Combinatorial control of adhesion of *Brucella abortus* 2308 to host cells by transcriptional rewiring of the trimeric autotransporter *btaE*

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Summary

Regulatory network plasticity is a key attribute underlying changes in bacterial gene expression and a source of phenotypic diversity to interact with the surrounding environment. Here, we sought to study the transcriptional circuit of HutC, a regulator of both metabolic and virulence genes of the facultative intracellular pathogen *Brucella*. Using *in silico* and biochemical approaches, we identified a novel functional HutC-binding site upstream of *btaE*, a trimeric-autotransporter adhesin involved in the attachment of *Brucella* to host extracellular matrix components. Moreover, we identified two additional regulators, one of which, MdrA, acts in concert with HutC to exert a combinatorial control of both *btaE* promoter activity and attachment of *Brucella* to HeLa cells. Analysis of *btaE* promoter sequences of different species indicated that this HutC-binding site was generated *de novo* by a single point mutation in a virulent *Brucella* strain, indicative of a transcriptional rewiring event. In addition to major domain organization differences existing between BtaE proteins within the genus *Brucella*, our analyses revealed that sequences upstream of *btaE* display high variability probably associated to intrinsic promoter structural features, which may serve as a substrate for reciprocal selection during co-evolution between this pathogen and its mammalian host.

Introduction

Brucella is the etiological agent of brucellosis, a zoonotic disease that affects a wide range of mammals including humans (Godfroid *et al.*, 2011). This genus of Gram-negative coccobacilli is classified within the α-2 subgroup of class Proteobacteria and comprises several species, each of which displays a characteristic animal host preference. Among the zoonotic species more commonly associated with human health and animal industry, *Brucella abortus*, *Brucella suis* and *Brucella melitensis* exhibit preference for cattle, swine, and goat, respectively (Whatmore, 2009). Although significant progress has been made in both identifying virulence determinants and genome sequencing of different species of this genus, the molecular bases of host specificity of *Brucella* remain to be elucidated.

In the animal host, brucellosis is transmitted sexually or through oral contact with aborted fetal or placental tissues. This disease severely affects reproduction of the infected animals by causing orchitis in males and abortion in pregnant females, being a major cause of important economic losses in livestock production. On the other hand, human brucellosis is a serious debilitating disease transmitted by the ingestion of unpasteurized dairy products or by contact of wounds or mucosa with fluids from infected animals (Atluri *et al.*, 2011). Once *Brucella* enters the host, it spreads systemically and invades a variety of cell types including both professional and non-professional phagocytes. In the absence of antibiotic treatment at early stages of the infection, *Brucella* eventually colonizes different tissues and organs, which in humans results in a chronic disease characterized by clinical manifestations such as arthritis, endocarditis, meningitis, and even death (Corbel, 1997).

Pathogenicity of *Brucella* depends on its ability to survive and replicate within macrophages of the host. This is achieved by a series of mechanisms that allow this bacterium to attach, internalize, and express different virulence factors that contribute to prevent lysosomal-mediated degradation and promote the biogenesis of an endoplasmic reticulum (ER)-like organelle, where *Brucella*

multiplies exponentially (Pizarro-Cerdá, Moreno, *et al.*, 1998; Pizarro-Cerdá, Méresse, *et al.*, 1998). Previous work on gene regulation of elements contributing to pathogenesis of *Brucella* identified a regulatory circuit that modulates expression of both a metabolic operon and a virulence determinant essential for intracellular replication (Sieira *et al.*, 2010). This transcriptional network is controlled by HutC, a GntR-type transcription factor known as the repressor of the histidine utilization (*hut*) genes. Hut systems are widely distributed metabolic pathways consisting of enzymes that convert histidine to glutamate, which confers the ability to use this amino acid as a sole carbon source (Smith and Magasanik, 1971). In addition to its previously known function as *hut* repressor, it was observed that HutC also acts as a co-activator contributing to modulation of expression of the *Brucella virB* operon (Sieira *et al.*, 2010). These genes code for a Type-IV Secretion System essential for *Brucella* to interact with ER-derived membranes and to promote biogenesis of the intracellular replication niche (O'Callaghan *et al.*, 1999). Based on our previous findings, we hypothesized that, in addition to the *hut* and *virB* promoters, HutC may also control expression of additional targets in the genome of *Brucella*.

Here, by using *in silico* and biochemical approaches, we identified additional HutC-target DNA-sequences in *B. abortus* 2308. Gene expression analyses showed that one of the newly identified motifs is a functional binding site for HutC in the promoter of *btaE*, a gene encoding an adhesin involved in the attachment of *Brucella* to the host cell surface (Ruiz-Ranwez *et al*., 2013a). In addition to HutC, we show that two other transcriptional regulators interact with the *btaE* promoter, one of which, MdrA, acts in concert with HutC to enhance transcription of the adhesin. Comparison of *btaE* promoter sequences among different *Brucella* species revealed that the novel HutC-binding site was generated *de novo* recently in the evolution of the genus, indicating that transcriptional rewiring contributed to fine-tuning of *btaE* promoter activity and modulation of attachment of *B. abortus* 2308 to host cells.

Results

Identification of novel HutC-target DNA-sequences in *B. abortus* 2308

Previous work showed that in the *Brucella hut* promoter, HutC binds to a palindromic sequence entirely conserved in other closely related α-2 proteobacteria (i.e, *Agrobacterium*, *Rhizobium*, and *Ochrobactrum*) (Sieira *et al.*, 2010). On the other hand, in the *Brucella virB* promoter, HutC interacts with an imperfect palindromic sequence slightly differing from that of the *Brucella hut* promoter, which displays a structure similar to that of HutC-binding sites of unrelated genera such as *Klebsiella* and *Pseudomonas* (Osuna *et al.*, 1994; Zhang and Rainey, 2007; Sieira *et al.*, 2010). In order to identify possible additional HutC target DNA-sequences in the genome of *Brucella*, a 12-bp degenerated consensus sequence was manually constructed based on the alignment of the two previously characterized HutC-binding sites. This consensus motif, WTGTATATAMRW (where W = A or T, R = A or G, and M = A or C), was applied in an *in silico* analysis of strain *B. abortus* 2308 using the Genome-scale DNA-pattern tool from RSAT (http://rsat.ulb.ac.be/rsat/) (Thomas-Chollier *et al.*, 2008). As a result, we identified two sequences that match the degenerated HutC-binding consensus motif and lie in intergenic regions. The first one is located upstream of locus *bab1_1656*, whereas the second sequence is repeated twice in the genome, upstream of loci *bab2_0138* and *bab1_0069*.

To determine whether HutC is able to interact with the *in silico* identified motifs, we performed electrophoresis mobility shift assays (EMSA) using three different radiolabeled DNA probes and a His-tagged *B. abortus* 2308 HutC recombinant protein (Sieira *et al.*, 2010). Using this procedure, we observed that the transcription factor failed to bind the probe corresponding to the upstream region of *bab1_1656*, whereas it specifically interacted with high affinity with both probes corresponding to *bab2_0138* (data not shown) and *bab1_0069* (Fig. 1B), indicating that HutC is able to recognize the sequence ATGTATATAAGA located upstream of these two latter loci.

bab2_0138 codes for a protein of unknown function that displays 49% identity with the putative transcriptional regulator SyrB of *Rhizobium meliloti* (Barnett and Long, 1997). On the other hand, *bab1_0069* codes for the orthologue of BtaE, an adhesin recently described in *B. suis* (Ruiz-Ranwez *et al.*, 2013a), which belongs to the trimeric autotransporter adhesin (TAA) family of proteins that translocate across the bacterial outer membrane (Cotter *et al.*, 2005; Dautin and Bernstein, 2007). Following secretion to the periplasm through the Sec system, TAA insert the C-terminal beta-barrel-forming anchor domain in the outer membrane in a β-barrel assembly machinery (Bam) complex-dependent manner (Lehr *et al.*, 2010; Leo *et al.*, 2012). Once assembled, the TAA C-terminal domain acts as a pore through which the N-terminal passenger, functional binding domain is translocated to the bacterial surface. In *B. suis* 1330, the BtaE protein was detected on the bacterial surface at the new cell pole, and was shown to be involved in the attachment of *Brucella* to host cell and components of the extracellular matrix (ECM) such as hyaluronic acid (HA) (Ruiz-Ranwez *et al*., 2013a).

Analysis of the architecture of the *btaE* promoter

Following identification of two new HutC-target DNA sequences in the genome of *Brucella*, hereafter, we decided to focus our work on the study of the promoter that is associated to a gene of known function. Accordingly, we performed a molecular analysis of the *B. abortus* 2308 *btaE* regulatory region. First, we asked whether the *btaE* promoter might also be recognized by other transcription factors that are known to act in concert with HutC. One of the two characterized targets of HutC in *Brucella*, the *virB* promoter, is regulated by the nucleoid-associated global regulatory protein Integration host factor (IHF) and by several promoter-specific transcription factors including the MarR-family regulator MdrA (Sieira *et al.*, 2004; Sieira *et al.*, 2012). Therefore, we performed EMSA to examine whether IHF and MdrA are able to bind the *btaE* promoter. To our surprise, we found that not only HutC but also IHF and MdrA specifically

interacted with the *B. abortus* 2308 *btaE* promoter in the same nanomolar-ranges as those previously determined in the *virB* regulatory region (Fig. 1B). Indeed, as previously observed for the *virB* promoter, either IHF or HutC produced a mobility shift corresponding to a single protein-DNA complex, suggesting that each of these two regulators interact with a single binding site in the *btaE* promoter (Fig. 1B). In contrast, MdrA interacted with the *B. abortus* 2308 *btaE* promoter in such a way that at produced least 8 distinct protein-DNA species (Fig. 1B). This laddering pattern clearly differed from that previously observed by EMSA for the interaction between MdrA and the *virB* (Sieira *et al.*, 2012) or the *mdrA* promoter (Supplementary Fig. S2), suggesting that the *btaE* contains a higher number of MdrA-binding motifs.

The *btaE* transcription start site (TSS) was identified by primer extension. As shown in Figure 2A, transcription of *btaE* starts at 40-bp upstream of the first ATG codon. To determine the target DNA-sequences for the transcriptional regulators interacting with the *B. abortus* 2308 *btaE* promoter, we performed DNAse I Footprinting experiments using a specific radiolabeled probe and HutC, IHF, or MdrA recombinant proteins. As shown in Figure 2B, HutC protected a 20-bp region that, as expected from the *in silico* predicted HutC-binding sequence, is centered at position -195 relative to the TSS. On the other hand, IHF protected a 26-bp region containing a sequence centered at postion -212, which partially matches the *Escherichia coli* IHF consensus motif (Fig. 2C and 2F). Noteworthy, the *btaE* promoter IHF- and HutC-DNase I protected regions overlap (Fig. 2B-C and 2F), suggesting that both proteins compete for the binding to DNA, as indicated by EMSA (Supplementary Fig. S1). DNAse I Footprinting experiments performed with MdrA revealed a 46-bp protected region spanning positions -138 to -183 (Fig. 2D). At higher protein concentrations, it was observed an additional 44-bp long protected region located between positions -32 and -75 (Fig. 2E). These results indicated that, regardless of the laddering pattern observed by EMSA, MdrA interacts with the *btaE* promoter at two discrete regions. This seems to be a common trait for the MdrA-target promoters, since this transcription factor was also shown to interact at two positions

with regulatory regions of both the *virB* operon (Sieira *et al.*, 2012) and the *mdrA* promoter (Fig. 3 and Supplementary Fig. S2). A sequence analysis of both MdrA-protected regions in the *btaE* promoter revealed the presence of several partially conserved MdrA-binding motifs (Fig. 2F). These observations support the notion that many MdrA dimers could bind to multiple sites within both MdrA-protected regions and may produce higher order complexes in the *btaE* promoter, which is in agreement with the laddering pattern observed by EMSA (Fig. 2B).

The *btaE* promoter displays high variability among different *Brucella* species and strains.

Alignment of sequences corresponding to the intergenic region upstream of *btaE* revealed the presence of point insertions, deletions (indels) and nucleotide substitutions among different representative species and strains of *Brucella*. Three out of five positions showing indels are located at homopolymer tracts of variable length (Fig. 4A), probably as a result of slipped-strand mispairing events during DNA replication (Dechering *et al.*, 1998; Deitsch *et al.*, 2009). On the other hand, positions showing nucleotide substitutions comprise seven transitions and one transversion (Fig. 4A). It is worth mentioning that all these mutations seem to have occurred randomly during evolutionary time scales, since phylogenetic relationships based on *btaE* promoter sequence analysis are consistent with whole-genome-based phylogeny of the genus *Brucella* (Supplementary Fig. S3) (Foster *et al.*, 2009). Figure 4A also shows that the HutC-binding site that we identified in *B. abortus* 2308 was interrupted by a cytosine in all other examined *Brucella* strains. In fact, EMSA showed that HutC was not able to interact with the *btaE* promoter of strain *B. suis* 1330, whereas IHF and MdrA showed a binding pattern similar to that observed in *B. abortus* 2308 (Fig. 4B). Thus, from this analysis it can be deduced that the HutC-binding site of the *btaE* promoter region was generated *de novo* in *B. abortus* 2308 by a single nucleotide deletion at position -198 relative to the TSS.

Unlike the *btaE* promoter, other 5' spacer regions (e.g., *hutC* and *virB*) display identical nucleotide sequences in almost all analyzed *Brucella* species and strains (data not shown), suggesting that the BtaE adhesin promoter region has an unusually high mutation rate. To explore this possibility, we studied the evolutionary dynamics of point mutations in the btaE 5' spacer region by comparing the mutation rate of all intergenic sequences of *Brucella* likely to contain promoters, using *B. abortus* 2308 as a reference strain. As shown in Supplementary Figure S4A, among all 1314 putative promoter regions of *B. abortus* 2308, 818 displayed a median of probability of nucleotide differences (*p*) equal to zero, indicating low or no variation among the analyzed species and strains, consistent with the observation that different *Brucella* species display high sequence identity at the nucleotide level (Halling *et al.*, 2005; Chain *et al.*, 2005). The rest of the 5' spacers, instead, showed higher values and displayed a skewed distribution according to which the *btaE* promoter falls into the 3 % of the promoters displaying higher variation levels, with a median of p of 1.03×10^{-2} (Supplementary Fig. S4A). Interestingly, the *btaE* promoter region also displayed a large abundance of homopolymeric tracts (19.06 %) (Supplementary Fig. S4B), which is a characteristic associated to genomic regions with nucleotide diversity (McDonald *et al.*, 2011). Therefore, the hight mutation rate of the BtaE regulatory region may be a consequence of intrinsic features of its promoter architecture, which could provide a potential source of diversity at the regulatory level.

Role of HutC and MdrA in the transcriptional regulation of *btaE*

The possible functionality of the binding sites identified in the *btaE* promoter was studied by focusing our analysis on the promoter-specific transcription factors HutC and MdrA. To this end, *lacZ* transcriptional fusions to the *btaE* promoter were generated in *B. abortus* 2308 and *B. suis* 1330 wild type strains and their corresponding Δ*hutC* or Δ*mdrA* isogenic mutants. In *B. abortus* 2308, expression of *btaE* was induced in the stationary phase of growth under standard laboratory

conditions in rich medium, whereas the Δ*hutC* and Δ*mdrA* mutants showed reduced β-Gal. activity levels along the growth curve (data not shown). Comparison of β-Gal. activity analyses of late-stationary phase cultures ($OD_{600} \geq 3.6$) showed that complementation of either *hutC* or *mdrA* restored β-Gal activity to wild type levels, confirming that these two transcription factors act as positive regulators of *btaE* in *B. abortus* 2308 (Fig. 5A). These results also showed that both regulators are required simultaneously to produce a regulatory effect on this promoter, suggesting that HutC acts in concert with MdrA to display a combinatorial mode of transcriptional control of *btaE* in *B. abortus* 2308. As expected, in *B. suis* 1330, the *hutC* deletion mutant showed the same expression levels as the wild type strain (Fig. 5B), consistently with the observation that this species lacks a HutC-binding site in the *btaE* promoter. Moreover, deletion of *mdrA* did not produce any observable effect on this promoter in *B. suis* 1330, in agreement with the notion that concerted action of both HutC and MdrA is required to modulate *btaE* promoter activity. To test this latter hypothesis, we introduced in *B. suis* 1330 the transcriptional fusion construct of the *lacZ* reporter gene under the control of a *btaE* promoter region containing the functional *B. abortus* 2308 HutC-binding site, generating the strain *B. suis* $P_{btaE\ Bab}$, which showed induction of *btaE* promoter activity in the stationary phase of growth (Fig. 5B) similarly to that observed in *B. abortus* 2308 (Fig. 5A).

Taken together, these results show that the HutC and MdrA target-DNA sequences identified in the *B. abortus* 2308 *btaE* promoter constitute functional binding sites capable of generating a transcriptional output. Moreover, our observations indicated that *de novo* generation of a HutC-binding site in *B. abortus* 2308 resulted in a transcriptional rewiring event that integrated expression of this TAA into an ancient regulatory network controlling both metabolism and virulence genes.

Impact of rewiring of the *btaE* promoter on adherence of *B. abortus* 2308 to the host cell surface

To date, BtaE function on attachment of *Brucella* to the host cell surface has been described in strain *B. suis* 1330, which exhibits a predicted BtaE protein architecture similar to that of *B. abortus* 2308 but different in size. Like many other members of the TAA family, BtaE orthologues show length polymorphism and display a passenger domain containing an N-terminal globular head and a stalk region with a repetitive architecture with a variable number of neck-head-Trp ring domain modules (Fig. 6). These intragenic repeats are encoded by 339 bp-long sequences that show 92 to 100% nucleotide identity among the different modules of the analyzed *Brucella* species. Such a repetitive structure allows the occurrence of recombination events that can lead to an increase or decrease in the number of intragenic repeats, combinatorial reshuffling of the different modules, or even gene inactivation. It has been proposed that plasticity of TAA modular structure contributes to the generation of new combinations that could lead to antigen variation, functional diversity, and may eventually favor adaptation to changing environmental conditions (Linke *et al.*, 2006). According to the annotation for trimeric autotransporter adhesins server (daTAA) (Szczesny and Lupas, 2008), the *B. abortus* 2308 BtaE orthologue displays 6 modular repeats, whereas that of *B. suis* 1330 is a shorter adhesion harboring a single repeat (see Figure 6).

The role of the *B. abortus* 2308 BtaE variant in the attachment of this bacterium to the host cell surface was examined by performing an adherence assay to HeLa cell monolayers. As shown in Figure 7A, the *B. abortus* Δ*btaE* deletion mutant strain displayed a 39 % reduction of adhesion to HeLa cells compared to *B. abortus* 2308. Since proper cloning of the complete *B. abortus* 2308 *btaE* gene was not possible (probably due to the high sequence identity existing between the six 339 bp repeats of this gene), we performed a heterologous complementation assay of the *B. abortus* Δ*btaE* mutant with a plasmid expressing the *B. suis* 1330 *btaE* ortholog (Ruiz-Ranwez *et al*., 2013a). As a result, complementation of *btaE* deletion led to a complete restoration of the wild type adhesion phenotype (Fig. 7A), thus demonstrating that the BtaE variant of *B. abortus* 2308 contributes to the interaction of *Brucella* with the host cell surface to a similar extent to that

observed for the *B. suis* 1330 ortholog (Ruiz-Ranwez *et al*., 2013a). Additionally, similarly to that observed in previous analysis from our group (Ruiz-Ranwez *et al*., 2013a; Ruiz-Ranwez *et al*., 2013b), the wild type *B. abortus* 2308 and Δ*btaE* mutant strains showed similar efficiencies of invasion, suggesting that *btaE* is involved in the initial attachment of *B. abortus* to HeLa cells rather than in internalization (data not shown). Taken together, and in line with previous reports on TAA function (Sheets and St Geme, 2011), our observations indicate that variation in five tandem repeats between *B. suis* 1330 and *B. abortus* 2308 *btaE* orthologs may not interfere with the TAA adhesive activity in this experimental model. However, we cannot rule out possible polymorphism-dependent differences, for example, in adhesive phenotypes to specific cell types of the natural host.

The observations described above showed that a single point mutation generated a functional HutC-binding site upstream of the *B. abortus* 2308 *btaE* gene. To determine whether emergence of a HutC- and MdrA-dependent *btaE* promoter activity has an impact on the adhesion phenotype of *B. abortus* 2308, we analyzed the effect of deletions of these regulators on the attachment to HeLa cells. As shown in Figure 7B, both Δ*hutC* and Δ*mdrA* regulatory mutants displayed a decrease of adherence of *B. abortus* to HeLa cells to a similar extent to that observed for the structural mutant Δ*btaE*, whereas *hutC* or *mdrA* knock-in complemented strains recovered adhesion levels. Moreover, the presence of the *B. suis* 1330 *btaE* ortholog in a multicopy plasmid also restored bacterial attachment of of either Δ*hutC* or Δ*mdrA* mutant strains to HeLa cells to wild type levels, strongly suggesting that the binding effect of the regulatory mutants was due to decrease in *btaE* expression. Taken together, our observations show that modulation of *btaE* promoter activity correlated with changes in the levels of adhesion of strain *B. abortus* 2308 to host cells, indicating that transcriptional rewiring of *btaE* generated phenotypic diversity at the regulatory level of adhesion components, which might contribute to reciprocal selection between *Brucella* species and their mammalian hosts.

Discussion

In this work, by means of *in silico* search of new HutC-binding motifs in the genome of the virulent strain *B. abortus* 2308, we found that this transcription factor is involved in the control of more functions than previously known. In addition to its role in regulation of histidine metabolism and expression of the Type-IV secretion apparatus, our experiments revealed that HutC also affects the attachment of *B. abortus* 2308 to host cells through modulation of *btaE* promoter activity.

As many other types of bacterial surface exposed structures, TAAs are known to display high amino acid sequence variability among genera and strains. Generation of new TAA phenotypic variants does not only depend on recombination-mediated reshuffling of modular repeats present in their coding regions, but also can be achieved by mechanisms that introduce heterogeneity at the gene expression level. For instance, UspA1 of *Moraxella catarrhalis* and NadA of *Neisseria meningitidis* show inter-strain variation of expression associated to changes in the length of homopolymeric or heteropolymeric nucleotide tracts at their respective promoters (Lafontaine *et al.*, 2001; Martin *et al.*, 2003; Martin *et al.*, 2005). Here, we observed that, in addition to major differences existing between BtaE protein sequences of different *Brucella* strains, the *btaE* promoter exhibits nucleotide diversity and a high proportion of homopolymeric tracts. This latter feature could explain the observed high mutation rate found in this promoter within the genus *Brucella*, since it has been proposed that genomic regions containing homopolymeric and/or repeat sequences are prone to replication fork stalling, with a consequent local recruitment of error-prone DNA polymerases (McDonald *et al.*, 2011). Moreover, nucleotide substitutions in the *btaE* promoter involve mainly transitions (Supplementary Fig. 5A), which is also a characteristic previously observed in repeat-rich regions of bacterial genomes (McDonald *et al.*, 2011). Taken together, all these observations suggest that homopolymer tract organization of the *btaE* promoter may itself favour the occurrence of mutations, which eventually resulted in the generation of a new HutC-target DNA sequence and reorganization of a transcriptional circuit. Comparison of *btaE* promoter

sequences among different species indicated that such modification was produced by a relatively recent point mutation event in the evolution of the genus *Brucella*. As confirmed by DNAse I Footprinting, the newly-acquired HutC-binding site is located far upstream of the TSS at a relative position similar to that observed in the promoter of the *virB* genes (Sieira *et al.*, 2010) (Fig. 3). According to this similar spatial arrangement, we observed a correlated positive regulatory role for HutC on expression of the *B. abortus* 2308 BtaE adhesin. In the *hut* promoters, instead, HutC acts as repressor through binding to DNA near the TSS, thus preventing access of RNA polymerase holoenzyme (Hu *et al.*, 1989).

In addition to HutC, in this work we found two other transcription factors shared between the *btaE* and *virB* genes. EMSA experiments showed that both the global regulator IHF and the MarR-type transcription factor MdrA interacted with the *btaE* to nanomolar-range affinities. DNAse I Footprinting experiments revealed that the IHF-binding site does not only display similar spatial arrangements in both promoters, but also exhibits an IHF DNAse I-protected region that overlaps the HutC-binding site (Fig. 4F). In agreement with this observation, and as previously found for the *virB* promoter, EMSA experiments indicated that IHF and HutC are not capable of binding simultaneously to the *btaE* regulatory region (Supplementary Figure S1).

Our EMSA experiments showed that in all studied promoters (i.e, *virB*, *mdrA*, and *btaE*), MdrA interacts with DNA at two discrete sites and probably requires IHF to exert its regulatory activity, since this global regulator was also found to bind to these three loci (Fig. 2-3, Supplementary Figure S2) (Sieira *et al.*, 2012). However, unlike that observed by EMSA in the *virB* and *mdrA* promoters, interaction between MdrA and the *btaE* regulatory region produced a DNA laddering pattern, which suggests that more than one MdrA dimer binds to each binding site. As observed by DNAse I Footprinting, both MdrA-binding sites identified at the *btaE* promoter are longer than those previously identified in the *virB* genes and contain a higher number of putative MdrA-binding motifs (Fig 2F) (Sieira *et al.*, 2012). Moreover, one of the DNase I protected regions of MdrA is

located near the *btaE* TSS and overlaps position -35 by 3 bp (Fig. 2E). This observation lead us to speculate that unlike most activators of the MarR family, which act by means of displacing repressors, MdrA could also participate in enhancing *btaE* promoter activity by direct transcriptional activation, similarly to that suggested for RovA, a regulator of virulence of *Yersinia pseudotuberculosis* (Tran *et al.*, 2005). Thus, both positioning and the specific architecture of the MdrA-binding sites could be responsible for the formation of higher-order complexes and for the mode of regulation exerted by MdrA on the adhesin promoter in *B. abortus* 2308.

The analyses of expression performed with *hutC* and/or *mdrA* deletion mutants revealed that both transcriptonal regulators exert positive regulatory roles on the *B. abortus* 2308 *btaE* promoter to extents similar to those previously reported for the *virB* genes. However, we observed different interplays between HutC and MdrA on *virB* and *btaE* promoter regions. The previous report showed that HutC and MdrA play redundant roles on *virB* expression since single *hutC* or *mdrA* mutants produced no effects on promoter activity, indicating that binding of either of these two regulators is a sufficient condition to elicit equivalent gene expression outputs (Sieira *et al.*, 2012). In the *btaE* promoter, instead, HutC and MdrA seem to act in a combinatorial manner. This notion is supported by the comparative analysis between two different *Brucella* species, which showed that *B. suis* 1330 lacks the HutC-binding site and is therefore not affected by deletion of *mdrA* under the assayed conditions (Fig. 5B). Together with data obtained by EMSA, these observations indicate that the ancestral *btaE* promoter contains two MdrA-binding sites, which seem to be unable to mediate a transcriptional output in the absence of a combinatorial partner. On the other hand, in *B. abortus* 2308, MdrA target-DNA sequences seem to be a necessary prerequisite for *btaE* to achieve stationary stationary phase-inducible promoter activity upon *de novo* generation of a HutC-binding site. However, regardless of the HutC-mediated regulatory circuit re-organization observed in *B. abortus* 2308, conservation of MdrA binding sites in the ancestral *btaE* promoter raises the question

whether this transcription factor could also modulate adhesin expression *per se*, or in concert with a combinatorial partner other than HutC, under particular *in vivo* conditions within the host.

So far, the mechanism by which HutC enhances *virB* and *btaE* gene expression from operators located far upstream the TSS still remains to be elucidated. It could be speculated that HutC somehow stabilizes and/or positions activators that bind to downstream elements. In this regard, it is also worth pointing out that in both the *virB* and *btaE* promoters, the HutC- and MdrA-mediated regulation do not only show different kinds of interplay, but also display different requirements in terms of nutrients, specific metabolites, and pH, probably due to differential availability of putative accessory regulatory proteins in each of the assayed conditions. Future work will be needed to determine the identity of possible additional elements that participate in the HutC- and MdrA-mediated enhancement of expression and the precise molecular interactions that dictate this particular regulatory mechanism.

The observations presented in this work indicate that, as a result of *cis* regulatory gain of function, the *btaE* promoter acquired the ability to fine-tune its transcriptional output in response to changes in environmental parameters such nutrient availability, as evidenced by the transition from an ancestral constitutive behavior to a stationary phase-inducible one in bacteriological medium (Fig. 5). As this transcriptional rewiring event was fixed in a virulent strain of the genus *Brucella*, it can be speculated that integration of *btaE* promoter activity to a preexisting regulatory network provided the bacterium with an adaptive advantage. Probably, the ability to respond to resembling environmental cues perceived at specific contexts within the natural host enabled *B. abortus* 2308 to spatio-temporally modulate its adhesion to defined cell types, contributing to a more efficient establishment and/or maintenance of chronic infection. On the other hand, it is worth mentioning that some of the BtaE target molecules such as HA and fibronectin are not only present in the ECM but were also found within *Brucella*-containing vacuoles (Gay *et al.*, 1986) or in eukaryotic compartments associated to the *Brucella* intracellular life cycle (i.e., early endosomes and

lysosomes) (Kan, 1990; Hascall *et al.*, 2004). Therefore, synchronization of *btaE* promoter activity with HutC-mediated regulation of other *Brucella* intracellular virulence factors may adjust bacterial attachment to intravacuolar substrates with possible positive effects on intracellular survival. As mentioned above, intrinsic features related with homopolymeric tract organization may be responsible for the propensity of the *btaE* promoter for the accumulation of mutations, which eventually resulted in transcriptional regulatory changes. In addition, it was found that species of the genus *Brucella* display a variable repertoire of monomeric and trimeric adhesin autotransporters, several of which also show inter- and intra-species variation at the protein sequence level (A. Zorreguieta *et al*, unpublished data). Taken together, all these processes may act as a substrate for the evolution of *Brucella* by providing different combination of both quantity and type of surface-exposed adhesive proteins, supporting variability to exploit different host niches through interaction with specific cell types or target organs with changing affinities. In view of the results from the current work and the previous lines of evidence, it will be interesting to determine the extent to which regulatory heterogeneity and the different combinations of adhesins contribute to host preference and cell-type specificity of interaction in the different species of the genus *Brucella*.

Experimental procedures

Bacterial strains and media

B. abortus 2308, *B. suis* 1330, and derivatives were grown in a rotary shaker (250 rpm) at 37°C in Tryptic Soy Broth (TSB) supplemented with nalidixic acid (5 μg ml^{-1}) or kanamycin (50 μg ml^{-1}) as needed. *E. coli* S17 strains were cultured in Luria-Bertani (LB) broth at 37°C in a rotary shaker at 250 rpm.

EMSA

Primers 0069up (5'-AGAACCGATTATGTGCTGG-3') and 0069down (5'-GGCATGTAAAATTTTTACCTTATA-3') were used to amplify a 311-bp region spaning postions -266 to 45 relative to the TSS of the *btaE* promoter using Taq polymerase (Invitrogen), [α-^{32}P]-CTP, and *B. abortus* 2308 or *B. suis* 1330 genomic DNA as template. Likewise, probes corresponding to the promoter region of loci *bab1_1656*, *bab2_0138* (*syrB* orthologue), and *bab1_0783* (*mdrA*) were constructed by the same procedure using primers 1656up (5'-CACGATCTTCGGTCTTGC-3') and 1656down (5'-TGCGCCGTCCTTCATTCA-3'), PsyrB2up (5'-GCCCGGAATTGAGGTAAAG-3') and PsyrB2down (5'-CGATGAATGCTCCATCTATAG-3'), or PmarRup (5'-CGAGCCTGCCGCTTTGAC-3') and PmarRdown (5'-CGGTGGTTTGTTTCTCACC-3'), respectively. Control probe was constructed with primers B10Qu and B10d2, as described previously (Sieira *et al.*, 2010). Recombinant *B. abortus* 2308 HutC, MdrA, and IHF proteins were induced and purified as described previously (Sieira *et al.*, 2004; Sieira *et al.*, 2010; Sieira *et al.*, 2012). Binding reactions were performed by incubating recombinant proteins at the indicated concentrations with each radiolabeled probe for 20 min in binding buffer (15 mM Tris-HCl [pH 8.0], 0.5 mM EDTA, 10 μg ml^{-1} bovine serum albumin [BSA], 1 mM dithiothreitol [DTT], 30 mM KCl, 6% glycerol, 50 μg ml^{-1} salmon sperm DNA as unspecific competitor). Samples were analyzed

by electrophoresis on 8% non-denaturing polyacrylamide gels at 220 V. Subsequently, gels were dried and exposed to X-ray films.

Primer extension

Total RNA from stationary phase-cultures (OD_{600} ~ 3.6) of *B. abortus* 2308 was purified using RNeasy mini kit (Qiagen). Primer Pext0069-166 (5'-CCATATGCATCCATCATCTG-3') was 5'-end labeled with [γ-^{32}P]-ATP and Polynucleotide kinase (New England Biolabs). 15 µg of purified RNA was incubated with the radiolabeled primer for 5 min at 68°C and chilled on ice. Primer extension reaction was performed with SuperScript III (Invitrogen) reverse transcriptase for 60 min at 52°C. Samples were run in 6% polyacrilamyde sequencing gels in parallel with a sequence reaction performed with primer Pext0069-166 by the Maxam and Gilbert method (Maxam and Gilbert, 1977) with piperidine. After electrophoresis, gels were dried and exposed to X-ray films.

DNAse I Footprinting

A radiolabeled probe corresponding to the *btaE* promoter region was constructed by DNA polymerase chain reaction (PCR) using Taq (Invitrogen), primer 0069up, the 5' ^{32}P-radiolabeled primer 0069down, and *B. abortus* 2308 genomic DNA as template. Labeling and binding reactions, DNase I digestion, purification of digested fragments, and electrophoresis were performed as described previously (Sieira *et al.*, 2012).

Bioinformatic analysis

Genomic coordinates of *B. abortus* 2308 and custom Perl scripts were used to extract itergenic nucleotide sequences likely to contain promoters, defined as 5' spacer regions ≥ 90 bp, from complete record FASTA files of chromosomes I and II of the organism defined in GenBank as *Brucella melitensis* biovar *abortus* 2308. The obtained list of 5' spacer regions was used as query to

perform nucleotide blast (blastn) search against complete record FASTA files of genomes of strains *B. abortus* biovar 1 strain 9-941, *B. abortus* S19, *B. melitensis* 16M, *B. suis* 1330, *B. suis* ATCC 23445, *Brucella canis* ATCC 23365, *Brucella microti* CCM 4915, and *Brucella ovis* ATCC 25840. For each *Brucella* strain, the resulting blast analysis was subsequently parsed to extract the percentage of identity and to calculate the probability of nucleotide differences p between each 5' spacer region compared to that of *B. abortus* 2308. The extent of variation for each 5' spacer region among the different analyzed *Brucella* species and strains was calculated as median of *p*. A custom Python script was developed in order to determine in *B. abortus* 2308 the percentage of homopolymeric sequences longer or equal to 4 nt for each of the 1315 5' spacers identified.

BtaE domain organization analysis

Amino acid sequences of BtaE orthologs from different *Brucella* species and strains was analyzed using the domain annotation of trimeric autotransporter adhesins (daTAA) toolkit (http://toolkit.tuebingen.mpg.de/dataa).

Phylogenetic analysis

The phylogenetic analysis was performed based on the Tamura 3-parameter model (Tamura, 1992) on 436-bp long 5' spacer nucleotide sequences of *btaE* orthologs of the 9 indicated *Brucella* species and strains using the MEGA5 software (Tamura *et al.*, 2011). The bootstrap consensus tree was inferred from 1000 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Initial tree for the heuristic search were obtained automatically by the Neighbor-Join and BioNJ methods to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. There were a total of 433 positions in the final dataset. Topology was selected with superior log likelihood value.

Construction of plasmids and strains

A *btaE* promoter-*lacZ* transcriptional fusion plasmid was constructed as follows: a 432-bp insert was amplified by PCR using primers 0069up-BamHI (5'-CGGGATCCTTCGATTCCTGTTATTTGTTC-3') and 0069down-BamHI (5'-CGGGATCCAAATTTTTACCTTATATATAAATAC-3'), Pfx polymerase (Invitrogen), and genomic DNA of *B. abortus* 2308 or *B. suis* 1330. The resulting PCR products were cloned in plasmid pGEM-T easy (Quiagen), excised with BamHI, and cloned in the same restriction site of plasmid pK18mob-*lacZ*, thus generating plasmids pK18mob-$P_{btaE\ Bab}$-*lacZ* and pK18mob-$P_{btaEBsu}$-*lacZ*.

Construction of strains containing single chromosomal *lacZ* transcriptional fusions was performed as follows: plasmid pK18mob-$P_{btaEBab}$-*lacZ* was transferred to strains *B. abortus* 2308, *B. abortus* Δ*hutC*, *B. abortus* Δ*hutC* -KI (Sieira *et al.*, 2010), *B. abortus* Δ*mdrA*, *B. abortus* Δ*hutC* Δ*mdrA*, and *B. abortus* Δ*mdrA* -KI (Sieira *et al.*, 2012) by biparental conjugation with *E. coli* S17. Subsequently, kanamycin-resistant colonies were selected as single-recombinants. Likewise, plasmid pK18mob-$P_{btaEBsu}$-*lacZ* was transferred by the same procedure to *B. suis* 1330, *B. suis* Δ*hutC*, *B. suis* Δ*mdrA* or *B. suis* Δ*hutC* Δ*mdrA*.

Construction of strain *B. suis* $P_{btaE\ Bab}$ was performed as follows: plasmid pK18mob-$P_{btaEBab}$-*lacZ* was transferred to *B. suis* 1330, and kanamycin-resistant single-recombinants were analyzed by colony PCR using primers 0069up-BamHI and lacZ6143 (Sieira *et al.*, 2010) followed by Sanger sequencing of the PCR products. Clones where the single-recombination events produced a *lacZ* transcriptional fusion under the control of a *btaE* promoter containing a functional HutC-binding site were selected for β-Gal. activity analysis.

Construction of strain *B. abortus* Δ*btaE*. Plasmid pK18mob-*sacB*-Δ*btaE* (Ruiz-Ranwez *et al.*, 2013a) was transferred to *B. abortus* 2308 by biparental conjugation. Kanamycin-resistant colonies were selected as single-homologous recombinants. Selection with sucrose, excision of plasmids, and generation of deletion mutants were performed as described previously (Sieira *et al.*, 2004).

Construction of strains *B. suis* Δ*hutC* and *B. suis* Δ*mdrA*. Plasmid pK18mob-*sacB*- Δ*hutC* (Sieira *et al.*, 2010) or pK18mob-*sacB*-Δ*mdrA* was transferred to strain *B. suis* 1330 by biparental conjugation. Mutants were obtained by counterselection with sucrose as described above.

Construction of the heterologous complemented strain *B. abortus* Δ*btaE*/pBBR2-*btaE*: Plasmid pBBR*btaE*, which contains the *B. suis btaE* ortholog cloned in the broad-host range plasmid pBBR1 MCS-2 (Kovach *et al.*, 1995), was transferred to *B. abortus* ΔΔ*btaE* by biparental conjugation with *E. coli* S17.

β-Gal. activity measurements

Bacteria were grown in rich medium (TSB) up to the stationary phase of growth ($OD_{600} \geq 3.6$). β-Gal. activity was determined with whole cells as described previously (Sieira *et al.*, 2004) with the following modifications: after incubation for 10 min with o-nitrophenyl-β-D-galactopyranoside, reaction mixtures were centrifuged before determinations of A^{420}. β-galactosidase activity was expressed in Miller units.

Cell infection assays

HeLa cells were seeded in 24-well plates (5×10^4 cells per well) with Dulbecco's Modified Eagle Medium (Gibco) supplemented with 5% fetal bovine serum. The indicated *B. abortus* strains were grown in TSB until stationary phase of growth ($OD_{600} \sim 3.6$) with the appropriate antibiotics. Plated cells were inoculated with bacteria at a multiplicity of infection of 100:1. Subsequently, plates were centrifuged for 10 min at 170 rcf and incubated for 1 h in a 5% CO_2 atmosphere at 37°C. To determine the total number of bacteria associated to the cells, wells were washed three times with PBS and treated for 10 min at 37°C with 0.1% Triton X-100. Lysates were serially diluted and subsequently plated on TSB agar with the appropriate antibiotics, and CFU were determined. The number of attached bacteria was calculated as the average number of total bacteria associated to the

cells from three independent biological replicates, and expressed as percentage relative to the wild-type strain, which was defined as 100%.

Statistical analysis

All statistical analyses were performed using the program InfoStat. Statistical differences were tested using one-way analysis of variance (ANOVA) and Tukey post hoc test on data obtained from three independent biological replicates.

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Legends to figures

Figure 1. Analysis of binding of HutC and additional transcription factors to the *B. abortus* 2308 *btaE* promoter. EMSA performed with a control probe (A), or a probe corresponding to the *btaE* promoter region of *B. abortus* 2308 (B). Radiolabeled probes were incubated with recombinant HutC, MdrA, or IHF at the indicated nanomolar concentrations.

Figure 2. Architecture of the *B. abortus* 2308 *btaE* promoter. A) Primer extension analysis performed with total RNA from *B. abortus* 2308 and the radiolabeled primer Pext0069-166. Arrow indicates position of the primer extension product. A + G line shows a Maxam-Gilbert sequencing reaction performed with piperidine using the above-mentioned primer. B) DNAse I Footprinting analysis performed with a radiolabeled probe corresponding to the *B. abortus* 2308 *btaE* promoter and recombinant HutC at the indicated concentrations. Open rectangle indicates position of the DNase I protected region relative to the TSS. Lanes C and T show Sanger sequencing reactions performed with primer 0069down. C) DNAse I Footprinting analysis performed with the same radiolabeled probe, sequencing reactions and IHF recombinant protein. D) DNAse I Footprinting analysis performed with the same radiolabeled probe, sequencing reactions, and a MdrA recombinant protein. E) DNAse I Footprinting analysis showing DNA molecules of higher mobility from the same experiment shown in (D). F) Schematic representation of the *B. abortus* 2308 *btaE* promoter. Numbers indicate positions relative to the TSS. Positions -10 and -35 are indicated. +1 indicates TSS. Capital letters indicate the first codon of *btaE*. Underlined letters indicate the 5' predicted coding region of *btaE*. Open rectangles indicate DNAse I-protected regions. Binding consensus motif sequences are indicated for each transcription factor as upper or lower sequences, where W = A or T and R = A or G. Shaded bases indicate sequences that match the binding motif for each transcription factor. Asterisks indicate position displaying nucleotide differences between *B. abortus* 2308 and other *Brucella* species and strains.

Figure 3. Schematic representation of the *B. abortus* 2308 *btaE* and *virB* promoter regions. Coding regions of both *btaE* and *virB* genes are indicated in grey. Solid line indicates non-coding regions. DNAse I protected regions of HutC, IHF, MdrA or the LuxR-type regulator VjbR (Arocena *et al.*, 2010), are indicated as boxes, with their corresponding relative positions to the TSS (+1).

Figure 4. The HutC-binding site of *B. abortus* 2308 was generated *de novo* by a single point mutation event. A) Alignment of sequences corresponding to the 5' spacer region of *btaE* of different representative *Brucella* species and strains. Grey boxes indicate nucleotide homopolymeric tracts displaying length ≥ 4. Open boxes contain the HutC-binding site, the sequence that partially matching the *E. coli* IHF consensus motif, and nucleotides that encompass putative MdrA-binding sequences, as indicated. Black shaded positions indicate nucleotide differences. Asterisks indicate positions of indels probably due to slipped-strand mispairing events during DNA replication. Nucleotide positions relative to the TSS (+1) are indicated. B) EMSA performed with a probe corresponding to the *btaE* promoter region of *B. suis* 1330 incubated with recombinant *B. abortus* HutC, MdrA, or IHF at the indicated nanomolar concentrations.

Figure 5. Role of HutC and MdrA on transcriptional regulation of *btaE*. β-Gal activity measurements of strains harbouring chromosomal *btaE* promoter-*lacZ* transcriptional fusions. A) Strains *B. abortus* 2308 P_{btaE}-*lacZ* (2308 wt), *B. abortus* Δ*hutC* P_{btaE}-*lacZ* (*hutC*), *B. abortus* Δ*mdrA* P_{btaE}-*lacZ* (*mdrA*), *B. abortus* Δ*hutC* Δ*mdrA* P_{btaE}-*lacZ* (*hutC mdrA*), and the Knock-in complemented strains *B. abortus* Δ*hutC* KI P_{btaE}-*lacZ* (*hutC hutC*+) or *B. abortus* Δ*mdrA* KI P_{btaE}-*lacZ* (*mdrA mdrA*+) were grown in rich medium (TSB) up to the stationary phase of growth (OD_{600} ≥ 3.6) and β-Gal activity was determined. B) Strains *B. suis* 1330 P_{btaE}-*lacZ* (1330 wt), *B. suis* Δ*hutC* P_{btaE}-*lacZ* (*hutC*), *B. suis* Δ*mdrA* P_{btaE}-*lacZ* (*mdrA*), *B. suis* Δ*hutC* Δ*mdrA* P_{btaE}-*lacZ* (*hutC*

mdrA), and a strain harboring a transcriptional fusion between *lacZ* and a *btaE* promoter containing a functional HutC-binding site (1330 P_{btaE}-HutC-bs+) up to the stationary phase of growth ($OD_{600} \geq 5$) and β-Gal activity was determined as in A). Values are means ± standard deviations of three independent biological replicates. ***, $P < 0.0001$.

Figure 6. Domain structure of BtaE in different representative species and strains of *Brucella*. Schematic representation of domain arrangements of BtaE proteins. Amino acid sequences of the indicated BtaE orthologs of different *Brucella* species and strains were analyzed using the domain annotation of trimeric autotransporter adhesins (daTAA) tookit (http://toolkit.tuebingen.mpg.de/dataa). All orthologues are predicted to harbor: a) an N-terminal head, b) a membrane anchor domain consisting of 4 β-strands per subunit which assemble into a 12-stranded β-barrel, c) a stalk region that combines two small stalk domains (FGG and YTD) interrupted by a head-like structure, and d) a 113 amino acid-long neck-head-Trp ring domain which shows variation in number among the genus *Brucella*, exhibiting 1, 2, 3, or 6 repeats.

Figure 7. Role of *btaE*, *hutC*, and *mdrA* on adhesion of *B. abortus* 2308 to the host cell surface. HeLa cells were infected with the virulent wild-type strain *B. abortus* 2308 (2308 wt) and strains *B. abortus* Δ*btaE* (*btaE*) or the complemented strain *B. abortus* Δ*btaE*/pBBR-$btaE_{Bsu}$ (*btaE btaE*+) (A); or strains *B. abortus* 2308 (2308 wt), *B. abortus* Δ*hutC* (*hutC*), *B. abortus* Δ*hutC*-KI (*hutC hutC*+), B. *abortus* Δ*mdrA* (*mdrA*), *B. abortus* Δ*mdrA*-KI (*mdrA mdrA*+), *B. abortus* Δ*hutC*/pBBR-$btaE_{Bsu}$ (*hutC btaE*+), *B. abortus* Δ*mdrA*/pBBR-$btaE_{Bsu}$ (*mdrA btaE*+). Percentage of attached bacteria determined as the total bacteria associated to the cells relative to the wild type *B. abortus* 2308 strain, defined as 100%. Values are means ± standard deviations of three independent biological replicates. Data were analyzed by one-way ANOVA followed by a Tukey post hoc test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

Supplementary Figure S1. IHF and HutC compete for the binding to the *btaE* promoter. Co-incubation experiment performed by EMSA with the probe correspondent to the *btaE* promoter and different combinations of 10 nM HutC, 200 nM IHF, or 20 nM MdrA.

Supplementary Figure S2. Analysis of binding of IHF and MdrA to the *B. abortus* 2308 *mdrA* promoter. EMSA performed with a radiolabeled probe corresponding to the *mdrA* promoter and recombinant IHF or MdrA at the indicated nanomolar concentrations.

Supplementary Figure S3. Phylogenetic analysis based on *btaE* promoter sequences of different *Brucella* species and strains. Unrooted maximum likelyhood tree based on 436-bp long 5' spacer nucleotide sequences of *btaE* orthologs of the 9 indicated *Brucella* species and strains using the MEGA5 software (Tamura *et al.*, 2011). The bootstrap consensus tree was inferred from 1000 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches.

Supplementary Figure S4. Global analysis of 5' spacer regions of *B. abortus* 2308. A) Frequency distribution of median of *p* (probability of nucleotide differences) between 5' spacer regions of *B. abortus* S19, *B. abortus* bv. 1 str. 9-941, *B. melitensis* 16M, *B. suis* 1330, *B. suis* ATCC 23445, *Brucella canis* ATCC 23665, *Brucella microti* ATCC 4915, *Brucella ovis* ATCC 25840, and *B. abortus* 2308 as a reference strain. Dashed line indicates interval containing the 5' spacer region corresponding to the *btaE* promoter. B) Frequency distribution of percentage of nucleotide homopolymeric sequences of length ≥ 4 in 5' spacer regions of *B. abortus* 2308. Dashed line indicates interval containing the 5' spacer region corresponding to the *btaE* promoter.

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Figure 1

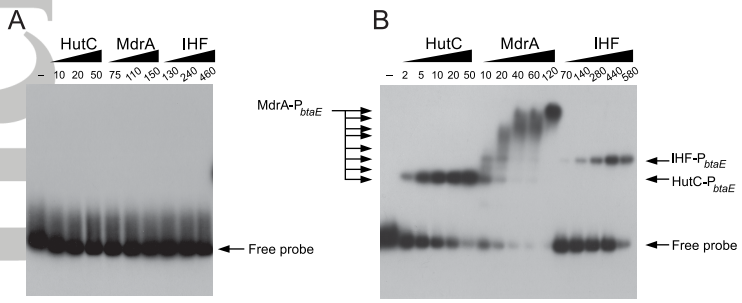


Figure 2

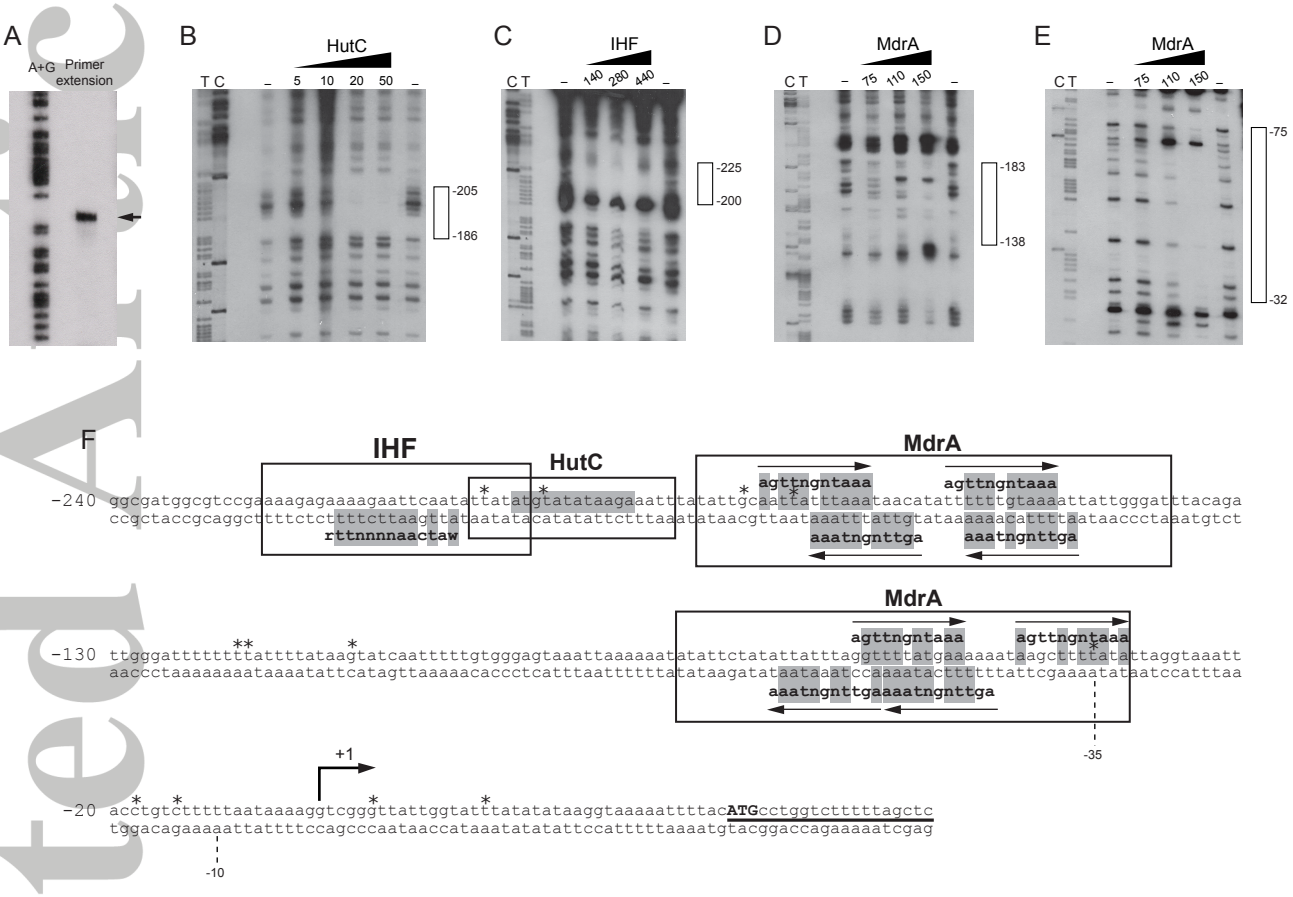


Figure 3

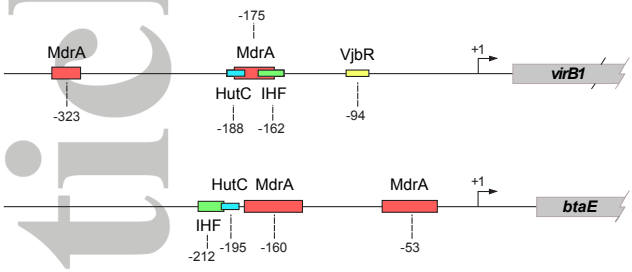


Figure 4

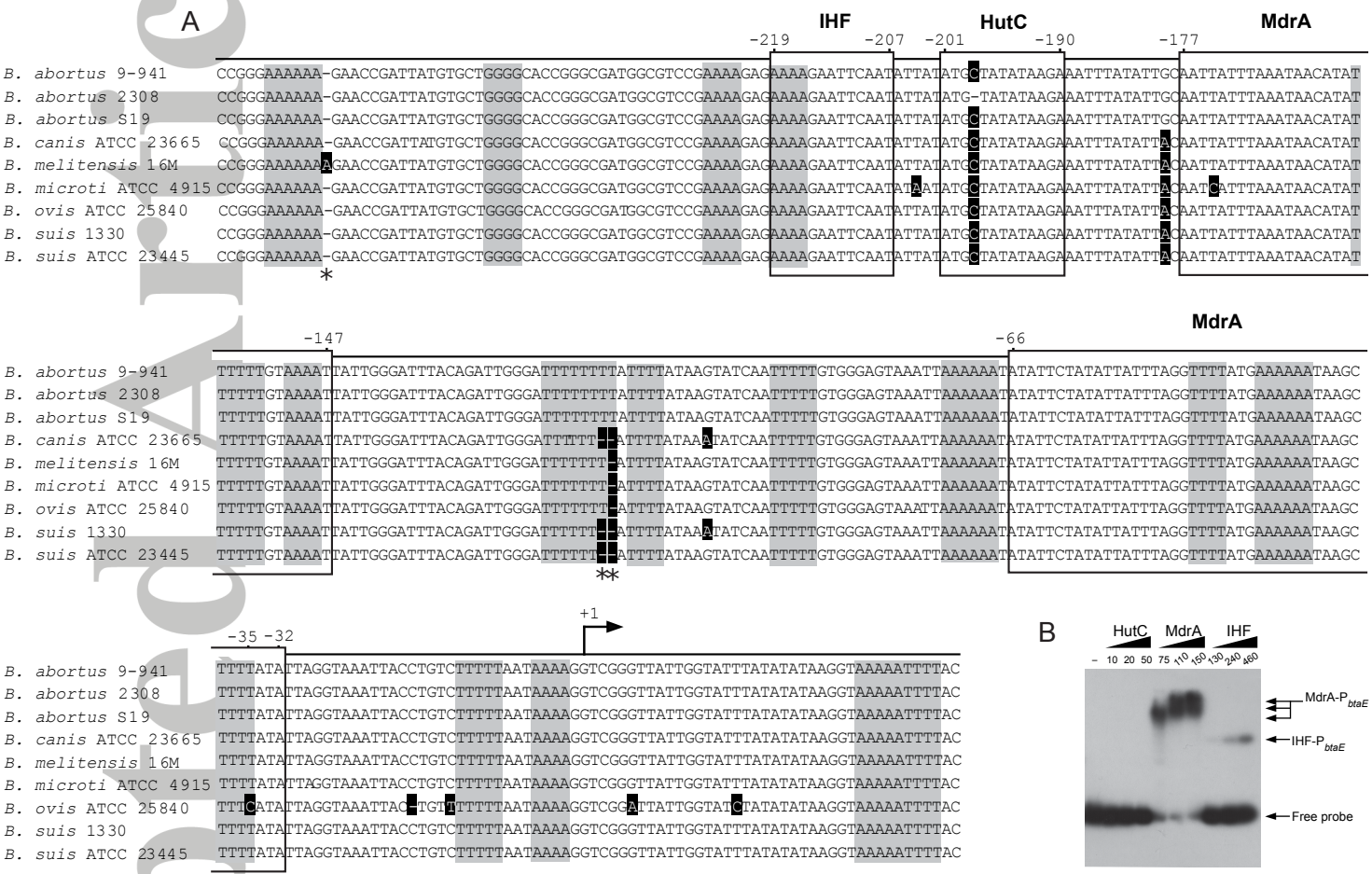
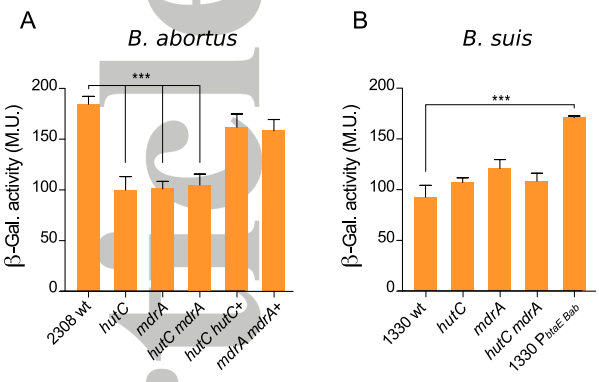


Figure 5



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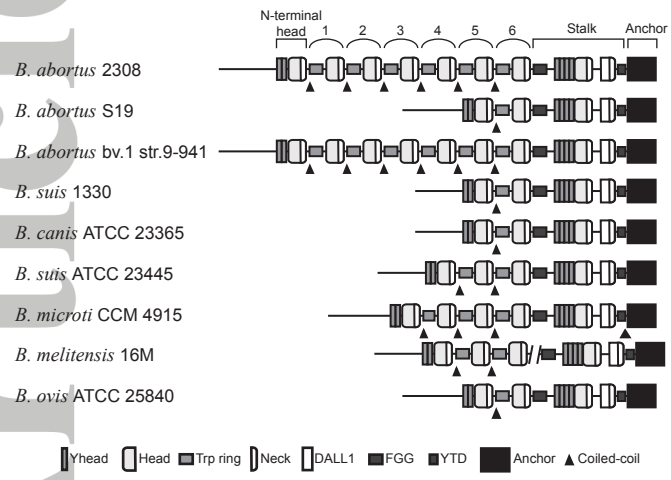
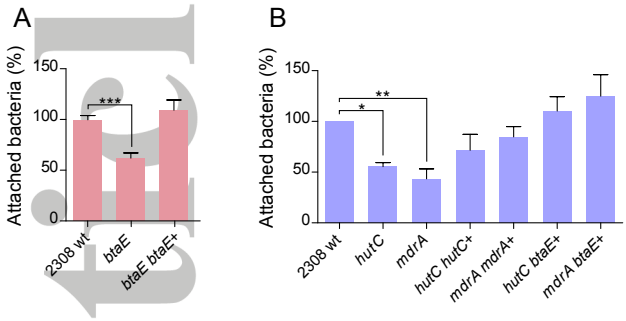
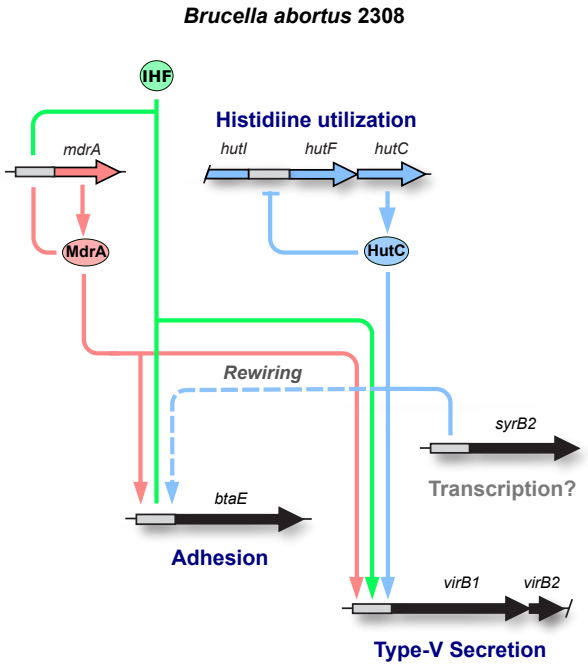


Figure 7



Graphical Abstract



Regulatory network plasticity is a key attribute underlying changes in bacterial gene expression and a source of phenotypic diversity to interact with the environment. Here, we identified a functional HutC-binding site upstream the *Brucella* adhesin *btaE*, which was generated *de novo* by a single point mutation in the *Brucella abortus* 2308 virulent strain. This transcriptional rewiring event integrated *btaE* promoter activity and bacterial adhesion into an ancient regulatory network controlling both metabolic and virulence genes, probably as a result of nucleotide variability associated to intrinsic *btaE* promoter structural features.