Journal of Invertebrate Pathology 146 (2017) 58–68

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

The genome sequence of Agrotis segetum granulovirus, isolate AgseGV-DA, reveals a new *Betabaculovirus* species of a slow killing granulovirus

Gianpiero Gueli Alletti[a], Marina Eigenbrod[a,b], Eric B. Carstens[a,c], Regina G. Kleespies[a], Johannes A. Jehle[a,*]

[a] Institute for Biological Control, Federal Research Centre for Cultivated Plants, Julius Kühn Institute, Heinrichstraße 243, 64287 Darmstadt, Germany
[b] Technische Universität Darmstadt, Karolinenplatz 5, 64289 Darmstadt, Germany
[c] Queen's University, Kingston, K7L 3N6 Alberta, Canada

ARTICLE INFO

Keywords:
Baculovirus
AgseGV
Histopathology
Next generation sequencing
Molecular phylogeny
Enhancin

ABSTRACT

The European isolate Agrotis segetum granulovirus DA (AgseGV-DA) is a slow killing, type I granulovirus due to low dose-mortality responses within seven days post infection and a tissue tropism of infection restricted solely to the fat body of infected *Agrotis segetum* host larvae. The genome of AgseGV-DA was completely sequenced and compared to the whole genome sequences of the Chinese isolates AgseGV-XJ and AgseGV-L1. All three isolates share highly conserved genomes. The AgseGV-DA genome is 131,557 bp in length and encodes for 149 putative open reading frames, including 37 baculovirus core genes and the *per os* infectivity factor *ac110*. Comprehensive investigations of repeat regions identified one putative non-*hr* like origin of replication in AgseGV-DA. Phylogenetic analysis based on concatenated amino acid alignments of 37 baculovirus core genes as well as pairwise distances based on the nucleotide alignments of partial *granulin, lef-8* and lef-*9* sequences with deposited betabaculoviruses confirmed AgseGV-DA, AgseGV-XJ and AgseGV-L1 as representative isolates of the same *Betabaculovirus* species. AgseGV encodes for a distinct putative enhancin, distantly related to enhancins from other granuloviruses.

1. Introduction

The family of *Baculoviridae* comprises occluded dsDNA viruses with rod-shaped, enveloped virions infecting larval stages of the insect orders Lepidoptera, Diptera and Hymoptera. Infections of insect larvae with these viruses have been described long before any knowledge of structure and mode of infection arose (Benz, 1986). Based on their phylogenetic relationship, baculoviruses are classified into four genera, which also reflect their host range and to a certain extent, occlusion body (OB) morphology. Members of the genera *Alphabaculovirus* and *Betabaculovirus* only infect Lepidoptera, whereas viruses from *Gammabaculovirus* and *Deltabaculovirus* are specific for Hymenopteran and Dipteran species, respectively (Herniou et al., 2003; Jehle et al., 2006a). In contrast to alpha-, gamma- and deltabaculoviruses, that can occlude multiple enveloped virions into a polyhedral occlusion body (OB), the single-nucleocapsid virions of betabaculoviruses are occluded into an ovo-cylindrical, granule-shaped OB (Gati et al., 2017). The term granulovirus is derived from that well-described OB morphology, previously used in baculovirus taxonomy (Theilmann et al., 2005). Currently, all granuloviruses are classified in the genus *Betabaculovirus* (Herniou and Jehle, 2007). Baculovirus phylogeny has been under intensive revision with the advent of molecular markers, based on conserved genes such as *polyhedrin/granulin*, *lef-8*, *lef-9* and/or *pif-2* (Herniou et al., 2003; Jehle et al., 2006b). Extensive enhancement of baculovirus molecular phylogeny is based on a set of baculovirus core genes, predictably present in all baculovirus species. Until now, 37 genes have been accepted as baculovirus core genes (Garavaglia et al., 2012) and their predicted amino-acid sequences are used for phylogenetic analyses. Aside from phylogeny-based classification, three types of granuloviruses have been identified by their pathogenesis in infected host larvae (Federici, 1997). Slow-killing type I granuloviruses include Trichoplusia ni granulovirus (TnGV), Pseudaletia unipuncta granulovirus (PsunGV), Xestia c-nigrum granulovirus (XecnGV) and Adoxophyes orana granulovirus (AdorGV) (Hilton and Winstanley, 2008a; Mukawa and Goto, 2008). In type I granulovirus infections, the production of OBs is restricted to the fat body of host larvae, and larval development remains largely unaffected, allowing infected larvae to grow to their final larval stages. In contrast, fast-killing type II granuloviruses such as Cydia pomonella granulovirus (CpGV) (Tanada and Leutenegger, 1968), cause killing within a few days after infection

* Corresponding author.
E-mail address: johannes.jehle@julius-kuehn.de (J.A. Jehle).

http://dx.doi.org/10.1016/j.jip.2017.04.008
Received 23 January 2017; Accepted 20 April 2017
Available online 22 April 2017
0022-2011/

and formation of OBs is not limited to the fat body but occurs systemically in multiple larval tissues, such as trachea, neurons, and others. Finally, infections caused by the type III granulovirus Harrisina brillians granulovirus (HbGV) have been so far observed only in larvae of *Harrisina brillians*. Their tissue tropism is limited to the midgut epithelium cells and, unlike other types of granuloviruses, the infected larvae discharge infectious granules and cells due to a diarrheic effect until they die within a week post infection (Federici, 1997).

Infections of *Agrotis* species with isolates of Agrotis segetum granulovirus (AgseGV) have been well described since the late 1960s from field collected infected larvae (Zethner, 1980; Zethner et al., 1987). Since then, AgseGV has been considered as a candidate for the biological control of the so-called cutworm larvae of the turnip moth, *Agrotis segetum* and the ipsilon moth, *Agrotis ipsilon*, which are severe pests of numerous crops in the world (Dugdale, 1995). According to historic records from the Julius Kühn Institute (JKI), specimens of a European isolate of AgseGV, which we termed AgseGV-DA, have been distributed from Germany to several research institutes in Europe, including the Horticulture Research International (Warwick, UK) and the Laboratory of Virology (Wageningen, the Netherlands). All these laboratory stocks apparently derived from the same original material: infected *A. segetum* larvae collected in Austria in 1964 and sent to Federal Biological Research Institute (now the JKI) in Darmstadt, Germany. Chinese AgseGV isolates have also been identified: AgseGV-XJ with the first complete AgseGV genome sequence, and AgseGV-L1, an isolate from Shanghai (Zhang et al., 2014). According to recent reports, the turnip moth, *A. segetum*, is invasive even to isolated areas of Chinese islands and considered as a pest species to various crops (Guo et al., 2015). With this background, the interest in AgseGV as a biological control agent has increased during the last decade, particularly in China (Yang et al., 2012).

In the current study, whole genome sequencing with Solexa Illumina techniques was used to complement genome characterizations of AgseGV and to identify geographical differences between the two Chinese isolates AgseGV-XJ and AgseGV-L1 and the European isolate AgseGV-DA. As little is known about the pathogenesis of AgseGV in general, histopathological investigations were performed and infectivity parameters (LD_{50}) were determined to assert the infection mode of AgseGV-DA in larvae of the turnip moth. These findings will help to facilitate the use of this betabaculovirus in biological control, irrespectively of its presence in mixed infections with Agrotis segetum nucleopolyhedrovirus B as described previously (Wennmann et al., 2015b) or used as single agent as in China (Yang et al., 2012).

2. Materials and methods

2.1. Insects

Mass-rearing of *A. segetum* was performed at the Institute for Biological Control (JKI) in Darmstadt as described previously (Wennmann and Jehle, 2014). In brief, neonate larvae were kept on semi-artificial diet (Ivaldi-Sender, 1974) at 22 °C with a 16/8 h light/dark photoperiod until they reached the fourth instar (L4). For pupation, groups of up to 30 individuals were then transferred to plastic boxes (18.3 cm × 13.6 cm × 6.4 cm) containing 3 cm thick layers of vermiculite (< 0.5 mm grain size). Additional diet was provided shortly before pupation. Pupae were collected every second day and incubated at 25 °C until the adults hatched. Adult moths were kept for two weeks in groups of about 30–50 individuals in transparent plastic cylinders (20 cm diameter, 25 cm height) that were covered inside with rough surfaced paper. Eggs were collected three times a week by replacing paper tissues and incubation at 25 °C in moist boxes for several days until hatching.

2.2. AgseGV lineage and virus propagation

A virus stock of AgseGV was provided by Doreen Winstanley, Horticulture Research International (HRI) collection, Warwick (UK). This virus originated from 112 infected *A. segetum* larvae that were collected in Vienna (Austria) by Otto Muhr in 1964. Occlusion bodies (OB) of this AgseGV isolate (termed AgseGV-DA) had been purified and sent to the Institute for Biological Control in Darmstadt in the late 1960s, from where it was handed over to the HRI by Ole Zethner in 1974. For the experiments presented in this study, virus was propagated with late third instars to early fourth instars of *A. segetum* fed with small pieces of artificial diet (8 mm^3) that were overlaid with 10^6 OB of AgseGV-DA. Larvae that had consumed the entire diet cube within 12 h were transferred to normal diet and incubated individually under standard rearing conditions. Dead larvae were collected on a daily basis and stored at −20 °C for OB purification. Frozen larvae were homogenized in 0.5% sodium dodecyl sulfate (SDS) and treated with an ultra-sonic pulse before being filtered through a sandwich-filter consisting of layers of gaze with mesh cotton. The filtrate was repeatedly washed with water and centrifuged at 12,000*g* for 15 min until the pellet had a whitish/light grey appearance. The pellet was resuspended in water and 2 ml of this suspension were loaded on a 55% - 80% glycerol gradient [gradient steps 80%/70%/60%/55% (v/v) glycerol/water] and centrifuged at 4000*g* at 12 °C in a swing-out rotor for 45 min to separate OB from cadaver debris. The OB band between 60% and 70% glycerol was recovered with a sterile plastic Pasteur pipette and washed twice in water by spinning down in an Eppendorf 5418 R tabletop centrifuge at 12,000*g* for 2 min and resuspending the pellet in water. Finally, the OB were recovered in sterile water and stored at -20 °C. OB concentration was enumerated with a Petroff-Hauser hemocytometer (2.5×10^{-3} mm^2 × 0.02 mm depth) and dark-field microscopy (Leica DM RBE).

2.3. Determination of median lethal dose (LD_{50})

To determine the median lethal viral dose-mortality response (LD_{50}) of AgseGV-DA, full range bioassays were performed with late third instars to early fourth instars (L3-L4) of *A. segetum*. The larvae were starved overnight and subsequently fed with small cubic pieces of artificial diet (8 mm^3) overlaid with serial 1:10 dilutions of AgseGV-DA suspensions of 83 to 8.3×10^6 OB/cube. Each treatment consisted of triplicates of 25 – 30 tested larvae and 50 – 60 uninfected control larvae. Larvae that did not ingest the diet overnight were excluded from the experiment. The tested larvae were then transferred individually into 50 well boxes with normal diet and mortality was scored 7 and 14 days post infection (dpi). Dose-response rates were estimated by probit analysis with ToxRat 3.2 software (ToxRat® Solutions).

2.4. Histopathological studies

To characterize the course of infection of AgseGV-DA in *A. segetum* larvae, selected L4 larvae from the full range bioassays were dissected for light microscopy, whereas L3 larvae of *A. segetum* fed with 2×10^3 OB of AgseGV-DA were used for transmission electron microscopy (TEM). In both cases, larvae were collected 7 dpi and anesthetized with ethyl acetate before embedding. For light microscopy, specimens were fixed in in Bouin's Gendre solution, embedded in paraffin (Histosec®) and a series of 6 µm longitudinal sections were produced with a SK4 rotary microtome (Leitz, Wetzlar). The serial sections were stained following the Heidenhain's iron hematoxylin technique (Eberle et al., 2012) and were evaluated on a DMRB light microscope (Leica Microsystems). For transmission electron microscopy, a modified protocol was used as described previously (Rose et al., 2013). For this purpose, fat-bodies and midguts of infected larvae were dissected and either fixed in 3% glutaraldehyde or in Karnovsky solution at 4 °C for 24 h. After post-fixation with 2% osmium tetroxide