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Establishment of an entirely plasmid-based reverse genetics system for Bluetongue virus

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ABSTRACT

Bluetongue virus (BTV), the type species of the genus *Orbivirus* within the family *Reoviridae*, has a genome consisting of 10 linear double-stranded RNA genome segments. Current reverse genetics approaches for engineering the BTV genome rely upon *in vitro* synthesis of capped RNA transcripts from cloned cDNA corresponding to viral genome segments. In an effort to expand the utility of BTV reverse genetics, we constructed a reverse genetics vector containing a T7 RNA polymerase promoter, hepatitis delta ribozyme sequence and T7 RNA polymerase terminator sequence. Viable virus was recovered following transfection of mammalian cells, expressing T7 RNA polymerase, with 10 plasmid constructs representing the cloned BTV-1 genome. Furthermore, the plasmid-based reverse genetics system was used successfully to isolate viable cross-serotype reassortant viruses and a mutant virus containing a defined mutation in the replicating viral genome. The new reverse genetics platform established here for BTV is likely applicable to other orbiviruses.

Introduction

Bluetongue virus (BTV), an arbovirus transmitted by a few species of biting midges of the genus *Culicoides*, is the causative agent of bluetongue disease of domesticated and wild ruminants. Among domestic species, sheep is the most susceptible and most severely affected host, but cattle and wild ruminants serve as reservoirs for the virus (Wilson and Mellor, 2009). Bluetongue disease is endemic in many parts of the world, including Africa and some regions of Asia, Australia and America (Walton, 2004; MacLachlan, 2011). However, there have been increasingly frequent outbreaks in European countries due to a northern expansion of the virus, with several outbreaks also occurring in the Balkans (Purse et al., 2005; Mellor et al., 2008; MacLachlan and Guthrie, 2010). Due to its severity, economic impact and ability to spread rapidly from endemic regions, bluetongue disease is listed by the World Organization for Animal Health (OIE) as a notifiable disease of livestock.

BTV is a member of the *Orbivirus* genus in the *Reoviridae* family and has a genome consisting of 10 segments of linear double-stranded RNA (dsRNA), designated from segment 1 (S1) to S10 in decreasing order of size (Verwoerd et al., 1970). The viral genome encodes seven structural proteins and four non-structural proteins (NS1–NS4) (Roy, 2007; Belhouchet et al., 2011; Ratinier et al., 2011). Structural proteins VP1, VP3, VP4, VP6 and VP7 form the virus core particle, which is surrounded by an outer capsid layer composed of VP2 and VP5 (Verwoerd et al., 1972). Shortly after cell entry by the virus, the outer capsid proteins are removed and the transcriptionally active core particle is released into the host cell cytoplasm. Within the core particle, each of the dsRNA genome segments is repeatedly transcribed by the core-associated enzymes VP1 (RNA-dependent RNA polymerase), VP4 (capping enzyme) and VP6 (helicase), resulting in extrusion of newly synthesized, capped, viral single-stranded RNA (ssRNA). The extruded transcripts, in turn, function as templates for the synthesis of viral proteins and also act as templates for the synthesis of genomic dsRNA following their encapsidation in progeny viral cores (Mertens and Diprose, 2004; Patel and Roy, 2014).

The development of reverse genetics systems for all major groups of RNA- and DNA-containing viruses is considered to be one of the most transformative technological advances in virology, having allowed for considerable progress to be made in understanding multiple aspects of virus biology and pathogenesis. Despite variations in their molecular design and methodology, these reverse genetics systems all share a common feature, which is the availability of cloned cDNA copies of the viral genomes that can be genetically modified and manipulated to generate live viruses containing precisely engineered changes in their genomes (Bridgen, 2012). In contrast to other RNA viruses, the construction

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of reverse genetics systems for members of the *Reoviridae* family has proven to be challenging, owing in part to the technical complexity of manipulating multi-segmented genomes (Komoto and Taniguchi, 2013; Trask et al., 2013). Reoviruses, which contain a 10-segmented dsRNA genome, were the first members of the *Reoviridae* family for which a plasmid-based reverse genetics method was developed (Kobayashi et al., 2007). This was followed by the establishment of reverse genetics systems for different members of the genus *Orbivirus* such as BTV (Boyce et al., 2008; Matsuo and Roy, 2013), African horse sickness virus (Kaname et al., 2013) and, more recently, epizootic hemorrhagic disease virus (Yang et al., 2015). In contrast to the plasmid-based reverse genetics system established for reovirus, the reverse genetics systems developed for the above orbiviruses all rely on the use of *in vitro*-synthesized and capped T7 RNA transcripts from cloned cDNAs corresponding to viral genomic segments. In this approach, permissive cells are transfected with expression helper plasmids that synthesize the inner core proteins and two non-structural proteins (NS1 and NS2), followed by transfection of the cells with all 10 synthetic T7 transcripts.

Here we describe the establishment of an entirely plasmid-based reverse genetics system for the prototype orbivirus, BTV, in which viable virus was generated solely from cDNA clones. The performance of this system was validated by isolation of a viable mutant virus containing a targeted marker mutation introduced into the viral genome, as well as viable directed cross-serotype reassortant viruses.

Results

Construction of a T7 RNA polymerase-based reverse genetics plasmid

It has been demonstrated previously that transfection of permissive cells with message-sense RNA synthesized *in vitro* from core particles of different orbiviruses leads to the production of viral progeny (Boyce and Roy, 2007; Matsuo et al., 2010). These data would suggest that an entirely plasmid DNA-based reverse genetics system for orbiviruses would be feasible if the transcripts produced from the recombinant plasmids mimicked the viral message-sense RNAs. To this end, we engineered a reverse genetics vector, designated pRG15, as described under *Materials and Methods*. The pRG15 vector contains a *Bsm*BI cloning site flanked by an upstream T7 RNA polymerase promoter sequence and downstream hepatitis delta virus ribozyme (HDVR) and T7 RNA polymerase terminator sequences (Fig. 1A). Considering that the T7 RNA polymerase promoter directs transcription initiation preferentially from a juxtaposed guanosine residue (Milligan et al., 1987) and that all BTV message-sense RNAs are terminated with a 5' guanosine (Rao et al., 1983), plasmid-generated transcripts were thus expected to possess native 5' ends (Fig. 1A). Moreover, self-cleavage of the HDV ribozyme fused to the 3' terminus of nascent transcripts is anticipated to generate RNAs with native BTV 3' termini (Fig. 1A). Plasmid transfection of BSR cells in which T7 RNA polymerase is constitutively expressed (Fig. 1B) should therefore yield 10 unique BTV mRNA species that are capable of serving as templates for viral protein synthesis and negative-strand synthesis to form the dsRNA genome segments present in progeny virus particles.

Generation of viable BTV-1 from cDNA plasmids

To determine whether infectious BTV could be generated with the plasmid-based reverse genetics vector system, cDNA copies of each BTV-1 genome segment were cloned into the pRG15 reverse genetics vector. The 10 BTV-1 constructs were co-transfected in T7 RNA polymerase-expressing BSR-T7 cells. The cells were harvested at 3–5 days post-transfection, lysed and plaque assays were performed. In contrast to mock-transfected cells, plaques were recovered from cells transfected with the 10 BTV-1 cDNA plasmids (Fig. 2A). The viral titers in transfection lysates were 1.4×10^4 pfu/ml and 9.1×10^5 pfu/ml at 3 days and 5 days post-transfection, respectively. To confirm virus recovery, viruses from individual plaques were amplified, after which dsRNA was extracted and analyzed by non-denaturing polyacrylamide gel electrophoresis. The electrophoresis pattern of dsRNA segments extracted from

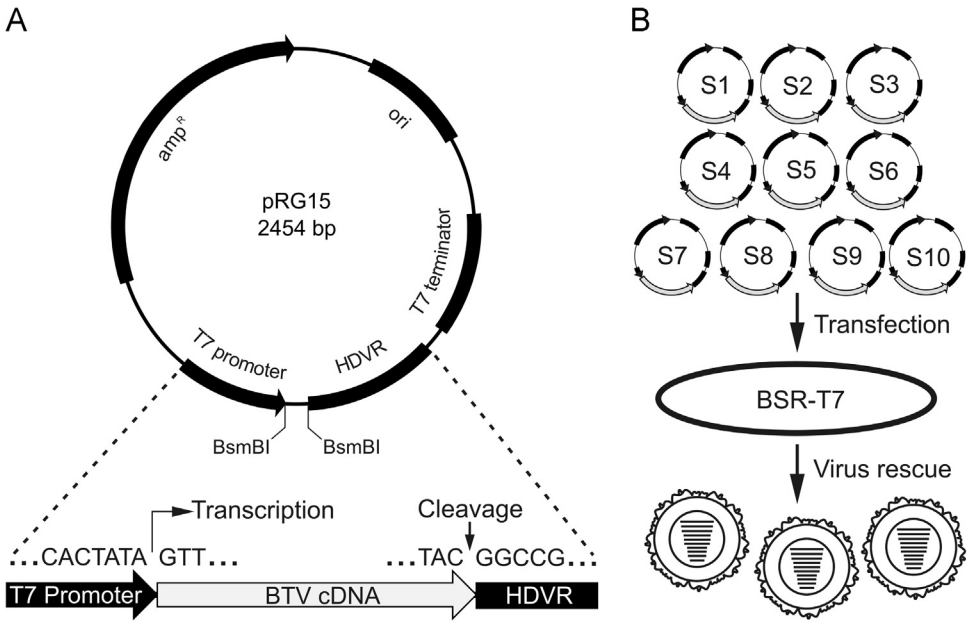

Fig. 1. Experimental strategy to generate BTV from cloned cDNAs. (A) Diagram of the pRG15 reverse genetics vector. In each recombinant plasmid, transcription of the BTV genome segment cDNA is driven by an upstream T7 RNA polymerase promoter, producing transcripts with a native 5′-terminal sequence. The transcripts are equipped with native 3′-terminal sequences through the autocatalytic cleavage of an HDV ribozyme sequence. (B) To generate BTV, the 10 recombinant plasmids, each harboring a BTV genome segment transcription cassette, are transfected into T7 RNA polymerase-expressing BSR-T7 cells. Cytoplasmic transcription of the cloned cDNAs yields native BTV positive-sense RNAs that serve as templates for protein synthesis and genome replication, resulting in the recovery of infectious virions.

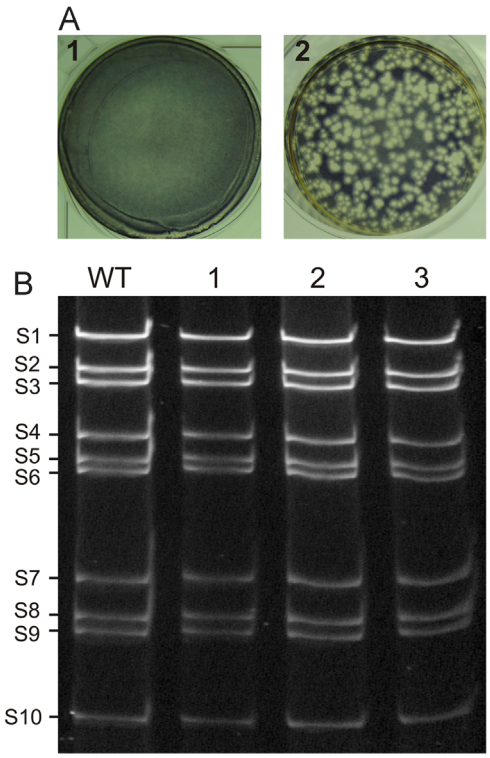

Fig. 2. Recovery of infectious BTV-1 by transfection of BSR-T7 cells with 10 cDNA plasmids. (A) Plaque assay of the transfection cell lysate performed at 72 h post-transfection (well 2). Mock-transfected cells (well 1) were included as a control. (B) Electropherotypes of wild-type (WT) BTV-1 and plasmid-derived recombinant BTV-1 (lanes 1–3). Genomic dsRNA were electrophoresed in an 8.5% non-denaturing polyacrylamide gel, followed by ethidium bromide staining to visualize the viral genome segments (S1–S10).

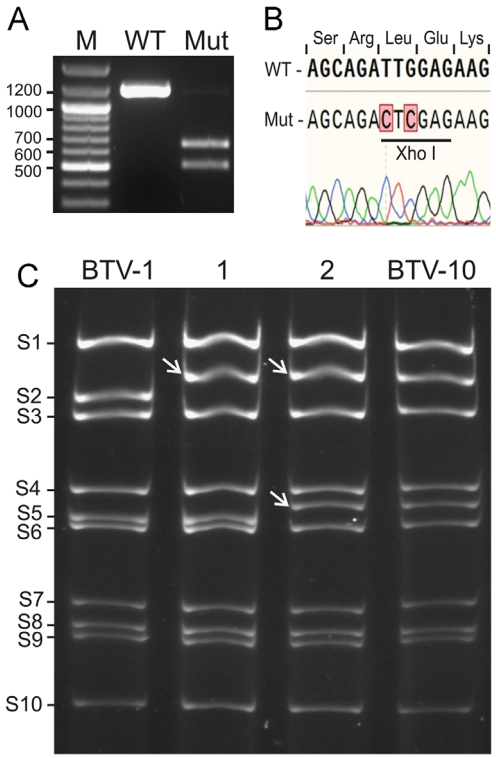

Fig. 3. Characterization of recombinant BTV-1 containing a mutation and recombinant cross-serotype reassortant viruses. (A and B) RT-PCR products of the S8 genome segment of wild-type (WT) BTV-1 and recombinant mutant BTV-1 (Mut) were analyzed by agarose gel electrophoresis after digestion of the products with *Xho*I (A), and the nucleotide sequence of the RT-PCR products were also determined and compared (B). Shown in (B) is the sequence chromatogram demonstrating the presence of the introduced T to C and G to C point mutations, resulting in an *Xho*I silent mutation. (C) Electropherotypes of the recombinant reassortant viruses $_{B1}$BTV-10^{VP2} (lane 1) and $_{B1}$BTV-10$^{VP2/VP5}$ (lane 2) obtained by electrophoresis of the genomic dsRNA in an 8.5% non-denaturing polyacrylamide gel. The reassortant viruses contain respectively genome segment S2 from BTV-10 and both genome segments S2 and S5 from BTV-10 in an otherwise BTV-1 genetic background. Arrows indicate the slower migrating BTV-10 S2 (lane 1) and S2 and S5 (lane 2) genome segments. Wild-type BTV-1 dsRNA and BTV-10 dsRNA marker lanes are indicated to the left and right of the figure, respectively.

plasmid-derived BTV-1 was identical to that of the wild-type BTV-1 derived from cell infection (Fig. 2B). The complete nucleotide sequence of plasmid-derived BTV-1 was also determined and matched that of the parental plasmids (data not shown). Thus, BTV, like reovirus, can be recovered from plasmid cDNAs expressing full-length positive-sense RNAs without the need for synthetic transcripts or helper plasmids that express viral proteins independently (Kobayashi et al., 2007).

Generation of directed mutant and reassortant viruses

To determine whether the plasmid-based reverse genetics system is sufficiently robust to enable the introduction of a specific mutation into the BTV genome, a unique *Xho*I restriction enzyme site was incorporated into the S8 genome segment of BTV-1 by the introduction of two silent point mutations into the pRG15-B1-S8 rescue plasmid: T to C at nucleotide 635 and G to C at nucleotide 637. BSR-T7 cells were subsequently co-transfected with the recombinant pRG15 reverse genetics plasmids in which the wild-type pRG15-B1-S8 construct was substituted with the mutant pRG15-B1-S8mut plasmid. Viruses resulting from the transfection were plaque-purified and dsRNA extracted from virus-infected BSR cells were used for cDNA synthesis. To confirm that the viruses contain the introduced mutation, the S8 genome segment was PCR amplified, digested with *Xho*I and the reaction mixtures analyzed by agarose gel electrophoresis. The results indicated that, in contrast to the non-mutated S8 genome segment which was not cleaved by the restriction enzyme, the S8 genome segment of mutant BTV-1 was cleaved and yielded two DNA fragments of the expected sizes (635 and 490 base pairs) (Fig. 3A). The nucleotide sequences of the purified amplicons were also determined, the results of which confirmed the presence of the unique *Xho*I restriction enzyme site (Fig. 3B).

We next attempted to modify the BTV-1 genome by exchanging the S2 or both the S2 and S5 genome segments of BTV-1 with those of BTV-10. These genome segments encode for the outer capsid proteins VP2 and VP5, respectively (Boyce et al., 2008). For this purpose, cDNA copies of the S2 and S5 genome segments from BTV-10 were cloned into the pRG15 reverse genetics plasmid to yield pRG15-B10-S2 and pRG15-B10-S5, respectively. BSR-T7 cells were subsequently co-transfected with mixtures of plasmid DNA in which pRG15-B10-S2 or both pRG15-B10-S2 and pRG15-B10-S5 were used to substitute the corresponding BTV-1 rescue plasmids. Reassortant viruses from randomly selected plaques were amplified in BSR cells, dsRNA was extracted and the genotype of the recovered viruses was initially determined by electrophoresis of the dsRNA on non-denaturing polyacrylamide gels. The electrophoretic pattern of a representative monoreassortant, $_{B1}$BTV-10^{VP2}, which contains the BTV-10 S2 genome segment in an otherwise BTV-1 background, clearly shows co-migration of the S2 RNA with that of BTV-10. Likewise, the double-reassortant virus $_{B1}$BTV-10$^{VP2/VP5}$ displayed an electropherotype consistent with eight genome segments derived from BTV-1 and two genome segments, S2 and S5, derived from BTV-10 (Fig. 3C). The identity of the S2 and S5 genome segments as originating from BTV-10 was

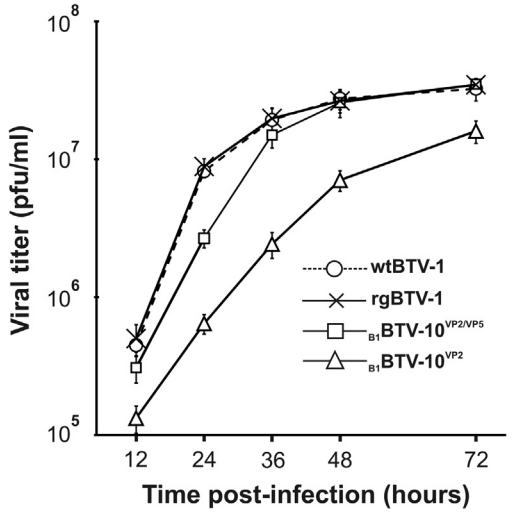

Fig. 4. Virus growth curves. BSR cells were infected with wild-type (WT) BTV-1, plasmid-derived BTV-1 (rgBTV-1) and the reassortant viruses $_{B1}$BTV-10^{VP2} and $_{B1}$BTV-$10^{VP2/VP5}$ at a MOI of 0.1 pfu/cell, and viral titers were determined by plaque assay at the times shown. Results are presented as the mean viral titers for four experiments and error bars indicate the standard deviation.

confirmed by nucleotide sequencing of cDNA copies of the respective genome segments (data not shown).

Growth properties of wild-type and recombinant viruses in mammalian cells

The replicative capacities of wild-type BTV-1 and plasmid-derived BTV-1 (hereafter designated wtBTV-1 and rgBTV-1, respectively) as well as the reassortant viruses $_{B1}$BTV-10^{VP2} and $_{B1}$BTV-$10^{VP2/VP5}$ were evaluated in BSR cells. Cells were infected with each indicated virus at a multiplicity of infection (MOI) of 0.1 pfu/cell and virus titers were determined at various time points post-infection. Essentially no difference in growth kinetics was observed between wtBTV-1 and rgBTV-1. The double-reassortant virus $_{B1}$BTV-$10^{VP2/VP5}$ grew slightly slower than wtBTV-1 or rgBTV-1 until 36 h post-infection, but reached a peak titer of 3.5×10^7 pfu/ml at 72 h post-infection which is similar to that of wtBTV-1 and rgBTV-1 (Fig. 4). In contrast, the monoreassortant virus $_{B1}$BTV-10^{VP2} achieved lower titers than any of the other viruses at each time point. Although the peak titer of $_{B1}$BTV-10^{VP2} was 1.6×10^7 pfu/ml at 72 h post-infection, this was approximately 2.1-fold lower than the other viruses included in the assay (Fig. 4).

Discussion

Current methods for engineering the segmented dsRNA genomes of orbiviruses, including BTV, are limited to the use of RNA-based reverse genetics systems, in which virus is recovered wholly from *in vitro*-synthesized capped T7 transcripts or, alternatively, by transfecting the full complement of capped synthetic T7 transcripts into cells previously transfected with helper expression plasmids to form the primary replication complex (Boyce et al., 2008; Matsuo and Roy, 2013; Kaname et al., 2013; Yang et al., 2015). In an effort to expand the utility of BTV reverse genetics, our objective in this study was to develop an unencumbered reverse genetics platform to recover recombinant BTV from plasmid cDNA only.

We constructed a reverse genetics vector, pRG15, into which cDNA of each BTV-1 genome segment was cloned. The cloned cDNA is flanked by a T7 RNA polymerase promoter sequence at the 5' end, and a HDV ribozyme and T7 RNA polymerase terminator at the 3' end. This strategy for the synthesis of BTV-1 mRNA species was aimed at overcoming problems inherent to the *in vitro* synthesis of T7 run-off transcripts. T7 RNA polymerase introduces considerable heterogeneity on the 3' end of the transcripts. Not only does premature termination of transcription produce shortened transcripts, but also the enzyme tends to add a few extra nucleotides at the 3' end of the transcript that are not present in the linearized DNA template (Milligan et al., 1987; Martin et al., 1988) and, in certain cases, may produce RNAs that are longer than expected (Cazenave and Uhlenbeck, 1994). Indeed, recovery of infectious BTV from 10 synthetic T7 transcripts was reported to be less efficient compared to the use of core-derived viral transcripts (Boyce et al., 2008). Thus, incorporation of the *cis*-acting HDV ribozyme into the RNAs transcribed directly from the plasmid-borne cDNA copies of the viral genome segments should overcome the above problems by producing transcripts with homogenous 3' ends (Schürer et al., 2002). Transfection of the 10 BTV-1 rescue plasmids into the cytoplasm of T7 RNA polymerase-expressing BSR cells consistently resulted in the recovery of recombinant BTV-1. Growth kinetics, total yield and the genomic electrophoretic signature were indistinguishable between wild-type BTV-1 and the recombinant BTV-1, thus validating retention of the native viral properties by the plasmid-derived viruses.

Although RNAs transcribed directly from the recombinant plasmids will not contain a 5' cap, the recovery of infectious BTV from plasmid cDNA alone demonstrates that the transcripts synthesized are functionally equivalent to authentic viral transcripts at all stages of the replication cycle. The transcripts are translated and selected during packaging and mediate negative-strand synthesis, resulting in infectious virions. These results are in accordance with those reported for reovirus, which indicated that recombinant reovirus could be recovered following transfection of BHK cells constitutively expressing T7 RNA polymerase with four rescue plasmids corresponding to the reovirus genome (Kobayashi et al., 2010). In this regard, it is interesting to note that Fuerst and Moss (1989) reported that uncapped and non-polyadenylated T7 transcripts originating from plasmids transfected into mammalian cells had a half-life of 75 min and represented up to 30% of the total cytoplasmic RNA after a 24-h period. Thus, the sustained synthesis of RNA transcripts and their accumulation to relatively high levels in the cell cytoplasm may account for the efficiency in BTV rescue. Indeed, at 3 days post-transfection, plasmid-derived BTV-1 reached a titer of 1.4×10^4 pfu/ml.

To evaluate the utility of the fully plasmid-based BTV reverse genetics system, we successfully generated a mutant BTV-1 containing a defined mutation in the replicating viral genome. This reverse genetics platform can therefore be used to introduce mutations into targeted proteins as a means to elucidate structure–function relationships in viral proteins and furthermore provides an opportunity to observe the effects of these mutations on BTV biology in the context of a replicating virus. We subsequently used the reverse genetics platform to generate reassortant viruses in which the VP2 protein or both the VP2 and VP5 outer capsid proteins of BTV-1 were replaced with those of BTV-10. Growth kinetics of the reassortant viruses indicated that the monoreassortant virus $_{B1}$BTV-10^{VP2} produced a lower titer than that of wild-type BTV-1, whereas the double reassortant virus $_{B1}$BTV-$10^{VP2/VP5}$ replicated to the same titer than wild-type BTV-1. In mammalian cells, BTV enters the cell through receptor-mediated endocytosis and is incorporated into early endosomes (Forzan et al., 2007). The outermost capsid protein VP2 is the cellular receptor binding protein, whereas VP5 is a membrane-penetrating protein that is thought to permeabilize the endosomal membrane, thereby releasing the transcriptionally active core-particle into the cell cytoplasm (Hassan et al., 2001; Zhang et al., 2010). Whether the observed growth difference is therefore due to

a structural difference in the VP2 (BTV-10)-VP5 (BTV-1) pair that may influence the receptor binding ability of VP2 or subsequent uncoating of the virus prior to release of the core particle into the cytoplasm requires further investigation. It is clear, however, that the plasmid-based reverse genetics system can be used to generate directed reassortant viruses. As such, the reverse genetics system can be exploited to generate directed reassortant viruses to investigate BTV virulence and pathogenicity (Coetzee et al., 2014; Celma et al., 2014), in addition to generating "serotyped" vaccine viruses (van Gennip et al., 2012; Feenstra et al., 2014; Nunes et al., 2014).

In conclusion, the entirely plasmid-based BTV reverse genetics system described here provides a new platform to augment existing platforms and has the added advantages of reduced reagent preparation time and expense. This could facilitate and accelerate basic studies of BTV biology, including aspects pertaining to replication, transmission, pathogenesis and immunity. Considering that orbiviruses have similar virion structures and replication mechanisms, the principles and techniques established in this study may be applied to the development of similar plasmid-based reverse genetics systems for other members of the *Orbivirus* genus.

Materials and methods

Cells and viruses

BSR cells (a clone of BHK-21 cells) were cultured at 37 °C and 5% CO_2 in Minimum essential medium (Eagle's MEM) supplemented with 2 mM L-glutamine, Earle's balanced salt solution (Lonza), 1% (v/v) non-essential amino acids (HyClone), 5% (v/v) fetal bovine serum (FBS) and antibiotics (10,000 U/ml penicillin G, 10,000 U/ml streptomycin, 25 μg/ml amphotericin B) (HyClone). BSR-T7 cells, which stably express bacteriophage T7 RNA polymerase (Buchholz et al., 1999), were maintained in the same manner as BSR cells except for the addition of 1 mg/ml of Geneticin (Invitrogen) with every second passage of the BSR-T7 cells. Both BSR and BSR-T7 cells are highly permissive for BTV growth.

BTV serotypes 1 (BTV-1) and 10 (BTV-10) were used for cell infections. BSR cell monolayers were rinsed twice with incomplete EMEM (lacking FBS and antibiotics) and then infected with virus at a multiplicity of infection (MOI) of 0.1 pfu/cell. Virus adsorptions were performed at 37 °C for 1 h, followed by incubation of the cell monolayers in complete EMEM for 3–4 days.

Construction of the pRG15 reverse genetics vector

The pRG15 reverse genetics vector was constructed in three stages. In the first stage, oligonucleotides were assembled to yield a transcription cassette comprising a T7 RNA polymerase promoter, followed by two inverted *Bsm*BI restriction enzyme sites, a hepatitis delta virus ribozyme (HDVR) sequence and a T7 RNA polymerase terminator sequence (Fig. 1A). Oligonucleotides RG-F1 through RG-R6 (Supplementary Table S1) were 51–64 nt in length and configured in such a way that, upon assembly, complementary oligonucleotides overlap by at least 20 nucleotides. The 5'-phosphorylated oligonucleotides were purchased from Inqaba Biotechnical Industries, re-suspended in sterile 10 mM Tris (pH 8) and stored at −80 °C. For oligonucleotide assembly, an overlap-extension PCR was performed (Stemmer et al., 1995). Briefly, 500 nM of the outer oligonucleotides (RG-F1 and RG-R6) and 25 nM of the internal oligonucleotides (RG-R2, RG-F3, RG-R4 and RG-F5) were added to a PCR reaction mixture containing 1 U of Phusion High-Fidelity DNA Polymerase (Thermo Scientific), 1 × HF buffer and 200 μM each of dNTP. The reaction was subjected to an initial denaturation step of 98 °C for 10 s, followed by 30 cycles of 98 °C for 20 s, 58 °C for 30 s and 72 °C for 30 s. The 257-bp product was purified from an agarose gel and cloned into pSMART LC Amp (Lucigen), according to the manufacturer's instructions, to yield pSMART-T7C. In the second stage, the reverse genetics vector backbone was prepared. To this end, pUC19 was used as the template in an inverse PCR reaction with primers ver2F and ver2R (Supplementary Table S1) to remove the *lacZ*' region inclusive of the multiple cloning site, as well as two *Bsm*BI restriction enzyme sites present on the pUC19 backbone. Finally, the transcription cassette was excised from pSMART-T7C with *Eco*RV and blunt-end ligated to the modified pUC19 genetic backbone to produce the reverse genetics vector pRG15. The nucleotide sequence of the cloned insert DNA was verified by automated sequencing procedures using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit v3.1 (Perkin-Elmer Applied Biosystems).

Cloning of BTV-1 cDNA

Total RNA was extracted from BTV-1-infected BSR cells with Tri-Reagent (Sigma-Aldrich) and ssRNA was subsequently removed by precipitation with 2 M LiCl and centrifugation at 17,000*g* for 15 min at 4 °C. First-strand cDNA was synthesized from 5 μg of dsRNA using the RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Scientific) according to the manufacturer's protocol. cDNA copies of each BTV-1 genome segment were amplified by PCR using appropriate primer pairs (Supplementary Table S1) and Phusion High-Fidelity DNA Polymerase (Thermo Scientific). Purified full-length PCR products were digested with *Bsm*BI (S1, S2, S3, S8 and S9 cDNA), *Bbs*I (S4, S6, S7 and S10 cDNA) or *Bsp*MI (S5 cDNA) and ligated into *Bsm*BI-digested plasmid pRG15. The derived recombinant plasmids were named pRG15-B1-S1 through pRG15-B1-S10. The complete nucleotide sequences of the cDNA clones were determined and exactly matched the database sequences (GenBank accession nos FJ969719-FJ969728; Boyce et al., 2008).

Construction of plasmids for use in targeted mutagenesis and reassortant experiments

Using a similar approach to that described above, reverse genetics plasmid clones were constructed for BTV-10 genome segment 2 (pRG15-B10-S2) and genome segment 5 (pRG15-B10-S5). Segment-specific cDNAs were amplified by PCR using primers B10VP2F and B10VP2R for S2, and primers B10VP5F and B10VP5R for S5 (Supplementary Table S1).

A mutant version of the BTV-1 S8 genome segment, containing an introduced *Xho*I restriction enzyme recognition sequence, was constructed by a megaprimer PCR-based mutagenesis strategy in which three primers and two PCRs were used (Sarkar and Sommer, 1990). In the first round of PCR, pRG15-B1-S8 was used as the template together with primer B1NS2R and the internal mutagenic primer NS2-mut (Supplementary Table S1). The purified amplicon was then used as a 'megaprimer' along with primer B1NS2F in the second round of PCR to generate the full-length S8 genome segment containing the newly introduced mutations. The PCR product was purified from an agarose gel, digested with *Bsm*BI and then cloned into the *Bsm*BI site of pRG15 to generate pRG15-B1-S8mut. The mutation inserted in the modified pRG15-B1-S8 plasmid was confirmed by nucleotide sequencing.

Plasmid transfections and recovery of infectious virus

Plasmid DNA used in cell transfections was purified with the GeneJET plasmid midiprep kit (Thermo Scientific), inclusive of the endotoxin removal procedure, according to the manufacturer's instructions. For recovery of BTV-1 from the plasmid cDNA clones, monolayers of BSR-T7 cells at 50% confluence in 25-cm^2 tissue culture flasks were co-transfected with the 10 recombinant pRG15 plasmid constructs representing the cloned BTV-1 genome using

Lipofectamine LTX reagent (Life Technologies). For this purpose, 1.1 μg of each recombinant plasmid was mixed in an Eppendorf tube, then diluted in 1.1 ml of OptiMEM I Reduced Serum Medium (Life Technologies) and 11 μl of PLUS Reagent (Life Technologies) was added. Following a 5-min incubation at room temperature, 28 μl of Lipofectamine LTX reagent was added and incubation was continued for 45 min at room temperature. The transfection mixture was added to BSR-T7 cells and OptiMEM I Reduced Serum Medium was added to a final volume of 3 ml. Four hours later, 5% (v/v) FBS was added to the medium. The same protocol was used for recovery of mutant BTV-1, except that pRG15-B1-S8 was substituted with the pRG15-B1-S8mut recombinant plasmid prior to transfection of the BSR-T7 cell monolayer. To recover directed reassortant BTV, the BSR-T7 cells were likewise co-transfected with a mixture consisting of the pRG15-B1 recombinant plasmids in which the pRG15-B1-S5 and/or pRG15-B1-S2 plasmids were substituted with recombinant plasmids pRG15-B10-S5 and/or pRG15-B10-S2, respectively. Following incubation of the transfected cells until the marked cytopathic effect (CPE) was observed (typically 3 days post-transfection), recombinant virus was isolated from the transfected cells by plaque purification on BSR cells (Oellermann, 1970).

Identification of plasmid-derived mutant and reassortant viruses

To identify mutant BTV-1, PCR amplification of synthesized cDNA was performed with the S8 genome segment-specific primer pair. The amplicons were subjected to *Xho*I restriction endonuclease digestion, followed by analysis of the digestion products on an agarose gel. As a final confirmation, the nucleotide sequence of the amplified S8 genome segments was also determined. To identify BTV reassortants, purified viral dsRNA samples were analyzed using non-denaturing 8.5% polyacrylamide gels. In addition, cDNA was synthesized from the viral dsRNA and PCR amplification was performed using S2 and S5 genome segment-specific primer pairs for BTV-10. The purified PCR products were subjected to nucleotide sequencing.

Virus growth curves

Confluent BSR cell monolayers in 6-well tissue culture plates were infected with wild-type BTV-1, plasmid-derived BTV-1 or cross-serotype reassortant BTV at a MOI of 0.1 pfu/cell. At different time points post-infection, the virus titers were determined by serial dilution and plaque assays on BSR cells.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.09.004.

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