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

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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 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.

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 pBBRpvirB–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 NdeI/BamHI and ligated into similarly digested pIN34 [13], named pBBRpvirB in the text for clarity, to yield pIN144 (pBBRpvirB–virB5). Plasmid pIN144 (pGLpvirB–virB5) was constructed by ligation of an XbaI/PstI fragment of pIN144 into pGL10. For expression from the lac promoter, the NdeI/KpnI

**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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fragment of plN144 was ligated into pSRKkm [14] to yield plN164 (pBBRplac–virB5).

A suicide vector was constructed by ligation of a 2.7 kb Bam HI/Xba I fragment of pSDM3005 [15] containing the sacR gene for negative selection, in pHSG398 (CmR) (TaKaRa Bio Inc), and named plN11.

2.3. Construction of a virB5 non-polar mutant

A mutant of B. suis 1330 with a non-polar deletion of the virB5 gene (binN1900, 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 BamHI/Ndel or Ndel/Xbol, respectively, and ligated simultaneously in BamHI/Xbol digested suicide vector plN11, resulting in plN143. After introduction of plN143 into 1330 by electroporation, chloramphenicol resistant colonies resulting from single crossover events were isolated and confirmed by PCR analysis.

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 (binN1900), 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 virB5 promoter using the medium copy number plasmid pBBR1-MCS (pBBRpvirB–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 (pGLpvirB–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...
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<sub>virB5</sub> 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<sub>virB5</sub>, an identical pBBR-based plasmid with the virB promoter, but lacking the virB<sub>5</sub> coding region. This strain was also attenuated in J774 macrophages, although significantly less attenuated than wild type 1330 with plasmid pBBR<sub>virB5</sub>–<sub>virB5</sub> (Fig. 2a). This clearly indicated that multiple promoter sequences partially contributed to the observed attenuation of 1330 (pBBR<sub>virB5</sub>–<sub>virB5</sub>), but that an additional effect of over expression of VirB5 contributed to the complete attenuation seen with pBBR<sub>virB5</sub>–<sub>virB5</sub> and possibly the inability of pBBR<sub>virB5</sub>–<sub>virB5</sub> 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<sub>virB5</sub>–<sub>virB5</sub>, we placed the virB5 gene under the control of a tightly regulated lac promoter in pSKKm<sub>14</sub>, which would not sequester virB specific transcription factors. Macrophages were infected with virB5 (pBBR<sub>lac</sub>–<sub>virB5</sub>) and virB5 expression was induced at different times with IPTG.
(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<sub>psppr</sub>. Immunoblot analysis showed that in wild type 1330 (pBBR<sub>psppr</sub>), levels of VirB5, VirB9 and VirB10 were indeed slightly reduced (Fig. 2b). However, additional over expression of VirB5 in 1330 (pBBR<sub>psppr</sub>-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<sub>psppr</sub>-virB<sub>5</sub>) and the inability of pBBR<sub>psppr</sub>-virB<sub>5</sub> to complement the virB5 mutant. In contrast, in virB5 (pGL<sub>psppr</sub>-virB<sub>5</sub>) with virulence restored to almost wild type levels, VirB5 and VirB10 levels were intermediate to those in 1330 and virB5 (pBBR<sub>psppr</sub>-virB<sub>5</sub>-virB<sub>10</sub>) (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 TASS in the bacterial envelope is a complex process in which many different, often transitory, protein–protein interactions occur. Often TASS 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 TASS 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 biosynthesis of the VirB pilus [29]. Disturbance of its regulation or stability may have dramatic effects on TASS 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 osmolality [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 TASS components and not to deregulate expression of the endogenous operon. The protein levels required to maintain stoichiometric levels; however, may be different for each TASS 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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