Review
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Regulation of virulence in *Brucella*: an eclectic repertoire of transcription factors defines the complex architecture of the *virB* promoter

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Many intracellular bacterial pathogens use type IV secretion systems to deliver effector molecules and subvert the eukaryotic host cell defenses. The genus *Brucella* comprises facultative intracellular bacteria that cause brucellosis, a disease affecting a wide range of mammals including humans. The *virB* operon codes for a type IV secretion system that plays a central role in intracellular survival and replication of *Brucella* within the host. Expression of the *virB* genes is under the control of various transcription factors that allow this system to respond to different types of environmental signals, and display binding site structures and arrangements that define the intrinsic complexity of the *virB* promoter. This review focuses on summarizing the current state of research concerning regulation of the *Brucella virB* operon, with special emphasis on describing the nature and function of the implicated regulatory elements and examining the involved protein–DNA interactions.

Type IV secretion systems (T4SSs) are multiprotein structures responsible for the secretion of different kinds of molecules across the bacterial cell envelope. The largest subfamily of T4SSs consists of machinery specialized for translocation of protein–DNA complexes in plasmid conjugation (e.g., Tra system of plasmids pR388, pRP4 and pKM101) or in transfer of oncogenic T-DNA (*Agrobacterium tumefaciens* VirB system) [1]. By contrast, other T4SSs have evolved to secrete specific protein substrates known as effectors, which participate in the establishment of the infection process of many bacterial pathogens. Owing to the prominent role of these machineries in the interaction with eukaryotic hosts, T4SS mutant strains display virulence-defective phenotypes in many bacterial genera such as *Brucella*, *Helicobacter*, *Legionella* and *Bordetella* [2–7].

Brucella is a genus of α-2 proteobacteria belonging to the order Rhizobiales, together with close relatives, such as the plant pathogen *Agrobacterium* and the symbiotic nitrogen-fixing genus *Rhizobium*. *Brucella* spp., are responsible for brucellosis, a worldwide-distributed zoonotic disease that affects a wide range of wild and domestic mammals. In the animal reservoir, brucellosis produces clinical signs that compromise the reproductive ability of both males and females, leading to significant economic losses in endemic countries mainly caused by *Brucella* spp. affecting livestock [8]. In humans, this disease can be acquired through inhalation of aerosols, by direct contact of mucosa or skin wounds with body fluids from infected animals or by consumption of contaminated dairy products. Human brucellosis manifests itself as a debilitating disease that produces symptoms such as undulant fever, sweating, fatigue and weight loss. The absence of treatment at early stages of human brucellosis leads to chronicity, which is characterized by persisting foci of infection and osteoarticular, cardiovascular or neurologic complications [9].

Brucella is essentially an intracellular pathogen. Following uptake and systemic spread, *Brucella* is able to internalize, persist and replicate within a variety of cell types of the host. After internalization, the bacterium is enclosed within the so-called '*Brucella*-containing vacuole' (BCV), which rapidly acquires early endosomal markers, contacts the endoplasmic reticulum (ER) membranes, and transiently interacts with lysosomes [10–12]. In macrophages, this latter interaction is tightly controlled in such a way that, although *Brucella* prevents lysosomal-mediated degradation, limited fusion is achieved leading to a content exchange with lysosomes that is consistent with the observed luminal acidification of BCVs [12,13]. Subsequently, *Brucella*

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promotes the maturation of BCVs into the replication compartment, an ER-derived organelle where the bacterium starts replicating exponentially at 12 h postinfection (p.i.) [11]. This process is dependent on the activity of the VirB T4SS, which is coded in an operon that was named after the homologous system of *A. tumefaciens*. The *Brucella virB* mutants cannot sustain interactions with the ER, fail to promote the biogenesis of the replication compartment and are inevitably degraded in phagolysosomes [10,14]. According to the role of the *Brucella* T4SS in the control of intracellular trafficking, mutation of the *virB* genes abrogates the ability of both the zoonotic and nonzoonotic (e.g., *Brucella ovis*) species to survive within the mammalian host [3,5,15,16].

After the discovery of the *virB* operon, both the lack of evidence of plasmid conjugation in *Brucella* and the absence of genes coding for conjugative T4SS-associated coupling elements supported the idea that this secretion apparatus may be involved in the translocation of protein substrates, which could act as effectors within the eukaryotic host cell. By using different strategies, many laboratories have confirmed this hypothesis through the identification of several proteins that are secreted into the host cell cytoplasm in a VirB-dependent manner [17,18]. In addition, it was also found that the VirB system is directly or indirectly involved in the intracellular secretion of RicA, an effector protein that recruits the GTPase Rab2 into BCVs and probably participates in the subversion of the host cell intracellular trafficking [19].

The first analyses of regulation of *virB* expression in *Brucella* indicated that this system is under the control of mechanisms that modulate transcription of the *virB* genes both in culture media and during intracellular infection of the host cells [5,20,21]. This supported the hypothesis that cross-talk exists between this pathogen and its host, where specific internal or external signals could be sensed by the bacterium to modulate *virB* expression accordingly. Over recent years, significant progress has been made in the identification of regulatory elements that influence transcription of the *virB* genes. The contributions of different research groups revealed that regulation of this system is not simple, but instead is susceptible to modulation by a variety of transcriptional regulators. The aim of this review is to summarize the current knowledge of the transcription factors that directly control expression of the *Brucella* T4SS, with special emphasis on updating some of the recent advances and discussing the information that can be extracted from the protein–DNA molecular interactions described in the regulatory region of the *virB* genes.

A first overview

Regulation of the *virB* genes was initially studied in *Brucella abortus* and *Brucella suis*, two zoonotic species affecting cattle and swine, respectively. Experiments performed with *lacZ* transcriptional fusions showed that the *B. abortus virB* operon is maximally expressed at the stationary phase in bacterial cultures grown in rich media [5]. On the other hand, *B. suis* displays a different growth-phase *virB*-expression pattern, since in this species, expression of the *virB* genes is induced at the early exponential phase and repressed in the stationary phase, both in minimal acidified medium and also, to a lesser extent, in rich medium at neutral pH [20]. These apparently controversial results were subsequently confirmed by a more in-depth analysis that demonstrated that expression of the VirB proteins differs among *Brucella* species. Using western blot experiments, Rouot *et al.* observed that the VirB expression pattern of *Brucella melitensis*, *B. ovis* and *Brucella canis* (whose preferred hosts are goat, sheep and dogs, respectively) resembles that of *B. abortus*, thus differing from *B. suis* [21]. It is noteworthy that the DNA sequence of the entire *virB* promoter is identical in all these *Brucella* species, suggesting that the growth-phase expression pattern of *B. suis* may rely on differences inherent to transcriptional regulators that control expression of the VirB system. However, both the molecular basis of such differences and its possible significance in intracellular survival or host specificity remain unknown.

Unlike the different patterns of *virB* expression observed in rich medium, a common feature shared by all the studied *Brucella* species is that the *virB* genes are induced in bacteria incubated under nutrient-deprived and acidic conditions, which are parameters that resemble those of the intraphagosomal environment that *Brucella* encounters within the host cell [21]. Accordingly, it was observed that transcriptional activity of the *virB* promoter is induced shortly after internalization within macrophages, and such induction was impaired by neutralization of intraphagosomal pH [20]. This finding confirmed that low pH is one of the signals that trigger the *in vivo* expression of a virulence factor essential for the biogenesis of the replication compartment, which is consistent with early observations that acidification of BCVs is necessary

for intracellular survival of *Brucella* [13]. Taken together, these observations indicate that during evolution, *Brucella* have managed to cope with the bactericidal mechanisms of the host cell by positively exploiting specific steps of its intracellular trafficking, inducing expression of the *virB* genes in response to signals provided by limited fusion events between BCVs and lysosomes [12].

It was observed that the intracellular induction of *virB* expression is transient. Following rapid activation after internalization in macrophages, the transcriptional activity of the *B. abortus virB* promoter reaches a maximum level at 5 h p.i. and is subsequently repressed prior to the onset of bacterial replication (Figure 1) [22]. This pattern of intracellular *virB* expression was coincident with the kinetics of translocation of BPE123, one of the protein substrates secreted via the VirB system. As demonstrated by analyses of intracellular CyaA activity, translocation of a BPE123–CyaA fusion protein from *B. abortus* toward the host cell cytoplasm produced an increase of the levels of cAMP, which also reached a maximum level at 5 h p.i. [18]. This finding suggests that both translocation and activity of the VirB-secreted effectors within the host cell may be determined by the timing of expression of the *virB* operon, and that the function of the VirB system is probably no longer required after the replication compartment has been reached. This is in agreement with a previous statement that expression of the T4SS at later stages of intracellular trafficking may be detrimental for *Brucella*, since overexpression of the entire *virB* operon in a multicopy plasmid caused intracellular multiplication defects

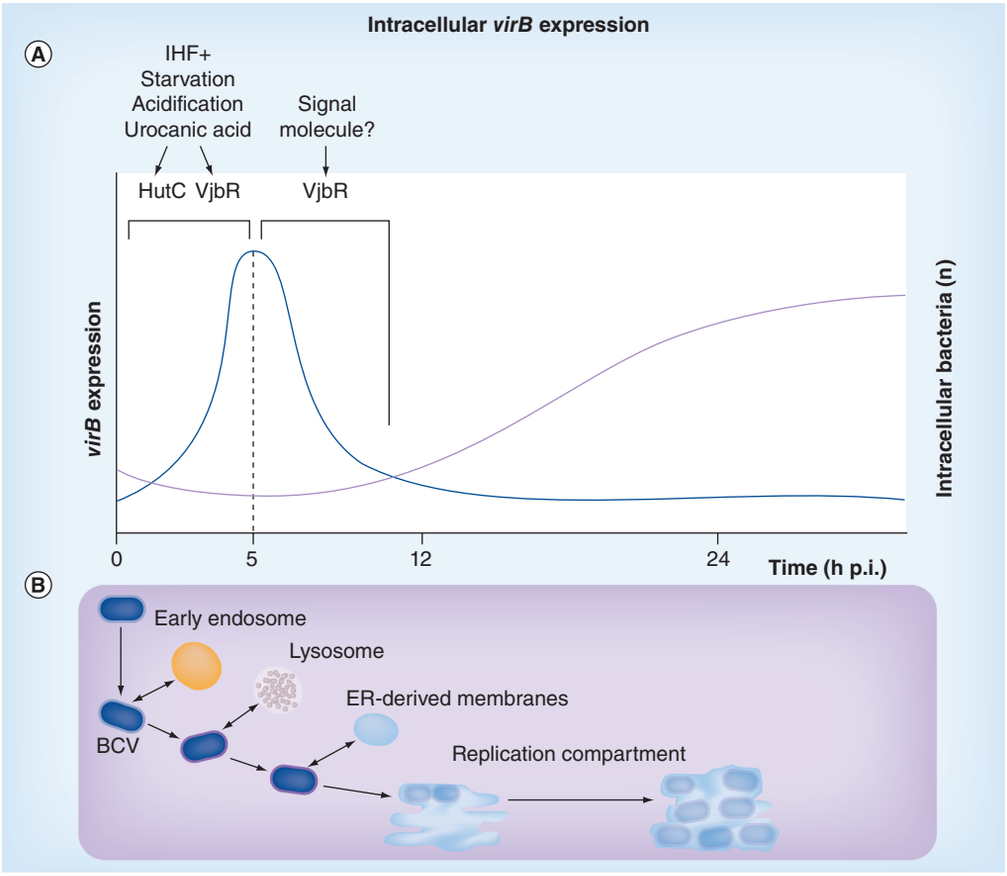

Figure 1. Intracellular regulation of *virB* expression. (A) Intracellular expression of the *Brucella abortus virB* genes. Blue line indicates transcriptional activity of the *virB* promoter based on different reports, indicating maximal promoter activity at 5 h p.i. [12,22,27]. Purple line indicates bacterial replication within the host cell. Positive regulatory contributions of VjbR, IHF and HutC are indicated. Possible negative effect of quorum sensing signaling molecules on VjbR activity is indicated. **(B)** Intracellular trafficking of *Brucella*. Following internalization the bacterium localizes within BCVs, which sequentially interact with vacuoles of the endocytic pathway, lysosomes and with ER-derived membranes. After 12 h p.i., the bacterium starts multiplying exponentially within the ER-derived replication compartment.
BCV: *Brucella*-containing vacuole; ER: Endoplasmic reticulum; IHF: Integration host factor; p.i.: Postinfection.

in *B. suis* [3]. Taken together, these findings revealed that expression of the *virB* operon is tightly controlled within the eukaryotic host cell, suggesting the existence of regulatory mechanisms that confer the capacity to rapidly activate or repress this secretion apparatus to *Brucella* and ensure bacterial intracellular survival and replication. The following sections will describe the nature and function of the regulatory elements of *Brucella* that were found to directly participate in the transcriptional regulation of *virB* expression.

VjbR

VjbR was identified by Letesson's group in a screening of mutants of *B. melitensis* defective for intracellular replication [23]. This transcription factor acts as the main regulator controlling expression of the *virB* operon and also modulates expression of hundreds of genes [24,25]. It was observed that deletion of *vjbR* abrogates both expression and function of the *Brucella* VirB system, displaying intracellular replication defects and a reduction of virulence in the mouse infection model [23].

VjbR belongs to the LuxR family, a group of transcriptional regulators involved in a cell-to-cell communication process called quorum sensing (QS). The QS system allows bacteria to sense changes in population density and coordinate adaptive responses accordingly, which involve regulation of various bacterial physiological functions, such as conjugation, biofilm formation, motility, symbiosis and virulence [26].

The canonical QS systems of Gram-negative bacteria act by means of a LuxI component that catalyzes the synthesis of diffusible *N*-acyl-homoserine lactone (AHL) signaling molecules. In an exponentially growing culture, the concentration of this autoinducer molecule increases proportionally to the bacterial cell density. After reaching a threshold concentration, AHL interacts with the LuxR response regulator, which undergoes a conformational change that enables it to bind DNA and modulate transcription of the target genes. By contrast, VjbR belongs to a small class of LuxR-type regulators that bind to DNA and regulate transcription in the absence of any autoinducer molecule. Moreover, as it occurs with the LuxR homologs ExpR of *Erwinia chrysanthemi* and EsaR of *Pantoea stewartii*, the addition of AHL dissociates VjbR from DNA, thus abrogating its positive regulatory activity on the *Brucella virB* genes [27]. In addition to this distinctive feature, many other observations raised interesting issues regarding functionality of AHL on the VjbR-mediated regulation of the *virB* promoter.

In *Brucella,* genes encoding enzymes that synthesize QS-related signaling molecules have not been identified, which places VjbR into the group of the so-called orphan LuxR-type regulators [28]. These transcription factors are not associated with any LuxI-type AHL synthase in the genome, but retain the ability to respond to AHL produced by the same microbe or by other bacterial species [29]. However, interestingly, the same group that originally described VjbR also isolated a *N*-dodecanoyl-AHL (C_{12}-AHL), which was detected at low levels in culture supernatants of *B. melitensis* incubated under specific conditions in a defined minimal medium [30]. However, despite extensive efforts by different laboratories, no C_{12}-AHL synthases have been described so far, and the identity of novel pathways for the production of QS signaling molecules in *Brucella* remains elusive.

Hitherto, a possible mechanism involving negative modulation of the activity of VjbR by a QS-related signaling molecule has been addressed, which could be responsible for the strong downregulation of the *virB* genes observed in intracellular bacteria after 5 h p.i. On the other hand, given the particular features of VjbR that allow it to bind to DNA and activate transcription in the absence of any signal, it was speculated that mechanisms of modulation of its positive regulatory activity probably exist. Regarding this possibility, a restricted set of conditions that, when they converge, trigger the expression of VjbR in cultured bacteria was recently identified [31]. These parameters include starvation and a specific pH value of 5.5, which resembles the conditions that *Brucella* encounters during the first stages of intracellular trafficking. Additionally, the presence of urocanic acid is also required. This metabolite is involved in the induction of HutC, a transcriptional regulator that also directly participates in the regulation of intracellular *virB* expression (see below). These findings indicate that modulation of the positive regulatory activity of VjbR could be achieved through regulation of the synthesis of the VjbR protein itself. It was observed that starvation, pH 5.5 and urocanic acid trigger expression of the VjbR protein through a post-transcriptional regulatory mechanism, which may confer to *Brucella* the ability to rapidly express this regulator without involving mechanisms of activation of the *vjbR* promoter [31]. These observations support a model whereby unspecific spatiotemporal expression of VjbR target genes could be prevented, and

may also explain why the *virB* genes are rapidly induced following internalization into the host cell in a VjbR-dependent manner.

The peculiarities of the VjbR-mediated regulation of the *virB* genes are not limited to positive and negative inputs on the activity of the LuxR-type regulator, but also involve unusual features regarding the interaction between this transcription factor and its target DNA sequence at the *virB* promoter. Using electrophoretic mobility shift assays (EMSAs), de Jong *et al.* reported that VjbR directly interacts with both the *virB* promoter and *virB1–virB2* intergenic regions [17]. The authors hypothesized that VjbR binds to a putative palindromic *lux* box-like element centered at position -37 relative to the transcription start site of the *virB* promoter, which is similar to the binding site consensus sequence for the well-studied LuxR-type regulator TraR of *A. tumefaciens* [17]. However, it was subsequently demonstrated, by DNase I footprinting, that VjbR recognizes a sequence that is located far upstream in the *virB* promoter at position -94 (Figure 2) [27]. Moreover, instead of being palindromic, the VjbR-binding site showed an unusual structure resembling a 'hemisite', which contains a 9-bp long sequence (GCCCCCTCA) identical to a half-binding site of MrtR, a LuxR-type regulator of *Mesorhizobium tianshanense* [27,32]. It was observed that this 9-bp sequence was necessary, but not sufficient, for VjbR to bind DNA, indicating that currently unidentified flanking sequences may also be important for VjbR to bind the *virB* promoter and other targets in the genome [27].

Both MrtR and its well-studied homologs CinR and BisR of *Rhizobium leguminosarum* are implicated in important functions such as symbiotic nodulation, nitrogen fixation and conjugation, respectively [33–35]. Additionally, all these LuxR-type regulators display similar palindromic target DNA sequences [32,36]. Thus, it is intriguing that the ortholog VjbR has evolved in *Brucella* to regulate expression of an important virulence factor through binding to a half-binding site, which led us to speculate about the possible adaptive advantages inherent in such a structure. The data obtained from DNase I footprinting experiments indicated that the affinity of VjbR for the binding to the *virB* promoter is relatively low, since the protected region was observed at a concentration of VjbR as high as 300 nM [27], which is considerably higher than that required for the binding of other LuxR-type

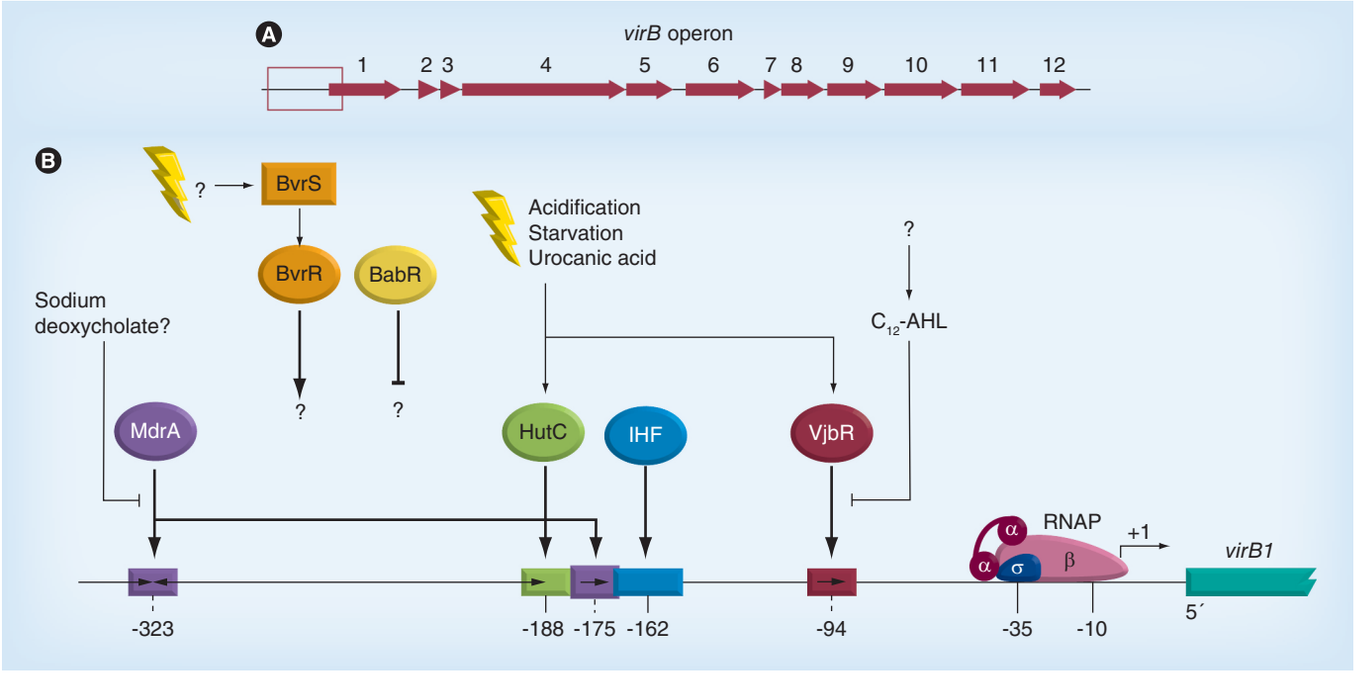

Figure 2. Architecture of the *virB* promoter. (A) *Brucella abortus virB* operon and its promoter region. Red arrows indicate the *virB1–12* genes. Rectangle indicates the inset detailed in **(B)** the *B. abortus virB* promoter. Binding sites for MdrA, HutC, IHF and VjbR are indicated as rectangles. Binding site-opposing arrows indicate palindromic sequences. Single binding site arrows indicate 'hemisite'-target DNA sequences. Positions relative to the transcription start site (+1) are indicated. Thick arrows indicate direct positive regulatory activity. Thick truncated line indicates negative regulatory activity. The negative effect of C_{12}-AHL on VjbR or sodium deoxycholate on MdrA is indicated by thin truncated lines. The 5′ region of the *virB1* gene is indicated. α, β, and σ subunits of RNAP are schematized.
AHL: *N*-acyl-homoserine lactone; IHF: Integration host factor; RNAP: RNA polymerase holoenzyme.

regulators [37–39]. Moreover, using EMSA, we did not observe binding of VjbR to a probe containing sequences of the native *virB* promoter, even using much higher VjbR protein concentrations [27]. This indicated that not only affinity, but also stability of VjbR for binding DNA is low, which probably does not allow the protein–DNA complex to withstand the electrophoretic conditions during EMSA. Interestingly, when an entire palindromic MrtR-binding site was generated in the *virB* promoter by duplication of the sequence GCCCCCTCA, a considerable increase of affinity was observed, since the DNase I-protected region was observed at 120 nM VjbR [17]. Moreover, the palindromic MrtR-binding site stabilized the binding of the regulator in such a way that the protein–DNA complex could be observed by EMSA with 250 nM VjbR [27]. Therefore, these findings confirmed that the nature of the binding sites of MrtR and VjbR are highly related and indicated that, for some reason, the low-affinity hemisite architecture of the VjbR-binding site was selected over its ancestral palindromic target DNA-sequences. Probably, a weak interaction between VjbR and DNA is required during intracellular trafficking to rapidly turn off the expression of the *virB* promoter, which may facilitate dissociation of the regulator in response to a QS-related signaling molecule after 5 h p.i.

The VjbR-binding site of the *virB* promoter lies much further upstream than the target DNA-sequences of other α-proteobacterial LuxR-type positive regulators, whose positions relative to the transcription start site range from position -45 to -66 [40,41]. Thus, in contrast to the majority of LuxR-type positive regulators, which are thought to induce transcription by directly contacting the RNA polymerase holoenzyme (RNAP) core or its α-carboxyterminal domains, VjbR most likely acts on regulation of *virB* expression by means of a noncanonical mode of activation. Consequently, an additional factor may be required to assist VjbR in activating transcription of the *Brucella virB* operon, probably by inducing changes in the chromatin structure in such a way that allows interaction of VjbR with RNAP. As described in the following sections, many other transcriptional regulators of a different nature were found to directly regulate the *virB* genes, which could act in concert with this primary LuxR-type activator as part of fine-tuning the mechanism of regulation of *virB* expression.

Integration host factor

Using EMSA, affinity chromatography and mass spectrometry, Ugalde's group implemented a strategy that allowed the identification of different DNA-binding proteins that specifically interact with sequences of the *Brucella virB* promoter [22,42,43]. One of them, integration host factor (IHF), belongs to the family of the so-called nucleoid-associated factors [44]. This small heterodimeric protein is widely distributed among bacteria, and participates in the control of transcription of a large number of well-studied promoters in either a positive or negative manner. Generally, IHF acts as an architectural factor that binds and bends its target DNA sequence, introducing structural changes in the double helix that can affect the function of other regulatory proteins. In addition, it was also shown that in some cases, binding of IHF lowers the energy of activation for the open complex formation [45].

The IHF-binding site of the *Brucella virB* promoter is centered at position -162.5 relative to the transcription start site (**Figure 2**) [22]. DNase I footprinting experiments revealed that the IHF-protected region contains two overlapping sequences that partially match the *Escherichia coli* IHF-consensus motif and are arranged at opposite sites of the DNA helix (**Table 1**) [22]. As in many other systems, IHF is able to exert an architectural role in the *virB* promoter, since it affects the structure of DNA *in vitro* by inducing an apparent bending angle of 50° [22]. Hence, one may speculate that this remodeling of the DNA structure could be involved in assisting the activity of the primary activator VjbR to induce transcription of the *virB* genes. It has recently been reported that IHF mediates interaction between the LuxR-type regulator SmcR and RNAP, thus inducing transcription of the virulence-associated *Vibrio vulnificus vvpE* gene [46]. In the *Brucella virB* promoter, however, the IHF-binding site is located upstream of that of VjbR (**Figure 2**), which does not fit with the model proposed for regulation of the *V. vulnificus vvpE* gene and for other bacterial promoters where IHF bends DNA allowing contact of distantly bound transcription factors with RNAP [46,47]. Therefore, further research will be required to determine the mechanism whereby IHF regulates *virB* expression and to assess the specific effect it exerts on the primary activator VjbR, with the possible participation of additional regulatory proteins.

Experiments performed with single-copy transcriptional fusions between the *virB* promoter and *lacZ* revealed that IHF plays a relevant role in the control of transcription of the *virB* genes under different conditions. Disruption of the

IHF-binding site in the *virB* promoter leads to a misregulated intracellular *virB* expression pattern and prevents maximal promoter activity at 5 h p.i. [22]. Moreover, it produces severe intracellular multiplication defects, indicating that the IHF-mediated regulation of the *virB* genes is necessary for the normal intracellular trafficking of *Brucella*. On the other hand, the analysis of promoter activity under laboratory conditions showed that IHF participates in the control of the *virB* genes in bacteria cultured at neutral pH in both rich or minimal media, whereas it does not seem to play any role in acidic minimal medium [22]. This latter observation is not consistent with the fact that IHF exerts a regulatory activity on the *virB* promoter in intracellular bacteria, which are enclosed in a compartment that is known to undergo an acidification process leading to intravacuolar pH values of 4.0–4.5 [13]. Therefore, the different behaviors observed between intracellular- and laboratory-acidic conditions may be indicative of a sequential succession of events that take place within the host cell and result in an intracellular IHF-mediated regulation of *virB* expression. In intracellular bacteria, IHF probably intervenes by inducing structural changes in the DNA at stages prior to acidification, which may provide the substrate for the loading of regulatory proteins at the *virB* promoter that acts at subsequent steps of the changing environment of BCVs. As discussed below, expression of the *virB* genes is positively regulated by additional factors that could sequentially participate in the modulation of the promoter activity.

Table 1. Summary of characteristics of the direct transcriptional regulators of the *Brucella virB* promoter.

Regulatory protein	Family	DNA-recognition sequence/postion†	Binding site structural characteristics	Effect on intracellular *virB* promoter activity	Ligand	Virulence-associated phenotype		Ref.
						Cultured cells?	*Mouse infection model?*	
VjbR	LuxR	gccccctca (-94)	Hemisite. Identical to half-binding site of *Mesorhizobium tianshanense* MrtR (gccccctcagatgagggggggg)	ND	C_{12}-AHL	Yes	Yes	[23,27]
IHF	Nucleoid-associated proteins	taaaaaagaatttt/ tattagaaaattc (-162.5)	Similar to *Escherichia coli* IHF-consensus motif (watcaannnnttr)	Yes	–	Yes	ND	[22]
HutC	GntR	cttgtatataagat (-188)	Palindromic, imperfect. Similar to the palindromic consensus of the *Klebsiella* or *Pseudomonas* (cttgtatatacaag) and α-2 proteobacterial *hut* promoters (tatgtatatacata)	Yes	Urocanic acid	No	Yes	[42]
MdrA	MarR	atttttgttaaa (site I, -175); (site II, -322)	Site I: hemisite Site II: palindromic	ND	Deoxycholate	No	ND	[43]
BvrR	OmpR	ND	–	ND	–	Yes	Yes	[65,67]
BabR/BlxR	LuxR	ND	–	ND	ND	Yes	Yes	[68,70]

†Relative to the distance between the center of the DNA-binding site and the virB *promoter transcription start site.*
–: No ligand has been found to be associated with this family of transcription factors; AHL: N-acyl-homoserine lactone; ND: Not determined.

HutC

The histidine utilization repressor HutC is the second regulatory protein of the *virB* genes that was identified by affinity-based purification and mass spectrometry [42]. It belongs to the large family of the GntR transcriptional regulators characterized by an N-terminal helix–turn–helix DNA-binding domain and a C-terminal effector-binding domain capable of interacting with specific effector molecules [48]. HutC acts as a repressor of the *hut* (histidine utilization) genes, which code for a widely conserved catabolic pathway that confers the ability to use histidine as a carbon source through conversion of this amino acid to glutamate, ammonia and formate or formamide [49]. In Gram-negative bacteria, the inducer of histidine utilization is urocanic acid, the first intermediate of the Hut catabolic pathway. This small molecule interacts with HutC and promotes its dissociation

from DNA, leading to derepression of the genes coding for the Hut catabolic enzymes and also *hutC* in the case of *Klebsiella*, *Pseudomonas* and *Brucella* [42,50,51].

In the *Brucella virB* promoter, HutC specifically interacts with a 14 bp imperfect palindromic sequence located at position -188 (Figure 2) [42]. Therefore, from such a distant location at the *virB* promoter, HutC would not be able to directly interact with RNAP, suggesting that the coactivator activity of HutC may rely on a concerted action with additional regulatory elements. As determined by affinity analyses performed with EMSA, the regulator binds to this target DNA sequence with high affinity, displaying an apparent dissociation constant (K_d) of 24 nM [42]. Interestingly, the HutC-binding site of the *Brucella virB* promoter is highly similar to that previously described in the *hut* promoters of *Klebsiella* and *Pseudomonas* [51,52].

In addition to the *virB* promoter, HutC is also able to bind to the *Brucella hut* promoter, but with 30-fold more affinity (K_d = 0.75 nM) [42]. Surprisingly, DNase I footprinting experiments demonstrated that the sequence recognized by HutC in the *hut* regulatory region differs from that of the *virB* promoter except for a 10 bp central core sequence, and displays a palindromic structure that is entirely conserved in all *hut* promoters of other closely related *Rhizobiales* (i.e., *Agrobacterium*, *Rhizobium* and *Ochrobactrum*) [42]. Consistent with the different affinities of the two HutC-binding sites characterized in *Brucella*, higher amounts of urocanic acid were required to dissociate HutC from the *hut* promoter (50 µM) than from the *virB* promoter (5 µM) [42].

Taken together, these findings constituted the first evidence that HutC is able to interact with regulatory regions other than that of the Hut pathway. They also revealed structural differences between two HutC-binding sites in the *virB* and *hut* promoters, which probably reflects different evolutionary origins: one derived from the ancestral α-proteobacterial *hut*-recognition motifs, whereas the other might have been acquired by a horizontal gene transfer event together with the acquisition of the entire *virB* operon in the genus *Brucella*.

Experiments performed with *virB* promoter–*lacZ* transcriptional fusions revealed that HutC exerts a positive regulatory role on *virB* expression in intracellular bacteria at 5 h p.i. The β-galactosidase activity levels in the *hutC*-deletion mutant background were 60% lower than that of the wild-type strain, indicating that HutC acts as a coactivator necessary for the fine-tuning intracellular regulation of the *virB* promoter [42]. *Brucella* is able to replicate intracellularly even in the absence of HutC, which indicates that this regulator performs an accessory regulatory role that serves to enhance expression of the *virB* genes under defined conditions. However, deletion of *hutC* caused a slight reduction of persistence in mice, showing a certain degree of contribution of HutC to *Brucella* virulence [42]. On the other hand, experiments performed with the *Brucella hut* promoter showed that HutC acts as a repressor of intracellular *hut* expression, thus confirming the negative regulatory role that HutC exerts on all studied Hut systems [42]. Altogether, these observations demonstrated that in *Brucella* this regulator can exert two different activities as a coactivator or as a repressor, depending on the target promoter. Moreover, experiments performed in cultured bacteria under specific laboratory conditions confirmed that HutC also coactivates *virB* expression whereas it represses the activity of the *hut* promoter [42]. Such conditions are also reminiscent of the intracellular environment found by *Brucella* within BCVs since they include starvation and an acidic pH value of 4.5. The presence of urocanic acid was an additional requirement necessary to observe a HutC-dependent *virB* expression in cultured bacteria, suggesting that induction of the Hut pathway is a prerequisite to coactivate transcription of the *virB* genes under these conditions [42]. At first glance, these latter results seemed to conflict with the fact that urocanic acid dissociates the regulator from DNA *in vitro*. However, the existence of two binding sites with different K_d values may support a model whereby the induction of the Hut pathway enables the sequential binding of HutC to both the *hut* and *virB* promoters. The model depicted in Figure 3 proposes that in the uninduced state, basal amounts of HutC would be sufficient to bind the *hut* promoter and repress transcription, but would not to be high enough to interact with the *virB* promoter. In the presence of an extracellular source of urocanic acid, the concentration of the inducer within the bacterial cytoplasm may increase until reaching amounts higher than 50 µM. This would dissociate HutC from the *hut* promoter, thus de-repressing the *hut* operon with the concomitant production of the Hut enzymes and also increasing the amounts of HutC itself. At this stage, the catabolic activity of the Hut enzymes could lower the

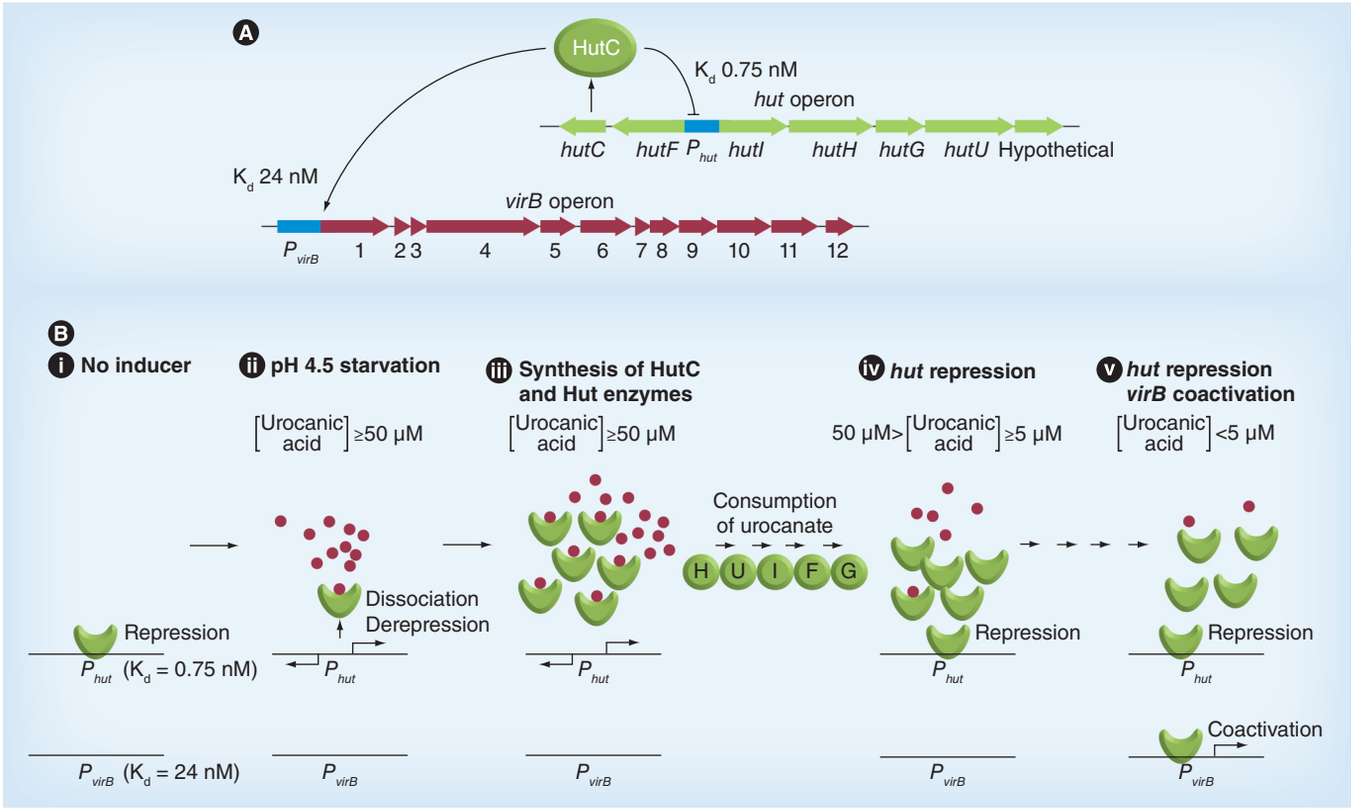

Figure 3. Proposed model of sequential interaction of HutC with the *virB* and *hut* promoters. (A) *hut* and *virB* operons. Green arrows represent the *hut* genes. Red arrows represent the *virB1–12* genes. K_d for the binding of HutC to each promoter is indicated. Truncated line indicates negative regulatory activity of HutC upon P_{hut}. Arrow indicates positive regulatory activity of HutC upon P_{virB}. **(B)** Proposed mechanism of urocanic acid-mediated induction of the Hut system and delayed coactivation of the *virB* operon. **(i)** Previous to induction of the Hut system, basal levels of the HutC protein are enough to bind to P_{hut} and repress transcription without affecting activity of P_{virB}. **(ii)** Intracellular accumulation of the inducer dissociates HutC from P_{hut}. **(iii)** Derepression of the *hut* operon induces the synthesis of the Hut enzymes and the regulator HutC, thus allowing catabolic degradation of urocanic acid. **(iv)** A decrease of the levels of urocanic acid to concentrations between 5 and 50 µM allow interaction of HutC with P_{hut}. **(v)** A further decrease of urocanic acid concentration below 5 µM allows HutC to interact with P_{virB} and coactivate *virB* expression.
K_d: Dissociation constant; P_{hut}: *hut* promoter; P_{virB}: *virB* promoter.

concentration of urocanic acid until reaching an equilibrium concentration, which may be determined by the rates of incorporation and consumption of the inducer molecule. Eventually, the equilibrium concentration of urocanic acid could be set below 5 µM, allowing HutC to bind the *virB* promoter and act as a coactivator of *virB* expression. Therefore, based on the biochemical features of the interaction of HutC with both promoters, this model represents a possible mechanism where the induction of the Hut pathway triggers a delayed enhancement of expression of the *virB* operon. Given the experimental evidence of a regulatory link between the Hut and VirB systems, one could speculate that the generation of a HutC-binding site in the *virB* promoter was, for some reason, positively selected to enhance the transcriptional activity in response to a stimulus provided by an extracellular supply of urocanic acid. Interestingly, it was previously shown that *Brucella* is able to incorporate and metabolize urocanic acid as efficiently as several other primary carbon sources but not histidine [42,53,54]. Moreover, since the discovery of the involvement of this metabolite in the regulation of *virB* expression at acidic pH, it was found that urocanic acid can also participate in regulation of VjbR and other virulence-associated factors of *Brucella*, as previously discussed [31]. It is known that urocanic acid accumulates in the skin of mammals to millimolar concentrations [55]. However, information regarding possible bacterial utilization of this metabolite is scanty and limited to a few examples of urocanic acid-degrading bacteria isolated from goat rumen or human skin [56,57]. Additionally, it was recently reported that urocanic acid can act as terminal electron acceptor and support anaerobic growth of *Shewanella oneidensis* [58]. Nevertheless, nothing is known about the availability of this metabolite in the

environment encountered by *Brucella* within the eukaryotic host, and further research will be required to investigate the possible role of urocanic acid in the *in vivo* gene regulation of this genus of facultative intracellular bacteria.

In the *virB* promoter, HutC specifically recognizes a sequence located adjacent to the IHF-binding site (Figure 2). EMSA experiments showed that HutC and IHF compete to bind to the *virB* promoter, probably as a consequence of steric hindrance owing to the close proximity of both binding sites [42]. Thus, the finding of two different positive regulators that cannot bind simultaneously to the promoter argues for possible sequential participation of these proteins on intracellular *virB* expression. This is supported by observations that indicate that in cultured bacteria, IHF acts as a positive regulator of the *virB* genes at neutral pH, whereas HutC participates only at acidic pH in the presence of urocanic acid [22,42]. Thus, these transcription factors probably act at different stages of the changing intracellular environment found by *Brucella* as BCV maturation proceeds.

MdrA

The multiple antibiotic resistance regulator (MarR)-type deoxycholate-responsive activator MdrA is the third transcription factor that was identified by affinity chromatography and mass spectrometry. It belongs to the MarR family of transcription factors involved in regulation of virulence and bacterial responses to antibiotic stress [59]. Using EMSA, it was initially observed that MdrA binds with high affinity to a DNA probe that contains sequences corresponding to the upstream region of the *virB* promoter (positions -430 to -202) [43]. In addition, incubation of MdrA with a DNA probe corresponding to the downstream region (positions -201 to +24) also resulted in a specific protein–DNA complex, indicating that this regulator recognizes two different binding sites in the *virB* promoter, which is a trait that was also found in other promoters regulated by MarR-type transcription factors. DNase I footprinting experiments indicated that MdrA protects two regions centered at positions -175 and -323, which were designated as MdrA-binding sites I and II, respectively (Figure 2). The analysis of the protected regions indicated that the MdrA-binding site II contains two partially conserved 11-bp motifs arranged as inverted repeats with dyad symmetry (Table 1). On the other hand, the downstream-located MdrA-binding site I showed no obvious dyad symmetry, but contains a similar 11-bp motif located at the center of the protected region [43].

As shown in Figure 2, the MdrA-binding site I overlaps both the IHF- and HutC-binding sites. According to these overlapping target DNA sequences, it was also observed that MdrA competes with IHF and HutC to bind to the promoter [43]. Interestingly, the superposition of the HutC- and MdrA-binding sites probably accounts for the functional interplay between these two transcription factors. Determination of the regulatory role of MdrA on *virB* expression has proven elusive owing to the functional redundancy between MdrA and HutC, since deletion of *mdrA* produced no effect on *virB* expression unless it was analyzed in a Δ*hutC* mutant background [43]. These redundant regulatory roles could be related to the fact that the center of both target DNA sequences recognized by MdrA (an 11-bp-long motif hemisite) or by HutC (a 12-bp-long nonperfect palindromic sequence) are spaced by 10 bp, which corresponds to one helical turn of DNA. This suggests that both regulators bind to the same side of the DNA double helix, which would enable MdrA or HutC to interact with the machinery involved in the activation of the *virB* expression with an equivalent orientation. Moreover, experiments performed by EMSA with different probes showed that, unlike IHF, both HutC and MdrA do not induce DNA bending upon binding to the *virB* promoter, thus suggesting that these latter two regulators exert similar structural roles [42]. Thus, both MdrA and HutC bind to the *virB* promoter at adjacent target DNA sequences, induce similar protein–DNA complex structures, and positively modulate expression of the *virB* genes to similar extents in a redundant manner. This allows speculation that the MdrA-mediated regulation of *virB* expression may be the result of an adaptation to enhance expression of the VirB T4SS when HutC is not acting.

The MdrA protein was detected in cultures of *B. abortus* at the exponential, but not in the stationary phase of growth in rich medium (tryptic soy broth). Accordingly, it was observed that MdrA acts as a coactivator of *virB* expression in cultured bacteria at the exponential phase of the growth in rich medium [43], or when bacteria are shifted to acidified minimal media from these initial conditions [Sieira R, Unpublished Data]. However, no MdrA dependence was observed for *virB* expression in intracellular bacteria in J774 macrophages and, therefore, the *in vivo* conditions in which MdrA intervenes in regulation of the *virB* genes remain to be determined.

Many members of the MarR family have the ability to bind specific small molecules that dissociate the regulator from the DNA with a consequent modulation of gene expression. Similar to that observed in MarR of *Salmonella enterica* serovar Typhimurium [60], MdrA interacts with sodium deoxycholate [43]. Micromolar concentrations of this component of bile specifically dissociate MdrA from DNA *in vitro*, whereas it does not have any effect on the binding of IHF or HutC. This leaves open the possibility that MdrA may sense the presence of deoxycholate and modulate *virB* expression accordingly. However, no effect on *virB* promoter activity was observed by the addition of sodium deoxycholate to bacteria cultured in rich medium at neutral pH, whereas in minimal acidified media-cultured bacteria, this bile salt produced a toxic effect that prevented an assessment of its possible participation on regulation of *virB* expression. Therefore, despite previous evidence indicating that *Brucella* is adapted for survival in environments in the presence of bile salts [61–63], we did not observe an MdrA-mediated effect of sodium deoxycholate in this system, and further research will be needed to assay additional conditions that could mimic some stage of the host infection where *Brucella* could be exposed to bile salts. On the other hand, the possibility that MdrA detects other cognate ligands cannot be ruled out.

As described above, MdrA binds far upstream of the transcription start site; therefore, additional factors may act in concert with this protein to achieve an MdrA-mediated enhancement of *virB* expression. As this situation is equivalent to its redundant partner HutC, it could be speculated that MdrA and HutC share the same regulatory mechanism of modulation of *virB* expression.

BvrR

The response regulator BvrR and the cognate sensor kinase BvrS constitute a two-component system (TCS) that plays a key role in maintaining the outer membrane homeostasis in *Brucella* [64]. TCSs are signal transduction systems that sense specific environmental stimuli and trigger autophosphorylation of the sensor kinase, which then transfers the phosphoryl group to the response regulator, thus activating this component to bind to its target promoters and induce transcriptional responses. To date, the specific environmental signal perceived by sensor histidine kinase BvrS has not yet been identified. However, many studies have shed light on the function of the TCS BvrR/BvrS, and the contribution of this system to both virulence and intracellular mutliplication has been well established.

Mutations of the BvrS/BvrR system lead to an increased sensitivity to polycations and surfactants, and produce pleiotropic effects that severely affect the intracellular trafficking and replication of *Brucella* within the host cell [64]. A transcriptome analysis of the *bvrR* mutant has revealed that this TCS directly or indirectly affects transcription of 127 loci including genes coding for outer membrane proteins, genes related to lipopolysaccharide biosynthesis and also affected expression of seven transcriptional regulators including VjbR [65]. Additionally, it was reported that both the VirB proteins and the levels of *virB* operon mRNA are diminished in a *B. abortus bvrR* mutant [65]. Moreover, as revealed by pull-down experiments that argue for a specific interaction between BvrS and the *virB* promoter, this protein has recently joined the list of transcription factors that directly control the expression of the *virB* genes [66]. These findings indicate that expression of the VirB T4SS is linked to a regulatory network responsible for the control of expression of many structural components of the *Brucella* cell envelope. However, the target DNA sequence of BvrR has not been described so far, and further research will be required to characterize the site of interaction between BvrR and the *virB* promoter and to determine whether this regulatory protein can act in concert with some of the aforementioned transcription factors directly involved in the control of *virB* expression.

BabR/BlxR

The transcription factor BabR (also known as BlxR) is the second QS-related regulator of *Brucella* that contains both the DNA- and AHL-binding domains characteristic of the LuxR-type proteins [67,68]. Transcriptomic and proteomic analyses revealed that VjbR and BabR/BlxR affect the transcription of overlapping sets of genes, and also influence expression of one another [24,25,67]. Deletion of *babR/blxR* affects virulence and intracellular survival of *Brucella*, although to a much lesser extent than VjbR [67]. It was observed that the addition of C_{12}-AHL produces an effect on the transcription profile of *Brucella* even in the Δ*vjbR* mutant, which suggests that BabR/BlxR may respond to this QS-related signaling molecule [24,25]. However, these observations are indirect, and further work will be required to determine

the precise role of C_{12}-AHL on modulation of BabR/BlxR activity.

Initially, BabR/BlxR was described in *B. melitensis* as a positive regulator of transcription of the *virB* operon and flagellar genes [67]. Additionally, in disagreement with this latter report, a subsequent analysis performed in the same species indicated that this regulator negatively modulates expression of the *virB* genes, and such discrepancies were attributed to differences between the experimental designs of both studies [24]. Subsequently, it was also reported that BabR/BlxR negatively modulates activity of the *virB* promoter in *B. abortus* [69]. The same authors showed by EMSA that BabR/BlxR specifically interacts with this promoter, thus demonstrating a direct negative regulatory activity on the VirB T4SS [69]. However, attempts to identify the BabR/BlxR-binding site by DNase I footprinting were unsuccessful, which leaves open the question of whether this LuxR-type transcription factor acts as a negative regulator by interfering with the positive function of some the previously described regulators of *virB* expression.

Conclusion & future perspective

Several lines of evidence revealed that a T4SS directly related to the pathogenesis of *Brucella* is under the control of varied regulatory pathways that affect activity of the *virB* promoter to different extents. Combination of the many transcriptional regulators directly acting on regulation of *virB* expression defines the intrinsic complexity of the *virB* promoter region, and accounts for the ability of this system to integrate environmental signals of a different nature.

The LuxR-type primary activator of the *virB* genes displays a mode of regulation that substantially differs from the classical QS systems. The positive regulatory activity of VjbR could be activated by mechanisms that trigger synthesis of the regulator itself, whereas its DNA-binding activity is negatively modulated by an AHL with a consequent downregulation of VjbR-mediated gene expression [23,27,31]. As VjbR is thought to act prior to the onset of the intracellular replication of *Brucella*, a QS-related signaling molecule may not act at the level of detection of changes in the bacterial population within BCVs. Taking into account the low diffusion rate of AHL with long acyl groups, it was hypothesized that an C_{12}-AHL signaling molecule could accumulate within the vacuole and serve to monitor the confinement state of *Brucella* during the early stages of intracellular trafficking [70].

In addition to VjbR, other regulatory elements were shown to play a role in fine tuning the transcriptional output of the *virB* operon by inducing structural changes in DNA or by enhancing promoter activity in response to specific environmental signals. One such signal is related to the induction of histidine catabolism, which was evidenced by the identification of HutC with affinity-based isolation procedures [42]. This regulatory link represents a connection between metabolism and regulation of *virB* expression, and was also shown to be involved in the control of expression of VjbR and other virulence-associated proteins [31]. However, to date, the physiological meaning of the link between urocanic acid and *virB* gene regulation remains elusive owing to limited information available about this metabolite in the context of an intracellular pathogen, and it will be worth investigating the adaptive benefit underlying such regulatory relationship. Although a direct regulatory effect on *virB* expression was not observed for sodium deoxycholate, this bile salt was able to dissociate the positive regulator MdrA from DNA *in vitro*, which represents a possible additional regulatory input directly affecting the *virB* promoter activity in response to environmental signals coming from the host [43]. The extracellular stimuli perceived by the TCS BvrR/BvrS have still not been determined. Nevertheless, the evidence available to date leads to the assumption that this signal transduction system is activated in response to environmental conditions that globally affect the bacterial cell wall components, which is consistent with a direct BvrR/BvrS-mediated regulation of a T4SS that is assembled into a macromolecular complex spanning both the inner and outer membranes. The aim of this review was to perform an analysis of transcription factors directly affecting the *virB* promoter activity. However, in addition to this level of control, it has been reported that the stringent response regulator RelA/SpoT homolog Rsh and components of the phosphotransferase system also modulate expression of the *virB* genes [71,72]. These findings revealed that regulation of the VirB T4SS is also linked to mechanisms affecting global gene expression in response to stressful conditions, starvation, and carbon and nitrogen metabolism. Moreover, it has recently been reported that post-transcriptional regulatory mechanisms are directly or indirectly involved in the modulation of expression of the *virB* genes [31,69]. According to all these observations, it may be speculated that codependence

on many transcriptional regulators and the involvement of post-transcriptional mechanisms could allow for the precise control of the expression levels of the VirB T4SS in response to both metabolic and environmental cues detected by the bacterium during the infection process.

Although many advances have been made in deciphering the regulatory pathways involved in regulation of *virB* expression in *Brucella*, many questions still need to be addressed. To date, it is not known how the primary activator VjbR is enabled to activate the *virB* promoter from a distant position. The discovery of additional activators further increase the complexity for ascertaining the answers to those questions, since the target DNA sequences identified so far are located distantly from the transcription start site. As the list of transcriptional regulators that directly bind the *virB* promoter continues to grow, so does the exciting expectation for discovering the molecular mechanism that constitutes the link between VjbR and the basal transcriptional machinery. The challenge for future research will be the characterization of the DNA-binding sites that remain to be identified and possible additional regulatory elements, which will allow us to achieve a comprehensive understanding of the regulatory mechanisms that govern the regulation of *virB* expression in *Brucella*.

Executive summary

The Brucella virB *operon*

- The *Brucella virB* operon is composed of 12 open reading frames that code for a type-IV secretion systems (T4SS), which constitutes perhaps the most important virulence factor of *Brucella*.
- Expression of the *Brucella virB* genes is tightly controlled and directly responds to different regulatory elements that modulate activity of the *virB* promoter to different extents.

VjbR

- This LuxR-type transcription factor is the main regulator of the *virB* operon and exhibits several unusual features, some of which are listed below.
- VjbR activates *virB* expression in the absence of any autoinducer molecule.
- The addition of C_{12}-*N*-acyl-homoserine lactone dissociates the VjbR from DNA and abrogates its regulatory activity.
- VjbR recognizes a 'hemisite' on the *virB* promoter, and activates *virB* expression from a position located 94 bp upstream of the transcription start site.

Integration host factor

- This widely distributed small heterodimeric protein performs an architectural role and, as in many other systems, it is thought to support a chromatin structure necessary for the activity of other elements that modulate activity of the *virB* promoter.

HutC

- In *Brucella*, the transcriptional repressor of the *hut (*histidine utilization) gene, HutC, is able to interact with both the *hut* and *virB* promoters.
- Beside repressing transcription of the *hut* operon, HutC also acts as a coactivator necessary for the fine tuning of intracellular *virB* expression.
- This regulatory link constitutes a direct connection between histidine metabolism and regulation of the *Brucella* VirB T4SS.

MdrA

- This MarR-type transcription factor interacts with the *virB* promoter at a sequence that overlaps the HutC-binding site.
- It was observed that MdrA exerts a redundant regulatory role with HutC as a coactivator protein that enhances *virB* expression under defined conditions.
- The bile-component deoxycholate is able to dissociate MdrA from DNA. However, no relationship to virulence has yet been established for this compound.

BvrR

- The response regulator BvrR is part of a two-component system that controls transcription of many genes and plays a major role in maintaining the outer membrane homeostasis of *Brucella*.
- Recently, it was reported that BvrR directly participates in the regulation of expression of the *virB* genes, which links expression of this T4SS to a regulatory network responsible for the control of several structural components of the bacterial cell envelope.

BabR/BlxR

- BabR/BlxR is a second LuxR-type transcription factor of *Brucella*.
- This regulator is responsible for the control of transcription of a set of genes that overlaps the VjbR regulon, and it was also shown that BabR/BlxR and VjbR influence expression of one another.
- Recent analyses showed that in *Brucella abortus*, BabR/BlxR directly modulates expression of the *virB* genes in a negative manner.

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