Identification and sequence determination of a novel double-stranded RNA mycovirus from the entomopathogenic fungus *Beauveria bassiana*

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Abstract

An isolate of the entomopathogenic fungus *Beauveria bassiana* was found to contain 5 double-stranded (ds) RNA elements ranging from 1.5 to more than 3 kbp. The complete sequence of the largest dsRNA element is described here. Analysis of the RdRp nucleotide sequence reveals its similarity to unclassified dsRNA elements, such as *Alternaria longipes* dsRNA virus 1, and its distant relation to the RNA-dependent RNA polymerases of members of the family *Partitiviridae*.

Introduction

Double-stranded (ds) RNA mycoviruses have been described in yeasts, mushrooms, and filamentous fungi, and are occasionally associated with hypovirulence [11]. They are classified into six families based on their virion structure and genome composition, but some are still unassigned to a genus or in some cases to a family. Recently, an increasing number of novel mycoviruses have been reported whose genomes are similar in sequence and organisation to plant viruses [7, 13] and animal viruses [10], together with dsRNA elements that cannot be definitively assigned to the officially recognised genera and families [14].

Entomopathogenic fungi are of great scientific interest because they enable analysis of virus-host interactions and can be used as biological control agents of insect pests. The deuteromycetous fungus *Beauveria bassiana* has a widespread geographical distribution and a wide host range, with individual isolates being more specific. In *B. bassiana*, the presence of dsRNA mycoviruses has been reported previously and two members of the genus *Victorivirus*, family *Totiviridae* have been fully sequenced [8, 12].

In this paper, we report the complete sequence of a novel dsRNA element from *B. bassiana*, provisionally designated as *B. bassiana* non-segmented virus (BbNV)-1.

Provenance of the virus material

B. bassiana isolate EABb 92/11-Dm, originally isolated in Spain from its insect host *Dociostaurus maroccanus*, family *Acrididae*, superfamily *Acridoidea*, order *Orthoptera* [6], was grown in liquid Czapek-Dox complete medium at 25^{o}C with shaking. Purification of virus particles was performed as described previously [2] and dsRNA was extracted from the particles using phenol/Sevag treatment. Five viral dsRNA elements were separated by electrophoresis on a 1% (w/v) agarose gel containing Tris-acetate-EDTA (TAE) buffer and 500 ng/ml ethidium bromide (Fig. 1a). The four dsRNAs, ranging from 1.3 to 2.5 kbp, constitute the genome of a mycovirus provisionally designated as *B. bassiana* multimycovirus-1 (Kotta-Loizou et al., unpublished data). The largest segment was extracted from the gel using the MinElute Gel Extraction Kit (Qiagen) and the dsRNA nature of the element was confirmed by its resistance to DNase I and S1 nuclease (Promega). Subsequently, the dsRNA was denatured with methyl mercuric hydroxide and used as template for reverse transcription and PCR amplification using random primers [4]. The sequence was completed by the use of sequence-specific primers, genome walking and RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) [3]. All products were cloned using the pGEM-T Easy vector system (Promega) and introduced into competent *Escherichia coli* XL10-Gold cells (Agilent). At least three different clones were sequenced covering the same part

of the genome. The effects of BbNV-1 on the host's phenotype and pathogenicity are currently unknown.

Sequence properties

The sequence of the BbNV-1 dsRNA element has been deposited in the GenBank/EMBL/DDBJ databases with accession number LN610699. The BbNV-1 genome consists of a single dsRNA of 3218 bp, which contains two open reading frames (ORFs), 945 bp and 1794 bp in length, respectively. The two ORFs do not overlap and the second ORF is located at a +2 position in relation to the first. No other ORFs of significant length were detected in either strand. The two ORFs are flanked by 5'- and 3'-untranslated regions (UTRs), 320 bp and 79 bp, respectively. The genomic organisation of BbNV-1 is shown in Fig. 1b.

The BbNV-1 ORF-1 has the potential to encode a protein of 315 aa (molecular mass 34 kDa). Sequence similarity searches of the GenBank, Swissprot and EMBL databases using the BLAST program [1], revealed that its amino acid sequence is most closely related to a protein of unknown function encoded by the recently published [9] unclassified *Alternaria longipes* dsRNA virus 1 (E-value e^{-46}, 41% identity, 58% similarity).

The BbNV-1 ORF-2 has the potential to encode a protein of 585 aa (molecular mass 66 kDa), which contains three partially conserved motifs (Fig. 1c), characteristic of one of the classified RNA-dependent RNA polymerase (RdRp) families of positive-strand, RNA eukaryotic viruses (RdRp_1, pfam00680). BLAST searches, showed that the BbNV-1 RdRp is most closely related to the RdRp of *A. longipes* dsRNA virus 1 (E-value 0, 58% identity, 71% similarity) and bears homology to RdRps of other unclassified mycoviruses; *Fusarium graminearum* dsRNA mycovirus-4 (E-value $2e^{-14}$, 24% identity, 40% similarity), *Zygosaccharomyces bailii* virus Z (E-value $1e^{-13}$, 24% identity, 40% similarity), *Heterobasidion* RNA virus 6 (E-value $4e^{-10}$, 25% identity, 41% similarity) and *Cryphonectria parasitica* bipartite mycovirus 1 (E-value $5e^{-10}$, 25% identity, 37% similarity). In addition, more distant similarities were noted with various members of the family *Partitiviridae*, genus *Gammapartitivirus*. Because of the extreme divergence of these sequences the construction of phylogenetic trees was considered inappropriate.

Secondary structure analysis of the 5'- and 3'-UTRs using the mfold server [15] predicted the presence of stem-loop structures (data not shown), which are common in mycoviruses and considered to be implicated in RdRp recognition and RNA replication [5]. Notably, the long 5'-UTR is a feature shared by *A. longipes* dsRNA virus 1 (318 bp).

In conclusion, since genomic organisation and sequence of BbNV-1 are similar to those described for *A. longipes* dsRNA virus 1, we propose that both viruses are members of an emerging mycovirus genus

('Unirnavirus'). Members of the genus possess one essential genome segment containing two large non-overlapping ORFs, the second located at a +2 position in relation to the first. The first ORF encodes a protein of as yet unknown function and the second ORF encodes the RdRp. The evolutionary relationships between the unirnaviruses, other similar unclassified viruses and established virus families such as *Partitiviridae*, *Totiviridae* and *Amalgaviridae* remain to be elucidated in the future.

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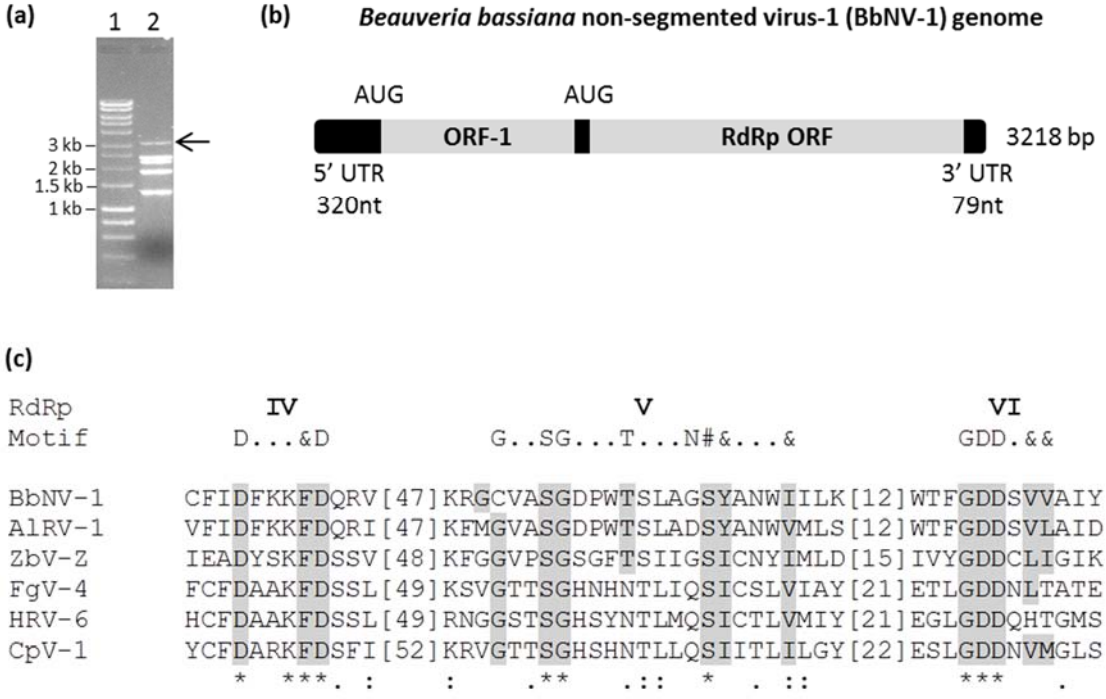

Fig. 1 (a) Agarose electrophoresis of viral dsRNA extracted from *B. bassiana* isolate EABb 92/11-Dm (lane 2). *Beauveria bassiana* non-segmented virus-1 is indicated by an arrow. Lane 1 contains the DNA marker Hyperladder I (Bioline), the sizes of with are shown to the left of the gel. **(b)** Schematic representation of the genome organisation of *Beauveria bassiana* non-segmented virus-1. BbV-1 dsRNA contains two ORFs (grey boxes) flanked by 5'- and 3'-UTRs (black boxes). **(c)** Comparison of the conserved motifs of the RdRP_1 family of RdRPs (pfam00680) in *Beauveria bassiana* non-segmented virus-1 (BbNV-1), *Alternaria longipes* dsRNA virus 1 (AlRV-1; AIJ01443.1), *Fusarium graminearum* dsRNA mycovirus-4 (FgV-4; YP_003288790.1), *Heterobasidion* RNA virus 6 (HRV-6; AHA82547.1), *Cryphonectria parasitica* bipartite mycovirus 1 (CpV-1; YP_007985675.1) and *Zygosaccharomyces bailii* virus Z (ZbV-Z; NP_624325.1). Numbers within the brackets indicate the number of amino acids not shown. In the RdRp motifs, the symbol '#' signifies S or T and the symbol '&' signifies bulky hydrophobic residues (I, L, V, M, F, Y, W). In the sequence alignment, asterisks signify identical amino acid residues, colons signify highly conserved residues and single dots signify less conserved but related residues. The residues than conform to the RdRp motifs are within grey boxes.