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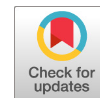
RESEARCH ARTICLE



Endoribonuclease YbeY Is Linked to Proper Cellular Morphology and Virulence in *Brucella abortus*

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ABSTRACT The YbeY endoribonuclease is one of the best-conserved proteins across the kingdoms of life. In the present study, we demonstrated that YbeY in *Brucella abortus* is linked to a variety of important activities, including proper cellular morphology, mRNA transcript levels, and virulence. Deletion of *ybeY* in *B. abortus* led to a small-colony phenotype when the bacteria were grown on agar medium, as well as to significant aberrations in the morphology of the bacterial cell as evidenced by electron microscopy. Additionally, compared to the parental strain, the Δ*ybeY* strain was significantly attenuated in both macrophage and mouse models of infection. The Δ*ybeY* strain also showed increased sensitivities to several *in vitro*-applied stressors, including bile acid, hydrogen peroxide, SDS, and paraquat. Transcriptomic analysis revealed that a multitude of mRNA transcripts are dysregulated in the Δ*ybeY* strain, and many of the identified mRNAs encode proteins involved in metabolism, nutrient transport, transcriptional regulation, and flagellum synthesis. We subsequently constructed gene deletion strains of the most highly dysregulated systems, and several of the YbeY-linked gene deletion strains exhibited defects in the ability of the bacteria to survive and replicate in primary murine macrophages. Taken together, these data establish a clear role for YbeY in the biology and virulence of *Brucella*; moreover, this work further illuminates the highly varied roles of this widely conserved endoribonuclease in bacteria.

IMPORTANCE *Brucella* spp. are highly efficient bacterial pathogens of animals and humans, causing significant morbidity and economic loss worldwide, and relapse of disease often occurs following antibiotic treatment of human brucellosis. As such, novel therapeutic strategies to combat *Brucella* infections are needed. Ribonucleases in the brucellae are understudied, and these enzymes represent elements that may be potential targets for future treatment approaches. The present work demonstrates the importance of the YbeY endoribonuclease for cellular morphology, efficient control of mRNA levels, and virulence in *B. abortus*. Overall, the results of this study advance our understanding of the critical roles of YbeY in the pathogenesis of the intracellular brucellae and expand our understanding of this highly conserved RNase.

KEYWORDS *Brucella*, *ybeY*, RNase, YbeY, ribonuclease

Received 21 February 2018 **Accepted** 3 April 2018

Accepted manuscript posted online 9 April 2018

Citation Budnick JA, Sheehan LM, Colquhoun JM, Dunman PM, Walker GC, Roop RM, II, Caswell CC. 2018. Endoribonuclease YbeY is linked to proper cellular morphology and virulence in *Brucella abortus*. J Bacteriol 200:e00105-18. https://doi.org/10.1128/JB.00105-18.

Editor Victor J. DiRita, Michigan State University

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Ribonucleases (RNases) are enzymes that catalyze the cleavage of myriad RNAs, be they mRNA, tRNA, rRNA, or small RNA (sRNA), and these enzymes are divided into two major classes called exoribonucleases and endoribonucleases depending on their

ability to cleave RNA strands at terminal or nonterminal nucleotides, respectively (1, 2). The “day-to-day operations” of RNases include degradation of RNAs during housekeeping turnover processes, but RNases also process longer RNA transcripts into shorter, functional RNAs. A classic example of RNA processing is the generation of the three major rRNAs (i.e., 23S, 16S, and 5S) and of tRNAs from precursor RNAs, a process catalyzed by several different RNases in bacteria (3, 4). As such, bacteria encode an extensive array of RNases to perform a wide variety of degradation and processing functions.

One of the more recently described bacterial RNases is the YbeY endoribonuclease (5). Interestingly, the structure of YbeY was studied prior to the availability of any insights into its biological functions. The crystal structure of the *Aquifex aeolicus* YbeY ortholog revealed resemblances to metal-dependent proteinases such as collagenases (6), while crystallization of the *Escherichia coli* YbeY protein as part of an NIH-funded Protein Structure Initiative program led to the suggestion that it is a metal-dependent hydrolase (7). Subsequently, the *Sinorhizobium meliloti* YbeY ortholog was found to be required for symbiosis, while *E. coli* YbeY drew attention because of its regulation as a heat shock protein (8). YbeY was then shown to participate in the maturation of ribosomal RNAs and the biosynthesis of ribosomes, and, more recently, evidence has been reported that YbeY functions as an endoribonuclease in rRNA maturation activities and 70S ribosome quality control (5, 9–11). Additionally, YbeY plays a significant role in the regulation and stability of bacterial sRNAs (12, 13). Not only has YbeY been linked to the capacity of *S. meliloti* to form an effective symbiotic relationship with its plant host alfalfa, but *ybeY* has been shown to be required for the full virulence of *Vibrio cholerae* and *Yersinia enterocolitica* (14–16). While RNases, including YbeY, are known to be important virulence determinants for several bacterial pathogens, very little is known about the role of RNases in the *Brucella* spp. (17).

The brucellae are small Gram-negative bacteria that cause significant disease in both humans and animals globally (18), and these bacteria are intracellular pathogens of macrophages and dendritic cells, where they reside in a vacuole-bound niche in close proximity to the endoplasmic reticulum (19, 20). Interestingly, the brucellae do not produce classical virulence factors, such as toxins or endotoxic LPS; rather, these bacteria are stealthy pathogens whose ability to cause disease is directly related to their capacity to survive and replicate inside the cells of the host (21, 22). As noted above, little is known about RNases in *Brucella* spp., and in fact, only two published reports describe RNases in *Brucella*, and neither of the described RNases is required for the infectivity of the brucellae (23, 24). We have recently investigated the contribution of the RNase YbeY to *Brucella* biology, and among several interesting observations, we have determined that YbeY is required for normal cellular morphology and wild-type virulence in *B. abortus* 2308. Overall, the current report defines and characterizes the importance of YbeY in *Brucella*; moreover, these data shed light on the significance of this endoribonuclease for intracellular bacterial pathogens.

RESULTS

YbeY is required for normal growth and cellular morphology of *Brucella abortus*. *Brucella abortus 1_2156* (*bab1_2156*; also known as *bab_rs26200*) is located on chromosome I of *Brucella melitensis* biovar Abortus 2308 between *bab1_2155* (*phoH*) and *bab1_2157* (*tlyC*) (Fig. 1A). The YbeY protein exhibits 56% identity and 68% similarity to the YbeY endoribonuclease from *Sinorhizobium meliloti* 1021. For this reason, and due to the results outlined in this report, here we refer to *bab1_2156* as *ybeY*.

An isogenic deletion of *ybeY* in *B. abortus* 2308 resulted in impaired growth *in vitro* and abnormal cellular morphology compared to the parental strain (Fig. 1). The *B. abortus* Δ*ybeY* deletion strain exhibited a small-colony phenotype when grown on agar medium, and this defect was genetically complemented when *ybeY* was provided in *trans* on plasmid pBBR-1MCS4 (Fig. 1B). When cultured in brucella broth (i.e., rich medium), the *ybeY* deletion strain was able to grow to similar maximum numbers of

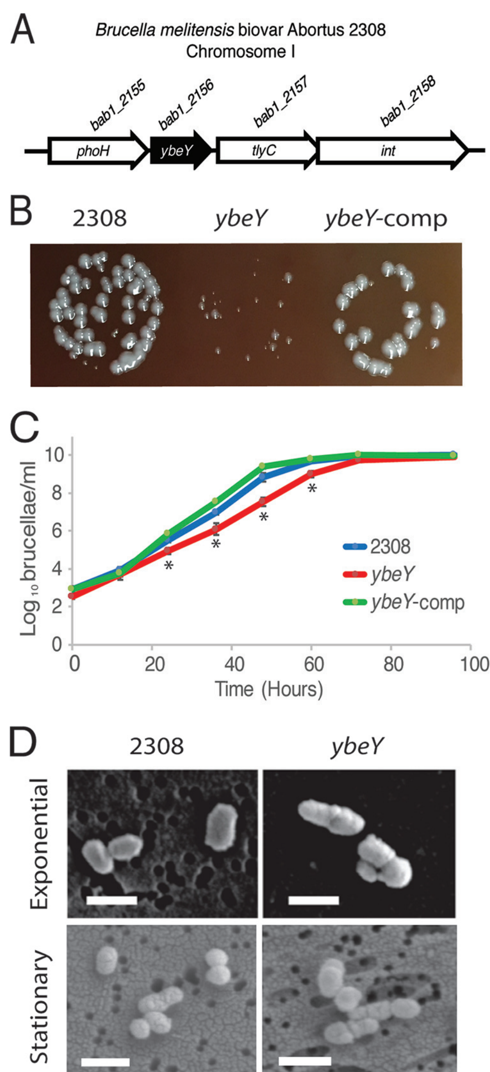

FIG 1 *In vitro* growth characteristics and cellular morphology of *B. abortus* 2308::Δ*ybeY*. (A) Genomic context of *ybeY*. The *ybeY* gene (*bab1_2156*; also known as *bab_rs26200*) is located on chromosome I in *B. abortus* 2308. *ybeY* is flanked by *phoH* (*bab1_2155*; also known as *bab_rs26195*) and a gene encoding a putative hypothetical protein (*bab1_2157*; also known as *bab_rs26205*). (B) Photograph of *B. abortus* colonies on SBA after 72 h of growth. (C) Growth curve of *Brucella abortus* strains in rich medium. *B. abortus* 2308, *B. abortus* 2308::Δ*ybeY*, and *B. abortus* 2308::Δ*ybeY*-comp were grown in brucella broth, and CFU counts per milliliter were monitored by serial dilution. The asterisk denotes a statistically significant difference ($P < 0.05$; Student's *t* test) between the *ybeY* deletion strain and parental strain 2308. (D) Electron microscopy of *Brucella abortus* cells. Exponential- and stationary-phase cells of *Brucella* strains were fixed and viewed using scanning electron microscopy (magnification, ×30,000). Bars = 1 μm.

bacteria as the parental strain, but the *ybeY* deletion strain had a decreased rate of growth during the exponential-growth phase (Fig. 1C). During the exponential-growth phase, *B. abortus* 2308 had a generation time of 2.2 h whereas the *ybeY* deletion strain had a generation time of 2.8 h. Importantly, the growth rate of the *B. abortus* Δ*ybeY* deletion strain was restored to a 1.9-h generation time by in *trans* complementation of *ybeY*.

Using scanning electron microscopy, the *ybeY* deletion strain was observed to have cellular morphology deformities when the bacteria were collected from the exponential and stationary phases of growth in brucella broth (Fig. 1D). As expected, *B. abortus* 2308 cells were coccobacilli in shape during the exponential phase of growth and cocci during the stationary phase of growth, with clear septa between dividing cells. The *ybeY* deletion strain, however, exhibited noticeable morphological irregularities, including

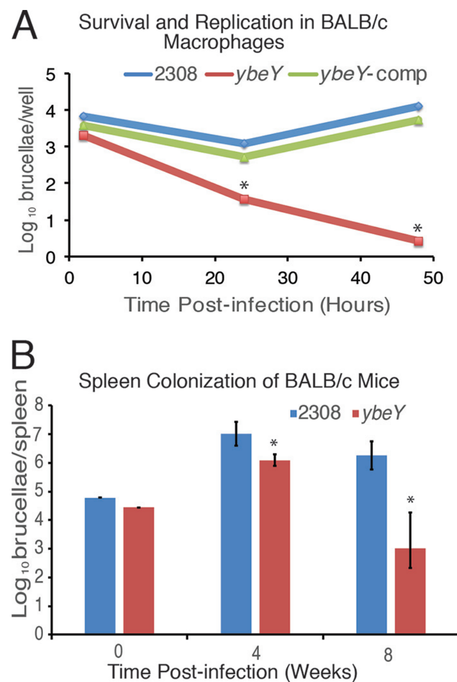

FIG 2 Virulence of *B. abortus* 2308 and Δ*ybeY* in peritoneally derived macrophages and BALB/c mice. (A) Macrophage survival and replication experiments. Cultured peritoneal macrophages from BALB/c mice were infected with *B. abortus* 2308, the isogenic *ybeY* deletion strain (*ybeY*), and the *ybeY* complemented strain (*ybeY*-comp). At the indicated times postinfection, macrophages were lysed, and the number of intracellular brucellae present in these phagocytes was determined by serial dilution and plating on agar medium. The asterisk denotes a statistically significant difference ($P < 0.05$; Student's *t* test) between the *ybeY* deletion strain and parental strain 2308 and between the *ybeY* deletion strain and the complemented strain at 24 and 48 h postinfection. (B) Mouse infection experiments. BALB/c mice (5 per strain) were infected intraperitoneally with *B. abortus* 2308 and with the isogenic *ybeY* deletion strain (*ybeY*). Mice were sacrificed at weeks 4 and 8 postinfection, and levels of brucellae/spleen were determined. The data are presented as average numbers of brucellae ± standard deviations of results from the 5 mice colonized with a specific *Brucella* strain at each time point. The asterisk denotes a statistically significant difference ($P < 0.05$; Student's *t* test) between the *ybeY* deletion strain and parental strain 2308 at 4 and 8 weeks postinfection.

occurrences of clusters of cells appearing to be unable to properly divide during both the exponential and stationary phases of growth. Taken together, these data demonstrate that YbeY is required for the efficient growth and cellular morphology of *B. abortus*.

YbeY contributes to *B. abortus* virulence in macrophages and experimentally infected mice. To characterize the importance of YbeY for *B. abortus* virulence, the *ybeY* deletion strain was assessed for the ability to infect peritoneal macrophages *in vitro* and BALB/c mice *in vivo* (Fig. 2). Peritoneally derived macrophages isolated from BALB/c mice were infected with *B. abortus* 2308 or *B. abortus* 2308::Δ*ybeY* or *B. abortus* 2308::Δ*ybeY*-comp at a multiplicity of infection (MOI) of 100. The *ybeY* deletion strain was strikingly less able to survive and replicate within the macrophage than the parental strain at 24 and 48 h postinfection, and this decrease in survival and replication was restored to wild-type levels in the *ybeY* complemented strain (Fig. 2A). Similarly, the *ybeY* deletion strain exhibited a substantially reduced ability to infect BALB/c mice compared to parental strain 2308, as significantly fewer bacteria were recovered from the spleens of mice infected with the *ybeY* deletion strain after both 4 and 8 weeks of infection (Fig. 2B). These experiments indicate that YbeY is necessary for the ability of *B. abortus* to sustain infection in macrophages and mice.

Deletion of *ybeY* in *B. abortus* leads to increased sensitivities to general stress and wide-ranging metabolic aberrations. Due to the decreased growth rate, defect in cell morphology, and reduced ability to infect in the *in vitro* and *in vivo* models of the

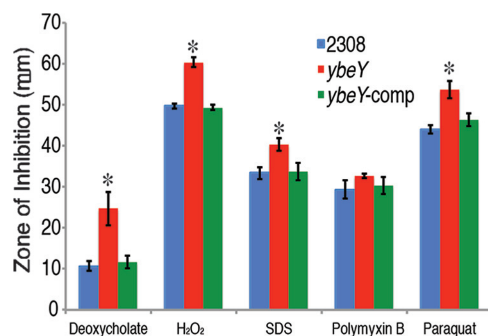

FIG 3 YbeY is required for optimal resistance to biologically relevant stresses. Sensitivity assay results are shown. *Brucella abortus* 2308, the *ybeY* deletion strain, and *ybeY*-comp complemented strain were assessed in a disk diffusion assay for their comparative susceptibilities to various sources of stress, including deoxycholate (10%), H_2O_2 (30%), SDS (20%), polymyxin B (10 mg/ml), and paraquat (0.25 M). The results are plotted as the average diameter (±standard deviation) of the zone of inhibition around a disk, and the results are from a single experiment that was performed in triplicate. Asterisks denote a statistically significant difference ($P < 0.05$; Student's *t* test) between the *ybeY* deletion strain and parental strain 2308 for a given condition.

ybeY deletion strain, we sought to gain insight into the link between YbeY and general stress in *B. abortus*. To achieve this, we employed disk diffusion assays in which *B. abortus* strains were exposed to a variety of stressors, including deoxycholate (10%), H_2O_2 (30%), sodium dodecyl sulfate (SDS) (20%), polymyxin B (10 mg/ml), and paraquat (0.25 M) (Fig. 3). In these experiments, the *ybeY* deletion strain was more sensitive than parental strain 2308 to deoxycholate, H_2O_2, SDS, and paraquat, and genetic complementation of *ybeY* in the deletion strain restored the zones of inhibition to the levels observed for strain 2308. Interestingly, deletion of *ybeY* had no effect on the ability of *B. abortus* to withstand killing by polymyxin B. These data demonstrate that YbeY is important for the ability of *B. abortus* to cope with general stress conditions.

The Biolog Phenotype MicroArray system provides an inexpensive and rapid means of testing microorganisms for the ability to grow under hundreds of sets of conditions. Here, we employed Biolog Phenotype MicroArrays to analyze the growth of *B. abortus* 2308 and *B. abortus* 2308::Δ*ybeY* in a wide variety of different nutrient sources and environments and in the presence of various stressors. Each Biolog Phenotype MicroArray plate was inoculated with 10^8 CFU/well of the appropriate *Brucella* strains and incubated for 84 h at 37°C. After 84 h of incubation, each individual well was measured at an optical density (OD) of 590 nm and visually monitored for growth, as indicated by metabolic activity (clear to purple) (see Data Set S1 in the supplemental material). Overall, we observed 27 differences in growth between *B. abortus* 2308 and the *ybeY* deletion strain (see Table S1 in the supplemental material). The conditions under which the 2308::Δ*ybeY* mutant grew more efficiently than strain 2308 are highlighted in green, and the conditions under which strain 2308 grew more efficiently than the *ybeY* strain are highlighted in red. With regard to carbon sources, the *ybeY* deletion strain was better able than parental strain 2308 to utilize malic acid and laminarin (a storage glucan). However, deletion of *ybeY* led to the inability of *B. abortus* to utilize butyric acid or caproic acid as a carbon source for growth. Compared to growth of *B. abortus* 2308, growth of the *ybeY* deletion strain was more sensitive to dodecyltrimethyl ammonium bromide, promethazine, alexidine, dichlofluanid, chloroxylenol, sodium m-periodate, lidocaine, josamycin, thioridazine, patulin, and tetrazolium violet. Conversely, growth of *B. abortus* 2308::Δ*ybeY* was more resistant to the presence of fusaric acid, 1-chloro-2,4-dinitrobenzene, 2-phenylphenol, antimony (III) chloride, pentachloro-phenol, azathioprine, phenethicillin, and lawsone. Taken together, the Biolog Phenotype MicroArray results underscored the diverse metabolic abnormalities that result from the deletion of *ybeY* in *B. abortus*.

YbeY impacts the levels of mRNA associated with a variety of cellular systems. The pleiotropic effects of *ybeY* loss on cellular RNAs have been well documented in

other bacteria (5, 9, 10, 12, 15, 25, 26), and as such, we hypothesized that deletion of *ybeY* would lead to changes in mRNA levels in *B. abortus*. Therefore, we employed microarray technology to identify mRNAs that are influenced by YbeY. This experiment was performed using RNA from cultures of *B. abortus* 2308 and *B. abortus* 2308::Δ*ybeY* grown in brucella broth to the late exponential phase (Data Set S2). Taking the results together, mRNAs from 84 genes exhibited differential expression levels (>3-fold difference) in the Δ*ybeY* mutant; of these, expression levels of 34 mRNAs were elevated in the *ybeY* deletion strain whereas those of 51 mRNAs were decreased in the *ybeY* deletion strain compared to the parental strain (Table 1). The mRNAs that displayed differential quantities in the *ybeY* deletion strain included those encoding membrane proteins and transport systems; proteins involved in DNA replication and transcriptional or translational regulation; proteins related to flagellar processes; proteins linked to metabolism, signaling, and enzymatic processes; and hypothetical proteins.

Interestingly, several of the mRNAs identified in the *ybeY* microarray encode proteins that have been previously characterized as being required for efficient *Brucella* infection or as being differentially expressed in the bacterium during intracellular trafficking of *Brucella*. Seven genes, *bab2_1099* (encoding FtcR, a flagellar transcriptional regulator), *bab2_1106* (encoding flagellin), *bab1_0303* (encoding UreG1, a urease accessory protein), *bab2_0583* (encoding an ABC transporter permease), *bab2_0584* (encoding an ABC transporter permease), *bab2_0585* (encoding UgpB, an ABC transporter periplasmic binding protein), and *bab1_1302* (encoding a hypothetical protein), have been implicated in *Brucella* virulence (27–32). Seven other genes, *bab1_1679* (encoding an ABC transporter ATPase), *bab1_1792* (encoding an ABC transporter periplasmic binding protein), *bab2_0282* (encoding an ABC transporter permease), *bab2_0700* (encoding an ABC transporter periplasmic binding protein), *bab1_1681* (encoding cell envelope biogenesis protein TonB), *bab2_0547* (encoding an ABC transporter periplasmic binding protein), and *bab2_0548* (encoding an ABC transporter permease), were shown previously to be differentially expressed in *Brucella* during intracellular infection (33, 34).

Contribution of YbeY-associated genes to *Brucella abortus* virulence. Given the large number of systems dysregulated in the *B. abortus ybeY* deletion strain, it is difficult to draw specific conclusions about the linkages between YbeY and individual mRNAs or systems and the observed phenotypes resulting from the deletion of *ybeY*. Therefore, to begin to define the YbeY-associated mRNAs that are required for virulence, we constructed strains harboring deletions in the nine genes that exhibited the greatest levels of mRNA difference in our microarray experiments (Table 1). Subsequently, peritoneally derived macrophages from BALB/c mice were infected with parental strain *B. abortus* 2308, as well as with the *B. abortus* strains with isogenic deletions of *bab2_0277* (encoding choline dehydrogenase and related flavoprotein), *bab2_0282* (encoding an ABC transporter permease), *bab2_0822* (encoding an ABC transporter periplasmic binding protein), *bab2_0548* (encoding an ABC transporter permease), *bab2_0830* (encoding an ABC transporter periplasmic binding protein), *bab2_1109* (encoding an ABC transporter periplasmic binding protein), *bab2_0700* (encoding an ABC transporter periplasmic binding protein), *bab1_0265* (encoding a hypothetical protein), and *bab1_1070* (encoding NADPH dehydrogenase) (Fig. 4). Of the deletion strains tested, mutants Δ*bab2_0822*, Δ*bab2_1109*, and Δ*bab2_0700* were less able to survive and replicate in the macrophages than parental strain 2308 at 48 h postinfection, while the other deletion strains displayed wild-type levels of infection. Interestingly, *bab2_0822*, *bab2_1109*, and *bab2_0700* all encode components of putative ABC transport systems, and these genes are discussed in more detail in the next section. Overall, these experiments demonstrated that several YbeY-associated systems are independently required for the full virulence of *B. abortus*.

DISCUSSION

In this study, we characterized the highly conserved YbeY protein in *Brucella abortus*. Our findings show that YbeY is necessary for proper cellular morphology, efficient

TABLE 1 Differential gene expression in *B. abortus* 2308::Δ*ybeY*[a]

Functional category and designation	Description	Fold expression change (Δ*ybeY* mutant vs strain 2308)
Membrane proteins and transport systems		
BAB1_0114	Glycosyl transferase	3.3
BAB1_0372	TRAP dicarboxylate transporter, DctM subunit	−3.2
BAB1_0373	TRAP-type mannitol/chloroaromatic compound transport system	−3.9
BAB1_1589	Major facilitator transporter	3.2
BAB1_1679	**MotA/TolQ/ExbB proton channel**	**3.5**
BAB1_1680	Biopolymer transport protein ExbD/TolR	3.5
BAB1_1681	**Cell envelope biogenesis protein TonB**	**3.8**
BAB1_1792	**Leu/Ile/Val-binding family protein**	**−4.5**
BAB2_0242	Putative sulfite oxidase subunit YedZ	3.9
BAB2_0277	Choline dehydrogenase and related flavoproteins	−16.1
BAB2_0278	ABC transporter, permease	−9.5
BAB2_0279	Inner membrane translocator	−10.3
BAB2_0280	Shikimate kinase	−8.2
BAB2_0281	ABC transporter ATPase	−9.9
BAB2_0282	**Leu/Ile/Val-binding family protein**	**−7.5**
BAB2_0300	Inner membrane translocator	−3.2
BAB2_0519	Periplasmic spermidine/putrescine-binding protein	−3.3
BAB2_0547	**Solute-binding family 1 protein**	**−3.7**
BAB2_0548	**Vacuolar H^+-transporting two-sector ATPase subunit C**	**−5.7**
BAB2_0583	**Aromatic amino acid permease**	**−3.2**
BAB2_0584	**Binding protein-dependent transport system inner membrane protein**	**−4.0**
BAB2_0585	**Solute-binding family 1 protein**	**−3.2**
BAB2_0593	Leu/Ile/Val-binding family protein	−3
BAB2_0700	**Solute-binding family 5 protein**	**−4.1**
BAB2_0822	Leu/Ile/Val-binding family protein	−6.9
BAB2_0827	ABC transporter ATPase	−4.1
BAB2_0828	Glutelin	−4.1
BAB2_0829	Inner membrane translocator	−5.6
BAB2_0830	Leu/Ile/Val-binding family protein	−5.4
BAB2_1109	D-Xylose ABC transporter	−4.8
DNA replication, transcription, and translation		
BAB1_0636	Response regulator receiver/transcriptional regulatory protein, C terminal	3.1
BAB1_1100	Phage integrase	3.0
BAB1_1362	Periplasmic binding protein/LacI transcriptional regulator	−3.3
BAB1_1588	MarR family regulatory protein	4.6
BAB2_0222	Response regulator receiver/transcriptional regulatory protein, C terminal	3.3
BAB2_1099	**Response regulator receiver/transcriptional regulatory protein, C terminal**	**3.2**
Flagellum related		
BAB2_0299	Flagellar hook-length control protein	−3.1
BAB2_1106	**Flagellin, C terminal; flagellin, N terminal**	**3**
Metabolism, signaling, and enzymatic processes		
BAB1_0204	Zinc-containing alcohol dehydrogenase	−4
BAB1_0303	**Urease accessory protein UreG**	**3.2**
BAB1_0459	Transglycosylase-associated protein	−3.0
BAB1_0577	Choline dehydrogenase	−3.5
BAB1_0637	ATPase-like ATP-binding protein	3.3
BAB1_0646	Endonuclease/exonuclease/phosphatase family protein	3.2
BAB1_0867	Hlyoxalase/bleomycin resistance protein/dioxygenase	3.4
BAB1_1070	NAD[P]H dehydrogenase	−4.0
BAB1_1299	Sugar fermentation stimulation protein A	3.1
BAB1_1461	SLT domain-containing protein	3.8
BAB1_1578	Glutathione S-transferase	3.3
BAB1_1855	GCN5-related *N*-acetyltransferase	3.6
BAB1_2001	Aquaporin Z	−3.1
BAB1_2052	Luciferase	3.5

(Continued on next page)

TABLE 1 (Continued)

Functional category and designation	Description	Fold expression change (Δ*ybeY* mutant vs strain 2308)
BAB2_0243	Putative sulfite oxidase subunit YedY	3
BAB2_0821	Zinc-containing alcohol dehydrogenase	−4.7
BAB2_0823	Aldehyde dehydrogenase	−4.9
BAB2_0824	Glucose-methanol-choline oxidoreductase; GMC oxidoreductase	−3.6
BAB2_0825	Shikimate/quinate 5-dehydrogenase	−4.6
BAB2_0826	3-Ketoacyl-(acyl carrier protein) reductase	−4.1
BAB2_0831	Zinc-containing alcohol dehydrogenase superfamily protein	−4.2
BAB2_0890	Ribonucleotide reductase stimulatory protein	−3.0
BAB2_0905	Cytochrome *c* heme-binding site; 4Fe-4S ferredoxin, iron-sulfur binding domain	−3.2
BAB2_0906	Nitrate reductase, delta subunit	−3.2
BAB2_0907	Nitrate reductase, gamma subunit	−3.3
BAB2_1073	Immunoglobulin/major histocompatibility complex	3.7
Hypothetical		
BAB1_0147	Hyp	3.5
BAB1_0265	Hyp	−7.0
BAB1_0266	Hyp	−3.6
BAB1_0418	Hyp	6.2
BAB1_0419	Hyp	3.4
BAB1_0420	Hyp	4.6
BAB1_1296	Hyp	−3.8
BAB1_1302	**Hyp**	**4.3**
BAB1_1341	Hyp	4
BAB1_1347	Hyp	3.3
BAB1_1509	Hyp	3.3
BAB1_1793	Hyp	−3.2
BAB1_1893	Hyp	−6.8
BAB1_2156 (*ybeY*)	Hyp	−12.1
BAB2_0223	Hyp	5.2
BAB2_0224	Hyp	4
BAB2_0276	Hyp	−5.0
BAB2_0732	Hyp	−3.2
BAB2_0740	Hyp	4.3
BAB2_0759	Hyp	−3.2
BAB2_0847	Hyp	−3.0

[a]Microarray analysis was performed using total cellular RNA from *Brucella* strains grown in rich media to late exponential phase, and those genes whose expression was shown to have been altered more than 3-fold in the *ybeY* deletion strain compared to strain 2308 are shown in the list. Boldface indicates data related to genes previously observed to be required for efficient *Brucella* infection or as being differentially expressed during intracellular trafficking of *Brucella*. Hyp, hypothetical protein.

in vitro growth, and full virulence of *B. abortus*. Moreover, we have defined the repertoire of mRNAs whose levels are connected to YbeY and subsequently determined that several YbeY-controlled genes are independently required for *B. abortus* virulence.

Generally, there are several similarities between the *B. abortus ybeY* deletion strain and other well-characterized *ybeY* deletion strains of other bacterial species. For example, the *B. abortus ybeY* deletion strain displays a significant growth defect when grown in nutrient-rich media (Fig. 1B), and similarly, *V. cholerae*, *E. coli*, *Y. enterocolitica*, and *S. meliloti* exhibit various degrees of growth inhibition when *ybeY* is mutated (9, 14–16). Interestingly, though, the *B. abortus* Δ*ybeY* strain also has pronounced cellular morphology defects (Fig. 1D) that have not been reported previously in other bacterial *ybeY* mutants. Finally, the *B. abortus ybeY* deletion strain is severely compromised in its ability to cope with biologically relevant stresses, such as bile acid, membrane perturbation, and oxidative stresses (Fig. 3), and this too is a phenotype reported for *ybeY* mutants of *V. cholerae* and *S. meliloti* (13, 15). Given the wide array of genes dysregulated in the *B. abortus ybeY* deletion strain, we cannot conclusively assign a specific YbeY-controlled gene or set of genes to the growth defect, morphological abnormalities, and/or increased sensitivities to external stresses observed in the *ybeY* deletion strain, but future experiments will be aimed at analyzing specific YbeY-associated genes for links to these phenotypic properties.

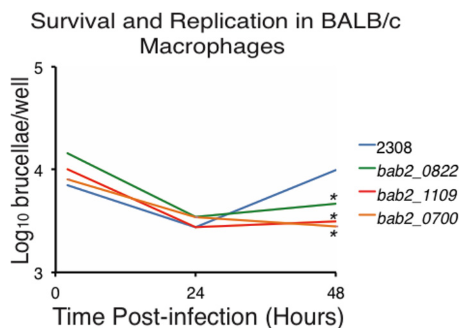

FIG 4 Virulence phenotypes associated with genes differentially expressed in the *B. abortus ybeY* deletion strain. Cultured peritoneal macrophages from BALB/c mice were infected with *B. abortus* 2308 or with isogenic deletion strain *B. abortus* Δ*bab2_0822*, *B. abortus* Δ*bab2_1109*, or *B. abortus* Δ*bab2_0700*. At 2, 24, and 48 h postinfection, the macrophages were lysed, and the number of intracellular brucellae present in these phagocytes was determined by serial dilution and plating on agar medium. The asterisk denotes a statistically significant difference ($P < 0.05$; Student's *t* test) between the isogenic deletion strains and parental strain 2308 at 48 h postinfection.

Regarding the transcriptomic analysis, we determined that a wide range of mRNAs exhibit significantly altered levels in the *B. abortus ybeY* deletion strain (Table 1). Due to the large number of mRNAs affected by the deletion of *ybeY*, it is difficult to ascertain which mRNAs are directly processed by YbeY and which mRNAs YbeY indirectly regulates. Interestingly, our analyses revealed five dysregulated genes in the *ybeY* deletion strain that encode putative transcriptional regulators, and it is possible that YbeY controls gene expression indirectly through these transcriptional regulatory proteins. Of particular interest is *bab2_1099*, which encodes the FtcR transcriptional regulator of flagellar genes, as *ftcR* mRNA levels were elevated >3-fold in the *ybeY* deletion strain. FtcR is the master transcriptional activator of the flagellar biosynthesis system in *B. melitensis*, and, importantly, inactivation of FtcR decreases virulence in a mouse model of infection (28). Additionally, expression of *fliC* (*bab2_1106*), encoding the major flagellin protein in *Brucella*, is also significantly elevated in the *ybeY* deletion strain, and because FtcR is required for FliC production, the observed increase in expression of *fliC* mRNA in the Δ*ybeY* strain might be due to increased levels of FtcR (27, 28). This is just one example of a possible indirect regulatory link between YbeY and dysregulated mRNAs in *B. abortus*, and more work is needed to completely characterize the regulatory circuitries associated with YbeY in *Brucella* strains.

Another prominent element of riboregulation often associated with bacterial YbeY proteins is that of regulatory small RNAs (sRNAs), as demonstrated in *S. meliloti*, *Y. enterocolitica*, and *V. cholerae* (13–15, 35). In these organisms, large variations in sRNA levels have been observed in the corresponding *ybeY* mutant strains. To date, comparatively few sRNAs have been identified and characterized in *Brucella* strains (36–40). Given the role of YbeY in bacterial sRNA stability, we assessed the levels of many of the presently known *Brucella* sRNAs, including AbcR1 and AbcR2, and we did not observe significant differences in sRNA levels between parental *B. abortus* strain 2308 and the *ybeY* deletion strain (data not shown). While this was surprising given the well-documented role of YbeY in bacterial sRNA stability and maturation, it is likely that other sRNAs are yet to be identified in *Brucella* strains, and these sRNAs may well show differences based on the presence of YbeY; however, while unlikely, it is also possible that YbeY in *Brucella* does not play a major role in sRNA stability and/or maturation. This is an active area of investigation in our laboratory, and future work is aimed at identifying novel *Brucella* sRNAs, as well as characterizing the effect of YbeY on sRNAs in *Brucella*.

Overall, it is not surprising that a deletion of *ybeY* decreases the ability of *B. abortus* to survive and replicate in macrophages and to colonize the spleens of mice (Fig. 2), as the Δ*ybeY* strain has pronounced growth and morphological defects (Fig. 1). Therefore, we sought to determine if individual YbeY-controlled genes could account for the

reduction in virulence independently of the growth aberrations resulting from deletion of *ybeY*. These experiments identified three genes, *bab2_0822*, *bab2_1109*, and *bab2_0700*, which are required for *B. abortus* to survive and replicate in murine macrophages (Fig. 4). Importantly, deletion of *bab2_0822*, *bab2_1109*, or *bab2_0700* did not result in growth inhibition of *B. abortus in vitro* (see Fig. S1 in the supplemental material). Thus, these genes are linked to YbeY-associated virulence mechanisms in *B. abortus* but are disconnected from the abnormal growth characteristics of the Δ*ybeY* strain. To date, no empirical information is available describing the function of BAB2_0822, BAB2_1109, and BAB2_0700, but each protein is predicted to act as a periplasmic binding protein likely connected to an ABC-type transport system. Questions remain about the biochemical activity of these proteins and the transport systems with which they function in concert, but our data clearly demonstrate that BAB2_0822, BAB2_1109, and BAB2_0700 are required for the full virulence of *B. abortus* in macrophages. In the future, it will be interesting to characterize both the regulatory link between YbeY and the mRNAs of *bab2_0822*, *bab2_1109*, and *bab2_0700* and the roles of BAB2_0822, BAB2_1109, and BAB2_0700 in the biology of *B. abortus*.

Taking together, the data show that YbeY is a highly conserved bacterial endoribonuclease, and deletion of *ybeY* in *B. abortus* results in a pleotropic phenotype characterized by growth abnormalities, increased sensitivities to multiple stresses, and attenuation in cellular and animal models of infection. Additionally, the *B. abortus* YbeY protein is linked to cellular mRNA levels of genes encoding proteins involved in a variety of processes, including metabolism, flagellar biosynthesis, nutrient transport, and transcriptional regulation. Future work is needed to fully elucidate individual genetic pathways associated with YbeY in the brucellae, as well as to biochemically characterize the endoribonuclease activity of the *B. abortus* YbeY protein. Moreover, the relationship between YbeY and sRNAs, if one exists, needs to be clearly defined in *Brucella*. In the end, this work provides important foundational information about YbeY in the brucellae and, furthermore, contributes to better understanding of the diversity of activities controlled by YbeY proteins in bacteria.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. *Brucella abortus* 2308 and derivative strains were routinely grown on Schaedler blood agar (SBA), which is composed of Schaedler agar (BD, Franklin Lakes, NJ) containing 5% defibrinated bovine blood (Quad Five, Ryegate, MT), or in brucella broth (BD). For cloning, *Escherichia coli* strain DH5α was grown on tryptic soy agar (BD) or in Luria-Bertani (LB) broth. When appropriate, growth media were supplemented with kanamycin (45 μg/ml) or carbenicillin (100 μg/ml).

Construction of *Brucella abortus* deletion strains and genetic complementation. The *ybeY* gene (*bab1_2156*; also known as *bab_rs26200*) in *Brucella abortus* 2308 was mutated using a nonpolar, unmarked gene excision strategy as described previously (41). Briefly, an approximately 1-kb fragment of the upstream region of each gene extending to the second codon of the coding region was amplified by PCR using primers *bab1_2156*-Up-For and *bab1_2156*-Up-Rev and genomic DNA from *Brucella abortus* 2308 as a template. Similarly, a fragment containing the last two codons of the coding region and extending to approximately 1 kb downstream of the *ybeY* open reading frame (ORF) was amplified with primers *bab1_2156*-Down-For and *bab1_2156*-Down-Rev. The sequences of all oligonucleotide primers used in this study can be found in Table 2, and the plasmids used in the study are listed in Table 3. The upstream fragment was digested with BamHI, the downstream fragment was digested with PstI, and both fragments were treated with polynucleotide kinase in the presence of ATP. Both of the DNA fragments were included in a single ligation mix with BamHI/PstI-digested pNTPS138 (M. R. K. Alley, unpublished data) and T4 DNA ligase (Monserate Biotechnology Group, San Diego, CA). The resulting plasmid (p*ybeY*) was introduced into *B. abortus* 2308, and merodiploid transformants were obtained by selection on SBA plus kanamycin. A single kanamycin-resistant clone was grown for ~6 h in brucella broth and then plated onto SBA containing 10% sucrose. Genomic DNA was isolated from sucrose-resistant, kanamycin-sensitive colonies and screened by PCR for loss of the *ybeY* gene. The method described above was used to construct isogenic mutations of *bab2_0277*, *bab2_0282*, *bab2_0822*, *bab2_0548*, *bab2_0830*, *bab2_1109*, *bab2_0700*, *bab1_1070*, and *bab1_0265* using the primers specified in Table 2.

Genetic complementation of the *ybeY* deletion was achieved by expressing the wild-type *ybeY* allele from its native promoter in pBBR1MCS-4 (42). The *ybeY* gene, along with the native *ybeY* promoter, was amplified by PCR using primers *ybeY*-RC-For and *ybeY*-RC-Rev (Table 2) and *Pfx* polymerase (Invitrogen). The resulting DNA fragment was treated with polynucleotide kinase and then ligated into SmaI-digested pBBR1MCS-4. This construct, p*ybeY*-comp, was introduced into the *B. abortus ybeY* deletion strain by electroporation, and colonies were selected on SBA plus carbenicillin.

TABLE 2 Oligonucleotide primers used in this study

Primer name	Primer sequence (5′→3′)[a]
bab1_2156-Up-For	GCGGATCCTTATGAAACATTGCAAAAGG
bab1_2156-Up-Rev	GATCATGATATCAATATGGATCG
bab1_2156-Down-For	GATTGACCATGGCTGAACA
bab1_2156-Down-Rev	CGCTGCAGTCCAATACGTGGAATTCATAACC
ybeY-RC-For	ATGTGGACGGCGCACTGCGCAT
ybeY-RC-Rev	GGAATGGCCTGAACCACTTCACC
bab2_0548-Up-For	TAGGATCCTTGCAGGAATTTGCCAAATATGA
bab2_0548-Up-Rev	CGGCATGCAATTCCGTCGTAAG
bab2_0548-Dn-For	CCATGAGCGTCCAATCGCAAGAT
bab2_0548-Dn-Rev	TACTGCAGACCAGAAACCCGCCTTCATCAA
bab2_0282-Up-For	TAGGATCCATATTTGCTGGCGATGAAATAAG
bab2_0282-Up-Rev	TTTCATGAAGTGTTTCCTCCCAG
bab2_0282-Dn-For	CAGTAAGAGGCTGGTTTGATGAA
bab2_0282-Dn-Rev	TACTGCAGTTTGCGGATAATGCCCATGATG
bab2_0277-Up-For	TAGGATCCAAATGCGGCTTACAGCAAGGC
bab2_0277-Up-Rev	GGTCATGATTCTATATCCAGTAA
bab2_0277-Dn-For	CGGTGAACGGGTTTTCCATCG
bab2_0277-Dn-Rev	TACTGCAGAACCAGTGCCTTCACCCAAGG
bab1_1070-Up-For	TAGGATCCTAGGACATGACCGATCTCCTTCC
bab1_1070-Up-Rev	CATCTGACATCTCCGTTAATCG
bab1_1070-Dn-For	ATTACCGCGAAACTGCATGGCT
bab1_1070-Dn-Rev	TACTGCAGATATGCGAAAGCTTGACCCG
bab2_1109-Up-For	TAGGATCCTTTGAGCGCGGCAGCGATGCA
bab2_1109-Up-Rev	TTTCATGCACGTTTCCTCCAA
bab2_1109-Dn-For	AAATAAACCTTCTGTTCTGC
bab2_1109-Dn-Rev	TACTGCAGAAACATCGTCGACCACCTTGCG
bab2_0830-Up-For2	TAGGATCCGGTCCTGAAGTTCTTGAGCTCGTT
bab2_0830-Up-Rev	TCTCATTCTTTTCTCCCTCAA
bab2_0830-Dn-For	AAATGATCCTGTGTGGGCG
bab2_0830-Dn-Rev	TACTGCAGTTATTCATGCCGGCGCGGTCTAT
bab2_0822-Up-For	TAGGATCCTTGGTGCAGGCTGTTCCGTG
bab2_0822-Up-Rev	TTCCAATTTTCCCTCCTCTT
bab2_0822-Dn-For	CAGTAACAGTCGTCACCGAGGTG
bab2_0822-Dn-Rev	TACTGCAGCGAATGGATTTTTCTTCCGCCAC
bab1_0265-Up-For	TAGGATCCAAACCAAAAGCCCACAATGAACC
bab1_0265-Up-Rev	ACTCAGGTACATAGATTTGTTCC
bab1_0265-Dn-For	GAATGAAACCCGACCGTCTTTC
bab1_0265-Dn-Rev	TACTGCAGAATTTTCTTCACGACATATGA
bab2_0700-Up-For	TAGGATCCTAAGGTCAACTGGATACCTTTCG
bab2_0700-Up-Rev	AACCATCGAAAACTCCCATA
bab2_0700-Dn-For	AACTAACAAAACGAAACCCCTT
bab2_0700-Dn-Rev	TACTGCAGAATGCCGGGAATGCCGAAAAT

[a]Underlined sequences indicate a restriction endonuclease recognition site.

All *Brucella* strains generated during this study were tested using the crystal violet exclusion assay in order to assess whether a given strain produced a smooth or rough form of lipopolysaccharide (LPS) (43). Briefly, *Brucella* strains were grown on tryptic soy agar for 72 to 96 h, and the plates were flooded with a dilute (1:1,000) solution of crystal violet for ~25 s. Cells of parental strain *B. abortus* 2308 were included as smooth-LPS-producing controls, while *B. abortus* RB51 served as a rough-LPS-producing control.

Electron microscopy. *Brucella* strains were grown to the appropriate phase of growth in brucella broth with constant shaking (200 rpm) at 37°C. When cells reached the exponential and/or stationary phase, cultures were spun down at 16,000 × *g* for 10 min. Supernatants were discarded, and pellets were washed once with cold H_2O followed by vigorous vortex mixing. Cells were spun down for a second time, and supernatants were discarded. The pellets were then fixed in 2.5% to 5% glutaraldehyde, and kill cultures were carried out for 10 days to ensure that no viable bacteria were removed from biosafety level 3 (BSL3) containment. Fixed samples of brucellae were submitted to the Electron Microscopy Services at the Virginia-Maryland College of Veterinary Medicine (VMCVM) for scanning electron microscopy. Samples were then fixed in 0.1 M sodium cacodylate buffer and dehydrated with 15%, 30%, 50%, 70%, 95%, and 100% ethanol. The samples were then mounted on stubs and sputter coated with gold. Cells were then viewed using a Carl Zeiss EVO 40 microscope.

Growth in Biolog Phenotype MicroArray plates. Phenotype MicroArray plates (Biolog, Inc., Hayward, CA) were utilized to determine phenotypic differences between different *B. abortus* strains. Strains were grown on SBA plates to produce a lawn of bacteria. Bacteria was collected and suspended in IF-0a GN/GP base (Biolog). The protocol "PM procedures for GN fastidious bacteria" provided by Biolog was followed, and Biolog Phenotype MicroArray plates 1 to 20 were inoculated at a final concentration of 10^8 CFU/well. Plates were grown without shaking at 37°C for and measured after 84 h of incubation at an OD of 590 nm.

TABLE 3 Plasmids used in this study

Plasmid name	Plasmid description[a]	Reference or source
pBBR1MCS-4	Broad-host-range cloning vector; Amp^r	42
pNPTS138	Cloning vector; contains *sacB* gene; Kan^r	M. R. K. Alley (unpublished data)
p*ybeY*	In-frame deletion of *ybeY* plus 1 kb of each flanking region in pNPTS138	This study
p*ybeY*-comp	*ybeY* locus, including the entire promoter region in pBBR1MCS-4	This study
p*bab2_0277*	In-frame deletion of *bab2_0277* plus 1 kb of each flanking region in pNPTS138	This study
p*bab2_0282*	In-frame deletion of *bab2_0282* plus 1 kb of each flanking region in pNPTS138	This study
p*bab2_0822*	In-frame deletion of *bab2_0822* plus 1 kb of each flanking region in pNPTS138	This study
p*bab2_0548*	In-frame deletion of *bab2_0548* plus 1 kb of each flanking region in pNPTS138	This study
p*bab2_0830*	In-frame deletion of *bab2_0830* plus 1 kb of each flanking region in pNPTS138	This study
p*bab2_1109*	In-frame deletion of *bab2_1109* plus 1 kb of each flanking region in pNPTS138	This study
p*bab2_0700*	In-frame deletion of *bab2_0700* plus 1 kb of each flanking region in pNPTS138	This study
p*bab1_0265*	In-frame deletion of *bab1_0265* plus 1 kb of each flanking region in pNPTS138	This study
p*bab1_1070*	In-frame deletion of *bab1_0265* plus 1 kb of each flanking region in pNPTS138	This study

[a]Amp^r, ampicillin resistance; Kan^r, kanamycin resistance.

Sensitivity of the *B. abortus* Δ*ybeY* strain to stressors determined using disk diffusion assays. *Brucella* strains were grown on SBA at 37°C under 5% CO_2 for 48 to 72 h, and the bacterial cells were harvested into phosphate-buffered saline (PBS) and suspended at a concentration of ~10^8 CFU/ml in brucella broth containing 0.6% agar (maintained at 55°C). A 4-ml volume of this suspension was overlaid onto brucella agar plates, and after solidification of the overlay, a sterile 7-mm-diameter Whatman disk was placed in the center of each plate. A 7-μl volume of a deoxycholate (10%), H_2O_2 (30%), SDS (20%), polymyxin B (10 mg/ml), or paraquat (0.25 M) was applied to each filter disk, and the plates were incubated at 37°C with 5% CO_2 for 72 h. Zones of inhibition around each disk were then measured in millimeters.

Microarray analysis. RNA was isolated from *Brucella* cultures grown to the late exponential phase in brucella broth (36), and contaminating genomic DNA was removed by treatment with RNase-free DNase I (36). Ten micrograms of each RNA sample from *B. abortus* 2308 and *B. abortus* Δ*ybeY* was reverse transcribed, fragmented, and subjected to 3′ biotinylation as previously described (44). The labeled cDNA (1.5 μg) was hybridized to custom-made *B. abortus* GeneChips (PMD2308a520698F) according to the manufacturer's recommendations for antisense prokaryotic arrays (Affymetrix, Santa Clara, CA). Signal intensities were normalized to the median signal intensity value for each GeneChip, subjected to averaging, and analyzed with GeneSpring X software. RNA species exhibiting a ≥3-fold change in expression between *B. abortu*s 2308 and the Δ*yebY* strain, as determined by Affymetrix algorithms to be statistically differentially expressed (*t* test; $P < 0.05$), were identified. The microarrays used in this study were developed based on *B. melitensis* biovar *abortus* 2308 and on all of the *B. abortus* GenBank entries that were available at the time of design. In total, the predicted open reading frames and intergenic regions were represented on PMD2308a520698F.

Northern blot analysis. RNA was isolated from *Brucella* cultures as described previously (36). Ten micrograms of RNA was separated on a denaturing 10% polyacrylamide gel containing 7 M urea and 1× TBE (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA). A low-molecular-weight DNA ladder (New England BioLabs, Ipswich, MA) was labeled with [γ-^{32}P]ATP and polynucleotide kinase, and this radiolabeled ladder was also separated on the polyacrylamide gel. Following electrophoresis in 1× TBE buffer, the ladder and RNA samples were transferred to an Amersham Hybond-N$^+$ membrane (GE Healthcare, Piscataway, NJ) in 1× TBE buffer. The samples were UV cross-linked to the membrane, and the membrane was prehybridized in ULTRAhyb-Oligo buffer (Ambion, Austin, TX) for 45 min at ~42°C in a rotating hybridization oven. The oligonucleotide probes were end labeled with [γ-^{32}P]ATP and polynucleotide kinase. The radiolabeled probes were incubated with the prehybridized membranes at ~42°C in a rotating hybridization oven overnight (~12 h). The membranes were then washed three times for 10 min each time with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× SSC, and 0.5× SSC, respectively, at ~42°C in a rotating hybridization oven. All SSC wash buffers contained 0.1% sodium dodecyl sulfate (SDS). The membranes were then exposed to X-ray film and visualized by autoradiography.

Virulence of *Brucella* strains in cultured murine macrophages and experimentally infected mice. Experiments to test the virulence of *Brucella* strains in primary murine peritoneal macrophages were carried out as described previously (45). Briefly, resident peritoneal macrophages were isolated from BALB/c mice and seeded in 96-well plates in Dulbecco's modified Eagle's medium with 5% fetal bovine serum, and the following day, the macrophages were infected with opsonized brucellae at an MOI of 100:1. After 2 h of infection, extracellular bacteria were killed by treatment with gentamicin (50 μg/ml). For the 2-h time point, the macrophages were then lysed with 0.1% deoxycholate–PBS, and serial dilutions were plated on Schaedler blood agar (SBA). For the 24- and 48-h time points, the cells were washed with PBS following gentamicin treatment, and fresh cell culture medium containing gentamicin (20 μg/ml) was added to the monolayer. At the indicated time point, the macrophages were lysed, and serial dilutions were plated on SBA. Triplicate wells were used for each *Brucella* strain tested.

Infection and colonization of mice by *Brucella* strains were performed as described previously by Gee et al. (45). BALB/c mice (5 per *Brucella* strain) were infected intraperitoneally with ~5×10^4 CFU of each *Brucella* strain in sterile PBS. The mice were sacrificed at 4 and 8 weeks postinfection, and serial dilutions of spleen homogenates were plated on SBA to determine CFU counts of brucellae/spleen.

Accession number(s). The GEO database accession number for the microarray data is GSE113222.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00105-18.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 3, XLSX file, 0.2 MB.

ACKNOWLEDGMENTS

We thank Kathy Lowe of the Virginia-Maryland College of Veterinary Medicine for assistance in performing scanning electron microscopy.

This study was supported by grants from the National Institute of Allergy and Infectious Diseases to C.C.C. (AI117648) and R.M.R. (AI48499) and by a grant from the National Institute of General Medical Sciences to G.C.W. (GM31030). G.C.W. is an American Cancer Society Professor.

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