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Restoring virulence to mutants lacking subunits of multiprotein machines: functional complementation of a *Brucella virB5* mutant

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ABSTRACT

Complementation for virulence of a non-polar *virB5* mutant in *Brucella suis* 1330 was not possible using a pBBR-based plasmid but was with low copy vector pGL10. Presence of the pBBR-based replicon in wildtype *B. suis* had a dominant negative effect, leading to complete attenuation in J774 macrophages. This was due to pleiotropic effects on VirB protein expression due to multiple copies of the *virB* promoter region and over expression of VirB5. Functional complementation of mutants in individual components of multiprotein complexes such as bacterial secretion systems, are often problematic; this study highlights the importance of using a low copy vector.

1. Introduction

Type IV secretion systems (T4SS) are multiprotein complexes which can mediate the transfer of nucleoprotein and protein substrates across the bacterial cell envelope to bacterial recipients for plasmid spread, and to eukaryotic hosts for survival during establishment of pathogenic or symbiotic relationships [1]. T4SS are major virulence factors for several pathogens of plants and animals, including *Brucella*. *Brucella* causes brucellosis, a major bacterial zoonosis resulting in abortion in animals and a serious disease with chronic undulant fever in humans [2]. The virulence of *Brucella* requires its VirB T4SS, which is essential for the establishment of its intracellular niche in macrophages and epithelial cells [3–5]. The VirB system is equally important for virulence in the mouse model of infection [6] and in natural hosts [7], and thus a major target of study to unravel its precise role in virulence.

Structure/function studies have centred on the prototype VirB/D4 T4SS of the plant pathogen *Agrobacterium tumefaciens* and the Tra system of plasmid pKM101. The current model predicts a dynamic multiprotein machinery [8–10], with a pilus like structure exposed at the bacterial surface. This pilus is built up of the major subunit VirB2, and the minor component VirB5, which is localised at the pilus tip [11]. VirB5 is essential for *Brucella* virulence [12, this work]. We encountered difficulties in complementing a non-polar deletion *virB5* mutant using a pBBR-based vector. Here we show that both multiple copies of the *virB* operon promoter sequence and over expression of VirB5 had pleiotropic effects that disrupted VirB function.

2. Materials and methods

2.1. Bacterial strains and plasmids

All bacterial strains, plasmids and primers used in this study are listed in Table 1. Unless stated, *Brucella suis* was grown in Trypticase Soy (TS) broth, and *Escherichia coli* in Luria-Bertani (LB) broth. Expression from the *lac* promoter in $pBBR_{plac-virB5}$ was induced with 1 mM IPTG.

2.2. Plasmid constructions

The *virB5* gene was amplified using *B. suis* 1330 chromosomal DNA as a template with primers virB5-1 and virB5-2 (Table 1). For expression under control of the *virB* promoter, the PCR fragment was digested with *Nde*I/*Bam*HI and ligated into similarly digested pIN34 [13], named $pBBR_{pvirB}$ in the text for clarity, to yield pIN144 ($pBBR_{pvirB-virB5}$). Plasmid pIN146 ($pGL_{pvirB-virB5}$) was constructed by ligation of an *Xba*I/*Pst*I fragment of pIN144 into pGL10. For expression from the *lac* promoter, the *Nde*I/*Kpn*I

Abbreviations: bp, base pairs; CFU, colony forming units; hpi, hours post-infection; LB, Luria-Bertani; TS, Trypticase Soy; T4SS, type IV secretion system; MOI, multiplicity of infection

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Table 1
Strains, plasmids and primers used in this study.

	Synonym used in text	Relevant characteristics	Source or reference
B. suis strains			
1330	WT 1330	Wild type	ATCC 23444^T
bIN1929		1330 (pIN34)	This study
bIN1909		1330 (pIN144)	This study
bIN1900	*virB5*	1330 *virB5* non-polar mutant	This study
bIN1901		1330 *virB5*, (pIN 144)	This study
bIN1908		1330 *virB5*, (pIN 146)	This study
E. coli strain			
DH5α		*F*$^{-\varphi}$ *lacZΔM15 Δ(lacZYA-argF)U169 endA1 recA1 hsdR17* ($r_K^- m_K^+$) *supE44 thi-1 ΔgyrA96 relA1*	
Plasmids			
pIN11		Suicide vector, Sucr^s Cm^r	This study
pIN143		pIN11-derivative carrying 400 bp flanks of *virB5*, resulting in precise *virB5* deletion, Sucr^s Cm^r	This study
pBBR1-MCS		Broad host range plasmid, medium copy number (10–12 copies per genome equivalent [32] Cm^r	[33]
pIN34	$pBBR_{pvirB}$	pBBR1-MCS derived plasmid carrying 1.1 kb fragment of the *B. suis virB* promoter, Cm^r	[13]
pIN144	$pBBR_{pvirB-virB5}$	pIN34-derived plasmid containing *virB5* translationally fused to *pvirB*, Cm^r	This study
pGL10		Broad host range RK2-based low copy number cloning vector, low copy number (2–4 copies per genome equivalent), Km^r	[30]
pIN146	$pGL_{pvirB-virB5}$	pGL10 derivative carrying a *pvirB–virB5* expression unit, Km^r	This study
pSRKKm		pBBR1-derived plasmid containing a reengineered *lacI*^{Q}–promoter–*lac* operator complex in which cloned genes are strongly repressed in the absence of inducer	[14]
pIN164	$pBBR_{plac-virB5}$	pBBR derived plasmid containing *plac–virB5* expression unit, Km^r	This study
Primers			
virB5-1		GAGGAATTCCATATGAAGAAGATAATTCTCAGCTTC, *Nde*I site underlined	
virB5-2		GACGGGATCCTTAATAGGCGGCTTCCAGTGC, *Bam*HI site underlined	
B5MutUF		CGGGATCCCTACTGGATGGACGAGGCG, *Bam*HI site underlined	
B2MutUR		GGAATTCCATATGAGTGTCACCTTCCTGTTGATT, NdeI site underlined	
B5MutDF		GGAATTCCATATGCTACCCGACTAAGGAGTAGA, *Nde*I site underlined	
B5MutDR		GCTCTAGATGCGATGTTCCACCCCTTG, *Xba*I site underlined	

fragment of pIN144 was ligated into pSRKKm [14] to yield pIN164 ($pBBR_{plac-virB5}$).

A suicide vector was constructed by ligation of a 2.7 kb *Bam*HI/*Sph*I fragment of pSDM3005 [15] containing the *sacR* gene for negative selection, in pHSG398 (CmR) (TaKaRa Bio Inc), and named pIN11.

2.3. Construction of a virB5 non-polar mutant

A mutant of *B. suis* 1330 with a non-polar deletion of the *virB5* gene (bIN1900, *virB5* in the text) was constructed as described previously for *virB8* [13]. Both 500 base pair (bp) flanking regions of *virB5* were amplified by PCR so that ligation of the fragments would result in a precise deletion of *virB5* using primer sets B5MutUF/B2MutUR and B5MutDF/B5MutDR. The PCR fragments were digested with *Bam*HI/*Nde*I or *Nde*I/*Xba*I, respectively, and ligated simultaneously in *Bam*HI/*Xba*I digested suicide vector pIN11, resulting in pIN143. After introduction of pIN143 into 1330 by electroporation, chloramphenicol resistant colonies resulting from single crossover events were isolated and confirmed by PCR analysis. Positive colonies were grown overnight in LB medium (lacking NaCl and antibiotics) and plated on medium containing 6% (w/v) sucrose to select for a second excisional recombination event, resulting in precise deletion of *virB5*. Chloramphenicol sensitive/sucrose resistant colonies were subjected to PCR and Southern blotting, followed by sequence analysis to verify the precise deletion of *virB5*. The non-polar *virB5* mutant was named bIN1900.

2.4. Cell infections

Murine J774 A.1 macrophage-like cells (ATCC) were cultivated and infected with *Brucella* with a multiplicity of infection (MOI) of 50 in a standard gentamicin protection assay as described previously [3]. The number of colony forming units (CFU) per well for each time point was expressed as the geometric mean (±standard error of the mean, S.E.M.) of three wells. All experiments were performed at least 3 times. A Student's *t*-test (with two-tailed distribution and equal variance) was performed to determine whether two strains differed significantly ($P < 0.05$).

2.5. Analysis of VirB expression

To analyse VirB expression, *B. suis* strains were grown in minimal medium at pH 4.5 as described [16]. Western blot analysis was performed to detect VirB1, VirB5, VirB9 and VirB10; Bcsp31 was used as a control for equal loading.

3. Results and discussion

3.1. Successful complementation of a virB5 mutant to wild type virulence levels depends on plasmid copy number

We constructed a non-polar deletion of *virB5* (bIN1900), which was strongly attenuated for virulence in macrophages at 24 h post-infection (hpi) and 48 hpi (Fig. 1). However, we were unable to restore virulence when we complemented the *virB5* deletion mutant with the *virB5* gene under the control of the *virB* promoter using the medium copy number plasmid pBBR1-MCS ($pBBR_{pvirB-virB5}$) (Fig. 1), despite restoration of VirB5 production (Fig. 2c). In contrast, virulence was restored when the gene was carried on the low copy number plasmid pGL10 ($pGL_{pvirB-virB5}$; Fig. 1). Since genetic complementation studies with individual components of multiprotein complexes are a recurrent problem, we analysed this in more detail for VirB5.

3.2. Multiple copies of the virB promoter sequence and overproduction of VirB5 attenuate virulence of wild type B. suis

The expression of the *Brucella virB* operon is controlled through several layers of regulation [17–22]. We have previously suggested

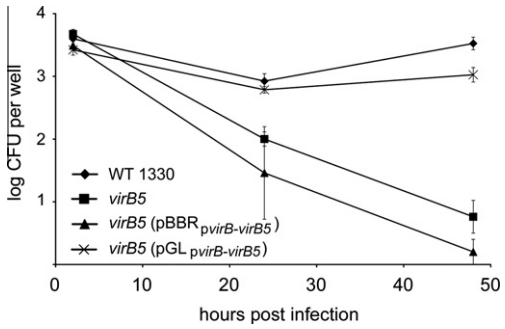

Fig. 1. $pGL_{pvirB-virB5}$ but not $pBBR_{pvirB-virB5}$ complements a *B. suis* 1330 *virB5* mutant. Replication kinetics of *B. suis* 1330, *virB5* mutant and complemented mutant (with $pBBR_{pvirB-virB5}$ or $pGL_{pvirB-virB5}$) in J774A.1 macrophages. A representative experiment is shown. Wild type 1330 differed significantly from the *virB5* mutant, and *virB5* ($pBBR_{pvirB-virB5}$) ($P = 0.003$, 0.084 at 24 hpi and $P = 5 \times 10^{-4}$, 2.5×10^{-5} at 48 hpi, respectively). $pGL_{pvirB-virB5}$ complemented the *virB5* mutant significantly ($P = 0.003$, 0.001 at 24 hpi and 48 hpi, respectively), although not to full virulence levels of the wild type (48 hpi ($P = 0.021$)). *virB5* ($pBBR_{pvirB-virB5}$) was as attenuated as *virB5* ($P = 0.53$, 0.267 at 24 hpi and 48 hpi, respectively).

that the presence of multiple copies of the *virB* promoter sequence might sequester regulatory factors essential for expression of the chromosomal *virB* operon or possibly of other genes that are co-regulated with the *virB* operon and essential for virulence [3]. A second possibility is that non-stoichiometric (high) levels of VirB5 could interfere with correct T4SS biogenesis and/or function [12], as shown for VirB6 of *A. tumefaciens* [23].

We introduced $pBBR_{pvirB-virB5}$ into wild type 1330, finding that it had a dominant negative effect, completely abolishing the virulence of the wild type strain (Fig. 2a). To determine the individual contribution of the presence of multiple *virB* promoter sequences that might result in the sequestration of transcription factors, we analysed the virulence of wild type 1330 carrying $pBBR_{pvirB}$, an identical pBBR-based plasmid with the *virB* promotor, but lacking the *virB5* coding region. This strain was also attenuated in J774 macrophages, although significantly less attenuated than wild type 1330 with plasmid $pBBR_{pvirB-virB5}$ (Fig. 2a). This clearly indicated that multiple promoter sequences partially contributed to the observed attenuation of 1330 ($pBBR_{pvirB-virB5}$), but that an additional effect of over expression of VirB5 contributed to the complete attenuation seen with $pBBR_{pvirB-virB5}$ and possibly the inability of $pBBR_{pvirB-virB5}$ to fully complement the *virB5* mutant. To further investigate the sequestration of transcription factors, we constructed plasmids carrying the putative binding sites for VjbR and HutC but saw no effects on virulence (data not shown), not unexpectedly due to the complex regulation of the *virB* operon.

3.3. Controlled expression of virB5 from a lac promoter partially complements the virB5 mutant

To further dissect the reason for the observed attenuation of wild type 1330 by the presence of $pBBR_{pvirB-virB5}$, we placed the *virB5* gene under the control of a tightly regulated *lac* promoter in pSRKKm [14], which would not sequester *virB* specific transcription factors. Macrophages were infected with *virB5* ($pBBR_{plac-virB5}$) and *virB5* expression was induced at different times with IPTG

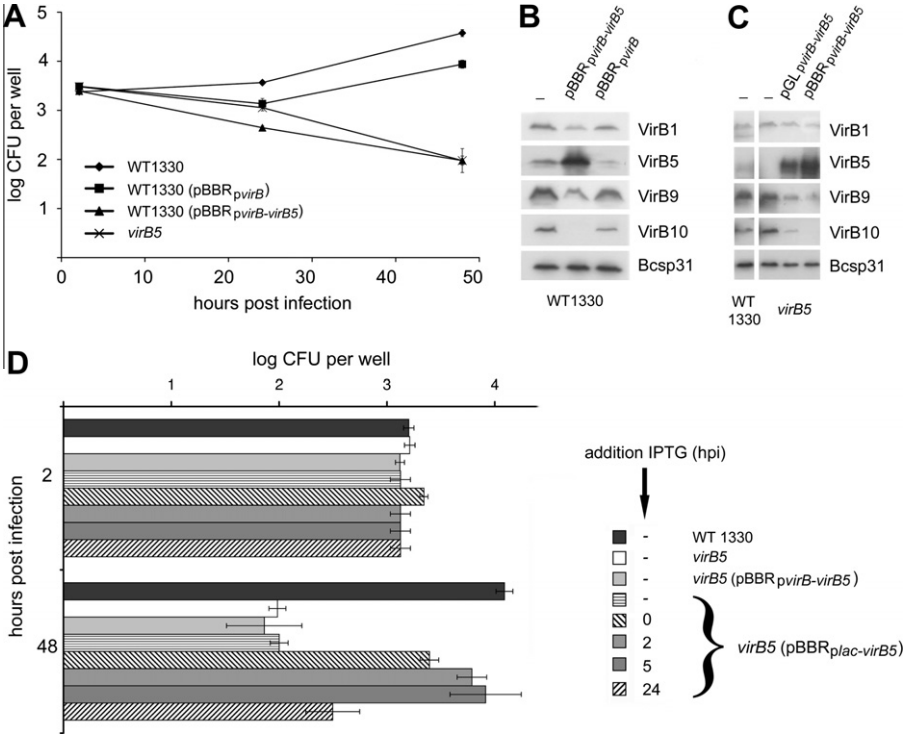

Fig. 2. Overproduction of VirB5 and presence of multiple copies of the *virB* promoter region attenuate wild type *B. suis*. (A) Replication kinetics of wild type 1330, *virB5*, 1330 ($pBBR_{pvirB}$) or ($pBBR_{pvirB-virB5}$) in J774A.1 macrophages. A representative experiment is shown. The *virB5* mutant and wild type 1330 ($pBBR_{pvirB-virB5}$) were significantly attenuated ($P = 0.001$, 2.7×10^{-5} at 24 hpi and $P = 2.5 \times 10^{-5}$, 5.2×10^{-4} at 48 hpi, respectively). 1330 is significantly more virulent than 1330 ($pBBR_{pvirB}$) ($P = 0.011$ at 24 hpi and $P = 0.001$ at 48 hpi). (B) Immuno blot analysis of wild type 1330, 1330 ($pBBR_{pvirB-virB5}$) and 1330 ($pBBR_{pvirB}$). All strains were grown under acid growth conditions. Expression was detected with polyclonal antisera raised against VirB5, VirB9, VirB10 and Bcsp31 (loading control). (C) Immuno blot analysis of wild type 1330, the *virB5* mutant, *virB5* ($pGL_{pvirB-virB5}$), and *virB5* ($pBBR_{pvirB-virB5}$). Expression was detected with polyclonal antisera raised against VirB5, VirB9, VirB10 and Bcsp31. D. Replication kinetics in J774 macrophages of wild type 1330, the *virB5* mutant, *virB5* ($pBBR_{pvirB-virB5}$) and *virB5* ($pBBR_{plac-virB5}$), the latter induced at different time points (0, 2, 5, and 24 hpi) with IPTG. At 48 hpi, wild type 1330 and *virB5* ($pBBR_{plac-virB5}$) induced at 0 hpi, 2 hpi and 5 hpi are significantly different from *virB5*, *virB5* ($pBBR_{plac-virB5}$) and *virB5* ($pBBR_{plac-virB5}$) non-induced or induced at 24 hpi ($P = 0.027$ between *virB5* ($pBBR_{plac-virB5}$) induced at 0 hpi and *virB5* ($pBBR_{plac-virB5}$) induced at 24 hpi).

(Fig. 2d). To restore virulence even partially, VirB5 production had to be induced within the first 5 h after infection, which is fitting with previous studies showing intracellular induction of the *virB* operon at 3–4 h after uptake, and the importance of early phagosome acidification to induce the *virB* operon [16,24]. Within those 5 h, better complementation correlated with later time points of induction of VirB5 expression, suggesting that either increasing levels of VirB5 reduce virulence or that virB5 expression from the *lac* promoter must be coordinated with induction of the rest of the chromosomal *virB* operon from its own promoter.

3.4. Pleiotropic effects on VirB protein expression during complementation

As several regulators have either positive or negative effects on *virB* expression by binding to specific sequences in the promoter region [17,25], we would expect that an effect on endogenous *virB* transcription by the presence of multiple *virB* sequences would result in a general reduction of *virB* expression in the wild type carrying $pBBR_{pvirB}$. Immunoblot analysis showed that in wild type 1330 ($pBBR_{pvirB}$), levels of VirB5, VirB9 and VirB10 were indeed slightly reduced (Fig. 2b). However, additional over expression of VirB5 in 1330 ($pBBR_{pvirB–virB5}$) led to a greater reduction of VirB9, and even undetectable VirB10 (Fig. 2b). This reduction in VirB protein levels correlates with the complete attenuation of 1330 ($pBBR_{pvirB–virB5}$) and the inability of $pBBR_{pvirB–virB5}$ to complement the *virB5* mutant. In contrast, in *virB5* ($pGL_{pvirB–virB5}$), with virulence restored to almost wild type levels, VirB5 and VirB10 levels were intermediate to those in 1330 and *virB5* ($pBBR_{pvirB–virB5}$) (Fig. 2c). Importantly, VirB10 was still detectable and VirB5 levels were still higher than those in wild type 1330, suggesting that some variation in VirB protein levels is tolerated to reach almost WT levels of complementation.

Other studies have shown that the assembly of a T4SS in the bacterial envelope is a complex process in which many different, often transitory, protein–protein interactions occur. Often T4SS genetic complementation studies are difficult and do not result in full functional complementation to wild type virulence levels [12,23,26]. The presence of one protein is often required to stabilize another; VirB5 was shown to interact in *Agrobacterium* with VirB9 and VirB10 [27,28] and co-expression of the T4SS components VirB7 and VirB8 is essential to restore virulence of individual null mutants [26]. In *Agrobacterium*, VirB10 plays an essential role in both substrate translocation and biogenesis of the VirB pilus [29]. Disturbance of its regulation or stability may have dramatic effects on T4SS function. Alternatively, an indirect effect on production of VirB9, which was shown in *A. tumefaciens* to be essential to stabilize VirB10 under specific conditions of low osmolarity [28], may play a role in the attenuation of our VirB5 overproducing strain. Overproduction of VirB5 might also result in mislocalisation of the protein at the pilus tip [11].

Our data highlight that the choice of promoter and plasmid replication origin are critical components to ensure optimal levels of protein of individual T4SS components and not to deregulate expression of the endogenous operon. The protein levels required to maintain stoichiometric levels; however, may be different for each T4SS component under investigation. An easy assay to determine whether the original multiprotein complex will be deregulated is to verify virulence of the wild type strain containing the complementing plasmid. A low copy plasmid, with the gene expressed from its natural promoter is effective in complementation of a *B. suis virB5* mutant. This approach has also been used with other proteins for which over expression may have inhibitory effects on bacterial physiology such as the CcrM protein [30]. An alternative way to ensure 'perfect' complementation is to recombine the complementing gene back into the chromosomal *virB* operon, a strategy used to complement a *B. abortus virB2* mutant [31]. However, this method will be too time consuming for studies requiring complementation with multiple variant alleles, and unfeasible for certain bacterial species that are difficult to manipulate.

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