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VLPs of *Leptopilina boulardi* share biogenesis and overall stellate morphology with VLPs of the *heterotoma* clade

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Abstract

Viruses and virus-like particles (VLPs) of insect parasitoids modify host-parasite interactions. The *Drosophila* wasp, *Leptopilina heterotoma*, produce 300 nm spiked VLPs that bind to the host's blood cells via surface projections. *L. heterotoma* is a generalist wasp that attacks over a dozen *Drosophila* species. Oviposition introduces VLPs into the hemolymph of *Drosophila* larvae. VLPs lyse hemocytes and obliterate immune signaling in infected larval hosts. *L. boulardi*, a member of a distinct *Leptopilina* clade, is a specialist, whose host range is limited to the *melanogaster* group. As a step toward understanding a potential relationship between venom contents and host range in these wasps, we used electron microscopy to characterize VLPs from the virulent *L. boulardi*-17 (*Lb*-17) strain. While the *Lb*-17 VLPs can neither lyse blood cells nor suppress host defense, their biogenesis is surprisingly similar to that of *L. heterotoma*. Like *L. heterotoma* VLPs, *L. boulardi* VLPs are stellate; but they have fewer spikes, each spike being significantly longer than the spikes in *L. heterotoma* VLPs. The VLP possess a dimple, making them clearly distinct from *L. heterotoma* VLPs. We discuss the significance of these cross-clade differences in VLP morphologies in relation to their biological activities and the host range of the wasp.

Keywords

Innate immunity; immune evasion; signaling; virulence; *Leptopilina boulardi*; virus-like particles

1. Introduction

Virus-like particles (VLPs) possess structural properties of viruses, but because they lack genetic material, they are non-infectious. In recent years, VLPs have gained value in vaccine development and as delivery systems for active small molecules and nucleic acid. VLPs are also found naturally in plant and animal cells. In this study we report biogenesis and morphology of *L. boulardi* VLPs, and compare them with biogenesis and morphology of VLPs from the distinct, *heterotoma* clade. Parasitoids constitute a very large number of

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insects that develop outside or within other insects, killing their host. Females insert their eggs into hosts mostly at the egg or larval stage.

Parasitoids utilize various strategies to avoid or overcome host immunity. Immune reactions against parasitoids have been well studied in *Drosophila* (Paddibhatla et al., 2010; Rizki and Rizki, 1984; Rizki and Rizki, 1992; Russo et al., 1996; Schlenke et al., 2007; Sorrentino et al., 2002). Because of extensive genetic tools, this system is particularly well-suited for detailed analysis of immune suppressive strategies.

The *Leptopilina* genus is divided in three clades (*heterotoma*, *boulardi* and *longipes*) (Allemand et al., 2002; Schilthuisen et al., 1998). These wasps attack larvae of *Drosophila* spp. and inject VLPs into the larval hemolymph during oviposition (Chiu et al., 2006; Dupas et al., 1996). Oviposition perturbs global transcription. While *L. boulardi*-17 (*Lb*-17), a specialist parasitoid modifies host transcription extensively, including activating immune signaling, *L. heterotoma*-14, a generalist parasitoid, with a significantly wider host range, has milder effects on host transcription. This disparity is attributed to differences in the venom proteins and VLPs of the two species (Colinet et al., 2009; Labrosse et al., 2005; Schlenke et al., 2007).

The venom of two sister species of the *heterotoma* clade, *L. heterotoma* and *L. victoriae*, contains several proteins and 300 nm-wide spiked VLPs (Chiu et al., 2006; Morales et al., 2005). Electron microscopy revealed that VLPs have a pentagonal or hexagonal core with short spike-like projections. Projections end in knobs that interact with blood cells (or hemocytes) of *D. melanogaster* larvae (Chiu et al., 2006; Morales et al., 2005). Oviposition triggers the differentiation of a class of large hemocytes, called lamellocytes (Rizki and Rizki, 1984; Sorrentino et al., 2002). Lamellocytes wrap around the wasp egg to encapsulate it and block its development. Accumulating evidence suggests that *L. heterotoma*/*L. victoriae* VLPs target lamellocytes for destruction to inhibit encapsulation (Chiu and Govind, 2002; Morales et al., 2005; Rizki and Rizki, 1984; Rizki and Rizki, 1991; Rizki et al., 1990). Purified VLPs induce lamellocyte lysis *in vitro* (Morales et al., 2005; Rizki and Rizki, 1992; Schlenke et al., 2007).

A virulence protein (p40/p47.5), produced in secretory cells of the *L. heterotoma*/*L. victoriae* venom gland remains associated with maturing VLPs, enters lamellocytes, and interferes with lamellocyte function and viability (Chiu et al., 2006; Ferrarese et al., 2009). Thus, both *L. heterotoma* and *L. victoriae* share structural features and biochemical factors for the suppression of their host's cellular immune response (Chiu et al., 2006; Morales et al., 2005).

L. boulardi infection also suppresses *D. melanogaster* cellular immune response (Colinet et al., 2009; Labrosse et al., 2005; Schlenke et al., 2007). However, the lytic activity associated with the VLPs of *L. heterotoma* and *L. victoriae* has not been reported in the venom secretions of *L. boulardi*, including *Lb*-17, even though several *L. boulardi* strains harbor VLPs (Dupas et al., 1996; Schlenke et al., 2007). A cross-clade comparison of VLP structures will provide insights into the potentially common and possible viral origin of these particles. These studies will also explain why VLPs of the same genus evoke such different responses in their hosts and how they contribute to the success of the wasps.

Previous fine structure analysis of *L. boulardi* VLPs (Dupas et al., 1996; Schlenke et al., 2007) is fragmentary as it does not describe VLP biogenesis and the final three-dimensional structures of the particles. Using electron microscopy, we characterized the venom gland content of *Lb*-17, a North American virulent strain (Schlenke et al., 2007). We report the existence of VLP precursors at different stages of biogenesis within the supracellular canals

and long gland lumen of the wasp's venom gland. Mature *Lb-17* VLPs are large with extensive side projections that are clearly distinguishable from those of *L. heterotoma/L. victorae* VLPs. We further propose that the shared morphological features and the similarities in the mode of biogenesis are consistent with a potential viral nature of *Leptopilina* VLPs. However, the morphological specificities contribute to differences in *Leptopilina-Drosophila* interactions and thus the wasp's host range.

2. Materials and methods

2.1. Wasp maintenance

Lb-17 wasps were originally sampled in California, North America (Schlenke et al., 2007). They are maintained in the lab on *yellow white* (*y w*) strain of *D. melanogaster* at 23°C.

2.2 Electron microscopy

2.2.1. Ultrastructure of VLPs and venom gland—For VLP ultrastructure, both transmission (TEM) and scanning (SEM) electron microscopy were used according to methods described in (Morales et al., 2005). Long gland/reservoir complexes from around 500 *Lb-17* wasps were dissected in PBS. Samples were fixed in 0.085 M cacodylate buffer for 1 hour, washed twice and fixed again in 1% osmium tetroxide prepared in 0.085 M cacodylate buffer for 1 hour. Samples were filtered on polycarbonate with 0.1µm pores, dehydrated in a graded series of ethanol and stored in 70% ethanol.

For SEM, filters were rinsed twice in amyl acetate, dried, mounted on an SEM pin and kept at 60°C for more than 2 hours. They were then coated with gold/palladium to observe with a Zeiss Supra 55 VP microscope. For TEM, samples were embedded in EMBeb-812 and sectioned. Sections were stained with 4% uranyl acetate for 4 h, followed by 0.6% lead citrate for 30 min. Sections were observed using a Zeiss EM 902 microscope.

2.2.2. VLPs in host hemolymph—To document morphologies of mature VLPs, 5 day-old *y w* larvae were infected with *Lb-17* for 1 hour and dissected in 4% glutaraldehyde; their hemolymph was quickly transferred into a tube with the fixative. Samples were fixed on ice for 1 hour. 2% osmium tetroxide was then added and samples were kept 10 more minutes on ice. Samples were then processed according to the details described above for SEM.

2.3 VLPs measurement

At least 10 VLPs were measured for each class of structures. For each VLP, both the core and its corresponding spikes were measured.

3. Results

L. boulardi exhibits considerable intraspecific variation in virulence and superparasitism behavior (Dubuffet et al., 2009; Varaldi et al., 2009). Superparasitism in this species is linked to a filamentous virus (*Leptopilina boulardi* Filamentous Virus - *LbFV*) found in the females oviduct (Varaldi et al., 2009). Using behavioral assays (as described in (Patot et al., 2009)), we found that *Lb-17* does not superparasitize *D. melanogaster* (data not shown). PCR experiments (Patot et al., 2009) also failed to amplify the two genomic markers described for the filamentous virus (data not shown). These observations suggest that *Lb-17* is free of the filamentous virus reported in other *L. boulardi* strains (Patot et al., 2009; Varaldi et al., 2009) simplifying the interpretation of the electron microscopy results below.

3.1. Ultrastructure of venom gland and VLP biogenesis in *Lb-17*

The *Leptopilina* venom gland is a tripartite structure with the long gland, the reservoir and the ovipositor (Fig. 1A). We prepared sections from *Lb-17* long gland and reservoir for fine structure analysis (Fig. 1B-H). The long gland lumen includes: (1) long filamentous structures ranging between 500–1000 nm in length (Fig. 1B-C, arrowheads). Their length approaches the size of projections of fully-formed VLPs suggesting that they could be added onto the geometric core at a subsequent step of VLP biogenesis (see below). These structures also appear similar to the long VLPs of the Ichneumonid wasp *Diadegma terebrans* (Krell, 1987). (2) Spherical or somewhat more elongated structures with an ovoid core and 1–2 projections (Fig. 1C–D, arrows). These structures range from 200-to-350 nm in diameter. The fact that a section through the middle of an ovoid/elongate core generates a spherical morphology suggests that these particles represent a single class of structures.

The electron micrographs revealed the existence of a supracellular system of canals that we recently reported from the long glands of *Leptopilina* wasps in whole-mount confocal experiments (Ferrarese et al., 2009). This study showed that in both *L. heterotoma* and *L. victorinae*, VLPs undergo assembly and maturation within this supracellular canal system (Ferrarese et al., 2009). In the present study, we found a comparable array of structures in *Lb-17*: rough canals surrounded by a matrix (Fig. 1E, stars) and microvilli (Fig. 1E, mv), and smooth canals (Fig. 1F) that end into the long gland lumen (Fig. 1G). Moreover, VLP precursors are found in cross sections of both the rough and smooth canals (Fig. 1E-G, arrows) strongly suggesting that the canal system is involved in *Lb-17* VLPs biogenesis.

Finally, sections of the *Lb-17* reservoir reveal only ovoid particles similar to those described previously (Fig. 1H) (Dupas et al., 1996). At this stage of biogenesis, electron-light regions, called “vesicles” by (Dupas et al., 1996), are also found in *Lb-17* VLPs. Sections of *Lb-17* reservoir did not contain filamentous structures (Fig. 1H). Altogether these results confirm previous reports of VLPs in other *L. boucardi* strains (Dupas et al., 1996) and strongly suggest that VLP assembly and morphogenesis is shared by at least three *Leptopilina* species.

3.2. Three-dimensional morphology of *Lb-17* VLPs

VLPs can influence host defense only if they are introduced into the host during oviposition. To understand the relationship between the *Lb-17* VLPs and mature VLPs of *L. heterotoma*/*L. victorinae*, we examined their three-dimensional morphology from preparations of the venom gland fluid and host larval hemolymph post infection (Fig. 2). Structures found in venom gland extracts include (a) clusters of spheres (Fig. 2A–B) of 506 ± 62 nm in diameter, some of which have a depression or dimple (Fig. 2B, black star). Most have 2-to-3 long spikes measuring 619 ± 195 nm, which is the length of filamentous structures observed in the transmission electron micrographs of the long gland lumen (Fig. 1B–C, arrowheads); (b) Pleomorphic structures (Fig. 2C, arrowheads) with a core measuring 343 ± 65 nm X 304 ± 94 nm. These bear 3 to 4 long spikes whose length is 312 ± 74 nm; they also possess a dimple (Fig. 2C, black star). We refer to these spiked structures as stellate VLPs; and (c) collapsed VLPs (Fig. 2D) with spikes wrapped on the core, but nevertheless with one long and thick extension. Interestingly, while differing in their core morphology, all these particles possess at least one spike terminating in a knob (Fig. 2A–B, E, white stars) and one dimple (Fig. 2B–C, E–F, black stars). Dimples have not been reported for VLPs of other *Leptopilina* species and their function in *Lb-17* VLPs is unclear. The stellate structures found in the host hemolymph (Fig. 2E–F) likely correspond to the morphology of mature *Lb-17* VLPs.

4. Discussion

4.1 Putative function of *Lb-17* VLPs in immune suppression

The VLPs from all three *Leptopilina* species studied to date possess an ovoid core extending into spikes. Each spike ends in a knob-like expansion (Fig. 2, 3; (Chiu et al., 2006; Morales et al., 2005)). These common features suggest that all VLPs share a mechanism for interacting with their target host cells. VLPs likely deliver associated virulence proteins or toxins of the venom gland. This interpretation is based on the association of putative virulence proteins p40/p47.5 with *L. heterotoma*/*L. victorae* VLPs (Chiu et al., 2006). It will therefore be of interest to examine if virulence factors from *L. boulardi* strains (e.g., a serpin in the G486 strain or LbGAP from the G431 strain) (Colinet et al., 2009; Labrosse et al., 2005) that disrupt or prevent encapsulation in fly hosts, also associate with the mature *L. boulardi* VLPs. Such a relationship will shed light on the precise biological functions of VLPs and associated proteins.

4.2 Diversity and biogenesis of *Leptopilina* VLPs

Lb-17 VLPs are clearly distinct from VLPs from the other two species: (a) *Lb-17* VLPs are larger (a core of around 350 nm compared to 300 nm in *L. heterotoma*), (b) have longer spikes (up to 800 nm compared to around 50 nm in the other two *Leptopilina* sp.) and, (c) some are dimpled, a feature not observed in VLPs from the other *Leptopilina* species (Fig. 1–2, (Morales et al., 2005)). It is quite likely that the unique features of *Lb-17* VLPs described in this study are shared among VLPs of other *L. boulardi* strains. Furthermore, we expect to find stellate VLPs in *L. orientalis* and *L. freyae*, wasps of the *boulardi* clade (Fig. 3). We propose that unique VLP morphologies among *Leptopilina* species contribute to specificities in the biological effects and host range among these species (Morales et al., 2005; Schlenke et al., 2007).

The secretory unit of the *Leptopilina* venom gland seems to be particularly conserved among the Figitid wasps (this study and (Ferrarese et al., 2009)). However the molecules produced in these units appear to be diverse in both their morphology and effects on the host (this study, (Chiu et al., 2006; Morales et al., 2005; Schlenke et al., 2007)) suggesting a diversity of biochemical and macromolecular processes supporting their genesis. Despite these differences, it appears that the cell biological context in which VLPs arise and assemble in the venom gland is similar in wasps of the *Leptopilina* genus. Because of similarities in their final morphologies and the way VLPs assemble within secretory canals of the venom gland of all three *Leptopilina* species, we speculate that these particles were already present before the divergence of the *L. heterotoma* clade from the *L. boulardi* clade (Fig. 3, black arrow). Alternatively, these VLPs were independently acquired in the two clades (Fig. 3, red arrows).

4.3 Possible viral origin of *Leptopilina* VLPs?

A class of parasitoids of Lepidopteran hosts relies on symbiotic polydnnaviruses (PDVs) to counteract host immunity. Polydnnaviruses are not infectious in the wasps, but encode virulence factors that are expressed in their wasps' hosts (Bezier et al., 2009a; Bezier et al., 2009b; Summers and Dib-Hajj, 1995; Webb and Strand, 2005). PDVs constitute the first example of a virus being involved in an obligatory mutualism with an insect species (Bezier et al., 2009a; Stoltz and Whitfield, 2009). Unlike PDVs, the presence of nucleic acid has not been reported from VLPs. However, they share common features with PDVs: (1) they are injected into the wasp's larval host during oviposition; (2) they are necessary for successful parasitism, i.e., they are immune suppressive; and (3) they are produced in a specialized organ associated with the ovipositor (venom gland for *Leptopilina* VLPs and calyx for PDVs).

Although, at this time, we cannot link *Leptopilina* VLPs to a family of known viruses, it is clear that they share morphological features with known viruses. The diameter of VLP core is in 250–350 nm - a size range that is comparable to arenavirus (ICTV 00.003.0.01), vaccinia virus (ICTV 00.058.1.01.001), or poxvirus (ICTV 00.058) (ICTVdB - The Universal Virus Database). Furthermore, spike-like extensions are observed in arenavirus and poxvirus (ICTVdB - The Universal Virus Database). The shared morphological features of *Leptopilina* VLPs identified in this study therefore support the "virus-like" nature of these particles.

Where did the VLPs come from and do they have any relationship with viruses? Do they, like PDVs, have a viral origin? Or are, VLPs simply the results of the wasp's maternal secretions produced in specialized organelles, the secretory units, for the explicit purpose of the delivery of venom factors? Our findings in this study will allow purification of *L. boulardi* VLPs following the protocols used for VLPs from *L. heterotoma* and *L. victorinae* (Morales et al., 2005; Chiu et al., 2006). VLP purification is an essential step toward proteomic and molecular analyses that will ultimately lead to addressing the evolutionary origin of these structures.

5. Conclusion

The exact biological function of *Leptopilina* VLPs is still mysterious. We suspect that differences in VLP composition and morphology contribute to differences in their ability to succeed on *Drosophila* spp. VLP components are likely to be encoded in the respective parasitoid genomes. Characterization of VLPs and their wasps will provide insights into the differences in their morphological and molecular specificities against host cells, potentially defining their host range.

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Highlights

- VLPs are essential virulence factors in some *Drosophila* parasitoids
- *Leptopilina*'s VLPs are stellate and share a similar biogenesis in wasp venom gland
- The stellate structures found in the host hemolymph
- *L. boulardi* VLPs are distinct from *L. heterotoma*'s, possess a dimple and long arms
- *L. boulardi* and *L. heterotoma* VLPs may have a similar, potentially viral, origin

Figure 1. Transmission electron micrographs of sections of *L. boulandi-17* venom gland

A. Schematic showing the organization of the long gland, reservoir and ovipositor complex of the wasp's venom gland. Arrows point to the regions from where the ultrathin sections were prepared. (B–D) Contents of *Lb-17* long gland. B. Fine structure of the long gland region with secretory cells (SC) and lumen (L). C. The lumen contains numerous long filamentous structures (arrowheads) and immature VLPs (arrows). D. Immature VLPs at higher magnification (arrows). (E–G) One cytoplasmic canal originates from each secretory cell and empties into the long gland lumen (see Ferrarese et al., 2009 for more details). E. Cross section from the rough canal portion with distinct microvilli (mv), proximal to the secretory cell. The rough canal has immature VLPs (arrow) in its lumen. The rough canal lumen is surrounded by a mesh-like matrix (white stars). Arrowheads indicate electron-dense particles between the microvilli and inside the matrix. F. Cross section of a smooth canal (no microvilli, distal to the secretory cell, and joins the lumen-see G.) with immature VLPs (arrow). Arrowhead points to the cuticle surrounding the canal. G. Smooth canal (cuticle-lined – arrowhead) ending into the gland lumen. VLPs (arrows) from the canal are dumped into the gland lumen (L) towards the reservoir.

H. Contents of the venom gland reservoir. The reservoir contains VLPs ranging from 200–450 nm in diameter. VLPs at this stage of biogenesis contain electron-light regions.

Figure 2.

(A–D) Scanning electron micrographs of structures recovered from *Lb-17* venom gland, or (E–F) from *D. melanogaster* hemolymph post-parasitization. A. Clusters of VLPs recovered from the wasp venom gland. (B–D) VLP morphologies in the venom gland. Stellate particles are found in both the venom gland (C) and in the host hemolymph compartments (E–F). Black stars indicate VLPs dimple, white stars indicate knobs at the end of the extensions and arrowheads point to spikes.

Figure 3. Phylogeny of *Leptopilina heterotoma* and *boulardi* clades (adapted from (Allemand et al., 2002)) superimposed with VLP morphologies

The first, more parsimonious, hypothesis is that wasps of the clade ancestral to the *heterotoma* and *boulardi* clades possessed stellate VLPs (black arrow). After the divergence of these clades, VLPs evolved independently acquiring distinct morphologies. In an alternative scenario, VLPs in both clades arose independently (red arrows). VLPs from *L. longipes* have not been reported yet. Studies of VLPs from additional species of the *Leptopilina* genus will help to distinguish these hypotheses. Panels to the right of the phylogeny allow comparison of surface morphologies of *L. heterotoma* (top) and *Lb-17* (bottom) VLPs.