Proteomic analyses of baculovirus *Anticarsia gemmatalis* multiple nucleopolyhedrovirus budded and occluded virus

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Baculoviruses infect insects, producing two distinct phenotypes during the viral life cycle: the budded virus (BV) and the occlusion-derived virus (ODV) for intra- and inter-host spread, respectively. Since the 1980s, several countries have been using *Anticarsia gemmatalis* multiple nucleopolyhedrovirus (AgMNPV) as a biological control agent against the velvet bean caterpillar, *A. gemmatalis*. The genome of AgMNPV isolate 2D (AgMNPV-2D) carries at least 152 potential genes, with 24 that possibly code for structural proteins. Proteomic studies have been carried out on a few baculoviruses, with six ODV and two BV proteomes completed so far. Moreover, there are limited data on virion proteins carried by AgMNPV-2D. Therefore, structural proteins of AgMNPV-2D were analysed by MALDI- quadrupole-TOF and liquid chromatography MS/MS. A total of 44 proteins were associated with the ODV and 33 with the BV of AgMNPV-2D. Although 38 structural proteins were already known, we found six new proteins in the ODV and seven new proteins carried by the AgMNPV-2D BV. Eleven cellular proteins that were found on several other enveloped viruses were also identified, which are possibly carried with the virion. These findings may provide novel insights into baculovirus biology and their host interaction. Moreover, our data may be helpful in subsequent applied studies aiming to improve AgMNPV use as a biopesticide and a biotechnology tool for gene expression or delivery.

INTRODUCTION

Two distinct phenotypes occur during the life cycle of baculoviruses: the budded virus (BV) responsible for intra-host systemic infection, and the occlusion-derived virus (ODV) that is essential for inter-host and oral primary infection in the environment (Keddie et al., 1989). At the end of the viral cycle, the ODV form is occluded in a paracrystalline protein matrix, which forms the occlusion bodies (OBs) (Blissard & Rohrmann, 1990; Castro et al., 1997; King & Possee, 1992). Members of the family *Baculoviridae* are insect-specific viruses and due to their high specificity have become widely used as biological control agents of pests in agriculture and forestry (Moscardi, 1999; O’Reilly et al., 1993; Szewczyk et al., 2006; Tanada & Kaya, 1993). In addition to their classical use as biopesticides, some baculoviruses are also used for recombinant protein production (Maeda, 1989; Smith et al., 1983), and more recently as vectors for human gene therapy (Airenne et al., 2013; Kost et al., 2005).

Baculoviruses have a dsDNA genome contained in a rod-shaped nucleocapsid, coding for 100–180 proteins (Herniou et al., 2003; Slack & Arif, 2007). Members of the family *Baculoviridae* are divided into two groups based...
on their OB morphology: nucleopolyhedrovirus (NPV) and granulovirus (GV). The lepidopteran NPVs are subdivided into groups I and II, following phylogenetic studies done with the polyhedrin gene (polh) (Zanotto et al., 1993), a larger set of conserved genes (Garavaglia et al., 2012; Herniou et al., 2003) or complete genomes (Garcia-Maruniak et al., 2004; Lauzon et al., 2006; Oliveira et al., 2006; Wolff et al., 2008). Distinct fusion proteins are required to invade host cells (Monsma et al., 1996; Pearson et al., 2000). Furthermore, baculoviruses are grouped in four genera following the insect order to which they belong: Alphabaculovirus (lepidopteran NPVs), Betabaculovirus (lepidopteran GV), Gammapaculovirus (hymenopteran NPVs) and Deltabaculovirus (dipteran NPVs) (Jehle et al., 2006).

Proteomic tools and MS are essential for studying proteins encoded by or associated with viruses in general and baculoviruses in particular. The first study that applied this methodology identified the structural proteins of the ODV phenotype of the baculovirus type species Autographa californica multiple NPV (AcMNPV) (Braunagel et al., 2003). Currently, the ODV protein complements of the following baculoviruses have also been studied: Culex nigripalpus NPV (CuniNPV) (Perera et al., 2007), Helicoverpa armigera NPV (HearNPV) (Deng et al., 2007; Hou et al., 2013), Bombyx mori NPV (BmNPV) (Liu et al., 2008), Chrysodeixis chalceis NPV (Xu et al., 2011) and Pieris rapae GV (PrGV) (Wang et al., 2011). However, so far, only the BV proteins of AcMNPV and HearNPV have been identified (Hou et al., 2013; Wang et al., 2010). However, there are limited protein data on the ODV and BV of Anticarsia gemmatalis MNPV (AgMNPV), a group I Alphabaculovirus of economic importance.

Several Latin American countries use AgMNPV as a biological control agent against the velvet bean caterpillar, A. gemmatalis (Lepidoptera: Noctuidae), which is a significant pest for soya bean crops (Moscardi, 1999; Moscardi et al., 2011). The AgMNPV isolate 2D (AgMNPV-2D) was chosen as prototype for basic studies (Maruniak, 1989; Maruniak et al., 1999; Ribeiro et al., 2001; Zanotto et al., 2002), since it was the most frequent genotype isolated from a genetically heterogeneous wild viral population obtained from infected velvet bean caterpillars from Campinas, Brazil in 1972 (Allen & Knell, 1977). The complete genome sequence (132239 bp) of AgMNPV-2D revealed 152 ORFs (GenBank NC_008520.1), with 24 ORFs that possibly encode structural proteins (Oliveira et al., 2006). Recently, the gene expression profile of AgMNPV-2D was determined in two different insect cell lines, showing that at least 149 ORFs are transcribed during the viral replication cycle (Oliveira et al., 2013). In the present study, structural and associated proteins of both the AgMNPV-2D ODV and BV were analysed by two complementary MS techniques. Peptides were identified by correlation of tandem mass spectra against the coding capacity of the AgMNPV-2D genome. A total of 44 proteins were identified in the ODV and 33 in the BV of AgMNPV-2D. These data improve our understanding of viral multiplication, morphogenesis and the possible involvement of cell-encoded proteins in the virion structure.

**RESULTS**

**ODV and BV structural and associated proteins from MS/MS analyses**

Two analytical methods were used to identify the AgMNPV-2D ODV proteins: (i) two-dimensional electrophoresis (2-DE) and MALDI-quadrupole-TOF (MALDI-Q-TOF) and (ii) SDS-PAGE and liquid chromatography–tandem MS (LC-MS/MS). A total of nine 2-DE runs were carried out including biological triplicates. The image analyses using Image Master 2D Platinum detected 85 spots present in all ODV 2-DE gels that were analysed by MALDI-Q-TOF (Fig. S1, available in the online Supplementary Material). However, from the MS analyses of the ODV spots, only 21 proteins were identified (Fig. S1, Table S1). Two proteins stained more intensely in the centre of the gel (spots 1 and 10) were identified as polyhedrin [POLH (ag1)] and CG30 (ag85), respectively (Fig. S1, Table S1). As expected, large amounts of POLH were found as a contaminant (Coulibaly et al., 2009), despite the fact that OBs were treated with alkaline solution and sucrose gradient purified twice. Additionally, intensely stained spots were also identified as VP39, ODV-E25, P49 and ODV-EC27, each of which was also present in several spots. Furthermore, three proteins were determined using only 2-DE and MALDI-Q-TOF methodologies: DNA polymerase (ag65), CG30 (ag85) and Alk-Exo (ag129). Unfortunately, more than half of the spots had a low matching score for sequence coverage against the theoretical ORF database of AgMNPV-2D using this MS method alone. Secondly, triplicate SDS-PAGE runs were carried out for each three using ODV protein samples. The 31 bands were manually excised (Fig. S2a) and analysed by LC-MS/MS, identifying a total of 42 structural and associated proteins in the ODV. Some of these proteins had been confirmed previously during other studies (Braunagel et al., 2003; Deng et al., 2007; Hou et al., 2013; Liu et al., 2008; Perera et al., 2007; Wang et al., 2011; Xu et al., 2011). Nevertheless, in our study 13 proteins were identified as being associated with the nucleosome, 14 proteins with the nucleocapsid structure and 10 were found to be part of the ODV envelope (Fig. 1a). Predictably, polyhedrin and P10 were found in the polyhedra (Fig. 1a). In addition to what was known, we further identified eddysteroid uridine 5’-diphosphate (UDP)-glucosyltransferase (EGT), AG15 and AG20, all of which have unknown functions, associated with the ODV (Fig. 1a, Table S1).

SDS-PAGE analyses were carried out in triplicate for BV protein samples, from which 19 bands were extracted, digested in the gel (Fig. S2b) and then subjected to LC-MS/MS using a QSTAR elite instrument. The 19 bands yielded a total of 33 structural and associated proteins that were identified as part of BV of AgMNPV-2D (Fig. 1b, Table S2). Of these, seven proteins were found to be associated with the
nucleosome, 13 with the nucleocapsid and seven with the envelope (Fig. 1b). Furthermore, six proteins that were previously thought to be present only in the ODV were also found in the BV: PP31, Desmoplakin, ODV-E25, PEP/PP34, ODV-E18 and ODV-E56 (Fig. 1b), similarly to two other reported BV proteomes of NPVs (Hou et al., 2013; Wang et al., 2010). Concerning the ODV, we also detected six proteins with unknown function on the BV (Fig. 1b, Table S2). We did not detect the 6.9 kDa basic protein P6.9 in the BV proteome. It is possible that P6.9 was cleaved into several oligopeptides, one of 11 aa and other smaller oligopeptide fragments as reported for other baculovirus proteomes (Wang et al., 2010). This could explain why it was not detected by electrospray ionization during LC-MS/MS. It appears that we did not have detectable contaminants, because BSA, a major component of cell growth medium, was present in very low amounts (below threshold levels when searching against the contaminant database).

**Novel identified proteins in the AgMNPV-2D proteome**

Our data revealed six proteins in the AgMNPV-2D ODV that have not been reported in other ODV proteome studies: PTP-1, EGT, AG20, PP31, IAP-1 and PK1. PTP-1 is a protein tyrosine phosphatase produced only by group I
NPV, which preferentially uses RNA as a substrate (Takagi et al., 1998). EGT reduces moulting and metamorphosis in insect larvae (O'Reilly & Miller, 1989). We also identified a 38 kDa protein coded by the AG20 ORF using both MS approaches, which suggests an abundance of this protein in the AgMNPV-2D ODV. PP31 was found in the ODV and is critical for late gene expression (Guarino et al., 2002); this protein was also found in the AcMNPV BV proteome (Wang et al., 2010). IAP-1 was identified by MALDI-Q-TOF and LC-MS/MS; this protein was initially classified as anti-apoptotic, but it is possible that it has an opposite function in AgMNPV, as in other group I NPV (Ikeda et al., 2011). The last unique protein identified in the ODV was the protein kinase PK1, a serine/threonine kinase required for polh transcription in AcMNPV (Mishra et al., 2008). Similarly, seven new structural proteins appear to be part of the BV: AG15, LEF-3, P33, P48, helicase, PNK/PNL and AG130. Both AG15 and AG130 were detected by MS analysis, producing two proteins of 41 kDa and 30 kDa, respectively, whereas LEF-3, P33 and helicase are part of the nucleosome of the BV binding with viral DNA, playing an important role in DNA replication (Hang et al., 1995; Kool et al., 1994). PNK/PKL has two described domains: kinase and T4 RNA ligase (Durantel et al., 1998), and is possibly involved in nucleic acid metabolism.

Western blot analyses

During the MS/MS experiments, PTP-1, LEF-1, ODV-E26 and PIF-3 from the ODV had a low score and low match against these four proteins to further check our proteome results (Fig. 2). These results confirmed our MS/MS data by indicating the presence of all four proteins in the ODV but not in the BV; PIF-3 and LEF-1 were only identified by Western blotting.

Comparing AgMNPV-2D proteins to those found in other baculoviruses

By combining all methodologies (MALDI-Q-TOF, LC-MS/MS and Western blotting) we identified 22 viral proteins shared by both phenotypes of AgMNPV-2D (Fig. 3). Most of these proteins were part of the nucleocapsid that is shared by both phenotypes. We also identified several BV/ODV common structural proteins present in the envelope, such as ODV-E25, ODV-E18, PEP and ODV-E56 (Fig. 3). ODV-E18 plays an important role during BV assembly (McCarthy et al., 2008). ODV-E25 and ODV-E56 have a transmembrane motif on their N and C terminus, respectively (Braunagel et al., 1996; Russell & Rohrmann, 1993). These two proteins are more abundant in the BV and are not essential for the production of the BV (Braunagel et al., 1996; Russell & Rohrmann, 1993). We detected PEP, an OB envelope phosphoprotein (Whitt & Manning, 1988), in both phenotypes. These data corroborated findings on the AcMNPV BV proteome (Wang et al., 2010). Almost half of the ODV proteome was encoded by baculovirus core genes (Fig. 3, Table S1) (Garavaglia et al., 2012; Herniou et al., 2003). Twelve BV proteins were highly conserved (core genes) (Fig. 3, Table S2) (Hou et al., 2013; Wang et al., 2010).

Host proteins associated with the AgMNPV-2D virions

The MS/MS data, MALDI-Q-TOF and LC-MS/MS supplied additional information on host proteins associated with both BV and ODV phenotypes of AgMNPV-2D infecting UFL-AG-286 cells (Fig. 3). Eleven cellular proteins were identified in ODV: actin, α-tubulin, elongation factor 1α (EF-1α), elongation factor thermo-unstable (EF-Tu), chaperone proteins HSP70 and HSP90, ribosomal protein S9, ADP/ATP translocase, ATP synthase, histone H4-like protein and GTP-binding nuclear protein RAN (RAS-related nuclear protein) (Fig. 3, Table S3). However, we could identify only actin and HSP90 in the BV (Fig. 3). The associated host proteins were classified according to function based on searches on both National Center for Biotechnology Information (NCBI) and Uniprot databases (http://www.uniprot.org/) as (i) mitochondrion inner membrane, (ii) cytoplasm and (iii) cytoplasm/nucleus. Importantly, these virion-associated host proteins have been identified previously in other enveloped viruses such as vaccinia virus (VV) (Resch et al., 2007), herpesviruses [alcelaphine herpesvirus 1 (AIHV-1) (Dry et al., 2008), herpes simplex virus (HSV) (Loret et al., 2008), Kaposi’s sarcoma-associated herpesvirus (KSHV) (Zhu et al., 2005).
and Epstein–Barr virus (EBV) (Johannsen et al., 2004), human cytomegalovirus (HCMV) (Varnum et al., 2004), respiratory syncytial virus (RSV) (Radhakrishnan et al., 2010), influenza virus (IV) (Shaw et al., 2008) and human immunodeficiency virus type 1 (HIV-1) (Chertova et al., 2006). Moreover, some of these cellular proteins, such as actin, α-tubulin, HSP70, HSP90, EF-1α, histone H4-like protein and the GTP-binding nuclear protein RAN, were identified in other baculoviruses (Hou et al., 2013; Wang et al., 2010).

**DISCUSSION**

By using different proteomic methodologies we identified the structural proteins of the AgMNPV-2D. Our proteomics and Western blotting experiments confirmed 44 and 33 proteins present in the ODV and BV, respectively (Fig. 3). Additionally, 11 host proteins were possibly ODV associated and two others were BV associated.

**Complete MS/MS analyses of AgMNPV**

We combined 2-DE and MALDI (Table S1) with SDS-PAGE and LC-MS/MS methods to obtain viral proteome data (Tables S1 and S2). This approach also yielded more information on other baculoviruses (Braunagel et al., 2003; Deng et al., 2007; Hou et al., 2013; Liu et al., 2008; Perera et al., 2007; Wang et al., 2010, 2011; Xu et al., 2011). Of the methodologies used, MALDI-Q-TOF was the least sensitive, but three proteins were exclusively reported as ODV components: DNA polymerase (ag65), CG30 (ag85) and Alk-Exo (ag129). It was noticeable that the virus-encoded DNA polymerase (ag65) was present in the ODV, while it was not found in virions of other large DNA viruses such as herpesviruses (Dry et al., 2008; Johannsen et al., 2004; Zhu et al., 2005) and HCMV (Varnum et al., 2004). Nevertheless, the DNA polymerase is well conserved in all baculovirus genomes and it was also found as an ODV component in AcMNPV, HearNPV and PrGV (Braunagel et al., 2003; Deng et al., 2007; Wang et al., 2011), possibly suggesting that the virus is trying to maintain some autonomy of its DNA replication from cellular transcription control, despite the fact that its early genes are transcribed by cellular RNA polymerase II. In addition, CG30 (ag85) and Alk-Exo (ag129) were described only in the AcMNPV ODV proteome (Braunagel et al., 2003). In baculovirus, CG30 has no clear role but, as it harbours both a zinc-finger-like and a leucine zipper motif, it could be involved in the regulation of gene expression (Passarelli & Miller, 1994), which is also suggestive of a viral strategy to maximize DNA replication autonomy. Alk-Exo (ag129) is a homologue of an alkaline nuclease; it has 5′ to 3′ exonuclease/endonuclease activity and is involved in DNA recombination and replication (Mikhailov et al., 2003, 2004). To overcome the limitations imposed by MALDI and 2-DE, SDS-PAGE was used for both ODV and BV within the LC-MS/MS analyses.

![Venn diagram showing the phenotypic associations among all AgMNPV-2D proteins. Total protein composition in the two phenotypes from AgMNPV-2D as obtained from 2-DE and MALDI-Q-TOF, SDS-PAGE and LC-MS/MS and Western blotting from an infection by Anticarsia gemmatalis UFL-AG-286 cells. Twenty-two proteins were shared by both phenotypes. The proteins in bold represent the core genes conserved in all baculoviruses sequenced to date.](https://www.microbiologyresearch.org)
Western blotting of AgMNPV proteins

In the ODV proteome, we noted no MS detection of either PIF-3 or LEF-1, and in the BV proteome we did not detect the P6.9 basic protein. Regardless of the sensitivity of MS, our failure to detect some proteins is to be expected due to: (i) their degradation at different rates, (ii) their presence in low relative amounts, (iii) low MS sensitivity and even (iv) the presence of more abundant proteins that hamper detection of others in lower amounts during MS analysis, which will only focus on the most abundant species (Braunagel et al., 2003; Steen & Mann, 2004; Wang et al., 2010, 2011; Xu et al., 2011).

Novel identified proteins and cell modulation proteins found in the AgMNPV-2D

Major efforts have been made to identify structural and auxiliary proteins of baculoviruses that interact directly with host factors, as they may be key to understanding mechanisms of host–virus interaction. Accordingly, our proteome data from AgMNPV-2D helped elucidate the structural proteins present in both phenotypes and brought new insights concerning six new proteins which modulate and regulate cellular and systemic activity, and that were associated with the ODV: PTP-1, EGT, AG20, PP31, IAP-1 and PK1. Both PTP-1 and EGT appear to have been acquired from ancestral hosts (Hoover et al., 2011). PTP-1 is a protein tyrosine phosphatase present only in group I NPVs and was recently described in behavioural hypermobility of B. mori infected with BmNPV (Katsuma et al., 2012). Likewise, EGT is involved in the reduction of systemic ecdysone titres, delaying moulting and metamorphosis, thus allowing the virus to better exploit larval tissues (O’Reilly et al., 1993). EGT was also shown to cause the ‘tree-top disease’ behaviour in the caterpillar Lymantria dispar infected with L. dispar MNPV (LdMNPV) (Hoover et al., 2011). Moreover, during the first day after LdMNPV infection, L. dispar larvae still displayed tree-top disease behaviour, even with the EGT gene knocked out (Hoover et al., 2011). This suggests that other factors, such as PTP-1 carried inside the ODV particles, may have a role in this virus-induced host behavioural change that facilitates predation and consequently, increased viral dispersal. We further identified a 38 kDa protein (encoded by Ag20 ORF) using both MS methodologies. Interestingly, its homologues are conserved in all alphabaculoviruses but with an unclear role (Miele et al., 2011). Curiously, we found phosphoprotein PP31 in both phenotypes, but in AcMNPV it was only present in the BV (Wang et al., 2010). This DNA-binding protein was confirmed as a critical factor for late gene expression in experiments with RNA silencing during AcMNPV infection (Guarino et al., 2002; Schultz & Friesen, 2009). Additionally, we consistently observed IAP-1 using both MS methodologies. This protein, coded by iap genes, has been classified as anti-apoptotic and is conserved in all NPVs from group I sequenced so far (Clem, 2007). Nevertheless, IAP-1 was recently also described as an important pro-apoptotic protein. It induces caspase-dependent apoptosis in late infection by Hyphantria cunea MNPV, AcMNPV, BmNPV and Orgyia pseudotsugata MNPV (Ikeda et al., 2011). As we detected this protein in the ODV of the AgMNPV, it may have an important role, probably shutting off host protein synthesis. This would facilitate viral replication and propagation of ODVs during late stages of infection or virus egression from cells. The last protein identified in the AgMNPV-2D ODV was PK1, a serine/threonine kinase that was associated only with the BV of HearNPV (Hou et al., 2013). Mutations associated with pk1 in AcMNPV and BmNPV blocked very late gene expression, hence showing it was essential for baculovirus replication (Katsuma et al., 2007; Mishra et al., 2008; Reilly & Guarino, 1994). It may also play an important role in G2 arrest during cell division (Ikeda & Kobayashi, 1999), like ODV-EC27. In AgMNPV, PK1 possibly phosphorylates the P6.9 basic protein (P. M. A. Zanotto, unpublished results), but PK1 as an ODV structural protein suggests the need for phosphorylation of host or viral proteins after the onset of infection.

Furthermore, seven new proteins were identified as part of the BV: AG15, LEF-3, P33, P48, helicase, PNK/PNL and AG130. Both AG15 and AG130 were detected by MS analysis. Experiments carried out with AcMNPV did not show a clear role for AG15 (Wang et al., 2007). Likewise, AG130 has no function described as yet. In contrast, LEF-3, P33 and helicase (P143) are very important for DNA replication (Hang et al., 1995; Kool et al., 1994). Interestingly, LEF-3 and helicase were tested with RNA silencing in AcMNPV and the absence of both proteins had a strong negative result at the early phase of apoptosis signalling (Schultz & Friesen, 2009). The P33 protein also targets the host protein P53, inhibiting apoptosis or progression of the insect cell cycle (Prihod’ko et al., 1999). Gene knockout experiments with P33 (Ac92) in the AcMNPV system showed that its absence hinders BV production after DNA replication (Wu & Passarelli, 2010). As in the ODV, it seems that the BV carries proteins that influence apoptosis assisting viral replication. PNK/PKL, which may be part of an RNA repair pathway, and P48 were identified in both the BV and ODV of AgMNPV-2D. Homologues of PNK/PKL with unclear functions were found in five other baculovirus genomes including AgMNPV, three of which are closely related to AcMNPV (Durantel et al., 1998). The P48 protein has orthologues in alpha-, beta- and gammabaculoviruses and was also shown to interfere with BV assembly during knockout experiments (Yuan et al., 2008).

Enveloped large DNA viruses selectively acquire cellular protein

Most of the host proteins associated with AgMNPV-2D have been reported previously in the virion structure of other enveloped viruses such as VV (Resch et al., 2007), AIHV-1 (Dry et al., 2008), HSV (Loret et al., 2008), KSHV
(Zhu et al., 2005), EBV (Johannsen et al., 2004), HCMV (Varnum et al., 2004), RSV (Radhakrishnan et al., 2010), IV (Shaw et al., 2008) and HIV-1 (Chertova et al., 2006). Moreover, RNA suppression subtractive hybridization data showed that most of these cellular proteins had their mRNA level upregulated during AgMNPV-2D infection (Oliveira et al., 2013). Similar cellular proteins were also detected in proteomics that compared uninfected and AcMNPV-infected Spodoptera frugiperda Sf9 cells (Carinhas et al., 2011). We found several virion-associated host proteins, differing in function and sources, ranging from the cytoskeleton structure (e.g. actin, α-tubulin, EF-Tu, HSP90, RPS9 and EF-1α), DNA binding (e.g. H4) and mitochondria (e.g. ANT1 and ATP-syntabN) to the nucleus (e.g. RAN and HSP70). We also found, during our MS analyses, chaperone proteins such as HSP70 and HSP90. These two abundant proteins were identified in the ODV but we only found HSP90 in the BV. HSPs were found to play a significant role in the protein metabolism of several viruses (Carinhas et al., 2011). The ribosomal protein S9, found in AgMNPV-2D ODV, was also the most abundant protein detected in Sf9 cells upon infection by AcMNPV (Carinhas et al., 2011). Cytoskeleton actin was also found to be copious by our MS analysis. This could be explained by the fact that it is required for the movement of the nucleocapsid from the cytoplasm to the nucleus during the early stages of infection (Gandhi et al., 2012; Ohkawa et al., 2010).

It is quite interesting to note that phylogenetically unrelated enveloped viruses appear to sequester a similar set of host proteins in their virions. This functional convergence may indicate a fundamental, general role of specific cell functions in the early stages of the virus life cycle, and possibly new roles for envelope-associated functions. However, the protein assortativity we observed may be a consequence of recurrent, common and particularly strong protein–protein interactions.

In summary, we have presented the structural proteins from ODV and BV of AgMNPV-2D, an important member of the family Baculoviridae. This work has brought new insights into virion-associated host proteins, which will underpin subsequent investigations on their function and action mechanisms, within both basic and applied research contexts. We have also described proteins that could have pro-apoptotic activity and/or be involved in the host behaviour manipulation during infection, helping viral transmission. Notwithstanding, functions for most of the proteins described herein for AgMNPV-2D were based on what is known from other baculoviruses. Additional experiments with AgMNPV are needed to better define their localization, functions and molecular interactions.

**METHODS**

**Cell culture, virus infection, ODV and BV purification and quantification.** The A. gemmatalis cell line UFL-AG-286 (Sieburth & Maruniak, 1988), which supports growth of AgMNPV-2D, was maintained at 28 °C in Grace’s insect medium (Invitrogen) supplemented with 10% (v/v) FBS. UFL-AG-286 cells were infected with AgMNPV-2D at an m.o.i. of 1. Purification was performed according to Braunagel & Summers (1994) to avoid cell protein contamination. Briefly, at 48 h post-infection (p.i.) the supernatant with BV was collected and centrifuged on a sucrose cushion and then purified on a continuous (25–60%, w/w) sucrose gradient. ODVs were collected at 72 h p.i. and the OBs were purified. Subsequently, the ODVs were released by alkaline treatment (pH 10.9) and then purified by continuous (40–65%, w/w) sucrose gradient centrifugation. Both pellets within BV and ODV virions were resuspended in 0.1 x TE (pH 7.4) with protease inhibitor (GE Healthcare) to prevent any possible degradation of virion components. All samples were obtained in triplicate.

**2-DE, SDS-PAGE and in-gel digestion.** Experiments with 2-DE gels were performed in triplicate with 300 µg ODV proteins quantified using a 2-DE Quant kit (GE Healthcare). Briefly, proteins were solubilized in a 690 µl rehydration solution containing 7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, 0.5% IPG buffer and 0.002% bromophenol blue, followed by a hydration step of three IPG dry strips [pH 3–10 linear (180 x 30 x 0.5 mm)] overnight. Isoelectric focusing was conducted in an IPGphor system (GE Healthcare) in gradient mode with four steps, and a total of 17,250 V h were accumulated. The second dimension was run onto a 12% uniform SDS-polyacrylamide gel. All 2-DE gel experiments were done in triplicate and spot analyses were normalized with the Imagemaster 2D Platinum software version 7.0 (GE Healthcare). Alternatively, a 500 µg sample of ODV and BV from each three was quantified by the method of Bradford (1976) and proteins were separated in triplicate SDS-PAGE runs. Subsequently, the gels were fixed and stained with Coomassie Brilliant Blue R-350 (GE Healthcare). All spots and bands from the triplicate SDS-PAGE gels were manually excised, stained, reduced, alkylated and digested in gel with trypsin-modified sequencing-grade reagents (Promega) according to Deng et al. (2007) and Paes Leme et al. (2012).

**Protein identification by MS.** We used two complementary MS techniques for the tryptic in-gel digested peptides. First, a MALDI-Q-TOF Premier mass spectrometer (Waters) was used to analyse ODV spots enzymatically digested from 2-DE triplicate gels. For MALDI-Q-TOF analyses, each ODV tryptic peptide was mixed with 8.6 mg z-ciano MALDI matrix ml⁻¹ and spotted onto the sample plate; it was then mixed, dried and analysed in delayed extraction and reflectron modes using polyethylene glycol glycol oligomers as external calibrants. Subsequently, the peptides from in-gel digestion of the ODV and BV 12% SDS-polyacrylamide gel were analysed by LC-MS/MS. For Q-TOF an Ultima mass spectrometer (Waters), the peptides from SDS-PAGE (4.5 µl) were loaded and then separated using a C₁₈ column (100 µm x 100 mm) coupled with a nanoelectrospray source set at 3.5 kHz with a cone voltage of 30 V, at a flow rate of 0.6 µl min⁻¹. The instrument was operated in the ‘top three’ mode in a data-dependent analysis, in which one MS spectrum is acquired followed by MS/MS of the three most intense peaks detected. For the QSTAR elite hybrid Q-TOF mass spectrometer (Applied Biosystems), some of the enzymically digested samples from ODV and all tryptic peptide mixture from BV were injected into a capillary trap (LC Packings PepMap) and desalted for 5 min using 0.1% (v/v) acetic acid at a flow rate of 3 µl min⁻¹. Samples were then loaded onto an LC Packing C₁₈ PepMap nanoflow HPLC column. LC-MS/MS analyses were carried out using the information-dependent acquisition (IDA) mode in which a survey scan from m/z 400 to 1800 was acquired followed by collision-induced dissociation of the four most intense ions. Survey and MS/MS spectra for each IDA cycle were accumulated for 1 and 3 s, respectively. All peptides were analysed at the Laboratory of Mass Spectrometry, Brazilian Biosciences National Laboratory, Brazil, and at the proteomic facility of the
Data analysis and protein search algorithm. The spectra were acquired using MassLynx v.4.1 software and the raw data files were converted by Mascot Distiller v.2.3.2.0, 2009 software (Matrix Science). All data were searched against our AgMNPV-2D ORF database consisting of 152 ORFs coding for 40,427 residues and a non-redundant insect protein database obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) using Mascot 2.0 online software (Matrix Science). Moreover, to conduct our downstream analysis (Foster, 2011; Knudsen & Chalkley, 2011), we assembled a special database containing proteins from Lepidoptera, Hymenoptera and Diptera with a minimal length of 60 aa. Mascot software was set with a fragment ion mass tolerance of 0.1 Da and a parent ion tolerance of 0.1 Da. Iodoacetamide derivative of Cys, deamidation of Asn and Gln, and oxidation of Met, were specified in Mascot as variable modifications. During the analyses, our samples were checked against a contaminant database supplied by Applied Biosystems. The BV proteins were further confirmed with Scaffold-03-00-04 software (Proteome Software) to validate MS/MS-based peptide and protein identification. All validated proteins had at least two independent spectra with a minimal length of 10 aa, with greater than 99.0 % probability estimated by the Peptide Prophet algorithm (Keller et al., 2002) of being encoded by AgMNPV-2D or were present the insect database as at least two unique peptides (Nesvizhskii et al., 2003). Moreover, the classification and function of the proteins found were determined manually using the NCBI Uniprot resource (http://www.uniprot.org/) and the known baculovirus proteins in the literature to date.

Antisera generation and Western blot analyses. Our MS results were further confirmed by Western blotting. For this, we designed polyclonal antibodies against PTP-1, LEF-1, ODV-E26 and IF-3. Briefly, these four genes from AgMNPV-2D were amplified by PCR, and a Gateway Cloning Technology (Life Technologies) kit was used for cloning the PCR products in E. coli DH5-α according to the manufacturer’s instructions. The bacteria were collected by centrifugation and used for protein purification according to the method of Anandaraao et al. (2006). Polyclonal antibodies were raised in rats against the isolated proteins by standard methods. Western blot analyses were performed individually with the four antisera produced. Proteins were loaded on a 12 % SDS-polyacrylamide gel using the Mini Protean Tetra Cell apparatus (Bio-Rad) following the manufacturer’s instructions. The immobilized proteins were transferred onto nitrocellulose membranes (Sigma) using the Trans-Blot Semi Dry Transfer Cell (Bio-Rad). The membranes were then blocked in 1 × PBS containing 5 % skimmed milk, washed three times with PBS, 0.05 %Tween 20 and probed with the desired antiserum followed by incubation with the alkaline phosphatase-conjugated anti-mouse/rat secondary antibody (Promega). Blots were developed using the NBT/BCIP (Sigma) substrate dissolved in alkaline phosphatase buffer.

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