Characterization of a new densovirus infecting the German cockroach, Blattella germanica

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A new DNA virus (Parvoviridae: Densovirinae, Densovirus) was isolated and purified from descendants of field-collected German cockroaches, Blattella germanica. Viral DNA and cockroach tissues infected with B. germanica densovirus (BgDNV) were examined by electron microscopy. Virus particles, about 20 nm in diameter, were observed both in the nucleus and in the cytoplasm of infected cells. Virus DNA proved to be a linear molecule of about 1·2 μm in length. BgDNV isolated from infected cockroaches infected successfully and could be maintained in BGE-2, a B. germanica cell line. The complete BgDNV genome was sequenced and analysed. Five open reading frames (ORFs) were detected in the 5335 nt sequence: two ORFS that were on one DNA strand encoded structural capsid proteins (69–7 and 24–8 kDa) and three ORFs that were on the other strand encoded non-structural proteins (60–2, 30–3 and 25–9 kDa). Three putative promoters and polyadenylation signals were identified. Structural analysis of the inverted terminal repeats revealed the presence of extended palindromes. The genome structure of BgDNV was compared with that of other members of the family Parvoviridae; the predicted amino acid sequences were aligned and subjected to phylogenetic analyses.

INTRODUCTION

The family Parvoviridae includes autonomously replicating DNA viruses belonging to two subfamilies: Parvovirinae, members of which infect vertebrates, and Densovirinae, which infect invertebrates (Fédière, 2000). Three genera are recognized within the Densovirinae: Densovirus, Iteravirus and Brevidensovirus. Densovirus infect arthropods, mostly insects, in which densovirus infection has been detected in species of six insect orders (Lepidoptera, Diptera, Orthoptera, Dictyoptera, Odonata and Hemiptera) and in decapod crustaceans (Tijssen & Bergoin, 1995; Shike et al., 2000; van Munster et al., 2003; Wang et al., 2005). Their name, densonucleosis virus or densovirus (DNV), refers to histopathological and ultrastructural characteristics of infected cells, the nuclei of which become hypertrophied and contain dense, dark, Feulgen stain-positive virion masses (Vago et al., 1966). In most cases, DNV infection causes death of the host (Tanada & Kaya, 1993).

Icosahedral, non-enveloped DNV particles are 18–26 nm in diameter and harbour a single-stranded, linear DNA of about 4000–6000 nt. Some DNVs (e.g. AeDNV, isolated from the mosquito Aedes aegypti) contain predominantly an mRNA-complementary DNA strand in their particles, whereas others (e.g. JcDNV, isolated from the butterfly Junonia coenia) produce particles with different strands in similar amounts (Bergoin & Tijssen, 2000). When both plus and minus strands are packed in particles, total DNA isolation at high ionic strength results in annealing of the complementary strands, yielding double-stranded DNA (dsDNA).

The viral coding sequences are flanked by inverted terminal repeats (ITRs) of about 500 nt that assume hairpin configurations at the ends of DNV DNA and may form secondary-structural elements. The nucleotide sequences of the two ITRs may be identical (e.g. JeDNV; Dumas et al., 1992), as in vertebrate adeno-associated viruses, or different (e.g. AeDNV; Afanasiev et al., 1991). ITRs play an important role in autonomous replication and packaging of virus DNA (Bergoin & Tijssen, 2000).
The DNV genomes sequenced so far each contain several open reading frames (ORFs). ORFs occur in one or in both DNA strands, depending on the virus type, and encode structural proteins or viral capsid polypeptides (VP) and non-structural proteins (NS) (Bergoin & Tijsen, 2000).

DNVs tend to be highly host-specific; they infect most tissues of their hosts, they do not appear capable of infecting vertebrates and they resist extreme environmental conditions, thus making them effective biological-control agents against populations of agricultural and medically important pests (Corsi et al., 1996; Afanasiev & Carlson, 2000; Carlson et al., 2000). In addition, DNVs provide convenient transduction vectors for delivery of foreign genes that can be used to genetically manipulate insect populations (Afanasiev & Carlson, 2000; Carlson et al., 2000; Bossin et al., 2003). A large portion of the virus genome can be substituted with foreign genes, as long as the terminal sequences, which are required for replication and packaging, remain intact.

Here, we report on the isolation of a new DNV (Blattella germanica DNV or BgDNV) from the cockroach Blattella germanica and describe its ultrastructure, pathology, histopathology and infectivity for cockroaches and B. germanica cell lines. We have cloned and sequenced the viral DNA and compared its genome structure and predicted amino acid sequences with those of other paroviruses. We conclude that this virus is a new member of the genus Densovirus.

**METHODS**

Total DNA was isolated from B. germanica individuals of a cockroach colony (P6) that originated from a natural population in a pig farm in North Carolina, USA. Isolation of total and plasmid DNA, endonuclease digestion, gel electrophoresis and fragment elution from agarose gel were carried out according to published protocols (Sambrook et al., 1989; Mukha et al., 1995).

**Cell culture and preparation of infectious inoculum.** B. germanica cell lines BG-E-1 and BG-E-2, isolated previously from embryonic tissues (Kurtti & Brooks, 1977), were used to propagate BgDNV. Cells were grown in antibiotic-free L-15B medium supplemented with fetal bovine serum (5%), tryptose phosphate broth (5%) and bovine lipoprotein concentrate (0-1%), pH 7-0 (Munderloh & Kurtti, 1989). Cultures were incubated at 25°C and fed weekly by replacing spent medium with fresh medium. Subcultures were made every 3–4 weeks with an initial seeding density of approximately 5 x 10^5 cells ml^-1. Cultures were inoculated with virus prepared from insects infected with the P6 virus strain. Individual infected insects were bisected; one part of the body was kept in the freezer, whilst the other was used for total DNA extraction and analysis by agarose-gel electrophoresis (see below). Frozen tissues from the second half were homogenized in cell-free cell-line medium, passed through a 0.22 µm filter and the filtrate, which presumably contained virus particles, was inoculated into flasks containing B. germanica cells.

**Electron microscopy.** DNA preparations were examined by transmission electron microscopy (TEM; magnification × 30,000). A mixture of virus and plasmid DNA (2:1) was prepared as described in the section on DNA (plasmid) preparation for TEM by Dykstra & Reuss (2003).

For TEM of cells, B. germanica tissues were fixed with 4F:1G fixative, rinsed in phosphate buffer, osmicated, dehydrated in an ethanolic series culminating in acetone, infiltrated with Spurr’s resin and polymerized at 70°C. Ultrathin sections were stained with methanolic uranyl acetate and Reynolds’ lead citrate (Dykstra & Reuss, 2003).

**PCR.** PCR amplifications were carried out by using Taq DNA polymerase (Promega) and a PTC-100 Thermal Cycler (MJ Research Inc.). Two primers (5’-CAGGATTGCCATATAAGG and 5’-CATCAT-CTTGTTAGACGTGC) were used for PCR amplification of an approximately 500 bp fragment, spanning the middle region of the virus genome. Each reaction contained 0.1 µg DNA template, 1.5 mM MgCl2, 1 mM each dNTP and 0.2 pmol each primer. The PCR regimen was as follows: initial template denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 1 min, and a final 7 min elongation step at 72°C.

**Virus-genome cloning.** Virus dsDNA was digested with PstI to two fragments and resolved in 0.7% agarose gel (Sambrook et al., 1989). The tailing mixture (200 µl), containing 0.3 µg virus DNA fragment, 100 mM potassium cacodylate (pH 7.2), 2 mM CoCl2, 0.2 M dithiothreitol (DTT), 0.1 M dGTP and 30 U terminal deoxynucleotidyl transferase (Gibco-BRL), was incubated at 37°C for 1 h. Plasmid DNA (pUC19) was digested with PstI and resolved in 0.7% agarose gel. The linearized DNA was tagged with poly(dC). The reaction mixture (50 µl), containing 0.1 µg plasmid DNA, 100 mM potassium cacodylate (pH 7.2), 2 mM CoCl2, 0.2 M dDTT, 0.02 mM dCTP and 15 U terminal deoxynucleotidyl transferase, was incubated at 37°C for 30 min. Virus and plasmid DNAs were precipitated with ethanol, washed with 70% ethanol and dissolved in 50 µl water.

The annealing mixture (10 µl) contained 0.25 µg virus DNA, 0.05 µg plasmid DNA, 10 mM Tris/HCl (pH 8.0), 0.1 M NaCl and 1 mM EDTA. The mixture was incubated at 65°C for 5 min and at 57°C for 1 h. After incubation, 5 µl of the mixture was used to transform competent Escherichia coli XL2-Blue MRF’ cells (Strategene).

**Sequencing and sequence analysis.** The plasmids containing fragments of the virus genome (see Results) were sequenced according to Sanger et al. (1977) with a dGTP BigDye Terminator kit (Applied Biosystems) on an ABI PRISM 377 sequencer.

Promoters, poly(A) tracts, ORFs and their protein products were predicted and multiple and pairwise comparisons were done with the BCM Search Launcher software package (Smith et al., 1996) (http://searchlauncherbcm.tmc.edu). Secondary structure analysis of the alignments was done with the FOLD program (Zuker & Stiegler, 1981). Thermal denaturation profile of BgDNV dsDNA was computed according to Poland’s algorithm (Poland, 1974; Steger, 1994) with a program available at http://www.biophysics.uni-duesseldorf.de/local/POLAND/poland.html.

**Phylogenetic reconstruction.** Multiple alignments were performed with CLUSTALW 1.75 (Thompson et al., 1994). The sequences used in the alignments correspond to the NS-1 amino acid fragment located between two highly conserved replication-initiation and helicase motifs (van Munster et al., 2003) of the following viruses: Diaatraea saccharalis DNV (DsDNV), JdDNV, Galeria mellonella DNV (GmDNV), Aedes albopictus DNV (AaDNV), Bombyx mori DNV (BmDNV-5), infectious hypodermal and hematopoietic necrosis virus (IHHNV), Caspiaia extraeana DNV (CeDNV), Periplaneta fali- ginosa DNV (PrDNV) and BgDNV.

Phylogenetic trees were constructed by using the cluster algorithm TreeTop (http://www.genebee.msu.su/services/phdtree_reduced.html). In the clustering algorithm, the distance between groups of sequences is used for setting of the branching order. This distance is defined as the arithmetic mean of pairwise distances between elements of the two
RESULTS AND DISCUSSION

Virus detection, purification, pathology and histopathology

Electrophoresis of cockroach total DNA in 0.7% agarose gel revealed an additional DNA band of approximately 5 kb (Fig. 1, lane 2) in some B. germanica individuals. The cockroach colony (P6) was maintained in the laboratory for 5 years and it originated from cockroaches captured in an infested pig farm in North Carolina, USA. Most of the individuals possessing the ~5 kb band also displayed several symptoms of pathology, including lethargy, flaccidity, poorly coordinated movements and partial or complete paralysis of the hind legs. Similar symptoms have been reported for DNV infection of other insects, including the cockroach Periplaneta fuliginosa and the cricket Acheta domestica (Meynadier et al., 1977; Suto et al., 1979; Tanada & Kaya, 1993; Hu et al., 1994).

Electron microscopy of ultrathin tissue sections of infected individuals revealed that this virus exhibits polytropic pathology, infecting cells of the digestive system and fat body. Cells of different tissues were similar in cytopathological features, all having unusually structured chromatin with most of the nucleoplasm occupied by virus particles. Particles of approximately 20 nm in diameter were observed both within the nucleus and in the cytoplasm of infected cells. In the nucleus, virus particles were closely packed in an electron-dense virogenic stroma (Fig. 2a), which occasionally formed paracrystalline arrays. Virus particles could be divided into two types based on ultrastructural detail: those having an electron-transparent core surrounded by an electron-dense wall and others exhibiting electron-dense spheres (Fig. 2b). All of these features are characteristic of DNVs (Garzon & Kurstak, 1976; Chao et al., 1984, 1985).

Fig. 2. Electron-microscopic image of BgDNV-infected cells of the B. germanica digestive tract. (a) Virogenic stroma and (b) virus particles having an electron-transparent core and an electron-dense wall (thick arrows) or seen as electron-dense spheres (thin arrows). Bars, 1 μm (a); 100 μm (b).

The ~5 kb fraction was isolated from agarose gels and purified (Fig. 1, lane 3). TEM indicated that the purified fraction contained linear DNA of approximately 1.2 μm in length (Fig. 3). The DNA proved to be double-stranded; its restriction map was constructed with BglII, EcoRI and PstI (Fig. 4).

Infection of B. germanica cell lines

Maintenance of the virus, analysis of its life cycle and studies of integration into the host genome are best done with host-cell lines. We infected, by using virus particles, two virus-free B. germanica cell lines, BGE-1 and BGE-2 (Kurtti & Brooks, 1977). Line BGE-1 was isolated from embryos in the germ-band stage and BGE-2 from embryos undergoing dorsal...
closure. Originally isolated in a culture medium patterned after cockroach haemolymph (Landureau & Jolles, 1969), these cell lines were adapted to L-15B, a medium designed specifically for tick-cell culture (Munderloh & Kurtti, 1989), but which supports cells from a wide range of different arthropod species (Kurtti et al., 2005).

The filtrate prepared from infected P6 cockroaches was infectious for cultured B. germanica BGE-2 cells, but not for BGE-1 cells. The amount of virus replication in these two different cell lines is shown in Fig. 5. The quantity of BgDNV DNA (relative to the genomic DNA) increased dramatically during the first 20 days after infection of the BGE-2 cells. However, no virus DNA was found in total DNA extracts of infected BGE-1 cells. The results with BGE-2 show conclusively that BgDNV alone is responsible for the infection.

The two lines derived from two different embryonic stages are made up of distinctly different cell types. The tetraploid BGE-1 cells are mainly spindle-shaped or multipolar, whereas the diploid BGE-2 cells are round, smaller and contain many cytoplasmic inclusions. We are presently investigating the details of BgDNV cell and tissue specificity.

**Virus DNA cloning**

Full-length DNV genomes are difficult to clone in a circular plasmid vector, because of instability of their extended terminal palindromic regions when both (left and right) are present in the plasmid together (Deiss et al., 1990). An EcoRI–EcoRI fragment (Fig. 4a) spanning the middle part of the virus genome was cloned by standard methods (Sambrook et al., 1989) in pUC19 linearized by the restriction enzyme EcoRI. To clone virus fragments containing the ITRs, we used the following approach.

Virus dsDNA, isolated from agarose gel, was digested with PstI. The fragments containing the ITR regions (Fig. 4b, c) were cloned separately in the pUC19 vector. The fragments (Fig. 4b, c) and pUC19 DNA, linearized by the restriction enzyme PstI, were 3’-tailed with poly(dG) and poly(dC), respectively, annealed and then used to transform E. coli. The cloned fragments, each containing one end of the virus genome, remained stable in the recombinant plasmids maintained in E. coli XL2-Blue MRF’ cells.

The resulting plasmids, containing fragments a, b and c (Fig. 4a–c), were used to sequence the B. germanica virus genome.
Nucleotide sequence of BgDNV genome and structure of its ITRs

The BgDNV genome is 5335 nt (GenBank accession no. AJ189948). Pairwise comparisons with other DNV sequences available from GenBank/EMBL revealed only low similarity (48–51 %) with the BgDNV genome (data not shown). Nevertheless, several motifs proved to be evolutionarily conserved (100 % identity) among DNVs isolated from insects of two distant orders, Lepidoptera (moths and butterflies) and Blattodea (cockroaches) (Fig. 6). The motifs are in the region of nt 2923–3221 of the BgDNV genome (Fig. 6) and correspond to the 3’ end of ORF3 (Fig. 4). Therefore, motifs 1 and 2 may be used to construct universal degenerate primers suitable for seeking new insect DNVs.

The right and left ends of the BgDNV genome are, respectively, 216 and 217 nt and form ITRs (Fig. 7). Analysis with the BLAST program (Altschul et al., 1990) did not reveal any appreciable similarity between these and other virus sequences available from GenBank/EMBL (data not shown). Imperfect palindromes of 192 nt that are contained in the sequences available from GenBank/EMBL (data not shown)

Possibly, changes resulting in a new ITR conformation take place in early divergence, suggesting saltatory evolution of the taxon (Altukhov, 2003).

The G+C content of BgDNV ITRs (59–60 mol%) is higher than that of the entire virus genome (39–40 mol%), which may contribute to the stability of the ITR secondary structure. A thermal-denaturation profile was constructed for the first 950 nt (Fig. 7, on the left) of BgDNV DNA according to Poland’s algorithm (Poland, 1974; Steger, 1994). The central region of the virus genome proved to have a denaturation temperature of approximately 80 °C, whilst two peaks (approx. 95 °C) were observed for ITRs (see Supplementary Fig. S2, available in JGV Online). Hence, the palindromes possibly play an important role in replicative dsDNA stabilization (Siegl & Tratschin, 1987). Whilst the G+C content of the virus generally reflects the G+C content of its host cells (Bergoin & Tijssen, 2000), the ITR G+C content is usually high in members of the family Parvoviridae, being 60 % in PJDNV (Yamagishi et al., 1999), 50 % in BmDNV (Bando et al., 1990) and 46–60 % in autonomously replicating paroviruses (Bloom et al., 1988). An exception is AeDNV, with ITRs having only 27 mol% G+C (Afanasiev et al., 1991).

Genome organization

ORFs and deduced amino acid sequences. The BgDNV genome contains five ORFs, two (1 and 2) on one strand and three (3–5) on the other (Fig. 4). The ORF arrangement is typical for the genus Densovirus (Bergoin & Tijssen, 2000). ORF1 (1887 nt; nt 922–2808) encodes a predicted protein (PP) of 628 amino acid residues, corresponding to a molecular mass of 69 kDa. ORF2 (690 nt; nt 243–932) encodes a PP of 262 residues (25.5 kDa). ORF3 (1594 nt; nt 4404–2811) encodes a PP of 530 residues (60.1 kDa). ORF4 (790 nt; nt 4397–3608) encodes a PP of 229 residues (24.5 kDa). ORF5 (652 nt; nt 5055–4404) encodes a PP of 91 residues (9.5 kDa). ORF4 and ORF5 have a polyadenylation signal (AATAAA) at their 3′ end. The amino acid sequences and
putative functions of the deduced protein products are listed in Supplementary Table S1, available in JGV Online.

The predicted BgDNV protein sequences were compared with protein sequences of other DNVs with an adapted version of the BLAST program (Altschul et al., 1997). The total similarity was very low. However, several relatively short motifs showed substantial homology, being evolutionarily conserved among various insect DNVs, including BgDNV, PfDNV, JcDNV, GmDNV, DsDNV and Myzus persicae DNV (MpDNV) (see Supplementary Table S1, available in JGV Online). The putative functions of the PPs corresponding to the ORFs are as following: ORF1 and ORF2, capsid proteins; ORF3, non-structural protein (NS-1), which is important for virus replication (Bergoin & Tijssen, 2000); ORF4 and ORF5, non-structural proteins with unknown function.

Promoters. We used two algorithms for predicting putative transcription-regulatory sequences: Bucher’s algorithm (Bucher & Trifonov, 1986; Bucher, 1990) and NNPP, the Neural Network Promoter Prediction program (Reese, 2001; http://www.fruitfly.org/seq_tools/promoter.html). A putative promoter, P1, with a score of 0-1.0, was revealed at nt 200–249, with a transcription-initiation start point at nt 240 (Fig. 7). P1 probably controls transcription of ORF1 and ORF2. Both algorithms revealed a second putative promoter sequence, P2, on the second strand between nt 5136 and 5086, with a transcription-initiation start point at nt 5095 (Fig. 7). The transcription of ORF3–ORF5 is probably controlled by P2. A third promoter, P3, with a score of 0-88 in the NNPP program, is on the same strand as P2 between nt 4553 and 4504, with a transcription-initiation start point at nt 4513; P3 might control the transcription of ORFs 3 and 4.

Polyadenylation signals. Polyadenylation signals were found in positions 937–942 and 2818–2823 on the DNA strand containing ORF1 and ORF2. The complementary strand contains an adenylation signal at position 2810–2815. Like other DNVs, BgDNV contains an ambisense-coding genome, with the structural and non-structural proteins being encoded by several genes in opposite directions (Fig. 4). Nevertheless, the genome structure of BgDNV has
several unique features compared with that of other insect DNVs, especially concerning ORFs encoding capsid proteins. The two BgDNV ORFs encoding structural proteins each contain a polyadenylation signal, whereas synthesis of JcDNV, GmDNV and Mythimna loreyi DNV (MIDNV) capsid proteins may be initiated from several codons of one ORF (Dumas et al., 1992; Gross et al., 1990; Tijssen et al., 2003; Fédière et al., 2004). As in JcDNV, GmDNV, MIDNV and PfDNV (Yamagishi et al., 1999), one promoter, P1, controls transcription of the structural genes in the BgDNV genome. The genome of MpDNV (van Munster et al., 2003), as in BgDNV, has two ORFs encoding structural proteins, but each ORF is under the control of its own promoter. Possibly, in BgDNV, two mRNAs are synthesized from the P1 promoter. Transcription starts from a common site and is terminated downstream of the polyadenylation site at nt 937–942 in the case of the ORF2 mRNA or at nt 2818–2823 in the case of the other, larger mRNA. Translation of the large mRNA may be initiated at several codons; one of the proteins may also be synthesized from the ORF2 mRNA. To explain such an unusual genome structure, it is reasonable to assume that the protein encoded by ORF2 is prevalent in the BgDNV capsid.

Transcription of the non-structural ORFs in BgDNV is controlled by two promoters, P2 and P3 (Fig. 7). The polyadenylation signal is at position 2810–1815, exactly at the end of ORF3. Translation of the large mRNA may be initiated at several codons; one of the proteins may also be synthesized from the ORF2 mRNA. To explain such an unusual genome structure, it is reasonable to assume that the protein encoded by ORF2 is prevalent in the BgDNV capsid.

Stages of the virus life cycle, only P3 is active and only ORF3 and ORF4 are expressed. It should be noted that such a structure is not common for all DNVs. Thus, the PfDNV (Yamagishi et al., 1999) genes for non-structural proteins are transcribed from two promoters, but in JcDNV, GmDNV, MpDNV and MIDNV (Bergoin & Tijssen, 2000; Tijssen et al., 2003; van Munster et al., 2003; Fédière et al., 2004), genes for non-structural proteins are transcribed from one promoter. Clearly, comparative genome analysis of insect DNVs may contribute greatly to our understanding of DNV evolution.

Densonucleosis pathology and genomic features (single-stranded DNA, ITRs and two strands that encode regulatory and structural proteins, respectively) suggest that the new pathogen that we discovered in B. germanica is a member of the genus Densovirus in the subfamily Densovirinae, family Parvoviridae. Phylogenetic analysis of the deduced amino acid sequences of a highly conserved region of NS-1 confirmed this taxonomic placement: BgDNV clustered with other members of the genus Densovirus, whereas Iterovirus and Brevidensovirus clustered separately as more distantly related genera (Fig. 8).

B. germanica is an important pest of residential and other structural environments and a major aetiological agent of allergic disease and asthma, especially among inner-city children (Rosenstreich et al., 1997). The tendencies of B. germanica to aggregate and to engage in coprophagy and cannibalism (Kopanic et al., 2001) might make it especially susceptible to horizontal BgDNV transmission. We initiated
screening of cockroach populations in the USA and Russia. Total DNA was extracted from cockroaches collected at three separate swine farms (NC, USA) and at 27 separate apartments in Moscow, Russia. Thirty individuals were analysed from each population. Two primers (see Methods) were used for PCR amplification of an approximately 300 nt fragment, spanning the middle region of the virus genome. We found virus-infected cockroaches in two swine farms, with 50 and 20 % infected insects (data not shown). Virus-infected cockroaches were not found in the third swine farm or in the any of the Moscow populations. The mechanisms of BgDNV spreading into uninfected cockroaches are unknown. Two ways of virus transmission are conceivable: via virus particles (epidemiologically) and via chromosomes with integrated virus (genetically), similar to retroviruses and retroelements. We are presently investigating whether BgDNV integrates into the host genome and the possible mechanisms of virus activation.

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