Molecular control of gene expression by *Brucella* BaaR, an IclR-type transcriptional repressor

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Julien Herrou§, Daniel M. Czyż§, Aretha Fiebig§, Jonathan W. Willett§∥, Youngchang Kim¶, Ruiying Wu¶, Gyorgy Babnigg†, and Sean Crosson‡§∥

From the Departments of §Biochemistry and Molecular Biology and ‡Microbiology, University of Chicago, Chicago, Illinois 60637, the ¶Howard Taylor Ricketts Laboratory, University of Chicago, Argonne, Illinois 60439, and the ‡Argonne National Laboratory, Argonne, Illinois 60439

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The general stress response sigma factor $\sigma^{E1}$ directly and indirectly regulates the transcription of dozens of genes that influence stress survival and host infection in the zoonotic pathogen *Brucella abortus*. Characterizing the functions of $\sigma^{E1}$-regulated genes therefore would contribute to our understanding of *B. abortus* physiology and infection biology. $\sigma^{E1}$ indirectly activates transcription of the IclR family regulator Bab2_0215, but the function of this regulator remains undefined. Here, we present a structural and functional characterization of Bab2_0215, which we have named *Brucella* adipic acid-activated regulator (BaaR). We found that BaaR adopts a classic IclR-family fold and directly represses the transcription of two operons with predicted roles in carboxylic acid oxidation. BaaR binds two sites on chromosome II between *baaR* and a divergently transcribed hydratase/dehydrogenase (*acaD2*), and it represses transcription of both genes. We identified three carboxylic acids (adipic acid, tetradecanedioic acid, and $\epsilon$-amino-caproic acid) and a lactone (2-caprolactone) that enhance transcription from the *baaR* and *acaD2* promoters. However, neither the activating acids nor caprolactone enhanced transcription by binding directly to BaaR. Induction of *baaR* transcription by adipic acid required the gene *bab2_0213*, which encodes a major facilitator superfamily transporter, suggesting that Bab2_0213 transports adipic acid across the inner membrane. We conclude that a suite of structurally related organic molecules activate transcription of genes repressed by BaaR. Our study provides molecular-level understanding of a gene expression program in *B. abortus* that is downstream of $\sigma^{E1}$.

Microbes use numerous mechanisms to modulate their physiology in response to changes in the intracellular and extracellular environments (1). A fundamental mechanism required for physiological adaptation is the regulation of gene expression. The Gram-negative bacterium, *Brucella abortus*, is a facultative intracellular pathogen that can cause abortion in mammals and is among the most common zoonotic pathogens globally (2, 3). *B. abortus* encodes an extracytoplasmic function-type sigma factor, $\sigma^{E1}$, which directly and indirectly regulates transcription of ~100 genes in response to environmental perturbation. $\sigma^{E1}$-dependent transcription confers resistance to multiple environmental stressors and is required for maintenance of chronic *B. abortus* infection in a mouse model (4–7). Assigning physiological and/or biochemical functions to $\sigma^{E1}$-regulated genes is therefore important for understanding *B. abortus* stress physiology and infection biology.

Although several genes in the *B. abortus* $\sigma^{E1}$ regulon have been characterized (4, 5, 7–10), the functions of the majority of $\sigma^{E1}$-regulated genes remain undefined. Gene *bab2_0215*, which encodes a predicted IclR-family transcriptional regulator, was identified as having significantly reduced expression in a $\sigma^{E1}$ deletion strain (*ArpoE1*) (4). IclR proteins are conserved across bacteria and archaea and have been reported to control the transcription of genes involved in a range of processes (11–13), including carbon catabolism (14–20), biofilm formation (21), quorum sensing (22, 23), virulence (24–26), stress response (27), antibiotic resistance (28, 29), amino acid and secondary metabolite biosynthesis (30–32), motility (33), and sporulation (34, 35). However, the functional roles of IclR-family regulators in *Brucella* spp. remain largely unexplored. Herein, we present a structural and functional characterization of Bab2_0215, which we have named *Brucella* adipic acid-activated regulator, or BaaR. The experimental data presented in this study define BaaR as a transcriptional repressor, identify the regulatory targets of BaaR, and reveal chemical signals that derepress transcription of genes inhibited by BaaR *in vivo*.

Specifically, we have solved an X-ray crystal structure of BaaR, which revealed a classic IclR fold (11) with a C-terminal ligand-binding domain (LBD) and an N-terminal helix-turn-
Transcription regulation by Brucella BaaR

Figure 1. Transcriptional regulation of bab2_0215 (baaR) under oxidative stress. A, fragments/kb of transcript per million mapped reads (FPKM) values for baaR determined by RNA-seq in the B. abortus WT strain and a rpoE1 deletion strain (ΔrpoE1) under oxidative stress conditions (5 mM H2O2). Error bars represent the standard deviations of three independent experiments. B, relative survival of WT, ΔbaaR, and ΔrpoE1 strains after treatment with 5 mM H2O2 for 1 h. Error bars represent the standard deviations.

Helix (HTH) DNA-binding domain. We demonstrate that deleting baaR (∆baaR) results in strong transcriptional up-regulation of four genes located adjacent to baaR on chromosome II of B. abortus 2308. This regulated gene set shares sequence similarity with characterized dicarboxylic acid (dca) β-oxidation operons from Acinetobacter and Pseudomonas (36–39). We found that BaaR functions as a transcriptional repressor by binding to two conserved palindromic motifs between bab2 and bab2_0216 (previously annotated as acdA2 (gch-C0A dehydrogenase 2) (40)). A set of structurally related organic molecules enhanced transcription from a BaaR-dependent transcriptional reporter in vivo. Activation of transcription by one of these molecules, acetic acid, required the transporter gene bab2_0213, providing evidence that bab2_0213 encodes an acetic acid transporter. However, none of the small molecules that activate transcription in vivo were found to bind directly to purified BaaR in vitro. We thus conclude that the molecular signal(s) that directly control BaaR-dependent transcription in the cell are distinct from the activating molecules we identified via selective addition to the growth medium.

Results

Regulation of baaR by σE1 is likely indirect, and baaR does not contribute to B. abortus hydrogen peroxide (H2O2) resistance

Previously, wild-type (WT) B. abortus strain 2308 and B. abortus strain 2308 harboring an in-frame deletion of the general stress regulator rpoE1 (encoding σE1) were subjected to H2O2 stress, and differences in gene expression between the two strains were assessed by RNA-Seq (RNA-seq) (4). Transcription of baaR was decreased 2-fold in the ΔrpoE1 strain relative to the WT strain (Fig. 1A), suggesting that baaR is directly or indirectly activated by σE1. We could not identify a σE1-binding site in the baaR promoter region, which suggests that this regulatory effect is indirect. To evaluate the contribution of baaR to σE1-dependent protection against H2O2 stress, we measured the survival of the WT, ΔrpoE1, and ΔbaaR strains after treatment with 5 mM H2O2. Survival of ΔrpoE1, assessed by enumerating colony-forming units (CFU) on solid medium after hydrogen peroxide treatment, was reduced by ~1 order of magnitude, whereas ΔbaaR survival did not differ from WT survival (Fig. 1B). We thus concluded that decreased expression of baaR in ΔrpoE1 does not contribute to the viability defect of ΔrpoE1 under the assayed condition.

BaaR adopts a classic IclR family fold

To better understand the structure and function of BaaR, we expressed, purified, and crystallized the BaaR protein and solved its structure to 1.95 Å resolution (Rwork = 21.2% and Rfree = 17.7%) (see Table S1 for data collection and refinement statistics). Two BaaR dimers were present in the crystallographic asymmetric unit, and each monomer adopted the classic fold of IclR proteins (11). The monomeric protein consisted of an N-terminal winged HTH domain (residues Phe-39–Asp-101), α-helical linker (α4, residues Ile-102–Leu-115), and C-terminal LBD (residues Met-116–Pro-284) (Fig. 2, A–C). The HTH DNA-binding domain consists of three α-helices (α1, α2, and α3) and two β-strands (β1 and β2) (Fig. 2, A and C). Remarkably, compared with typical IclR protein sequences, BaaR has an unusually long N-terminal region. This extension is ~38 amino acids (from residue Met-1 to Gln-38) and is similar in size to the N termini of Acinetobacter DcaR and DcaS and Pseudomonas P1630 (Fig. 2C and Fig. S1). For crystallization, a trimmed version (residues Lys-21–Pro-284) of BaaR was used; the N terminus appeared to be mostly unstructured with the exception of residues Gly-31–Asp-36, which adopted an α-helical fold (α1’) in three of four monomers (Fig. 2, A and C). The functional relevance of this region is unknown; we cannot exclude the possibility that an alternative start codon is used for protein translation in vivo.

The BaaR LBD consists of five α-helices (α5, α6, α7, α8, and α9) and six anti-parallel β-strands (β3, β4, β5, β6, β7, and β8) arranged in a semi-circular β-scaffold. The β-scaffold is partially occluded by α6 and folds to form a cavity in the LBD (Fig. 2, A and C). In the LBD cavity of each IclR protein, we observed extra electron density consistent with a bound acetate molecule (Fig. 2, A and B). This molecule is likely derived from the crystallization solution, which contained 200 mM calcium acetate. Notably, the acetate occupied the same position as pyruvate or glyoxylate molecules that have previously been reported to bind to the LBD of Escherichia coli IclR (Fig. 2B) (41, 42). Additional structural alignments and a schematic of hydrogen bonding to bound acetate (and its corresponding electron density in the BaaR cavity) are presented in Fig. S2.

Protein sequence alignment of BaaR, Acinetobacter DcaR (ACIAD1688) and DcaS (ACIAD1684), and Pseudomonas PA1630 revealed high sequence identity and common secondary structural features (Fig. 2C). All have HTH domains with long N termini; these proteins also have LBD cavities comprising very similar residues, suggesting that they recognize and respond to identical or similar small molecules and interact with target DNA in a comparable manner (Fig. 2C and Fig. S1). The two BaaR dimers present in the asymmetric unit are highly superimposable and displayed few structural differences (r.m.s.d = 0.76). IclR-family proteins have been found to adopt dimeric or tetrameric conformations in X-ray structures, in solution, or in their interactions with DNA (11, 41, 43–45).
Therefore, even if BaaR crystallizes as a dimer in vitro, it potentially exists in different oligomeric states in vivo.

**BaaR represses the transcription of a set of genes with similarities to the dca operon**

Our crystal structure clearly identified BaaR as an IclR-family transcriptional regulator capable of binding a carboxylic acid (acetate) via its LBD. To identify genes specifically regulated by BaaR, we measured the global transcriptomic profile of the /H9004 baaR strain using RNA-seq and compared it with that of the WT strain (Table S2). Among the differentially expressed genes, four genes were highly up-regulated in /H9004 baaR relative to WT (Fig. 3A). These four genes, bab2_0213, bab2_0214, bab2_0216, and bab2_0217, are contiguous and adjacent to baaR (bab2_0215). Genes bab2_0213, bab2_0214, and bab2_0216 are located on the opposite strand from bab2_0216 and bab2_0217, and the two gene sets are likely divergently transcribed (Fig. 3B). bab2_0213 is annotated as a MucK transporter, a member of the major facilitator superfamily (MFS). A transporter related to bab2_0213 in Acinetobacter sp. ADP1 is involved in cis,cis-muconic acid uptake (39). bab2_0214 is annotated as an acyl-CoA dehydrogenase; this family of flavo-
proteins catalyzes α,β-dehydrogenation of fatty acid acyl-CoA conjugates (46, 47). bab2_0216 and bab2_0217 are annotated as pseudogenes. However, this annotation appears to be incorrect: a search of Pfam (48) using the primary structures of these loci suggested that bab2_0216 (acaD2), is in fact two fused, in-frame genes that correspond to an enoyl-CoA hydratase/isomerase and a 3-hydroxyacyl-CoA dehydrogenase. Both of these enzymes are predicted to be involved in β-oxidation of fatty acids (49, 50). Our transcriptomic data corroborate a recent proteomic study in B. abortus in which a small peptide corresponding to the Bab2_0216 N terminus was identified by MS (51), suggesting that the full-length “fusion” protein is expressed. This hypothesis is supported by known examples of functional proteins (FadB and FadN in E. coli) that simultaneously contain an enoyl-CoA hydratase/isomerase and a 3-hydroxyacyl-CoA dehydrogenase in a single polypeptide (52).

**bab2_0217** encodes a protein with homology to the CoA-transferase family III, a class of enzymes that catalyzes reversible transfer reactions of CoA groups from CoA-thioesters to free acids (53). An early stop codon present in the sequence of this gene truncates the corresponding protein by 45 residues relative to related CoA-transferase family III proteins. The adjacent gene bab2_0218 includes these 135 nucleotides missing from bab2_0217; the functional significance of bab2_0217 truncation is not known.

Two additional gene sets were differentially regulated in ΔbaaR relative to WT. The first corresponds to 12 adjacent genes (bab2_1036–bab2_1047) (Fig. 3A) with predicted enzyme functions similar to those associated with the bab2_0213–bab2_0217 operon. Two of the most strongly up-regulated genes, bab2_1046 and bab2_1045, are annotated as enoyl-CoA hydratase/isomerase and 3-hydroxyacyl-CoA dehydrogenase, respectively, and may have functionally redundant roles with bab2_0216. Gene bab2_1036 is annotated as a CoA-transferase family III protein and may be redundant with bab2_0217. Expression of the second gene set, corresponding to bab2_0277–bab2_0282 (Fig. 3A), was significantly lower in ΔbaaR relative to WT. The majority of these genes encode components of an ATP-binding cassette (ABC) transport system predicted to be involved in branched-chain amino acid transport. Comparison of the amino acid sequence of the cognate periplasmic binding protein (PPB) with the sequences of PPBs that co-crystallize with *bona fide* ligands revealed that Bab2_0282 shares 54% identity and 72% similarity with a *Burkholderia mallei* PPB (Protein Data Bank (PDB) code 3I09) that co-crystalizes in a closed conformation with acetooacetate. Residues involved in the interaction with acetooacetate in this PPB are conserved in Bab2_0282 (Fig. S3), suggesting that the *B. abortus* bab2_0277–bab2_0282 ABC transporter operon is involved in uptake of acetooacetate or a related molecule. Acetooacetate is produced in the liver during ketogenesis. Under certain conditions, the acetyl-CoA formed in the liver from β-oxidation of fatty acids can be converted into ketone bodies (acetooacetate, β-hydroxybutyrate, and acetone) for export to other tissues (54, 55). This *Brucella* ABC transporter operon is proximal to a gene (bab2_0277) annotated as a glucose/methanol/choline oxidoreductase, which is also expressed at significantly lower levels in ΔbaaR. In *B. mallei*, a similar gene (BMA2933) also co-occurs with the related ABC transporter, suggesting that this system may have a function in ketone metabolism.

Finally, genes *bab1_0303* (UreG1 urease accessory protein), bab1_0578 (BtuB transcriptional regulator), bab1_0914 (DUF1127), and bab2_0548 (ABC transmembrane transporter) also showed significant differential regulation in our data set. The functional significance of these transcriptional changes is not known (see Table S2 for the full data set).
BaaR binds palindromic motifs in its own promoter region

To evaluate the mechanism of BaaR-dependent transcriptional repression, we measured binding between purified BaaR and a region of DNA corresponding to its promoter. Because the IclR protein family recognizes and specifically interacts with palindromic motifs (11), the DNA sequence corresponding to the promoter region between baaR and bab2_0216 was analyzed for palindromes. We identified two similar palindromic regions that were 44 and 36 nucleotides away from the bab2_0216 and baaR start codons, respectively. These two regions were named BaaR-binding site 1 (BBS1) and BaaR-binding site 2 (BBS2), and each consisted of two overlapping palindromic sequences (Figs. 4 and 5). A 375-nucleotide fluorescent DNA probe corresponding to the promoter region between baaR and bab2_0216 was used for gel-shift experiments. As expected, 5 ng of this “long” fluorescent DNA probe corresponding to the WT sequence (L W F , long, and WT, fluorescent) was shifted in size on the gel in the presence of increasing concentrations (2–500 nM) of purified BaaR, confirming that BaaR interacts with the DNA region between bab2_0215 and bab2_0216 (Fig. 4A, panel 1).

To determine whether this interaction was specific, we conducted a series of control experiments using unlabeled probes as well as mutated probes in which the palindromic motifs were randomized to maintain base content and length. As observed previously, 5 ng of the L W F probe were shifted in size when mixed with increasing concentrations (62–500 nM) of BaaR (Fig. 4A, panel 2). However, this shift was weakened when 5 ng of the L W F probe (long mutated and fluorescent) was used instead. The 1st lane (control) corresponds to 5 ng of L W F alone (i.e. without BaaR) and was used as a size reference. B, gel-shift assays in panels 1–5 were performed as described in A; however, instead of using a DNA probe targeting the full-length baaR–bab2_0216 intergenic region, a shorter (196 nucleotides long) WT fluorescent DNA probe (S W F ) targeting only half of this region, carrying BBS2 but not BBS1, was used. A schematic representation of the different DNA probes used in this assay is presented at the top of each panel.

Figure 4. Electrophoretic mobility shift assay of the interaction between purified BaaR protein (residues 9–283) and the bab2_0215–bab2_0216 intergenic region. A, panel 1, a long WT fluorescent DNA probe (L W F , 375 nucleotides long) corresponding to the intergenic region between baaR and bab2_0216, including BBS1 and BBS2, was mixed with increasing concentrations (2, 4, 8, 16, 31, 62, 125, 250, and 500 nM) of purified BaaR and run on a 5% native acrylamide gel. 1st lane represents 5 ng of L W F alone (i.e. without BaaR) and was used as a size control. Panel 2, the L W F (5 ng) was mixed with increasing concentrations (62, 125, 250, and 500 nM) of purified BaaR and run on a 5% native acrylamide gel. The 1st lane (control) represents 5 ng of L W F alone (i.e. without BaaR). Panel 3, the L W F (5 ng) was mixed with 500 nM purified BaaR and increasing concentrations (5, 50, 100, and 200 ng) of a long WT unlabeled DNA probe (L W U), corresponding to the intergenic region between baaR and bab2_0216, including BBS1 and BBS2. The 1st lane (control) represents 5 ng of L W F alone (i.e. without BaaR). Panel 4, the conditions used in this assay were similar to those used in panel 2, except that 50 ng of the L W U DNA probe (long mutated and unlabeled), corresponding to the intergenic region between baaR and bab2_0216 and carrying mutated BBS sequences, was added. The 1st lane (control) corresponds to 5 ng of L W F alone (i.e. without BaaR). Panel 5, the conditions used in this assay were similar to those used in panel 2, except that 5 ng of L W F probe (long mutated and fluorescent) was used instead. The 1st lane (control) corresponds to 5 ng of L MF alone (i.e. without BaaR) and was used as a size reference. B, gel-shift assays in panels 1–5 were performed as described in A; however, instead of using a DNA probe targeting the full-length baaR–bab2_0216 intergenic region, a shorter (196 nucleotides long) WT fluorescent DNA probe (S W F ) targeting only half of this region, carrying BBS2 but not BBS1, was used. A schematic representation of the different DNA probes used in this assay is presented at the top of each panel.
unlabeled) were mixed together. This mutated probe did not disrupt the BaaR/LMF interaction, suggesting BaaR binding requires the BBS1 and BBS2 regions (Fig. 4, panel 4). Finally, when we performed a gel-shift assay with a fluorescent DNA probe carrying mutated BBS1 and BBS2 (LMF, long, mutated, and fluorescent), no shift was observed, even at the highest BaaR concentrations (Fig. 4, panel 5). We conclude that BaaR constitutively and specifically interacts with the bab2_0215–bab2_0216 promoter region, and this interaction requires BBS1 and BBS2.

Equivalent gel-shift assays were conducted using a shorter DNA probe (196 nucleotides) harboring only one BBS (BBS2) (Fig. 4B). This probe behaved exactly as the long probe in the presence of the purified BaaR (Fig. 4B). Control experiments performed with the different unlabeled or mutated DNA probes also confirmed the specificity of this interaction (Fig. 4B).

As discussed above, BaaR contains ~38 additional amino acids at its N terminus. To assess the role of this N-terminal extension in the interaction between BaaR and its target DNA, we performed gel-shift assays using a BaaR mutant protein missing these additional N-terminal amino acids. We observed no significant differences in probe shift between the short (residues Val-40–Pro-284) and WT BaaR proteins; both resulted in shifts of the long (289 nucleotides, contains BBS1 and BBS2) and short (196 nucleotides, contains BBS2) fluorescent DNA probes (Fig. S4).

Palindromic regions between baaR and bab2_0216 are required for recognition by BaaR

To identify the motifs in the BBS region between baaR and bab2_0216 required for interaction with BaaR, each palindrome of BBS2 was independently mutated by randomizing the corresponding sequences. We then measured binding of the corresponding 196-nucleotide fluorescent DNA probe to BaaR by gel-shift assay. Compared with the WT BBS2-containing probe, no gel shift was observed when the first half of the first palindromic motif (region 1) was mutated, suggesting no interaction between the DNA probe and BaaR (Fig. 5, A and B). Mutation of BBS2 region 2, corresponding to overlapping regions between the first and second palindromic motifs, also ablated binding of the probe to BaaR (Fig. 5, A and B). Mutation of the second half of the second palindrome (region 3) resulted in an intermediate effect; a partial gel shift of the corresponding DNA probe was still observed with the highest BaaR concentrations (250 and 500 nM)(Fig. 5, A and B). As expected, mutating regions 1–3 completely ablated a gel shift (Fig. 5B). We conclude that regions 1 and 2, present in the TTTGC/GCGAAA palindrome, are required for interaction with BaaR and that region 3 plays a minor role in the BaaR/DNA interaction. We note that DNA regions upstream of bab2_0277–bab2_0282 and bab2_1036–bab2_1047 do not contain similar palindromic sequences recognized by BaaR. This suggests that the transcriptional regulation of these loci observed in our RNA-seq data are indirect.
BaaR interacts with BBS1 and BBS2 in vivo and regulates transcription of the bab2_0213–bab2_0217 operon

We next evaluated whether BaaR interacts with the baaR–bab2_0216 promoter region in vivo and whether both BBSs are required for transcriptional regulation in B. abortus 2308. We transformed WT and ΔbaaR strains with a plasmid carrying a transcriptional fusion of the intergenic region between bab2_0215 and bab2_0216 to lacZ. This lacZ transcriptional reporter construct (PbaaR–lacZ) contains the baaR promoter region, including both BBS1 and BBS2.

In the WT strain, β-gal activity was low, suggesting constitutive repression of transcription by BaaR. In the ΔbaaR strain, β-gal activity was increased by a factor of 10 relative to WT, suggesting that deletion of baaR derepresses transcription from
Transcription regulation by Brucella BaaR

its own promoter (Fig. 6A, panel 1). Surprisingly, randomizing the sequences of the BBS1 and BBS2 site (BBS1<sup>mut</sup>/BBS2<sup>Wt</sup>) resulted in low β-gal activity in both strains. It is likely that these tandem BBS1 and BBS2 mutations disrupt the ability of RNA polymerase to induce transcription from this promoter (Fig. 6A, panel 1).

Mutation of BBS1 alone (BBS1<sup>mut</sup>/BBS2<sup>Wt</sup>) had little effect on BaaR-dependent transcriptional repression; β-gal activity was low in the WT and high in the ΔbaaR strain (Fig. 6A, panel 1). However, in the WT strain, the measured β-gal activity was higher from the BBS1<sup>mut</sup>/BBS2<sup>Wt</sup> than from the BBS1<sup>Wt</sup>/BBS2<sup>Wt</sup> reporter. This indicates that the integrity of both BBSs is likely required for efficient repression by BaaR, perhaps by permitting multiple BaaR dimers to interact simultaneously. Mutation of BBS2 alone (BBS1<sup>Wt</sup>/BBS2<sup>mut</sup>) resulted in low β-gal activity in both the WT and ΔbaaR strains, further supporting that RNA polymerase cannot efficiently induce transcription from this sequence (Fig. 6A, panel 1). We conclude that the integrity of BBS2 is essential for the proper interaction of transcription factors at this promoter.

When half of the intergenic region present between baaR and bab2<sub>0216</sub> was fused to lacZ, the corresponding reporter gene (1/2P<sub>baaR</sub>−lacZ) behaved similarly to the BBS1<sup>mut</sup>/BBS2<sup>Wt</sup> reporter; β-gal activity was low in the WT and high in the ΔbaaR strain (Fig. 6A, panel 2). Again, transcription from the corresponding reporter with only one BBS (BBS2<sup>Wt</sup>) was not as repressed as that from the reporter containing both BBS sequences (BBS1<sup>Wt</sup>/BBS2<sup>Wt</sup>) (Fig. 6A, panel 2). Mutation of BBS2 (BBS2<sup>mut</sup>) resulted in low β-gal activity in both strains and could be attributed to a possible disruption in transcription (Fig. 6A, panel 2). To overcome this problem, we mutated smaller regions of BBS2 by randomizing the corresponding sequences. When BBS2 region 1 (BBS2<sup>R1</sup>) or 2 (BBS2<sup>R2</sup>) was mutated, the corresponding β-gal activity was high in both the WT and mutant strains (Fig. 6A, panel 2). From these data, we conclude that the integrity of the transcription initiation site is preserved in these constructs. This result also confirmed that BaaR in the WT strain fails to interact with BBS2<sup>R1</sup> or BBS2<sup>R2</sup>. When region 3 (BBS2<sup>R3</sup>) was mutated, intermediate β-gal activity was measured in both strains (Fig. 6A, panel 2), which confirmed our previous in vitro observations (Fig. 5B).

Taken together, these results suggest that BBS2 is required for BaaR-dependent transcriptional regulation of bab2<sub>0216</sub>, bab2<sub>0214</sub>, and bab2<sub>0213</sub>. However, the presence of BBS1 ensures even greater transcriptional repression. WT or ΔbaaR strains carrying the empty vector (pMR15) or the baaR complemented strain were used as controls (Fig. 6A, panel 3).

We next evaluated whether transcription from bab2<sub>0216–baaR</sub> exhibited a similar transcriptional profile to baaR. We constructed a new lacZ transcriptional reporter in which the reversed and complemented intergenic sequence between baaR and bab2<sub>0216</sub> was fused to lacZ (P<sub>baaR−lacZ</sub>). This construct was transformed into the WT or ΔbaaR strains. The β-gal activity in ΔbaaR was 5-fold higher than that in WT, providing evidence that BaaR represses transcription from this reporter as well (Fig. 6B, panel 1). The β-gal activity observed under this reporter was generally lower than that under P<sub>baaR−lacZ</sub>, suggesting weaker repression from this promoter. When BBS1 and BBS2 were mutated simultaneously (BBS1<sup>mut</sup>/BBS2<sup>mut</sup>) by randomizing the corresponding sequences, both the WT and ΔbaaR strains exhibited increased β-gal activity (Fig. 6B, panel 1). However, in the WT strain, these activities were higher and comparable with the β-gal activity levels measured in the ΔbaaR strain carrying the BBS1<sup>Wt</sup>/BBS2<sup>Wt</sup> reporter gene. In both strains, mutation of BBS2 only (BBS1<sup>Wt</sup>/BBS2<sup>mut</sup>) had no effect on β-gal activity, whereas mutation of BBS1 only (BBS1<sup>mut</sup>/BBS2<sup>Wt</sup>) induced greater activity (Fig. 6B, panel 1).

Finally, a reporter containing half of the bab2<sub>0216</sub> promoter region was evaluated (1/2P<sub>bab2_0216−lacZ</sub>) (Fig. 6B, panel 2). Transcriptional activity under this reporter in the WT strain was 5-fold lower than that in the ΔbaaR strain and was comparable with that in the BBS1<sup>Wt</sup>/BBS2<sup>mut</sup> reporter strains (Fig. 6B, panel 2). When BBS1 was mutated, activity increased in both strains and was comparable with that in the BBS1<sup>mut</sup>/BBS2<sup>mut</sup> reporter strains (Fig. 6B, panel 2).

Together, these results provide evidence that BBS1 is required for BaaR-dependent transcriptional regulation of bab2<sub>0216</sub> and bab2<sub>0217</sub>. BBS1 and BBS2 mutations did not disrupt the ability of RNA polymerase to induce transcription from this promoter, suggesting that the bab2<sub>0216−bab2_0217</sub> initiation site does not overlap with BBS1 or BBS2.

-λ-Aminocaproic acid derepresses transcription from a BaaR-regulated promoter

BaaR, like other IclR proteins, has a C-terminal LBD with a cavity accommodating small molecules. Interaction with specific molecules can positively or negatively modify the affinity of the protein for its target DNA (11, 14, 17, 41, 42, 44, 56). We screened for small molecules that affect transcription from a BaaR-regulated reporter plasmid. Specifically, we transformed WT Brucella ovis, a closely related Biosafety Level 2 (BSL2) surrogate for B. abortus, with the P<sub>baaR−lacZ</sub> reporter and inoculated this strain into 96-well plates containing 480 distinct, individual small molecules. A single molecule, -λ-aminoacaproic acid, activated transcription from the P<sub>baaR−lacZ</sub> reporter under this cultivation condition (Fig. 7A). We confirmed this hit in a B. abortus strain carrying the same reporter plasmid (Fig. 7B). -λ-Aminocaproic acid is a six-carbon molecule with a carboxyl and amine group. It is a lysine derivative and analog used in clinical settings to promote blood clotting (57, 58). In this same transcription induction screen, acetate and acetoacetate did not affect transcription (Table S3). We thus concluded that neither acetoacetate nor acetate are ligands that interact with the LBD in the BaaR crystal structure.

Adipic acid, tetradecanedioic acid, and caprolactone also derepress transcription

Given the results of our initial screen, we evaluated other related small molecules for their ability to activate transcription from a BaaR-regulated reporter. Selection of these molecules was based on their metabolic and physiological properties and
chemical and structural similarities shared with 6-aminocaproic acid (Fig. S5). To narrow the number of molecules to be tested, the sequence of BaaR was compared with those of IclR proteins previously described to regulate similar metabolic pathways. BaaR shares 59 and 54% identity with DcaS (ACIAD1684) and DcaR (ACIAD1688), two Acinetobacter IclR proteins potentially involved in the transcriptional regulation of a dca β-oxidation operon (36, 37, 59). BaaR also has high identity (66%) to the Pseudomonas DcaR protein (PA1630) involved in regulation of ε-caprolactam catabolism and β-oxidation (36, 38). In Acinetobacter ADP1 and Pseudomonas aeruginosa, dca operons have been previously described as essential for growth on adipic acid or ε-caprolactam as the sole carbon sources (36, 38). Interestingly, a cis,cis-muconic acid transporter (ACIAD1681) is proximal to the Acinetobacter dca operon (39); this transporter is 67% identical to Bab2_0213 in B. abortus. The ability of adipic acid, ε-caprolactam, and cis,cis-muconic acid to derepress transcription of P\textsubscript{bab2_0213-lacZ} was therefore investigated in B. abortus. Addition of cis,cis-muconic acid did not enhance transcription, but 4 mM adipic acid strongly activated transcription (Fig. 7B and Fig. S5). To evaluate the specificity of this activation, shorter or longer dicarboxylic acids (C3 to C14) were assessed as well. Only tetradecanedioic acid, a C14 dicarboxylic acid, significantly derepressed transcription from our reporter (Fig. 7B and Fig. S5). The trans,trans-muconic acid, a dicarboxylic acid closely related to cis,cis-muconic acid, was also evaluated, but it had no effect on transcription from the reporter (Figs. 7B and Fig. S5). Interestingly, the six-carbon fatty acid caproic acid had no effect on BaaR-dependent repression of P\textsubscript{bab2_0213-lacZ} reporter gene transcription, although its cyclic form, 6-aminocaproic acid (60), significantly derepressed transcription (Fig. 7B and Fig. S5). Conversely, ε-caprolactam, a cyclic ε-aminocaproic acid (61), had no effect on transcription (Fig. 7B and Fig. S5). As a control, we cultivated the reporter strain in the presence of DMSO, which was used to solubilize most of the small molecules evaluated. Transcription from this reporter was
unchanged by addition of the DMSO (Fig. 7B). We also confirmed that the different molecules had no effect on an empty vector control strain (Fig. 7B). All molecules were also assessed in a *B. abortus* strain carrying the P\_baaR\_lacZ reporter plasmid. The \(\beta\)-gal activity was significantly enhanced in the presence of 4 mM \(\beta\)-aminocaproic acid, adipic acid, tetradecanoic acid, and \(\epsilon\)-caprolactone (Fig. 7C).

**Evidence that adipic acid is transported by Bab2_0213, an MFS transporter**

We next evaluated whether the molecules found to induce transcription from a BaaR-dependent reporter (P\_baaR\_lacZ) are specifically transported by the MucK-like transporter Bab2_0213. We also tested whether Bab2_0214 (acyl-CoA dehydrogenase), Bab2_0216 (enoyl-CoA hydratase/isomerase and 3-hydroxyacyl-CoA dehydrogenase), and Bab2_0217 (CoA-transferase family III) affect BaaR-regulated transcription. We generated in-frame deletions of each of these genes in *B. abortus* and transformed the corresponding null mutant strains with the P\_baaR\_lacZ reporter plasmid. Each deletion strain was grown in the presence of increasing concentrations of the different activating molecules, and \(\beta\)-gal activity was measured under each condition.

Transcription reporter activity in all four null mutant strains was the same as that in the WT in the presence of \(\beta\)-aminocaproic acid, tetradecanoic acid, or \(\epsilon\)-caprolactone (Fig. 8A–C). However, the strain harboring the bab2_0213 deletion exhibited significantly lower transcriptional activity than the WT strain and other deletion strains in the presence of adipic acid (Fig. 8D). Complementation of \(\Delta\)bab2_0213, by reintroducing a WT copy of bab2_0213, restored the WT transcriptional phenotype (Fig. 8E). We conclude that, at the concentrations tested, Bab2_0213 is involved in transport of adipic acid. It is not known how \(\beta\)-aminocaproic acid, tetradecanoic acid, or \(\epsilon\)-caprolactone is transported. We further conclude that deletion of bab2_0214, bab2_0216, or bab2_0217 did not affect the response of *B. abortus* to these inducing molecules. This provides evidence that none of these enzymes are required for BaaR-dependent transcriptional regulation by adipic acid, \(\beta\)-aminocaproic acid, tetradecanoic acid, or \(\epsilon\)-caprolactone.

**Adipic acid does not support growth of *B. abortus* in GMM**

A physiological role for adipic acid in *Brucella* cell physiology has not been defined. To test whether adipic acid can support growth of *B. abortus*, we cultivated the WT 2308 strain in Gerhardt’s minimal medium (GMM) lacking one of its potential carbon sources (glycerol, lactate, or glutamate) and supplemented with 1 mM adipic acid (Fig. 8A). We observed substantial growth after 72 h in GMM containing all three molecules (glycerol, lactate, and glutamate) or missing glycerol. No growth was observed in GMM missing lactate or glutamate. Addition of adipic acid did not improve or rescue growth of *B. abortus* in any condition tested, suggesting it cannot be used as a major carbon source under these cultivation conditions.

We postulated that constitutive expression of the bab2_0213–bab2_0217 locus might provide a growth advantage to *B. abortus* in a minimum medium containing adipic acid, so we repeated these same growth experiments with the baaR deletion strain. Again, we observed no growth in GMM lack-
ing lactate or glutamate, with or without 1 mM adipate (Fig. S6B).

**Adipic acid, ε-aminocaproic acid, tetradecanedioic acid, and ε-caprolactone do not interact directly with BaaR**

Given the ability of particular organic acids to derepress transcription from a BaaR-dependent reporter in *vivo*, we next evaluated whether adipic acid, ε-aminocaproic acid, tetradecanedioic acid, or ε-caprolactone modify BaaR binding to its target DNA. We mixed a fluorescent DNA probe corresponding to the BBS2 region with purified BaaR and (separately) 1 mM of each small molecule. We conducted this experiment using a range of BaaR concentrations.

None of the molecules tested had any effect on BaaR binding to the probe (Fig. 9, A–E). However, the possibility that these molecules interact with the BaaR LBD without affecting the interaction of BaaR with DNA could not be ruled out in this assay. Therefore, we also performed isothermal titration calorimetry (ITC) measurements, which allow characterization of the affinities between IclR proteins and small molecules at micromolar levels (41, 56, 62). Specifically, we performed ITC measurements between purified BaaR LBD and adipic acid; 1 μM of 10 mM adipic acid solution was injected into a cell containing 200 μl of a 50 μM protein solution. A total of 20 injections were performed.

**Deleting baaR does not affect B. abortus intracellular entry or replication**

As outlined earlier, the general stress response sigma factor, $\sigma^{E1}$, controls the transcription of dozens of genes and is required for *B. abortus* survival under stress conditions in *vivo* and chronic infection conditions in a mouse disease model (4, 5, 7, 9, 10). In this study, we present structural and functional studies of a transcriptional regulator of the IclR family, BaaR, which is indirectly activated by $\sigma^{E1}$. Our experiments provide molecular-level understanding of *baaR* regulatory function in *B. abortus* but demonstrate that this gene is not a major contributor to oxidative stress survival or mammalian cell colonization.

BaaR strongly represses transcription from two divergently transcribed operons (Figs. 3 and 6) homologous to the *dca* β-oxidation operons of *Acinetobacter* spp. and *Pseudomonas* spp., which have been implicated in growth on adipic acid or ε-caprolactam as a sole carbon source (Fig. 10) (36–38). In *Acinetobacter* and *Pseudomonas*, it has been postulated that IclR transcriptional regulators control *dca* operon expression, although this hypothesis has not been tested experimentally. When compared with other IclR proteins, BaaR is closely related to *Acinetobacter* DcaR (ACIAD1688; 54% identity) and DcaS (ACIAD1684; 59% identity) and *Pseudomonas* DcaR (PA1630; 66% identity) (Fig. 10). All four of these proteins possess an N-terminal extension compared with other IclR proteins, and very similar LBD cavities (Figs. 2C and Fig. S1), suggesting they recognize structurally related small molecules. The function of the extended N termini in these related proteins is
Transcription regulation by Brucella BaaR

**Figure 10. Genomic context and protein sequence identity of the β-oxidation operons in Acinetobacter, Pseudomonas, and B. abortus.** Genes annotated as IclR proteins are in brown; 3-hydroxyacyl-CoA dehydrogenases are in red; CoA transferase family III is in orange; MucK-like transporters are in yellow; acyl-CoA dehydrogenases are in green; and enoyl-CoA hydratases are in blue. Other genes present in the Acinetobacter dca operon that are not regulated by BaaR in B. abortus are in gray. Genes not related to β-oxidation and present in a different locus are shown in white. Gene annotation numbers are presented above each gene. Below each gene, the percentage of identity between the corresponding protein and the equivalent Bab2_0213-Bab2_0217 protein is presented.

unclear, and deletion of the N terminus of BaaR did not affect DNA binding in vitro (Fig. S4). It is possible that in a cellular context, this structural region affects protein/DNA interactions or protein stability in the presence of specific molecular signals.

In vivo, BaaR represses transcription of the bab2_0213–bab2_0217 locus (including its own gene, bab2_0215). Adipic acid and other related organics relieve this repression, resulting in initiation of bab2_0213–bab2_0217 transcription. It remains unclear how transcription of this operon is maintained despite increasing concentrations of BaaR in the cell. It is conceivable that like the TtgV protein of *Pseudomonas putida* (64, 65) and other proteins belonging to the IclR family (14, 15, 66, 67), reduced affinity of BaaR for DNA upon ligand binding is sufficient to overcome the effects of increased concentrations in the cell.

When compared with the *Acinetobacter* dca locus, the *B. abortus* bab2_0213–bab2_0217 dca-like operon (regulated by BaaR) appears incomplete. Indeed, genes essential for the β-oxidation of dicarboxylic acids in *Acinetobacter* are degenerate or absent in *B. abortus* (Fig. 10). It is therefore difficult to conclude that bab2_0213–bab2_0217 is truly involved in β-oxidation metabolism. The RNA-seq analysis of WT *B. abortus* strain also revealed a predicted ABC transport system (bab2_0278–bab2_0282) that was indirectly activated by BaaR strain also revealed a predicted ABC transport system (bab2_0278–bab2_0282) that was indirectly activated by BaaR (Fig. 3). The primary structure of the PBP in this system (Bab2_0282) is 54% identical and 72% homologous to a *Burkholderia mallei* PBP that co-crystallized with bound acetocetate (PDB code 3I09). Residues involved in this interaction with acetocetate are also present in Bab2_0282 (Fig. 53), suggesting that Bab2_0282 may transport acetocetate or a closely related molecule. In mammals, acetyl-CoA formed in the liver during fatty acid β-oxidation can be converted into acetocetate and released into the bloodstream as an energy source during periods of starvation or intense physical activity (54, 55). We have no evidence that exogenous acetocetate is actively transported by *B. abortus*, although a metabolic connection between this ABC transporter and the bab2_0213–bab2_0217 locus might
exist. As discussed earlier, the bab2_0213–bab2_0217 locus is missing a thiolase enzyme required to perform the last step of a $\beta$-oxidation reaction that leads to the production of an acetyl-CoA and an acyl-CoA molecule (Fig. S8) (52). None of the five putative thiolases (bab1_0486, bab1_1783, bab2_0443, bab2_0606, and bab2_0790) present in the B. abortus genome have altered expression in the $\Delta$baaR background (Table S2), suggesting that these genes are not involved in performing an ultimate thiolysis step. Instead, as a last step, we propose that the CoA-transferase family III enzyme (Bab2_0217) present in the bab2_0213–bab2_0217 locus may catalyze a reversible transfer of CoA from CoA-thioesters to free acids (Fig. S8) (53, 68), leading to the formation of ketones. Such a reaction has been previously described in an engineered E. coli strain producing isopropanol (69) and in acetone production in Clostridium acetobutylicum (70). In these strains, a CoA-transferase was involved in the conversion of acetoacetyl-CoA into acetone, using butyrate or acetate as CoA acceptors (69, 70).

Such a reaction in B. abortus may explain why the bab2_0278—bab2_0282 ABC transport system is down-regulated when the bab2_0213–bab2_0217 locus is overexpressed. Cytoplasmic accumulation of ketones, such as acetoacetate, may repress expression of the bab2_0278—bab2_0282, which would be no longer needed for ketone transport.

It remains unclear what role adipic acid would play in this hypothetical metabolic process. As shown in Fig. 9, adipic acid does not directly interact with BaaR, suggesting that a degradation product is instead the real activating signal. However, deletion of the bab2_0213–bab2_0217 enzymes did not affect the ability of adipic acid to induce the transcription of our reporter gene (Fig. 8). Assuming that these enzymes are indeed functional, our data provide evidence that Bab2_0214, Bab2_0216, and Bab2_0217 are not involved in its synthesis. In a defined medium, adipic acid does not support growth of WT or $\Delta$baaR strains (Fig. S6). In a previous study (36), it was shown that Acinetobacter can use adipic acid as a sole carbon source and that this required the presence of the acyl-CoA dehydrogenase DcaA, the enoyl-CoA hydratase DcaE, and the 3-hydroxyacyl-CoA dehydrogenase DcaH (which respectively share 78, 55, and 52% identity with Bab2_0214 and Bab2_0216 (Fig. 10)). The presence of a thiolase (DcaF) in this dca operon might explain the differences in growth between B. abortus and Acinetobacter in presence of adipic acid (Fig. 10) (36).

In conclusion, we have demonstrated that adipic acid and the related molecules $\epsilon$-aminocaproic acid, tetradecanedioic acid, and $\epsilon$-caprolactone activate transcription of the bab2_0213–bab2_0217 locus in B. abortus, although these molecules do not activate transcription through a direct interaction with BaaR. This suggests that the actual activating molecule in vivo is either a metabolic product of these compounds or that these inducing compounds activate some undefined metabolic pathway involved in the synthesis of the BaaR-activating signal. The possible relevance of such molecules in the life cycle of B. abortus and the functional significance of baaR transcriptional activation by $\sigma^{E1}$ remain undefined. Adipic acid is produced by oxidation of fatty acids but is not naturally abundant. We note that very little is known about the B. abortus life cycle outside the host. In the wild, B. abortus can persist for weeks in aborted fetuses, a major source of contagion (71). However, B. abortus can also persist for weeks in soil or on vegetation. How B. abortus survives in these harsh and competitive environments is unknown, and it is possible that the genes investigated in this study enable B. abortus to metabolize unusual substrates found outside the mammalian host.

To conclude, we propose a model (Fig. 11) whereby dimeric BaaR constitutively interacts with the DNA region between bab2_0213 and bab2_0216, repressing divergent transcription on both strands. When present in the environment, adipic acid is likely transported by the MFS MucK transporter Bab2_0213, resulting in derepression of transcription from the BaaR-inhibited promoters. The uptake of structurally related molecules, including $\epsilon$-aminocaproic acid, $\epsilon$-caprolactone, and tetradecanedioic acid, also induces transcription, although our data provide evidence that these molecules are transported by a genetically distinct system. Once in the cytoplasm, these molecules are predicted to interact with an unknown metabolic/regulatory process that leads to production of an intracellular ligand that binds to and regulates BaaR. Interaction with a ligand likely induces structural changes in BaaR that result in dissociation from DNA. The subsequent derepression of the bab2_0213–bab2_0217 locus may increase adipic acid uptake, creating a positive feedback loop. Derepressed expression of the bab2_0213–bab2_0217 locus also indirectly enhances expres-
Transcription regulation by Brucella BaaR

sion of a paralogous gene set, bab2_1036–bab2_1047, while attenuating expression of a potential ketone ABC transport system (bab2_0278–bab2_282).

Materials and methods

All experiments using live B. abortus 2308 were performed in Biosafety Level 3 facilities according to United States Centers for Disease Control (CDC) select agent regulations at the University of Chicago Howard Taylor Ricketts Laboratory.

Chromosomal deletions in B. abortus

The different B. abortus deletion strains (Δbab2_0213, Δbab2_0214, ΔbaaR (i.e. bab2_0215), Δbab2_0216, and Δbab2_0217) were constructed using a double-recombination strategy. Briefly, after PCR amplification using KOD Xtreme Hot start DNA polymerase (EMD Millipore) with B. abortus chromosomal DNA as a template, the corresponding PCR products were purified using the GeneJET PCR purification kit (Thermo Fisher Scientific). The in-frame deletion alleles (carrying 5′ and 3′-flanking sequences of B. abortus locus tags) were digested with restriction enzymes (New England Biolabs) before ligation using T4 DNA ligase (New England Biolabs) or digested with restriction enzymes (New England Biolabs) to remove any co-purified genomic DNA, 10 μl of DNase (RNase) free water. The reaction was incubated at maximum speed for 30 min at 4 °C, and the pellets were washed twice with 70% ethanol, air-dried, and resuspended in 80 μl of RNase-free water. To remove any co-purified genomic DNA, 10 μl of 10× buffer and 10 μl of Turbo DNase (Thermo Fisher Scientific) were added to each sample for 2 h at 37 °C. The samples were then purified using purification columns (from the RNeasy MinElute Cleanup kit; Qiagen) and eluted with 30 μl of DNase (RNase)-free water. The eluate was further digested on the column by adding 7 μl of Turbo DNase, 7 μl of 10× buffer, and 56 μl of RNase-free water. The reaction was incubated at room temperature for 30 min and purified using a Qiagen RNA purification kit according to the manufacturer’s protocol.

For RNA-seq analysis, RNA was depleted from WT and ΔbaaR samples using Ribo-Zero rRNA Removal (Gram-negative bacteria) kit (Epizentech). Libraries were prepared with Illumina TruSeq RNA kit according to manufacturer’s instructions and were then quantified using a 2100 Bioanalyzer (Agilent) and sequenced on a HiSeq2500 (Illumina). The obtained RNA-seq reads were aligned to the genome sequence of B. abortus 2308 (RefSeq AM040265) using the readmapper tool in CLC Genomics Workbench (Qiagen) (mismatch cost = 2; insertion cost = 3; deletion cost = 3; length fraction = 0.8; and similarity fraction = 0.8). Differential expression analysis of normalized data and false-discovery rate (FDR) p values were calculated in CLC Genomic Workbench. RNA-seq data sets are available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) accession number GSE107825.

lacZ transcriptional reporter construction

The different lacZ reporter genes used in this study were built as follows: the WT DNA fragments corresponding to the different portions of the bab2_0215 (baaR–bab2_0216 intergenic region were PCR-amplified using B. abortus chromosomal DNA as a template. A gBlocks-synthesized DNA fragment (Integrated DNA Technology, IDT) corresponding to the baaR–bab2_0216 intergenic region carrying mutated BBSs was used as a PCR template to introduce mutations in BBS1 and BBS2. To mutate BBS1 and BBS2, the corresponding palindromic sequences were randomized to maintain the length and base content equivalent to the native sequences. DNA frag-

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4 M. R. K. Alley, unpublished data.
ments corresponding to baaR and bab2_0216 promoter regions carrying a WT and a mutated BBS were generated by overlapping PCRs. Mutation of BBS2 region 1, region 2, or region 3 were introduced using overlapping primers carrying specific mutations. Primer sequences and information are available in Table S4. As described previously, PCRs were performed using with KOD Xtreme Hot start DNA polymerase (EMD Millipore), gel-purified with GeneJET PCR purification kit (Thermo Fisher Scientific), and digested with restriction enzymes (New England Biolabs). Ligation with the linearized pMR15 plasmid was performed using T4 DNA ligase (New England Biolabs). After ligation and transformation into an E. coli Top10 strain, single colonies carrying plasmids with the different inserts were PCR-screened and sent for sequencing. The plasmids were then purified with the GeneJET plasmid miniprep kit (Thermo Fisher Scientific) and transformed in WT B. ovis or in the different B. abortus genetic backgrounds (WT, Δbab2_0213, Δbab2_0214, ΔbaaR, Δbab2_0216, and Δbab2_0217) used in this study. Transformants were then selected on SBA plates supplemented with kanamycin (50 μg/ml). Primer, restriction enzyme, plasmid, and strain information are available in Tables S4 and S5.

Screening for ligands that derepress BaaR in vivo

A WT B. ovis strain carrying the pMR15-P\textsubscript{baaR}\textsuperscript{lacZ} reporter plasmid (see Table S5 for strain information) was harvested from a fresh SBA plate supplemented with kanamycin (50 μg/ml) and resuspended at OD\textsubscript{600} = 0.1 in 50 ml of BB containing 50 μg/ml of kanamycin. 100 μl of this B. ovis suspension was then used to inoculate each well of the phenotype MicroArray plates (Biolog). Biolog plates PM1, PM2A, PM3B, PM4A, and PM5 were used for this assay. After growth overnight at 37 °C in the presence of 5% CO\textsubscript{2}, the plates were removed from the incubator, and the OD\textsubscript{600} and β-gal activities were measured under each growth condition using the Tecan Spark 20 M plate reader.

Additional small molecule screening in Brucella

Depending on their solubility characteristics, all ligands used in this study were freshly prepared in DMSO or sterile water. When needed, the pH was adjusted to 7.4. Caproic acid, α-caprolactam, α-caprolactone, ε-aminocaproic acid, glutaric acid, adipic acid, pimelic acid, suberic acid, sebacic acid, and tetradecanedioic acid were purchased from Sigma. The cis,cis- and trans,trans-muconic acids were purchased from Acros-Organics. Succinic acid was purchased from Thermo Fisher Scientific. Malonic acid was purchased from MP-Biomedicals.

For β-gal transcriptional activity measurements, B. abortus liquid cultures were prepared as follows: B. abortus strains carrying the different lacZ transcriptional fusions were harvested from fresh SBA plates supplemented with kanamycin (50 μg/ml) (strain information is available in Table S5). Cells were resuspended in 1 ml of BB, and the corresponding OD\textsubscript{600} was measured by spectrophotometry (Thermo Fisher Scientific Genesys 20). These B. abortus suspensions were used to inoculate (at OD\textsubscript{600} = 0.1) culture tubes containing 2 ml of BB supplemented with kanamycin (50 μg/ml). These culture tubes also contained different concentrations of small molecules (from 50 μM to 4 mM). After overnight growth at 37 °C and 220 rpm shaking, β-gal activity was measured. Each condition was independently tested at least three times using different clones for each time.

Measurement of β-gal activity

In this study, all β-gal activity measurements were performed in 96-well plates, and the absorbance was measured using the Tecan Spark 20 M or Infinite 200 PRO plate reader.

To assess regulation of reporter gene transcription by BaaR, the different B. abortus strains were grown on SBA plates supplemented with 50 μg/ml kanamycin. After incubation for 2–3 days at 37 °C and 5% CO\textsubscript{2}, the cells were harvested and resuspended in 1 ml of BB; 200 μl of each culture tube were transferred to a clear Corning flat-bottom 96-well plate, and the OD\textsubscript{600} was measured using the Tecan plate reader. The β-gal activities of four different clones for each strain were independently measured at least twice. A representative data set is presented in Fig. 6.

For ligand screening on 96-well plates, Phenotype MicroArray (Biolog) plates were prepared as described earlier, and the OD\textsubscript{600} was measured using the Tecan plate reader. This initial screen was conducted once.

For ligand screening in culture tubes, 200 μl each culture tube were transferred to a clear flat-bottom 96-well plate, and the OD\textsubscript{600} was measured using the Tecan plate reader. In Figs. 7 and 8, each condition was independently tested at least three times using different clones for each time.

For β-gal activity measurements, between 2.5 and 10 μl of cell suspension were mixed and lysed with 25 μl of chloroform in a 96-well chloroform-resistant plate, and 125 μl of Z-buffer (60 mM Na\textsubscript{2}HPO\textsubscript{4}, 40 mM NaH\textsubscript{2}PO\textsubscript{4}, 10 mM KCL, adjusted to pH 7) were then added to each well. After addition of 42 μl of a 4 mg/ml O-nitrophenyl-β-D-galactopyranoside solution to each well, the reaction was developed at room temperature and stopped by adding 83 μl of a 1 M sodium carbonate solution. For each reaction, the incubation time was recorded; 200 μl of each reaction were then transferred to a clear flat-bottom 96-well plate, and the OD\textsubscript{420} was measured using the Tecan plate reader. Calculation of β-gal activity was performed using Equation 1,

\[
\frac{(A_{420} - \text{blankA}) \times 1000 \times 0.25}{(A_{600} - \text{blankB}) \times t \times \nu} = \text{Units of β-gal activity (Eq. 1)}
\]

where \(A_{420}\) is the absorbance measured at 420 nm of the β-gal reaction; blankA is a blank reaction containing no cells; 0.25 is the total volume of the reaction (in milliliters); \(A_{600}\) is the absorbance measured at 600 nm of the cell suspension; blankB is a blank containing Brucella broth alone; \(t\) is the incubation time (min); and \(\nu\) is the volume of cells used in the reaction (in milliliters).

Growth assays in Gerhardt’s minimal medium (GMM) in presence or absence of adipic acid

B. abortus WT and ΔbaaR strains were grown in GMM, pH 6.8 (NaCl 7.5 g/liter, K\textsubscript{2}HPO\textsubscript{4} 10 g/liter, sodium thiosulfate 0.1 g/liter, glycerol 30 g/liter, lactate 5 g/liter, L-glutamic acid 1.5
Transcription regulation by Brucella BaaR

g/liter, MgCl₂, hexahydrate 0.83 g/liter, FeSO₄ heptahydrate 5 mg/liter, MnCl₂ tetrahydrate 4 mg/liter, calcium pantothenate 0.4 mg/liter, thiamine HCl 2 mg/liter, nicotinic acid 2 mg/liter, biotin 0.001 mg/liter) (72). Four different GMM solutions were tested: with all three carbon sources present (glycerol, lactate, and glutamate), without glycerol, without lactate, and without glutamate. The same conditions were also tested but in presence of 1 mM adipic acid. In plastic culture tubes, 2 ml of each GMM solution were inoculated with the WT and ΔbaaR strains at a starting OD₆₀₀ of 0.05. Cultures were grown for 3 days at 37 °C/220 rpm, and optical densities of the cultures were assessed every 24 h by transferring 200 μl of each culture in 96-well plate and using a Tecan plate reader for measurements. Growth curves were performed in triplicate with two different strains.

Oxidative stress assay

B. abortus WT, ΔbaaR, and ΔroPE1 strains (strain information is available in Table S5) grown on SBA plates for 48 h were harvested and resuspended in GMM, pH 6.8 (72). Each sample was then adjusted to a final cell density of 1 × 10⁶ CFU/ml in 2 ml of GMM, pH 6.8. The test group was subjected to oxidative stress by the addition of 5 mM H₂O₂ (final concentration); the control group was mock-treated with sterile water. After 1 h of incubation in a shaking incubator at 37 °C, the cultures were serially diluted in PBS and plated on Tryptic Soy Agar (TSA) plates for viable CFU counting. Three independent cultures per condition were prepared for each strain.

Cell culture and macrophage infection assay

Human monocytic THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine. Differentiation of the cells into macrophages was induced by the addition of 40 ng/ml phorbol 12-myristate 13-acetate (Sigma) for 48 h at 37 °C in a 5% CO₂ atmosphere. Prior to infection, bacteria were harvested from freshly plated SBA plates and resuspended in sterile RPMI 1640 medium, and cell densities were adjusted. For infection assays, 5 × 10⁶ THP-1 cells were infected with 5 × 10⁶ cells of the WT or ΔbaaR B. abortus strains to achieve a multiplicity of infection of 1:100 in 96-well plates. To synchronize the infections after the addition of the Brucella cells, the plates were centrifuged at 200 × g for 5 min. After 1 h of incubation at 37 °C in a 5% CO₂ atmosphere, the medium was replaced with the RPMI 1640 medium supplemented with gentamicin (50 μg/ml) and incubated for 20 min at 37 °C in a 5% CO₂ atmosphere to kill extracellular bacteria. To determine the numbers of intracellular bacteria at 1, 24, and 48 h post-infection, the cells were washed once with PBS and lysed with 0.1 ml of PBS complemented with 0.1% Triton X-100. The lysate was serially diluted and plated on tryptic soy agar plates for CFU counting. This infection experiment was performed in triplicate using two different clones of each strain.

Protein expression plasmids

DNA fragments corresponding to the full-length BaaR (residues 1–284) protein, the BaaR protein deleted for the first 8 residues (9–283), 20 (21–284), or 39 (40–284) N-terminal amino acids, and the BaaR LBD (115–284) were amplified by PCR using KOD Xtreme Hot start DNA polymerase (EMD Millipore). The primers used for cloning are listed in Table S4. After purification using the GeneJET PCR purification kit (Thermo Fisher Scientific), the PCR products were directly Gibson assembled (New England Biolabs) or digested with restriction enzymes (New England Biolabs) and ligated using T4 DNA ligase (New England Biolabs) into a linearized pET28a plasmid. Fragments corresponding to BaaR (residues 9–283) and BaaR (residues 21–284) were cloned, respectively, into pMCSG81 and pMCSG73 plasmids using a ligation-independent procedure (73, 74). E. coli Top10 strains were then transformed with the different plasmids by electroporation, and transformants were selected on Luria broth (LB, Thermo Fisher Scientific) agar plates supplemented with 50 μg/ml kanamycin. After PCR screening and sequencing, the corresponding plasmids were purified using the GeneJET plasmid miniprep kit (Thermo Fisher Scientific) and transformed into E. coli BL21-Gold(DE3) or Rosetta(DE3)(pLysS) strains (Stratagene) for protein expression (see Table S5 for strain information).

Protein expression and purification

An overnight LB (Thermo Fisher Scientific) pre-culture (100 ml) was used to inoculate 1 liter of LB supplemented with the appropriate antibiotics. Overexpression of the different His-tagged BaaR proteins was induced at an OD₆₀₀ of ~0.8 (37 °C, 220 rpm) by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (GoldBio). After 5 h of induction, cells were harvested by centrifugation at 11,000 × g for 20 min at 4 °C. The cell pellets were resuspended in 20 ml of a buffer containing 10 mM Tris-Cl and supplemented with 50 μl of a DNase I solution at 5 mg/ml and one-half tablet of complete protease inhibitor mixture (Roche Applied Science). The cells were then disrupted by one passage in a microfluidizer (Microfluidics LVI). The resulting cell lysate was clarified by centrifugation at 39,000 × g for 20 min at 4 °C. Purification of the His-tagged proteins was performed by nickel affinity chromatography (nitritriacetic acid resin; GE Healthcare). After binding of the clarified lysate to the column, three washing steps were performed using 10, 30, and 75 mM imidazole Tris-NaCl buffers, followed by elution with 200 and 500 mM imidazole Tris-NaCl buffers. All purification steps were carried out at 4 °C. Protein purity was assessed by running the eluate on a 14% SDS-polyacrylamide gel and staining the gel with Coomassie Blue. Proteins were then dialyzed overnight at 4 °C against 2 liters of Tris-NaCl buffer (10 mM Tris, pH 8.8, 150 mM NaCl, 1.5 mM EDTA). The protein concentrations were estimated using a colorimetric Bradford protein assay method kit (Thermo Fisher Scientific). Protein samples were concentrated using a centrifugal filter (~kDa molecular mass cutoff, Amicon-Millipore). If necessary, protein aliquots were flash-frozen in liquid nitrogen after addition of glycerol to a final concentration of 50%.

Electrophoretic mobility shift assay (EMSA)

For gel-shift assays, fluorescent DNA probes corresponding to different portions of the WT bab2_0215–bab2_0216 inter-
genic region were PCR-amplified using *B. abortus* chromosomal DNA as a template. Upstream primers positioned before BBS1 or BBS2 and a downstream fluorescent primer positioned at the beginning of the *baaR* gene were used to generate the “long” (375 or 289 nucleotides) and “short” (196 nucleotides) fluorescent DNA probes. The fluorescent primer (Integrated DNA Technology, IDT) was labeled with the Alexa Fluor 488 dye with an excitation wavelength of 492 nm and an emission wavelength of 517 nm. Using the same set of primers, a gBlocks DNA fragment (Integrated DNA Technology, IDT) corresponding to the bab2_0215–bab2_0216 intergenic region carrying mutated BBSs was also used as a PCR template to introduce mutations in BBS1 and BBS2. To maintain the length and base content of the mutated regions, the nucleotide sequences of regions 1–3 of BBS1 and the nucleotide sequences of regions 1–3 of BBS2 were randomized. The resulting PCR products corresponded to the long (375 nucleotides) or short (196 nucleotides) fluorescent DNA probes. Mutations in BBS2 regions 1–3 were introduced using three different sets of overlapping primers containing BBS2 regions 1–3 mutations, respectively. The different fluorescent DNA probes were also used as templates to generate nonfluorescent DNA probes (short/long/WT/mutated) using a nonfluorescent downstream primer during PCR amplification. Primer sequences and information regarding the gBlocks gene fragment are available in Table S4.

The PCR products were run on a 1% agarose gel and gel-purified using the GeneJET PCR purification kit (Thermo Fisher Scientific). DNA concentrations were measured using the Nanodrop One (Thermo Fisher Scientific).

All EMSAs were performed using a BaaR protein corresponding to residues 9–283. Two additional constructs (a full-length (residues 1–284) and a shorter (residues 40–284) BaaR protein) were also used to evaluate the importance of the BaaR N-terminal region. All gel-shift assays were performed using previously published protocols (75, 76). BaaR protein was purified via Ni2+-affinity chromatography purification using a Bio-Rad Chemidoc MP imaging system with a 3-min exposure and the manufacturer’s parameters for Alexa Fluor 488 detection. Each specific condition was tested at least twice.

### ITC ligand–binding assay

All samples (proteins and ligands) were degassed for 10 min prior to ITC measurements, and final dilutions were made using dialysis buffer (10 mM Tris, pH 8.8, 150 mM NaCl, and 1.5 mM EDTA). The ligands were injected into a 200-μl sample cell containing 50 μM purified BaaR LBD (residues 115–284) (protein expression strain information is available in Table S5). A 1 mM adipic acid solution was prepared using the dialysis buffer and adjusted to pH 8.8. This same solution was then diluted to 10 mM using the same dialysis buffer. The ligand solution (1 μl) was injected into the cell every 2 min, with 20 injections total performed. Measurements were performed twice at 25 °C using an iTC200 micro-calorimeter (MicroCal, GE Healthcare).

### Protein expression and purification for crystallization

For crystallization, the *E. coli* strain carrying the pMCSG73 vector was used to overexpress BaaR (residues 21–284) (see strain information listed in Table S5). The pMCSG73 is a bacterial expression vector harboring a tobacco vein mottling virus- cleavable N-terminal NusA tag and a TEV-cleavable N-terminal His6 and StrepII tag (74). A 2-liter culture of enriched M9 medium was grown at 37 °C with shaking at 190 rpm. At OD600 ~1, the culture was cooled to 4 °C and supplemented with 90 mg of l-seleno-l-methionine (Se-Met, Sigma) and 25 mg of each methionine biosynthetic inhibitory amino acid (l-valine, l-isoleucine, l-leucine, l-lysine, l-threonine, and l-phenylalanine). Protein expression was induced overnight at 18 °C using 0.5 mM isopropyl β-D-1-thiogalactopyranoside. After centrifugation, cell pellets were resuspended in 35 ml of lysis buffer (500 mM NaCl, 5% (v/v) glycerol, 50 mM HEPES, pH 8.0, 20 mM imidazole, and 10 mM β-mercaptoethanol) per liter of culture and treated with lysozyme (1 mg/ml) and 3 ml of *E. coli* cells expressing the tobacco vein mottling virus protease. The cell suspension was sonicated, and debris was removed by centrifugation. The Se-Met protein was purified via Ni2+-affinity chromatography using the AKT Express system (GE Healthcare). The column was washed with 20 mM imidazole (lysis buffer) and eluted in the same buffer containing 250 mM imidazole. Immediately after purification, the His tag was cleaved at 4 °C for 24–48 h using a recombinant His-tagged TEV protease, resulting in an untagged protein with an N-terminal Ser-Asn-Ala peptide. A second Ni2+-affinity chromatography purification was performed to remove the protease, noncleaved protein, and affinity tag. The purified protein was then dialyzed against 20 mM HEPES, pH 8.0, 250 mM NaCl, and 2 mM DTT buffer. Protein concentrations were determined by UV absorption spectroscopy (280 nm) using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The purified Se-Met BaaR protein was concentrated to 44 mg/ml using a centrifugal filter (10-kDa MWCO, Amicon-Millipore).

### Crystallization

Initial crystallization screening was carried out using the sitting-drop, vapor-diffusion technique in 96-well CrystalQuick plates (Greiner Bio-one). Trays were prepared using a Mosquito robot (TTP LabTech) and commercial crystallization kits (MCSG-1–4, Anatrace). The drops were prepared by mixing equal volumes (0.4 μl) of the purified protein (44 mg/ml), and
the crystallization solution was equilibrated against 135 μl of the same crystallization solution. After 1 week at 16 °C, we obtained monoclinic crystals in condition no. 95 of the MScG-4 screen corresponding to 200 mm calcium acetate, 100 mm HEPES, pH 7.5, and 10% (w/v) PEG 8000. Prior to flash-freezing in liquid nitrogen, crystals were washed for a few seconds in the crystallization solution containing up to 12% ethylene glycol for cryoprotection.

Crystallographic data collection and data processing

Se-Met crystal diffraction was measured at a temperature of 100 K using a 2-s exposure/degree of oscillation. Crystals diffracted to a resolution of 1.95 Å and the corresponding diffraction images were collected on the ADSC Q315r detector with an X-ray wavelength near the selenium edge of 12.66 keV (0.97927 Å) for SAD phasing at the 19-ID beamline (SBC, Advanced Photon Source, Argonne, IL). Diffraction data were processed using the HKL3000 suite (77). The initial model was manually adjusted using COOT (78) and iteratively refined using COOT, PHENIX (79), and/or REFMAC (80); 5% of the total reflections was kept out of the refinement in both REFMAC and PHENIX. The final structure converged to an Rwork of 17.7% and Rfree of 21.2% and includes four protein chains (A, residues 21–284; B, 21–283; C, 21–284; and D, 20–284) forming two dimers, one ethylene glycol molecule, seven acetate molecules, one calcium ion, and 315 ordered water molecules. The BaaR protein contained three N-terminal residues (Ser-Asn-Ala) that remain from the cleaved tag and were not visible in the structure. The stereochemistry of the structure was checked using PROCHECK (81), and the Ramachandran plot and was validated using the PDB validation server. Coordinates of BaaR have been deposited in the PDB (PDB code 5WHM). Crystallographic data and refined model statistics are presented in Table S1. Diffraction images have been uploaded to the SBGrid data server (Data DOI: 10.15785/SBGRID/491).

Palindromic motif search, protein sequence alignment, and structural homology

Palindromic motifs were identified using the Palindromic Finder server (http://www.biophp.org/minitools/find_palindromes/demo.php) and the Geneious software.

Amino acid sequences were aligned using the M-COFFEE Multiple Sequence Alignment Server (82) and shaded using BoxShade. Figures of the structures, structural alignments, and r.m.s.d. calculations were performed using PyMOL (PyMOL Molecular Graphics System, version 1.7.4; Schrödinger, LLC). The XtalPred server (83) and Dali server (84) were used to identify proteins with the highest structural and sequence homologies. A structural model of Bab2_0282 based on the B. mallei PBP structure (PDB code 3I09) was generated using the ExPASy SWISS-MODEL server (85).

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