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The endoribonuclease YbeY is linked to proper cellular morphology and virulence in 2 Brucella abortus

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Abstract

25
26 The endoribonuclease YbeY is one of the most well conserved proteins across the kingdoms of
27 life. In the present study, we demonstrate that YbeY in *Brucella abortus* is linked to a variety of
28 important activities, including proper cellular morphology, mRNA transcript levels, and
29 virulence. Deletion of *ybeY* in *B. abortus* led to a small colony phenotype when the bacteria were
30 grown on agar medium, as well as significant aberrations in the morphology of the bacterial cell
31 as evidenced by electron microscopy. Additionally, compared to the parental strain, the $\Delta ybeY$
32 strain was significantly attenuated in both macrophage and mouse models of infection. The
33 $\Delta ybeY$ strain also showed increased sensitivities to several *in vitro* applied stressors, including
34 bile acid, hydrogen peroxide, SDS, and paraquat. Transcriptomic analysis revealed that a
35 multitude of mRNA transcripts are dysregulated in the $\Delta ybeY$ strain, and many of the identified
36 mRNAs encode proteins involved in metabolism, nutrient transport, transcriptional regulation,
37 and flagellum synthesis. We subsequently constructed gene deletion strains of the most highly
38 dysregulated systems, and several of the YbeY-linked gene deletion strains exhibited defects in
39 the ability of the bacteria to survive and replicate in primary murine macrophages. Altogether,
40 these data establish a clear role for YbeY in the biology and virulence of *Brucella*, and moreover,
41 this work further illuminates the highly varied roles of this widely conserved endoribonuclease in
42 bacteria.

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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 endoribonuclease YbeY for cellular morphology, efficient control of mRNA levels, and virulence in *B. abortus*. Overall, this study advances our understanding of the critical roles of YbeY in the pathogenesis of the intracellular brucellae and expands our understanding of this highly conserved ribonuclease.

Introduction

55
56 Ribonucleases (RNases) are enzymes that catalyze the cleavage of myriad RNAs, be it mRNA,
57 tRNA, rRNA, or sRNA, and these enzymes are divided into two major classes called
58 exoribonucleases and endoribonucleases depending on their ability to cleave RNA strands at
59 terminal or non-terminal nucleotides, respectively (1, 2). The “day-to-day operations” of RNases
60 include degrading RNAs during housekeeping turnover processes, but RNases also process
61 longer RNA transcripts into shorter, functional RNAs. A classic example of RNA processing is
62 the generation of the three major rRNAs (i.e., 23S, 16S, and 5S) and tRNAs from precursor
63 RNAs, a process catalyzed by several different RNases in bacteria (3, 4). As such, bacteria
64 encode an extensive array of RNases to perform a wide variety of degradation and processing
65 functions.

66 One of the more recently described bacterial RNases is the endoribonuclease YbeY (5).
67 Interestingly, the structure of YbeY was studied prior to any insights into its biological functions.
68 The crystal structure of the *Aquifex aeolicus* YbeY ortholog revealed resemblances to metal-
69 dependent proteinases such as collagenases (6), while crystallization of the *E. coli* YbeY protein
70 as part of an NIH-funded Protein Structure Initiative program led to the suggestion that it is a
71 metal-dependent hydrolase (7). Subsequently, the *Sinorhizobium meliloti* YbeY ortholog was
72 found to be required for symbiosis, while *E. coli* YbeY drew attention because of its regulation
73 as a heat-shock protein (8). YbeY was then shown to participate in the maturation of ribosomal
74 RNAs and the biosynthesis of ribosomes, and more recently, evidence has been reported that
75 YbeY functions as an endoribonuclease in rRNA maturation activities and 70S ribosome quality
76 control (5, 9-11). Additionally, YbeY plays a significant role in the regulation and stability of
77 bacterial sRNAs (12, 13). Not only has YbeY been linked to the capacity of *S. meliloti* to form

78 an effective symbiotic relationship with its plant host alfalfa, but *ybeY* is required for the full
79 virulence of *Vibrio cholerae* and *Yersinia enterocolitica* (14-16). While RNases, including
80 YbeY, are known to be important virulence determinants for several bacterial pathogens, very
81 little is known about the role of RNases in the *Brucella* spp. (17).

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

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Results

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98 **YbeY is required for normal growth and cellular morphology of *Brucella abortus***

99 *bab1_2156* (also known as *bab_rs26200*) is located on chromosome I of *Brucella melitensis*
100 biovar Abortus 2308 between *bab1_2155* (*phoH*) and *bab1_2157* (*tlyC*) (Fig. 1A). The YbeY
101 protein exhibits 56% identity and 68% similarity to the endoribonuclease YbeY from
102 *Sinorhizobium meliloti* 1021. For this reason, and due to the results outlined in this study, we will
103 hereafter refer to *bab1_2156* as *ybeY*.

104 An isogenic deletion of *ybeY* in *B. abortus* 2308 resulted in impaired growth *in vitro* and
105 abnormal cellular morphology compared to the parental strain (Fig. 1). *B. abortus::ΔybeY*
106 exhibited a small colony phenotype when grown on agar medium, and this defect was genetically
107 complemented when *ybeY* was provided in trans on the plasmid pBBR-1MCS4 (Fig. 1B). When
108 cultured in brucella broth (i.e., rich medium), the *ybeY* deletion strain was able to grow to similar
109 maximum numbers of bacteria as the parental strain, but the *ybeY* deletion strain had a decreased
110 rate of growth during the exponential growth phase (Fig. 1C). During exponential growth phase,
111 *B. abortus* 2308 had a generation time of 2.2 hours while the *ybeY* deletion strain had a
112 generation time of 2.8 hours. Importantly, the growth rate of *B. abortus::ΔybeY* was restored to a
113 1.9 hour generation time by in trans complementation of *ybeY*.

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

120 unable to properly divide during both exponential and stationary phases of growth. Altogether,
121 these data demonstrate that YbeY is required for the efficient growth and cellular morphology of
122 *B. abortus*.

123 **YbeY contributes to *B. abortus* virulence in macrophages and experimentally infected mice**

124 To characterize the importance of YbeY for *B. abortus* virulence, the *ybeY* deletion strain was
125 assessed for the ability to infect peritoneal macrophages *in vitro* and BALB/c mice *in vivo* (Fig.
126 2). Peritoneal derived macrophages isolated from BALB/c mice were infected with either *B.*
127 *abortus* 2308, *B. abortus* 2308:: $\Delta ybeY$, or *B. abortus* 2308:: $\Delta ybeY$ -comp at an MOI of 100. The
128 *ybeY* deletion strain was strikingly less able to survive and replicate within the macrophage
129 compared to the parental strain at 24 and 48 hours post-infection, and this decrease in survival
130 and replication was restored to wild-type levels in the *ybeY* complemented strain (Fig. 2A).
131 Similarly, the *ybeY* deletion strain exhibited a substantially reduced ability to infect BALB/c
132 mice compared to the parental strain 2308, as significantly fewer bacteria were recovered from
133 the spleens of mice infected with the *ybeY* deletion strain after both 4 and 8 weeks of infection
134 (Fig. 2B). These experiments indicate that YbeY is necessary for the ability of *B. abortus* to
135 sustain infection in macrophages and mice.

136 **Deletion of *ybeY* in *B. abortus* leads to increased sensitivities to general stress and wide** 137 **ranging metabolic aberrations**

138 Due to the decreased growth rate, defect in cell morphology, and reduced ability to infect the *in*
139 *vitro* and *in vivo* models of the *ybeY* deletion strain, we sought to gain insight into the link
140 between YbeY and general stress in *B. abortus*. To achieve this, we employed disk diffusion
141 assays in which *B. abortus* strains were exposed to a variety of stressors, including deoxycholate
142 (10%), H₂O₂ (30%), SDS (20%), polymyxin B (10 mg/mL), and paraquat (0.25 M) (Fig. 3). In

143 these experiments, the *ybeY* deletion strain was more sensitive than the parental strain 2308 to
144 deoxycholate, H₂O₂, SDS, and paraquat, and genetic complementation of *ybeY* in the deletion
145 strain restored the zones of inhibition to the levels observed for 2308. Interestingly, deletion of
146 *ybeY* had no effect on the ability of *B. abortus* to withstand killing by polymyxin B. These data
147 demonstrate that YbeY is important for the ability of *B. abortus* to cope with general stress
148 conditions.

149 The Biolog Phenotype MicroArray system provides an inexpensive and rapid means of
150 testing microorganisms for the ability to grow under hundreds of varying conditions. Here, we
151 employed Biolog Phenotype MicroArrays to analyze the growth of *B. abortus* 2308 and *B.*
152 *abortus* 2308:: Δ *ybeY* in a wide variety of different nutrient sources, environmental conditions,
153 and stressors. Each Biolog Phenotype MicroArray plate was inoculated with 10⁸ CFU/well of the
154 appropriate *Brucella* strains and incubated for 84 hours at 37°C. After 84 hours of incubation,
155 each individual well was measured at an O.D. of 590 nm and visually monitored for growth,
156 indicated by metabolic activity (clear to purple) (Dataset S1). Overall, we observed 27
157 differences in growth between *B. abortus* 2308 and the *ybeY* deletion strain (Table S1). The
158 conditions in which 2308:: Δ *ybeY* grew more efficiently than 2308 are highlighted in green, and
159 the conditions in which 2308 grew more efficiently than *ybeY* are highlighted in red. With
160 regards to carbon sources, the *ybeY* deletion strain was better able than the parental strain 2308 to
161 utilize malic acid and laminarin (a storage glucan). However, deletion of *ybeY* led to the inability
162 of *B. abortus* to utilize butyric acid or caproic acid as a carbon source for growth. Compared to
163 *B. abortus* 2308, growth of the *ybeY* deletion strain was more sensitive to dodecyltrimethyl
164 ammonium bromide, promethazine, alexidine, dichlofluanid, chloroxylenol, sodium m-periodate,
165 lidocaine, josamycin, thioridazine, patulin, and tetrazolium violet. Conversely, growth of *B.*

166 *abortus* 2308:: Δ *ybeY* was more resistant to the presence of fusaric acid, 1-chloro-2,4-
167 dinitrobenzene, 2-phenylphenol, antimony (III) chloride, pentachloro-phenol, azathioprine,
168 phenethicillin, and lawsone. Altogether, the Biolog Phenotype MicroArrays underscore the
169 diverse metabolic abnormalities that result from the deletion of *ybeY* in *B. abortus*.

170 **YbeY impacts the levels of mRNA associated with a variety of cellular systems**

171 The pleiotropic effects of *ybeY* loss on cellular RNAs has been well documented in other bacteria
172 (5, 9, 10, 12, 15, 25, 26), and as such, we hypothesized that deletion of *ybeY* would lead to
173 changes in mRNA levels in *B. abortus*. Therefore, we employed microarray technology to
174 identify mRNAs that are influenced by YbeY. This experiment was performed using RNA from
175 cultures of *B. abortus* 2308 and *B. abortus* 2308:: Δ *ybeY* grown in brucella broth to late
176 exponential phase (Dataset S2). Altogether, mRNAs from 84 genes exhibited differential levels
177 (>3 fold difference) in Δ *ybeY*, and of these, 34 mRNAs were elevated in the *ybeY* deletion strain,
178 while 51 mRNAs were decreased in the *ybeY* deletion strain compared to the parental strain
179 (Table 1). The mRNAs that displayed differential quantities in the *ybeY* deletion strain included
180 those encoding membrane proteins and transport systems; proteins involved in DNA replication,
181 transcriptional or translational regulation; proteins related to flagellar processes; proteins linked
182 to metabolism, signaling, and enzymatic processes; and hypothetical proteins.

183 Interestingly, several of the mRNAs identified in the *ybeY* microarray encode proteins
184 that have been previously characterized as being required for efficient *Brucella* infection or as
185 being differentially expressed in the bacterium during intracellular trafficking of *Brucella*. Seven
186 genes, *bab2_1099* (FtcR, flagellar transcriptional regulator), *bab2_1106* (flagellin), *bab1_0303*
187 (UreG1, urease accessory protein), *bab2_0583* (ABC transporter permease), *bab2_0584* (ABC
188 transporter permease), *bab2_0585* (UgpB, a ABC transporter periplasmic binding protein), and

189 *bab1_1302* (hypothetical protein) have been implicated in *Brucella* virulence (27-32). Seven
190 other genes, *bab1_1679* (ABC transporter ATPase), *bab1_1792* (ABC transporter periplasmic
191 binding protein), *bab2_0282* (ABC transporter permease), *bab2_0700* (ABC transporter
192 periplasmic binding protein), *bab1_1681* (cell envelope biogenesis protein TonB), *bab2_0547*
193 (ABC transporter periplasmic binding protein), and *bab2_0548* (ABC transporter permease)
194 were shown previously to be differentially expressed in *Brucella* during intracellular infection
195 (33, 34).

196 **Contribution of YbeY-associated genes to *Brucella abortus* virulence**

197 Given the large number of systems dysregulated in the *B. abortus ybeY* deletion strain, it is
198 difficult to draw specific conclusions about the linkages between YbeY, individual mRNAs or
199 systems, and the observed phenotypes resulting from the deletion of *ybeY*. Therefore, to begin to
200 define YbeY-associated mRNAs that are required for virulence, we constructed strains harboring
201 deletions in nine genes that exhibited the greatest levels of mRNA difference in our microarray
202 experiments (Table 1). Subsequently, peritoneal derived macrophages from BALB/c mice were
203 infected with the parental strain *B. abortus* 2308, as well as the *B. abortus* strains with isogenic
204 deletions of *bab2_0277* (choline dehydrogenase and related flavoprotein), *bab2_0282* (ABC
205 transporter permease), *bab2_0822* (ABC transporter periplasmic binding protein), *bab2_0548*
206 (ABC transporter permease), *bab2_0830* (ABC transporter periplasmic binding protein),
207 *bab2_1109* (ABC transporter periplasmic binding protein), *bab2_0700* (ABC transporter
208 periplasmic binding protein), *bab1_0265* (hypothetical protein), and *bab1_1070* (NAD[P]H
209 dehydrogenase) (Fig. 4). Of the deletion strains tested, $\Delta bab2_0822$, $\Delta bab2_1109$, and
210 $\Delta bab2_0700$ were less able to survive and replicate in the macrophages compared to the parental
211 strain 2308 at 48 hours post-infection, while the other deletion strains displayed wild-type levels

212 of infection. Interestingly, *bab2_0822*, *bab2_1109*, and *bab2_0700* all encode components of
213 putative ABC transport systems, and these genes will be discussed in more detail in the next
214 section. Overall, these experiments demonstrate that several YbeY-associated systems are
215 independently required for the full virulence of *B. abortus*.

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217

Discussion

218 In this study, we have characterized the highly conserved protein YbeY in *Brucella abortus*. Our
219 findings show that YbeY is necessary for proper cellular morphology, efficient *in vitro* growth,
220 and full virulence of *B. abortus*. Moreover, we have defined the repertoire of mRNAs whose
221 levels are connected to YbeY, and subsequently determined that several YbeY-controlled genes
222 are independently required for *B. abortus* virulence.

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

238 Regarding the transcriptomic analysis, we determined that a wide range of mRNAs
239 exhibit significantly altered levels in the *B. abortus ybeY* deletion strain (Table 1). Due to the

240 large number of mRNAs affected by the deletion of *ybeY*, it is difficult to ascertain which
241 mRNAs are directly processed by YbeY and which mRNAs YbeY indirectly regulates.
242 Interestingly, our analyses revealed five dysregulated genes in the *ybeY* deletion strain that
243 encode putative transcriptional regulators, and it is possible that YbeY may control gene
244 expression indirectly through these transcriptional regulatory proteins. Of particular interest, is
245 *bab2_1099*, which encodes the FtcR transcriptional regulator of flagellar genes, as *ftcR* mRNA
246 was >3-fold elevated in *ybeY* deletion strain. FtcR is the master transcriptional activator of the
247 flagellar biosynthesis system in *B. melitensis*, and importantly, inactivation of FtcR decreases
248 virulence in a mouse model of infection (28). Additionally, *fliC* (*bab2_1106*) encoding the major
249 flagellin protein in *Brucella* is also significantly elevated in the *ybeY* deletion strain, and because
250 FtcR is required for FliC production, the observed increase in *fliC* mRNA in the $\Delta ybeY$ strain
251 may be due to increased levels of FtcR (27, 28). This is just one example of a possible indirect
252 regulatory link between YbeY and dysregulated mRNAs in *B. abortus*, and more work is needed
253 to completely characterize the regulatory circuitries associated with YbeY in *Brucella* strains.

254 Another prominent element of riboregulation often associated with bacterial YbeY
255 proteins is that of small regulatory RNAs (sRNAs), as demonstrated in *S. meliloti*, *Y.*
256 *enterocolitica*, and *V. cholerae* (13-15, 35). In these organisms, large variations in sRNA levels
257 have been observed in the corresponding *ybeY* mutant strains. To date, comparatively few
258 sRNAs have been identified and characterized in *Brucella* strains (36-40). Given the role of
259 YbeY in bacterial sRNA stability, we assessed the levels of many of the presently known
260 *Brucella* sRNAs, including AbcR1 and AbcR2, and we did not observe significant differences in
261 sRNA levels between the parental *B. abortus* strain 2308 and the *ybeY* deletion strain (data not
262 shown). While this was surprising given the well-documented role of YbeY in bacterial sRNA

263 stability and maturation, it is likely that other sRNAs are yet to be identified in *Brucella* strains,
264 and these sRNAs may well show differences based on the presence of YbeY; however, while
265 unlikely, it is also possible that YbeY in *Brucella* does not play a major role in sRNA stability
266 and/or maturation. This is an active area of investigation in our laboratory, and future work is
267 aimed at identifying novel *Brucella* sRNAs, as well as characterizing the effect of YbeY on
268 sRNAs in *Brucella*.

269 Overall, it is not surprising that a deletion of *ybeY* decreases the ability of *B. abortus* to
270 survive and replicate in macrophages and colonize the spleens of mice (Fig. 2), as the $\Delta ybeY$
271 strain has pronounced growth and morphological defects (Fig. 1). Therefore, we sought to
272 determine if individual YbeY-controlled genes could account for the reduction in virulence
273 independently of the growth aberrations resulting from deletion of *ybeY*. These experiments
274 identified three genes, *bab2_0822*, *bab2_1109*, and *bab2_0700*, which are required for *B.*
275 *abortus* to survive and replicate in murine macrophages (Fig. 4). Importantly, deletion of
276 *bab2_0822*, *bab2_1109*, or *bab2_0700* did not result in growth inhibition *in vitro* of *B. abortus*
277 (Fig. S1). Thus, these genes are linked to YbeY-associated virulence mechanisms in *B. abortus*,
278 but disconnected from the abnormal growth characteristics of the $\Delta ybeY$ strain. To date, no
279 empirical information is available describing the function of BAB2_0822, BAB2_1109, and
280 BAB2_0700, but each protein is predicted to act as a periplasmic-binding protein likely
281 connected to an ABC-type transport system. Questions remain about the biochemical activity of
282 these proteins and the transport systems they function in concert with, but our data clearly
283 demonstrate that BAB2_0822, BAB2_1109, and BAB2_0700 are required for the full virulence
284 of *B. abortus* in macrophages. In the future, it will be interesting to characterize both the

285 regulatory link between YbeY and the mRNAs of *bab2_0822*, *bab2_1109*, and *bab2_0700*, as
286 well as the role of BAB2_0822, BAB2_1109, and BAB2_0700 in the biology of *B. abortus*.

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

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Materials and Methods

Bacterial strains and growth conditions

Brucella abortus 2308 and derivative strains were routinely grown on Schaedler blood agar (SBA), which is composed of Schaedler agar (BD, Franklin Lakes, NJ USA) containing 5% defibrinated bovine blood (Quad Five, Ryegate, MT, USA), or in brucella broth (BD). For cloning, *Escherichia coli* strains (DH5 α) were grown on tryptic soy agar (BD) or in Luria-Bertani (LB) broth. When appropriate, growth media were supplemented with kanamycin (45 μ g/ml) or carbenicillin (100 μ g/ml).

Construction of *Brucella abortus* deletion strains and genetic complementation

The *ybeY* gene (*babI_2156*; *bab_rs26200*) in *Brucella abortus* 2308 was mutated using a non-polar, unmarked gene excision strategy as described previously (41). Briefly, an approximately 1-kb fragment of the upstream region of each gene to the second codon of the coding region was amplified by PCR using primers *babI_2156*-Up-For and *babI_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 to approximately 1 kb downstream of the *ybeY* ORF was amplified with primers *babI_2156*-Down-For and *babI_2156*-Down-Rev. The sequences of all oligonucleotide primers used in this study can be found in Table 2. The upstream fragment was digested with BamHI, while 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) and T4 DNA ligase (Monserate Biotechnology Group, San Diego, CA, USA). The resulting plasmid (*pybeY*) was introduced into *B. abortus* 2308, and merodiploid transformants were obtained by selection on SBA+kanamycin. A single kanamycin-resistant clone was grown for ~6 hours in

323 brucella broth, and then plated onto SBA containing 10% sucrose. Genomic DNA from sucrose-
324 resistant, kanamycin-sensitive colonies was isolated and screened by PCR for loss of the *ybeY*
325 gene. The method described above was used to construct isogenic mutations of *bab2_0277*,
326 *bab2_0282*, *bab2_0822*, *bab2_0548*, *bab2_0830*, *bab2_1109*, *bab2_0700*, *bab1_1070*, and
327 *bab1_0265* using the primers specified in Table 2.

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

335 All *Brucella* strains generated during this study were tested by the crystal violet exclusion
336 assay in order to assess whether a given strain produced a smooth or rough form of
337 lipopolysaccharide (LPS) (43). Briefly, *Brucella* strains were grown on tryptic soy agar for 72-96
338 hours, and the plates were flooded with a dilute (1:1000) solution of crystal violet for ~25 sec.
339 The parental strains *B. abortus* 2308 was included as smooth LPS-producing controls, while *B.*
340 *abortus* RB51 served as a rough LPS-producing control.

341 **Electron microscopy**

342 *Brucella* strains were grown to the appropriate phase of growth in brucella broth with constant
343 shaking (200 RPM) at 37°C. When cells reached exponential and/or stationary phase, cultures
344 were spun down at 16,000 x *g* for 10 minutes. Supernatants were discarded and pellets were
345 washed once with cold H₂O followed by vigorous vortexing. Cells were spun down for a second

346 time and supernatants were discarded. The pellets were then fixed in 2.5-5% glutaraldehyde, and
347 kill cultures were carried out for 10 days to ensure no viable bacteria were removed from BSL3
348 containment. Fixed brucellae samples were submitted to the Electron Microscopy Services at
349 VMCVM for scanning electron microscopy. Samples were then fixed in 0.1 M sodium
350 cacodylate buffer and then dehydrated with 15%, 30%, 50%, 70%, 95%, and 100% ethanol. The
351 samples were then mounted on stubs and sputter coated with gold. Cells were then viewed using
352 a Carl Zeiss EVO 40 microscope.

353 **Growth in Biolog Phenotype Microarray plates**

354 Biolog Phenotype Microarray plates (Biolog, Inc., Hayward, CA.) were utilized to determine
355 phenotypic differences between different *B. abortus* strains. Strains were grown on SBA plates
356 to produce a lawn of bacteria. Bacteria was collected and suspended in IF-0a GN/GP Base
357 (Biolog). The protocol “PM Procedures for GN Fastidious Bacteria” provided by Biolog were
358 followed and Biolog Phenotype Microarray plates 1-20 were inoculated at a final concentration
359 of 10^8 CFU/well. Plates were grown statically at 37°C for and measured after 84 hours of
360 incubation at O.D. 590 nm.

361 **Sensitivity of *B. abortus* $\Delta ybeY$ strain to stressors using disk diffusion assays**

362 *Brucella* strains were grown on SBA at 37°C under 5% CO₂ for 48-72 h, and the bacterial cells
363 were harvested into PBS and suspended a concentration of $\sim 10^8$ CFU/ml in brucella broth
364 containing 0.6% agar (maintained at 55°C). Four ml of this suspension was overlaid onto
365 brucella agar plates, and after solidification of the overlay, a sterile 7 mm Whatman disk was
366 placed in the center of each plate. Seven μ l of a deoxycholate (10%), H₂O₂ (30%), SDS (20%),
367 polymixin B (10 mg/ml), or paraquat (0.25 M) was applied to each filter disk and the plates were

368 incubated at 37°C with 5% CO₂ for 72 hours. Zones of inhibition around each disk were then
369 measured in millimeters.

370 **Microarray analysis**

371 RNA was isolated from *Brucella* cultures grown to late exponential phase in brucella broth
372 (Caswell *et al.*, 2012), and contaminating genomic DNA was removed by treatment with RNase-
373 free DNase I (36). Ten micrograms of each RNA sample, *B. abortus* 2308 and *B. abortus* $\Delta ybeY$,
374 were reverse transcribed, fragmented and 3' biotinylated as previously described (44). The
375 labeled cDNA (1.5 μ g) was hybridized to custom-made *B. abortus* GeneChips
376 (PMD2308a520698F) according to the manufacturer's recommendations for antisense
377 prokaryotic arrays (Affymetrix, Santa Clara, CA USA). Signal intensities were normalized to the
378 median signal intensity value for each GeneChip, averaging and analyzed with GeneSpring
379 Software X. RNA species exhibiting ≥ 3 -fold change in expression, as determined by Affymetrix
380 algorithms to be statistically differentially expressed (*t*-test; $P < 0.05$), between *B. abortus* 2308
381 and the $\Delta yebY$ strain were stated. The microarrays used in this study were developed based on *B.*
382 *melitensis* biovar *abortus* 2308 and all *B. abortus* GenBank entries that were available at the time
383 of design. In total, predicted open reading frames and intergenic regions were represented on
384 PMD2308a520698F. The microarray data is currently being submitted to GenBank.

385 **Northern blot analysis**

386 RNA was isolated from *Brucella* cultures as described previously (36). Ten micrograms of RNA
387 was separated on a denaturing 10% polyacrylamide gel containing 7 M urea and 1 \times TBE
388 (89 mM Tris-base, 89 mM boric acid and 2 mM EDTA). A low-molecular-weight DNA ladder
389 (New England BioLabs, Ipswich, MA, USA) was labelled with [γ -³²P]-ATP and polynucleotide
390 kinase, and this radiolabelled ladder was also separated on the polyacrylamide gel. Following

391 electrophoresis in 1× TBE buffer, the ladder and RNA samples were transferred to an Amersham
392 Hybond™-N⁺ membrane (GE Healthcare, Piscataway, NJ, USA) in 1× TBE buffer. The samples
393 were UV-cross-linked to the membrane, and the membrane was pre-hybridized in ULTRAhyb®-
394 Oligo Buffer (Ambion, Austin, TX, USA) for 45 minutes at ~ 42°C in a rotating hybridization
395 oven. The oligonucleotide probes were end-labeled with [γ -³²P]-ATP and polynucleotide kinase.
396 The radiolabelled probes were incubated with the pre-hybridized membranes at ~ 42°C in a
397 rotating hybridization oven overnight (~ 12 h). The membranes were then washed three times
398 for 10 min each with 2× SSC (300 mM sodium chloride and 30 mM sodium citrate), 1× SSC and
399 0.5× SSC, respectively, at ~ 42°C in a rotating hybridization oven. All SSC wash buffers
400 contained 0.1% sodium dodecyl sulfate (SDS). The membranes were then exposed to X-ray film
401 and visualized by autoradiography.

402 **Virulence of *Brucella* strains in cultured murine macrophages and experimentally infected**
403 **mice**

404 Experiments to test the virulence of *Brucella* strains in primary, murine peritoneal macrophages
405 were carried out as described previously (45). Briefly, resident peritoneal macrophages were
406 isolated from BALB/c mice and seeded in 96-well plates in Dulbecco's modified Eagle's
407 medium with 5% fetal bovine serum, and the following day, the macrophages were infected with
408 opsonized brucellae at an MOI of 100:1. After 2 hours of infection, extracellular bacteria were
409 killed by treatment with gentamicin (50 μ g/ml). For the 2-hour time point, the macrophages were
410 then lysed with 0.1% deoxycholate in PBS, and serial dilutions were plated on Schaedler blood
411 agar (SBA). For the 24- and 48-hour time points, the cells were washed with PBS following
412 gentamicin treatment, and fresh cell culture medium containing gentamicin (20 μ g/ml) was
413 added to the monolayer. At the indicated time point, the macrophages were lysed, and serial

414 dilutions were plated on SBA. Triplicate wells were used for each *Brucella* strain tested.

415 The infection and colonization of mice by *Brucella* strains was as described previously
416 by Gee *et al.*, 2005 (45). BALB/c mice (5 per *Brucella* strain) were infected intraperitoneally
417 with $\sim 5 \times 10^4$ CFU of each *Brucella* strain in sterile PBS. The mice were sacrificed at 4 and 8
418 weeks post-infection, and serial dilutions of spleen homogenates were plated on SBA to
419 determine CFU brucellae/spleen.

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References

- 430 1. **Deutscher MP.** 1985. *E. coli* RNases: making sense of alphabet soup. *Cell* **40**:731-732.
- 431 2. **Deutscher MP.** 1988. The metabolic role of RNases. *Trends Biochem Sci* **13**:136-139.
- 432 3. **Apirion D, Gegenheimer P.** 1981. Processing of bacterial RNA. *FEBS Lett* **125**:1-9.
- 433 4. **Gegenheimer P, Apirion D.** 1981. Processing of procaryotic ribonucleic acid. *Microbiol*
434 *Rev* **45**:502-541.
- 435 5. **Jacob AI, Kohrer C, Davies BW, RajBhandary UL, Walker GC.** 2013. Conserved
436 bacterial RNase YbeY plays key roles in 70S ribosome quality control and 16S rRNA
437 maturation. *Mol Cell* **49**:427-438.
- 438 6. **Oganesyan V, Busso D, Brandsen J, Chen S, Jancarik J, Kim R, Kim SH.** 2003.
439 Structure of the hypothetical protein AQ_1354 from *Aquifex aeolicus*. *Acta Crystallogr D*
440 *Biol Crystallogr* **59**:1219-1223.
- 441 7. **Zhan C, Fedorov EV, Shi W, Ramagopal UA, Thirumuruhan R, Manjasetty BA,**
442 **Almo SC, Fiser A, Chance MR, Fedorov AA.** 2005. The ybeY protein from
443 *Escherichia coli* is a metalloprotein. *Acta Crystallogr Sect F Struct Biol Cryst Commun*
444 **61**:959-963.
- 445 8. **Rasouly A, Schonbrun M, Shenhar Y, Ron EZ.** 2009. YbeY, a heat shock protein
446 involved in translation in *Escherichia coli*. *J Bacteriol* **191**:2649-2655.
- 447 9. **Davies BW, Kohrer C, Jacob AI, Simmons LA, Zhu J, Aleman LM, Rajbhandary**
448 **UL, Walker GC.** 2010. Role of *Escherichia coli* YbeY, a highly conserved protein, in
449 rRNA processing. *Mol Microbiol* **78**:506-518.
- 450 10. **Rasouly A, Davidovich C, Ron EZ.** 2010. The heat shock protein YbeY is required for
451 optimal activity of the 30S ribosomal subunit. *J Bacteriol* **192**:4592-4596.

- 452 11. **Grinwald M, Ron EZ.** 2013. The *Escherichia coli* translation-associated heat shock
453 protein YbeY is involved in rRNA transcription antitermination. PLoS One **8**:e62297.
- 454 12. **Pandey SP, Winkler JA, Li H, Camacho DM, Collins JJ, Walker GC.** 2014. Central
455 role for RNase YbeY in Hfq-dependent and Hfq-independent small-RNA regulation in
456 bacteria. BMC Genomics **15**:121.
- 457 13. **Pandey SP, Minesinger BK, Kumar J, Walker GC.** 2011. A highly conserved protein
458 of unknown function in *Sinorhizobium meliloti* affects sRNA regulation similar to Hfq.
459 Nucleic Acids Res **39**:4691-4708.
- 460 14. **Leskinen K, Varjosalo M, Skurnik M.** 2015. Absence of YbeY RNase compromises
461 the growth and enhances the virulence plasmid gene expression of *Yersinia enterocolitica*
462 O:3. Microbiology **161**:285-299.
- 463 15. **Vercruyse M, Kohrer C, Davies BW, Arnold MF, Mekalanos JJ, RajBhandary UL,**
464 **Walker GC.** 2014. The highly conserved bacterial RNase YbeY is essential in *Vibrio*
465 *cholerae*, playing a critical role in virulence, stress regulation, and RNA processing.
466 PLoS Pathog **10**:e1004175.
- 467 16. **Davies BW, Walker GC.** 2008. A highly conserved protein of unknown function is
468 required by *Sinorhizobium meliloti* for symbiosis and environmental stress protection. J
469 Bacteriol **190**:1118-1123.
- 470 17. **Lawal A, Jejelowo O, Chopra AK, Rosenzweig JA.** 2011. Ribonucleases and bacterial
471 virulence. Microb Biotechnol **4**:558-571.
- 472 18. **Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV.** 2006. The new
473 global map of human brucellosis. Lancet Infect Dis **6**:91-99.

- 474 19. **Gorvel JP, Moreno E.** 2002. *Brucella* intracellular life: from invasion to intracellular
475 replication. *Vet Microbiol* **90**:281-297.
- 476 20. **Fabrik I, Hartlova A, Rehulka P, Stulik J.** 2013. Serving the new masters - dendritic
477 cells as hosts for stealth intracellular bacteria. *Cell Microbiol* **15**:1473-1483.
- 478 21. **Roop RM, 2nd, Gaines JM, Anderson ES, Caswell CC, Martin DW.** 2009. Survival
479 of the fittest: how *Brucella* strains adapt to their intracellular niche in the host. *Med*
480 *Microbiol Immunol* **198**:221-238.
- 481 22. **Seleem MN, Boyle SM, Sriranganathan N.** 2008. *Brucella*: a pathogen without classic
482 virulence genes. *Vet Microbiol* **129**:1-14.
- 483 23. **Heaton BE, Herrou J, Blackwell AE, Wysocki VH, Crosson S.** 2012. Molecular
484 structure and function of the novel BrnT/BrnA toxin-antitoxin system of *Brucella*
485 *abortus*. *J Biol Chem* **287**:12098-12110.
- 486 24. **Wu CX, Xu XJ, Zheng K, Liu F, Yang XD, Chen CF, Chen HC, Liu ZF.** 2016.
487 Characterization of ribonuclease III from *Brucella*. *Gene* **579**:183-192.
- 488 25. **Saramago M, Peregrina A, Robledo M, Matos RG, Hilker R, Serrania J, Becker A,**
489 **Arraiano CM, Jimenez-Zurdo JI.** 2017. *Sinorhizobium meliloti* YbeY is an
490 endoribonuclease with unprecedented catalytic features, acting as silencing enzyme in
491 riboregulation. *Nucleic Acids Res* **45**:1371-1391.
- 492 26. **Jimenez-Zurdo JI, Robledo M.** 2017. RNA silencing in plant symbiotic bacteria:
493 Insights from a protein-centric view. *RNA Biol* **14**:1672-1677.
- 494 27. **Fretin D, Fauconnier A, Kohler S, Halling S, Leonard S, Nijskens C, Ferooz J,**
495 **Lestrade P, Delrue RM, Danese I, Vandenhoute J, Tibor A, DeBolle X, Letesson JJ.**

- 496 2005. The sheathed flagellum of *Brucella melitensis* is involved in persistence in a
497 murine model of infection. *Cell Microbiol* **7**:687-698.
- 498 28. **Leonard S, Ferooz J, Haine V, Danese I, Fretin D, Tibor A, de Walque S, De Bolle**
499 **X, Letesson JJ.** 2007. FtcR is a new master regulator of the flagellar system of *Brucella*
500 *melitensis* 16M with homologs in *Rhizobiaceae*. *J Bacteriol* **189**:131-141.
- 501 29. **Sangari FJ, Seoane A, Rodriguez MC, Agüero J, Garcia Lobo JM.** 2007.
502 Characterization of the urease operon of *Brucella abortus* and assessment of its role in
503 virulence of the bacterium. *Infect Immun* **75**:774-780.
- 504 30. **Lestrade P, Delrue RM, Danese I, Didembourg C, Taminiou B, Mertens P, De Bolle**
505 **X, Tibor A, Tang CM, Letesson JJ.** 2000. Identification and characterization of in vivo
506 attenuated mutants of *Brucella melitensis*. *Mol Microbiol* **38**:543-551.
- 507 31. **Rossetti CA, Galindo CL, Lawhon SD, Garner HR, Adams LG.** 2009. *Brucella*
508 *melitensis* global gene expression study provides novel information on growth phase-
509 specific gene regulation with potential insights for understanding *Brucella*:host initial
510 interactions. *BMC Microbiol* **9**:81.
- 511 32. **Caro-Hernandez P, Fernandez-Lago L, de Miguel MJ, Martin-Martin AI,**
512 **Cloekaert A, Grillo MJ, Vizcaino N.** 2007. Role of the Omp25/Omp31 family in outer
513 membrane properties and virulence of *Brucella ovis*. *Infect Immun* **75**:4050-4061.
- 514 33. **Lamontagne J, Forest A, Marazzo E, Denis F, Butler H, Michaud JF, Boucher L,**
515 **Pedro I, Villeneuve A, Sitnikov D, Trudel K, Nassif N, Boudjelti D, Tomaki F,**
516 **Chaves-Olarte E, Guzman-Verri C, Brunet S, Cote-Martin A, Hunter J, Moreno E,**
517 **Paramithiotis E.** 2009. Intracellular adaptation of *Brucella abortus*. *J Proteome Res*
518 **8**:1594-1609.

- 519 34. **Roset MS, Alefantis TG, DelVecchio VG, Briones G.** 2017. Iron-dependent
520 reconfiguration of the proteome underlies the intracellular lifestyle of *Brucella abortus*.
521 Sci Rep 7:10637.
- 522 35. **Bobrovskyy M, Vanderpool CK, Richards GR.** 2015. Small RNAs Regulate Primary
523 and Secondary Metabolism in Gram-negative Bacteria. Microbiol Spectr 3.
- 524 36. **Caswell CC, Gaines JM, Ciborowski P, Smith D, Borchers CH, Roux CM, Sayood
525 K, Dunman PM, Roop Ii RM.** 2012. Identification of two small regulatory RNAs linked
526 to virulence in *Brucella abortus* 2308. Mol Microbiol 85:345-360.
- 527 37. **Saadeh B, Caswell CC, Chao Y, Berta P, Wattam AR, Roop RM, 2nd, O'Callaghan
528 D.** 2015. Transcriptome-Wide Identification of Hfq-Associated RNAs in *Brucella suis* by
529 Deep Sequencing. J Bacteriol 198:427-435.
- 530 38. **Zhong Z, Xu X, Li X, Liu S, Lei S, Yang M, Yu J, Yuan J, Ke Y, Du X, Wang Z,
531 Ren Z, Peng G, Wang Y, Chen Z.** 2016. Large-scale identification of small noncoding
532 RNA with strand-specific deep sequencing and characterization of a novel virulence-
533 related sRNA in *Brucella melitensis*. Sci Rep 6:25123.
- 534 39. **Wang Y, Ke Y, Xu J, Wang L, Wang T, Liang H, Zhang W, Gong C, Yuan J,
535 Zhuang Y, An C, Lei S, Du X, Wang Z, Li W, Yuan X, Huang L, Yang X, Chen Z.**
536 2015. Identification of a Novel Small Non-Coding RNA Modulating the Intracellular
537 Survival of *Brucella melitensis*. Front Microbiol 6:164.
- 538 40. **Peng X, Dong H, Wu Q.** 2015. A new cis-encoded sRNA, BsrH, regulating the
539 expression of hemH gene in *Brucella abortus* 2308. FEMS Microbiol Lett 362:1-7.

- 540 41. **Caswell CC, Baumgartner JE, Martin DW, Roop RM, 2nd.** 2012. Characterization of
541 the organic hydroperoxide resistance system of *Brucella abortus* 2308. J Bacteriol
542 **194**:5065-5072.
- 543 42. **Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, 2nd, Peterson**
544 **KM.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS,
545 carrying different antibiotic-resistance cassettes. Gene **166**:175-176.
- 546 43. **White PG, Wilson JB.** 1951. Differentiation of smooth and nonsmooth colonies of
547 Brucellae. J Bacteriol **61**:239-240.
- 548 44. **Beenken KE, Dunman PM, McAleese F, Macapagal D, Murphy E, Projan SJ,**
549 **Blevins JS, Smeltzer MS.** 2004. Global gene expression in *Staphylococcus aureus*
550 biofilms. J Bacteriol **186**:4665-4684.
- 551 45. **Gee JM, Valderas MW, Kovach ME, Grippe VK, Robertson GT, Ng WL,**
552 **Richardson JM, Winkler ME, Roop RM, 2nd.** 2005. The *Brucella abortus* Cu,Zn
553 superoxide dismutase is required for optimal resistance to oxidative killing by murine
554 macrophages and wild-type virulence in experimentally infected mice. Infect Immun
555 **73**:2873-2880.
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558 Table 1: Differential gene expression in *B. abortus* 2308:: $\Delta ybeY$.

<u>Gene Designation</u>	<u>Description</u>	<u>Fold Change ($\Delta ybeY$ vs. 2308)</u>
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

Flagellar Related Proteins

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	glyoxalase/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
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/quininate 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 (<i>ybeY</i>)	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

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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 be more than 3-fold altered in the *ybeY* deletion strain compared to strain 2308 are shown in the list. Cells highlighted in grey represent genes previously observed to be required for efficient *Brucella* infection or as being differentially expressed during intracellular trafficking of *Brucella*.

566 Table 2. Oligonucleotide primers used in this study.

567 <u>Primer name</u>	<u>Sequence (5'→3')</u>
Primer Name	Sequence (5'→3')
<i>bab1_2156</i> -Up-For	GCGGATCCTTATGAAACATTGCAAAAGG
<i>bab1_2156</i> -Up-Rev	GATCATGATATCAATATGGATCG
<i>bab1_2156</i> -Down-For	GATTGACCATGGCTGAACA
<i>bab1_2156</i> -Down-Rev	CGCTGCAGTCCAATACGTGGAATTCATAACC
<i>ybeY</i> -RC-For	ATGTGGACGGCGCACTGCGCAT
<i>ybeY</i> -RC-Rev	GGAATGGCCTGAACCACTTCACC
<i>bab2_0548</i> -Up-For	TAGGATCCTTGCAGGAATTTGCCAAATATGA
<i>bab2_0548</i> -Up-Rev	CGGCATGCAATTCCGTCGTAAG
<i>bab2_0548</i> -Dn-For	CCATGAGCGTCCAATCGCAAGAT
<i>bab2_0548</i> -Dn-Rev	TACTGCAGACCAGAAACCCGCCTTCATCAA
<i>bab2_0282</i> -Up-For	TAGGATCCATATTTGCTGGCGATGAAATAAG
<i>bab2_0282</i> -Up-Rev	TTTCATGAAGTGTTCCTCCAG
<i>bab2_0282</i> -Dn-For	CAGTAAGAGGCTGGTTTGATGAA
<i>bab2_0282</i> -Dn-Rev	TACTGCAGTTTGCGGATAATGCCCATGATG
<i>bab2_0277</i> -Up-For	TAGGATCCAAATGCGGCTTACAGCAAGGC
<i>bab2_0277</i> -Up-Rev	GGTCATGATTCTATATCCAGTAA
<i>bab2_0277</i> -Dn-For	CGGTGAACGGGTTTCCATCG
<i>bab2_0277</i> -Dn-Rev	TACTGCAGAACCAGTGCCTTACCCAAGG
<i>bab1_1070</i> -Up-For	TAGGATCCTAGGACATGACCGATCTCCTTCC
<i>bab1_1070</i> -Up-Rev	CATCTGACATCTCCGTTAATCG
<i>bab1_1070</i> -Dn-For	ATTACCGCGAAACTGCATGGCT
<i>bab1_1070</i> -Dn-Rev	TACTGCAGATATGCGAAAGCTTGACCCG
<i>bab2_1109</i> -Up-For	TAGGATCCTTTGAGCGCGGCAGCGATGCA
<i>bab2_1109</i> -Up-Rev	TTTCATGCACGTTTCCTCCAA
<i>bab2_1109</i> -Dn-For	AAATAAACCTTCTGTTCTGC
<i>bab2_1109</i> -Dn-Rev	TACTGCAGAAACATCGTCGACCACCTTGCG
<i>bab2_0830</i> -Up-For	TAGGATCCGGTCCTGAAGTTCTTGAGCTCGTT
<i>bab2_0830</i> -Up-Rev	TCTCATTCTTTTCTCCCTCAA
<i>bab2_0830</i> -Dn-For	AAATGATCCTGTGTGGGCG
<i>bab2_0830</i> -Dn-Rev	TACTGCAGTTATTCATGCCGGCGCGGTCTAT
<i>bab2_0822</i> -Up-For	TAGGATCCTTGGTGCAGGCTGTTCCGTG
<i>bab2_0822</i> -Up-Rev	TTCCAATTTTCCCTCCTCTT
<i>bab2_0822</i> -Dn-For	CAGTAACAGTCGTCACCGAGGTG
<i>bab2_0822</i> -Dn-Rev	TACTGCAGCGAATGGATTTTCTTCCGCCAC
<i>bab1_0265</i> -Up-For	TAGGATCCAAACCAAAAGCCACAATGAACC
<i>bab1_0265</i> -Up-Rev	ACTCAGGTACATAGATTTGTTCC
<i>bab1_0265</i> -Dn-For	GAATGAAACCCGACCGTCTTTC
<i>bab1_0265</i> -Dn-Rev	TACTGCAGAATTTTCTTACGACATATGA
<i>bab2_0700</i> -Up-For	TAGGATCCTAAGGTCAACTGGATACCTTTCG

bab2_0700-Up-Rev AACCATCGAAAAC~~TCCC~~CATA
bab2_0700-Dn-For AACTAACAAAACGAAACCCCTT
bab2_0700-Dn-Rev TACTGCAGAAATGCCGGAATGCCGAAAAT

*Underlined sequences depict a restriction endonuclease recognition site.

568

569

570 Table 3. Plasmids used in this study.

571	<u>Plasmid name</u>	<u>Description</u>	<u>Reference</u>
572	pBBR1MCS-4	Broad-host range cloning vector; Amp ^R	(42)
573	pNPTS138	Cloning vector; contains <i>sacB</i> gene; Kan ^R	(M.R.K. Alley, unpublished)
574	<i>pybeY</i>	In-frame deletion of <i>ybeY</i> plus 1 kb of each flanking region in pNPTS138	This study
575	<i>pybeY-comp</i>	<i>ybeY</i> locus including the entire promoter region in pBBR1MCS-4	This study
576	<i>pbab2_0277</i>	In-frame deletion of <i>bab2_0277</i> plus 1 kb of each flanking region in pNPTS138	This study
577	<i>pbab2_0282</i>	In-frame deletion of <i>bab2_0282</i> plus 1 kb of each flanking region in pNPTS138	This study
578	<i>pbab2_0822</i>	In-frame deletion of <i>bab2_0822</i> plus 1 kb of each flanking region in pNPTS138	This study
579	<i>pbab2_0548</i>	In-frame deletion of <i>bab2_0548</i> plus 1 kb of each flanking region in pNPTS138	This study
580	<i>pbab2_0830</i>	In-frame deletion of <i>bab2_0830</i> plus 1 kb of each flanking region in pNPTS138	This study
581	<i>pbab2_1109</i>	In-frame deletion of <i>bab2_1109</i> plus 1 kb of each flanking region in pNPTS138	This study
582	<i>pbab2_0700</i>	In-frame deletion of <i>bab2_0700</i> plus 1 kb of each flanking region in pNPTS138	This study
583	<i>pbab1_0265</i>	In-frame deletion of <i>bab1_0265</i> plus 1 kb of each flanking region in pNPTS138	This study
584	<i>pbab1_1070</i>	In-frame deletion of <i>bab1_1070</i> plus 1 kb of each flanking region in pNPTS138	This study
585			

586

Figure Legends

587 **Figure 1. *In vitro* growth characteristics and cellular morphology of *B. abortus***

588 **2308:: Δ ybeY.**

589 A. Genomic context of *ybeY*. The *ybeY* gene (*babI_2156*; *bab_rs26200*) is located on
590 chromosome I in *B. abortus* 2308. *ybeY* is flanked by *phoH* (*babI_2155*; *bab_rs26195*) and a
591 gene encoding a putative hypothetical protein (*babI_2157*; *bab_rs26205*).

592 B. Photograph of *B. abortus* colonies on SBA after 72 hours of growth.

593 C. Growth curve of *Brucella abortus* strains in rich medium. *B. abortus* 2308, *B. abortus*
594 2308:: Δ ybeY, and *B. abortus* 2308:: Δ ybeY-comp were grown in brucella broth and colony
595 forming units/ml was monitored by serial dilution. The asterisk (*) denotes a statistically
596 significant difference ($P < 0.05$; Student's *t*-test) between the *ybeY* deletion strain and the parental
597 strain 2308.

598 D. Electron microscopy of *Brucella abortus* cells. Exponential and stationary phase cells of
599 *Brucella* strains were fixed and viewed under scanning electron microscopy, magnified 30,000x.

600 Bar = 1 μ m.

601 **Figure 2. Virulence of *B. abortus* 2308 and Δ ybeY in peritoneal derived macrophages and**
602 **BALB/c mice.**

603 A. Macrophage survival and replication experiments. Cultured peritoneal macrophages from
604 BALB/c mice were infected with *B. abortus* 2308, the isogenic *ybeY* deletion strain (*ybeY*), and
605 the *ybeY* complemented strain (*ybeY*-comp). At the indicated times post-infection, macrophages
606 were lysed, and the number of intracellular brucellae present in these phagocytes was determined
607 by serial dilution and plating on agar medium. The asterisk (*) denotes a statistically significant
608 difference ($P < 0.05$; Student's *t*-test) between the *ybeY* deletion strain and the parental strain

609 2308, and the *ybeY* deletion strain and the complemented strain at 24 and 48 hours post-
610 infection.

611 B. Mouse infection experiments. BALB/c mice (5 per strain) were infected intraperitoneally with
612 *B. abortus* 2308, and the isogenic *ybeY* deletion strain (*ybeY*). Mice were sacrificed at weeks 4,
613 and 8 post-infection, and brucellae/spleen was determined. The data is presented as the average
614 brucellae +/- the standard deviation from the 5 mice colonized with a specific *Brucella* strain at
615 each time point. The asterisk (*) denotes a statistically significant difference ($P < 0.05$; Student's
616 *t*-test) between the *ybeY* deletion strain and the parental strain 2308 at 4 and 8 weeks post-
617 infection.

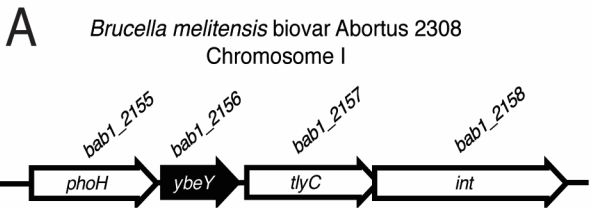
618 **Figure 3. YbeY is required for optimal resistance to biologically relevant stresses.**

619 A. Sensitivity assays. *Brucella abortus* 2308, the *ybeY* deletion strain, and *ybeY*-comp
620 complemented strain were assessed in a disk diffusion assay for their comparative susceptibilities
621 to various stress conditions, including deoxycholate (10%), H₂O₂ (30%), SDS (20%), polymyxin
622 B (10 mg/ml), and paraquat (0.25 M). The results are plotted as the average diameter (+/-
623 standard deviation) of the zone of inhibition around a disk, and the results are from a single
624 experiment that was repeated in triplicate. Asterisks (*) denote a statistically significant
625 difference ($P < 0.05$; Student's *t*-test) between the *ybeY* deletion strain and the parental strain 2308
626 for a given condition.

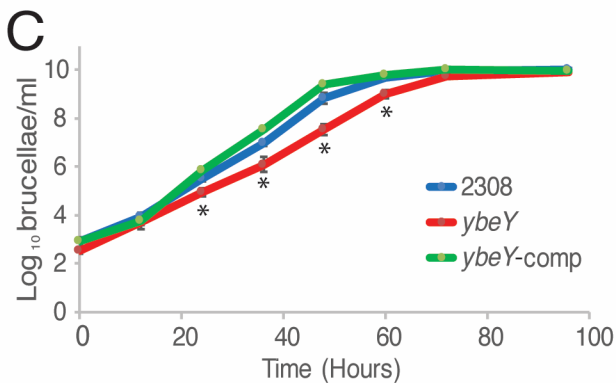
627 **Figure 4: Virulence phenotypes associated with genes differentially expressed in the *B.***
628 ***abortus ybeY* deletion strain.**

629 Cultured peritoneal macrophages from BALB/c mice were infected with *B. abortus* 2308, the
630 isogenic deletion strains $\Delta bab2_0822$, $\Delta bab2_1109$, or $\Delta bab2_0700$. At 2, 24, and 48 hours post-
631 infection, the macrophages were lysed, and the number of intracellular brucellae present in these

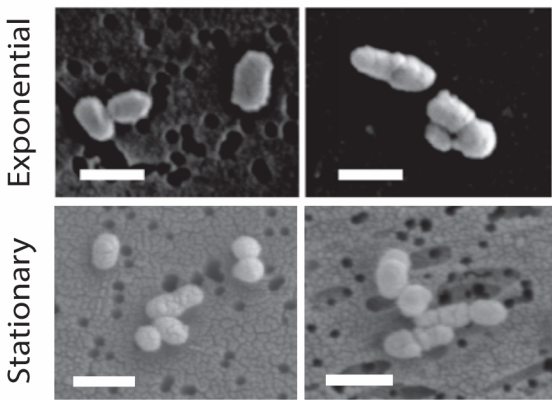
632 phagocytes was determined by serial dilution and plating on agar medium. The asterisk (*)
633 denotes a statistically significant difference ($P < 0.05$; Student's *t*-test) between the isogenic
634 deletion strains and the parental strain 2308 at 48 hours post-infection.



B 2308 *ybeY* *ybeY*-comp

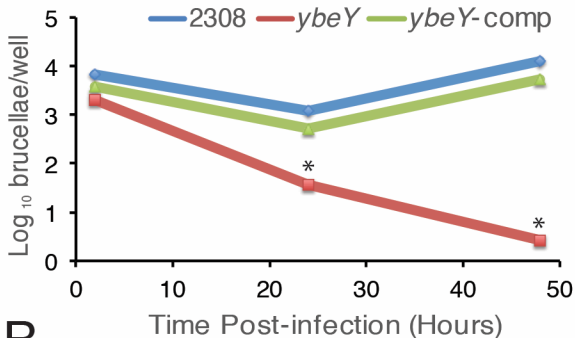


D 2308 *ybeY*

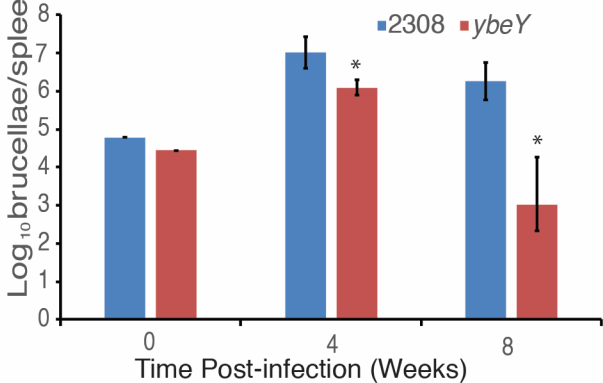


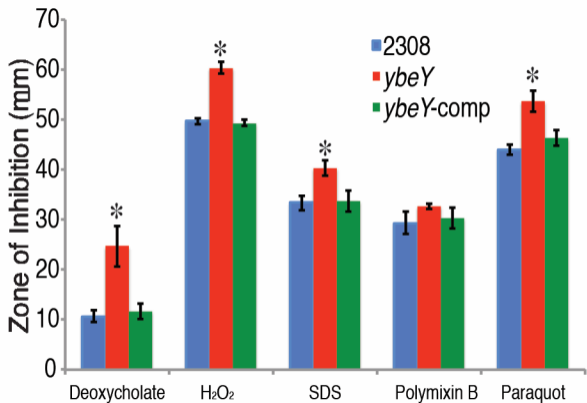
A

Survival and Replication in BALB/c Macrophages

**B**

Spleen Colonization of BALB/c Mice





Survival and Replication in BALB/c Macrophages

