysaccharide in *A. tumefaciens* (19, 87). A MucR mutant was found to be highly attenuated for survival in mouse and macrophage models, suggesting that the transcriptional network associated with MucR can provide insight into the genetic loci that are important for the survival of *B. melitensis* (158). Custom 70-mer oligonucleotide microarrays were used to compare the transcriptional profile of 16M wild type and 16MΔ*mucR* at a late exponential growth phase. Microarray analysis revealed that MucR regulates genes involved in nitrogen metabolism, iron sequestering, stress and stationary phase survival, outer membrane proteins and several proteases. Transcript

comparison revealed that MucR also regulates exopolysaccharide biosynthesis and *mucS* expression in *B. melitensis*, as in its closely related plant symbionts and pathogens.

MucR was found to regulate gene products associated with nitrogen metabolism, with all but one gene locus promoted by MucR. Positively regulated genes include; nitroreductase (BMEII 0246), *fixR* (BMEI 1832) a nitrogen fixation protein, two ammonium transporters (BMEI 0167 and 0645), *nosX* (BMEII 0967), a nitrous oxide reductase and pseudoazurin (BMEII 0965). A gene coding for *nolR*, a global transcriptional regulator important for optimal nodulation in *Sinorhizobium meliloti*, was found to be repressed by MucR (78). MucR positively influenced gene expression of *nosX* and pseudoazurin, which can function as an electron donor to a copper-containing nitrite reductase. These genes are located within an operon and may be co-transcribed with other *nos* genes not identified by the microarray analysis.

Nitrogen is a crucial element needed for the synthesis of many vital components of the cell including protein and DNA. *Brucella* spp., with the exception of *B. neotomae* is able to completely denitrify nitrate to dinitrogen gas, a unique and rare characteristic of animal pathogens (8). The importance of denitrification is two-fold. First, nitrate can serve as a terminal electron acceptor under anaerobic growth conditions, and further reduction of nitrite can accept a total of 6 additional electrons from metabolism (74). Secondly, the generation of

reactive nitrogen species by macrophage cells is an important defense against invading bacterial pathogens. *Brucella*'s ability to mitigate NO toxicity could be instrumental to intracellular survival and is shared by other bacteria pathogens. *Neisseria meningitidis* contains genes coding for nitrite and nitric oxide reductases that aid in mitigating NO toxicity (8). MucR may be a key regulator of genes coding for nitrogen metabolism and may suggest that *Brucella* spp. replicate in an environment low in oxygen, further supported by MucR's down regulation of a gene coding cytochrome O (*cyoD*). In *B. abortus*, it was found that in environments of lowered oxygen tension, cells switch to cytochrome *bo* oxidase to a cytochrome *bd* (74).

MucR's regulation of nitrogen metabolism is very interesting because this transcriptional regulator is found in both *Brucella* and the nitrogen-fixing bacterium *Sinorhizobium*. MucR has only been described to regulate the EPS genes in *S. meliloti* that are required for the proper nodule invasion and thus establishing a successful nitrogen-fixing symbiosis. It is not known if MucR also regulates nitrogen metabolic genes in *S. meliloti*; it would be interesting to determine if MucR also regulates nitrogen metabolism in family members of Rhizobiaceae or if this is a unique function of MucR in *Brucellae*.

An HlyD family efflux system (BMEI 0359) is promoted by MucR, and is often found to be involved in protein, drug and toxin transport across the cell envelope. In *E. coli*, HlyD is part of the type I translocater for hemolysin A (HlyA)

and is important in processing the final active confirmation of HlyA (113). *B. melitensis* has three hemolysin genes in the genome, it would be interesting to examine if HlyD is involved in their secretion.

Several genes associated with stress or stationary growth phase survival were found to be regulated by MucR. *YicC* (BMEI 1470) is promoted by MucR and is found to enhance, but is not required for, stationary growth phase survival, which could be important since our transcript examination was conducted at a culture interval close to the transition into stationary growth phase (116). Additionally, two genes associated with an acid shock response were found to be down regulated by MucR. Omp25 (BMEI 1830) is important for acid shock survival in *B. suis* and the chaperon HdeA was acid inducible in *E. coli* (10, 63). MucR may be important for turning off the stress response when conditions return to normal. It would be interesting to see if oxidative stress induces MucR expression and to examine the trafficking of the Δ*mucR* deletion strain to see if MucR is required following the early stages of the phagosome development when oxidative damage from the host may occur.

Iron is a vital element for many microorganisms and can be difficult to obtain in biological hosts or in aerobic environments. Bacteria have developed different strategies to obtain iron from hosts, including production and use of siderophores, outer membrane receptors for binding host iron-chelating proteins and transporters to take up ferrous iron and heme (73). In the host, iron is often

located intracellularly, chelated by high-affinity iron binding proteins or bound in heme or hemoproteins (73). MucR was found to regulate three genes that may be involved in iron sequestration. Three of these gene products have been characterized in *Brucella* spp. to be haemin-binding proteins (Omp25, BMEI 1830 and Omp31, BMEI 0402 and BMEII 0844). Omp31 has been found to be induced in iron-limiting conditions (24). Although only Omp31 was examined for haemin-binding, a genomic Blastp search of the *B. meltitensis* genome (DOE, JGI, IMG, http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) using the haemin binding protein b from *Bartonella quintana* as the query revealed that BMEI 1830, BMEI 0402 and BME II0844 all have significant amino acid identity, sharing 43, 34 and 30 % (respectively) identity. This strongly suggests that all three of these class 3 Omps may be involved in iron scavenging.

Additionally, MucR was found to decrease expression of an ABC transporter component, BMEII 1121, which transports the oxidized state of iron (ferric). Bacterioferritin (BMEII 0704) is an oligomeric protein for the storage of iron, containing both a binuclear iron center and haem b and is promoted by MucR. A study with *Salmonella enterica* sv. Typhimurium found that bacterioferritin was maximally induced when iron was abundant (148). Bacterioferritin mutations did not attenuate *B. melitensis* in human monocyte-derived macrophages, nor was *S. enterica* sv. Typhimurium attenuated in the mouse model, suggesting that the iron storage is not crucial or there are other

storage mechanisms that are able to replace the storage capabilities of bacterioferritin (30, 148).

In summary, MucR was found to regulate the expression of 105 genes, covering a diverse set of gene loci and was found to have a conserved function in the regulation of EPS biosynthesis in *B. melitensis.* Although *Brucella* does not fix nitrogen from the atmosphere, MucR regulates a number of genes involved in nitrogen assimilation and transport of nitrogen containing compounds. It is not clear if this function for MucR is conserved between *Brucella* and members of the closely related family of nitrogen-fixing Rhizobiaceae, but would be interesting to investigate. MucR was also found to regulate genes involved in sequestering and storing iron, a vital element for many microorganisms. Additionally, MucR regulates several stress and acid shock response genes, as well as a component of a putative type I secretion system, the only MucR regulated gene locus found to be involved in protein secretion identified in this screen. This study reveals that MucR is an important regulator of gene expression at the late exponential growth phase by regulating genes in nitrogen metabolism, iron sequestering, stress response and virulence.

CHAPTER V

SUMMARY AND CONCLUSIONS

SUMMARY

This study focused on identifying genetic loci and products that are required for the survival of the facultative intracellular pathogen *B. melitensis*. Two different approaches were taken to identify such genetic loci: a random transposon mutagenesis bank screening, and transcriptional network profiling to define the regulons of two transcriptional regulators required for intracellular survival. These combined methods identified approximately 1,000 genetic loci that may be important for the survival of *B. melitensis*. While many of these genetic loci have not been examined in *B. melitensis*, a few genes have been confirmed for their contribution to the survival of the bacteria in other work, confirming the approaches taken for the identification of genetic loci required for the intracellular replication of the pathogen.

CONCLUSIONS

Signature tagged transposon mutagenesis is a successful and useful technique to screen for gene products that are required for intracellular survival. Seventy-eight genes were identified in this study that are indispensable for the intracellular replication of *B. melitensis*. Many of the mutants identified have

metabolic defects, suggesting that the ability to synthesize many metabolites is crucial to intracellular survival and provides insight into the nature of the brucellosome, the intracellular replicative vacuole for *Brucella* spp. in the host cell. Transposon mutagenesis also identified classical virulence genes; however, metabolic defects were far more prominent and supports the notion that *Brucella* spp. are stealthy pathogens that are able to replicate in the host cell by being able to synthesize all of the necessary metabolites (75).

The identification of transcriptional regulators in this screen was of particular interest, due to the diverse environments that *B. melitensis* is able to inhabit and the lack of detailed information on transcriptional networks that regulate virulence genes. Seven transcriptional regulators were identified in the transposon mutagenesis study among which was VjbR, a LuxR homologue. VjbR mutants were found to be highly attenuated for survival in mouse and macrophage models of infection. LuxR homologues have been linked to the regulation of virulence determinates in a number of different organisms and are of great interest in the role of pathogenesis in *B. melitensis*. Very little is known about the transcriptional networks that exist in *Brucella* spp., beyond the identification of transcriptional regulators that are attenuated for survival and possibly a few transcriptional targets.

The second goal of this study was to identify the transcriptional targets of two transcriptional regulators that were identified as attenuated in transposon

mutagenesis studies. Identification of the regulons associated with these transcriptional regulators could provide further insight into genes required for survival and virulence of *B. melitensis*. Transcript comparisons by microarray technology were used to determine gene targets regulated by both transcriptional regulators.

Microarray analysis of the quorum sensing components VjbR and the AHL chemical signal C_{12}-HSL revealed that both are global regulators contributing to the virulence and survival of *B. melitensis.* Combined, these two quorum sensing components were found to regulate over 25 % of the *B. melitensis* genome. VjbR and C_{12}-HSL were found to regulate a number of genes with many different metabolic functions, as well as virulence genes such as adhesins, proteases, lipoproteins and several potential secretion systems and effector proteins. Seventy-nine genes found to be regulated by VjbR and/or C_{12}-HSL have been identified as attenuated for intracellular survival in transposon mutagenesis screens, conducted here or elsewhere, confirming the importance of this transcriptional regulon for the intracellular survival of *B. melitensis*.

Beyond the identification of regulatory targets for VjbR and C_{12}-HSL, it was observed that VjbR was able to regulate gene expression at both an exponential growth and during the transition into stationary phase. VjbR was found to regulate different sets of virulence genes at these two time points, suggesting these two distinct regulons may confer different advantages and may

be expressed differently as needed during the course of the infection. The *virB* operon and presumably the stationary phase regulated genes may be required early in the infection, based on the trafficking of Δ*vjbR* and its inability to maintain the replicative brucellosome early during the course of infection of the host cell (25). *FliF* and potentially the genes regulated during the exponential growth phase may be needed later during the infection of the host and are required to develop the chronic stages of the infection, as observed in mice with a Δ*fliF* deletion mutant (44). The observations made *in vitro* provide some insight to the gene regulation that may occur *in vivo* and is of great interest.

Currently, the activating signal for VjbR has not been described, but it identification could provide insight into the bi-phasic gene regulation observed with VjbR. A second AHL signal has been identified, potentially 3-oxo-C_{12}-HSL, but the structure of this second AHL signal remains unconfirmed by mass spectrophometry (144). It is possible that this signal may be the activator of VjbR, but this has not been tested. It will be interesting to identify not only the activating signal for VjbR, but also the environmental cue(s) that allow for the bi-phasic gene response and the differentiation between the two distinct regulons.

Interestingly, by comparing the wild type microarray observations to the observations of the addition of exogenous C_{12}-HSL, it appears that the signal is either absent, actively degraded, or does not reach a physiological concentration at either time point examined. This observation was surprising, particularly at

the later time point when AHL concentration was expected to have reached a physiological level. Examination of *virB* expression over time shows that it is induced at the onset of stationary growth in wild type cells (which does not occur when exogenous C_{12}-HSL is added) and that after approximately 8 hours, its expression decreases (29). It would be very interesting to determine if the observed decrease in *virB* expression is a result of the accumulation of C_{12}-HSL and if so, whether it is mediated by C_{12}-HSL interactions with VjbR.

Lastly, the microarray analysis of the effects of addition of C_{12}-HSL to wild type and the Δ*vjbR* deletion mutant provides some insight into the activity of C_{12}-HSL *in vitro*. The first observation is that C_{12}-HSL appears to be an antagonist of VjbR activity. Examination of the genes found to be regulated by both conditions revealed that all but 3 genes have an inverse relationship. If VjbR was found to promote the expression of a particular gene, then C_{12}-HSL represses the expression of that gene. Furthermore, the microarray analysis revealed that the addition of C_{12}-HSL to wild type culture mimics the Δ*vjbR* deletion mutant to a certain degree, observed by the relationship of the co-regulated genes by microarray analysis and by examination of target genes.

Interestingly, qRT-PCR data (Table 12) revealed that C_{12}-HSL induces the expression of a second luxR homologue and this induction was even greater in the absence of VjbR. It would be interesting to determine if VjbR is able to repress the induction of BabR by binding C_{12}-HSL, and if C_{12}-HSL interaction

with BabR is responsible for the induction of itself, creating a positive feedback loop that has been observed in quorum sensing circuits in other bacteria (130). In addition to observing the high induction of BabR in the absence of VjbR, C_{12}-HSL had a remarkably different expression profile at the stationary growth phase, found to have only a positive affect on gene expression. It is hypothesized that the gene expression observed by C_{12}-HSL is occurring through interactions with BabR.

Based on all of the observations discussed here, it is proposed that there are two dueling quorum sensing circuits in *B. melitensis*, VjbR activated by an unknown signal(s) which activates gene expression during exponential growth and at the early stationary growth phase, and by the BabR and C_{12}-HSL induction of gene expression. This model is supported by the observations that VjbR is able to bind C_{12}-HSL, potentially to keep the second circuit disabled by binding the activating signal until the appropriate time when the genes regulated by BabR and C_{12}-HSL are needed. Conversely, it can't be ruled out that C_{12}-HSL binds VjbR to inactivate the activity of the VjbR until the appropriate time, placing the BabR circuit on the top of the hierarchical arrangement. Further research would need to be conducted to determine if a hierarchical arrangement does indeed exist and the ordering between the two. Currently, Δ*babR* mutants are only slightly attenuated in macrophage or mouse models of infection (120). It

would also be interesting to examine this mutant in the natural host and *in vitro* to determine the role and importance of this second quorum sensing circuit.

The second transcriptional regulator examined in this study, MucR, was found to regulate gene expression at the late exponential growth phase. MucR was found to regulate several genes associated with nitrogen metabolism and iron sequestering. Nitrogen is not only a crucial element for many metabolic compounds, but could be important anaerobic respiration, with nitrate serving as the terminal electron acceptor. This could be crucial to the survival of *B. melitensis* in the host cell in an environment with a low oxygen tension. *B. melitensis* is able to fully reduce nitrate to dinitrogen gas, with some genes found to be regulated by MucR (28). The full reduction of nitrate to dinitrogen gas is able to accept a total of 8 electrons from metabolism and could be a crucial element to *Brucella* intracellular survival. Additionally, this could suggest that the brucellosome in the host cell may be deprived of oxygen. In addition to the metabolic importance of denitrification, the ability to reduce reactive nitrogen species produced by the host cell could mitigate the detrimental damages and could contribute to the intracellular survival. It would be interesting to study *in vitro* the importance of this regulation in nitrogen rich and limiting media.

MucR was also found to regulate several genes involved in iron sequestration and storage. Iron and the relationship in the virulence of different pathogens has long since been noted, dating as far back as the 1940s (117). It

would be interesting to determine if MucR is a key regulator of iron sequestering and storage and how this relates to the virulence and survival of the organism.

MucR's involvement in the regulation of exopolysaccharide biosynthesis was confirmed in *B. melitensis*, showing a conserved role for MucR between members of the order Rhizobiales. It would be interesting to determine if any of the additional regulatory roles of MucR found in this study are conserved in other Rhizobiales members. Expression profiling of MucR revealed that this transcriptional regulator contributes to nitrogen metabolism, iron storage and sequestering, stress response and potential virulence determinates; as well as a conserved function in the regulation of *mucS* and exoploysaccharide production.

From the studies described here, the most interesting finding is in respect to the signaling activity of C_{12}-HSL. It was surprising to find that C_{12}-HSL acts as an antagonist to VjbR activity, as well as the different gene expression profile in the absence of VjbR. Also, it was very interesting to find that C_{12}-HSL induces the expression of a second LuxR homologue, BabR. The observed C_{12}-HSL antagonist activity to VjbR and promotion of BabR gene expression suggests that this signal may act as a cue to alter gene expression in *B. melitensis* and that the production of this AHL signal must be tightly regulated during the course of infection. Currently, the genetic basis of the production of AHL signals in *Brucella* is unknown and is crucial to beginning to understand to basis of quorum sensing in *B. melitensis*. In addition to regulating gene expression in bacteria

cells, 3-oxo-C_{12}-HSL has been shown to have a profound affect on the immune response of host, down regulating TNF-α and skewing the cell mediated immune response towards a Th2 cytokine response, aiding intracellular pathogen evasion of the host immune response (145). The immunomodulation property of 3-oxo-C_{12}-HSL could be very important for the establishment of *Brucella* in the resident host cell.

The findings of the research discussed in this dissertation leads to many questions concerning the role of the genetic loci described here and their contribution to the virulence and survival of *B. melitensis.* The observations in regard to the quorum sensing components VjbR and C_{12}-HSL were all conducted *in vitro*, and have lead to a proposed model of the hierarchical arrangement of up to 4 LuxR-like transcriptional regulators (Fig. 22). It will be important to continue the development of the model of quorum sensing *in vitro* and to apply those findings to develop a model of quorum sensing *in vivo*. Some of the questions to be addressed in continued research include: 1) Does the C_{12}-HSL signal inactivate VjbR, or does VjbR inactivate C_{12}-HSL and how does this alter the hierarchical arrangement of multiple quorum sensing circuits, 2) Does quorum sensing *in vivo* regulate gene expression in a density dependent manner or does it behave in more of a diffusion sensing manner, used to determine enclosure in a small vacuole, such as the early endosome or brucellosome, and 3) What is the nature of the signaling activity of C_{12}-HSL *in vivo*, does it remain

within the brucellosome and regulate gene expression in the bacteria cell, or does it diffuse out of the brucellosome and function to skew the cell mediated response of the host cell to favor intracellular survival? All of these studies are dependent on the ability to manipulate and control the AHL production by *B. melitensis* and is a current limitation; thus the identification of the genetic loci responsible for the production of AHLs must be a priority.

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