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A new approach for investigating venom function applied to venom calreticulin in a parasitoid wasp

Aisha L. Siebert [a, c, *], David Wheeler [b, c], John H. Werren [c]

[a] Department of Clinical and Translational Science, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, USA
[b] Institute of Fundamental Science, Massey University, Palmerston North, 4442, New Zealand
[c] Department of Biology, University of Rochester, Rochester, NY 14627, USA

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ABSTRACT

A new method is developed to investigate functions of venom components, using venom gene RNA interference knockdown in the venomous animal coupled with RNA sequencing in the envenomated host animal. The vRNAi/eRNA-Seq approach is applied to the venom calreticulin component (***v-crc***) of the parasitoid wasp *Nasonia vitripennis*. Parasitoids are common, venomous animals that inject venom proteins into host insects, where they modulate physiology and metabolism to produce a better food resource for the parasitoid larvae. vRNAi/eRNA-Seq indicates that *v-crc* acts to suppress expression of innate immune cell response, enhance expression of clotting genes in the host, and up-regulate cuticle genes. *V-crc* KD also results in an increased melanization reaction immediately following envenomation. We propose that *v-crc* inhibits innate immune response to parasitoid venom and reduces host bleeding during adult and larval parasitoid feeding. Experiments do not support the hypothesis that *v-crc* is required for the developmental arrest phenotype observed in envenomated hosts. We propose that an important role for some venom components is to reduce (modulate) the exaggerated effects of other venom components on target host gene expression, physiology, and survival, and term this venom mitigation. A model is developed that uses vRNAi/eRNA-Seq to quantify the contribution of individual venom components to total venom phenotypes, and to define different categories of mitigation by individual venoms on host gene expression. Mitigating functions likely contribute to the diversity of venom proteins in parasitoids and other venomous organisms.

1. Introduction

Parasitoid wasps are an abundant and diverse group of 100–400 thousand species that parasitize other insects and use venom to alter host physiology to create an improved environment for developing wasp offspring (Whitfield, 1998; Heraty, 2009; Quicke, 1997). The best-characterized parasitoid venom system is that of the small (<2.5 mm) ubiquitous parasitoid *Nasonia vitripennis*, which produces ~100 venom proteins (de Graaf et al., 2010; Werren et al., 2010). Approximately 25% of these proteins have no recognizable homology to proteins found in other organisms. The venom proteins cause developmental arrest and manipulate highly conserved metabolic pathways in several distantly related fly host pupae, including *Sarcophaga bullata* (de Graaf et al., 2010; Mrinalini et al., 2014; Rivers and Denlinger, 1994; Werren et al., 2010). The envenomated fly host remains alive and transcriptionally active for over five days (Rivers and Denlinger, 1995), and exhibits transcriptional changes that correspond to metabolic, immune response, and developmental phenotypes (Danneels et al., 2013; Martinson et al., 2014; Mrinalini et al., 2014).

Attributing functions to individual venom components is challenging because the envenomation phenotype is often driven by synergistic interactions among multiple peptides. Venom proteins from many animals often have redundant functions *in vivo* – comprising "cabals" with overlapping effects on critical host pathways (Jimenez et al., 2003). Functional characterization is further complicated by the observation that venom proteins often share little to no sequence homology with previously characterized proteins in other species (de Graaf et al., 2010). In addition, classical

* Corresponding author. 601 Elmwood Avenue, Box 181, Rochester, NY 14642, USA.
E-mail address: Aisha_siebert@urmc.rochester.edu (A.L. Siebert).

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methods that rely on purification of individual proteins followed by biological testing are difficult in parasitoids given the small volume of venom that can be practically isolated from these insects. However, advances in genomic, transcriptomic, and proteomic tools that can use smaller volumes of material now make broad characterization of parasitoid venoms and their effects more practical.

High-throughput genome and transcriptome sequencing is now widely recognized as a powerful tool for both hypothesis testing and hypothesis generation (Egan et al., 2012; Hawkins et al., 2010; Lee et al., 2014b; Mwenifumbo and Marra, 2013; Wittkop et al., 2013). Here we investigate venom function using a combination of genetic knockdown (**KD**) of wasp venom genes by RNA interference (**vRNAi**) followed by high-throughput RNA-sequencing (**eRNA-seq**) of the envenomated host. The method, henceforth called **vRNAi/eRNA-seq**, does not require *a priori* identification of target genes/pathways or purification of biologically active individual venom proteins. Functional redundancy of venom proteins can be addressed by calculating the relative contribution of individual venom components to the total venom phenotype (see Methods Section 2.11).

To demonstrate the efficacy of the vRNAi/eRNA-Seq method, we chose to investigate the function of calreticulin, a calcium-binding protein and molecular chaperone present in both the endoplasmic reticulum and mitochondria, as well as secreted in *N. vitripennis* venom. In other animals calreticulin performs a variety of functions, including regulation of steroid hormone genes, cell adhesion, low-affinity high-capacity calcium sequestration, signal-induced calcium release (Mesaeli et al., 1999), and immune regulation (Pockley, 2003).

The *N. vitripennis* genome contains only a single calreticulin gene that likely serves a dual function as an endogenous physiological regulator and as a venom protein. *N. vitripennis* venom calreticulin has significant sequence homology to the calreticulin protein encoded in other insects, including the fly host *S. bullata* (see Supplementary materials).

Previous *in vitro* studies suggest that in venom, calreticulin may play a role in calcium signaling, initiation of a colloid osmotic lysis mechanism of cell death (oncosis), and developmental arrest in envenomated fly hosts (Rivers and Brogan, 2008). In this study we investigated the function of calreticulin in venom by assessing phenotypic and transcriptional effects on hosts that were envenomated by *N. vitripennis* females with RNAi KD of calreticulin gene expression. This novel approach to remove calreticulin without disrupting other venom components has allowed us to identify a set of venom calreticulin-responsive host genes and develop additional hypotheses about its function. We also develop a general model for categorizing venom effect on host gene transcription, including measuring the proportional contribution of a component to a host expression phenotype, and its potential mitigation of the effects of other venom components.

2. Methods

2.1. Wasp and host rearing conditions

N. vitripennis and *S. bullata* hosts were reared according to the previously published protocols (Werren and Loehlin, 2009a, 2009b). For venom collection experiments, adult *N. vitripennis* females were first hosted for 24-h on *S. bullata* pupae at a ratio of 3:3 per tube to stimulate venom production. Adult *N. vitripennis* females used in the RNA-seq experiments were allowed to host feed for 8 h, hosts were then withheld overnight to encourage stinging during the experimental period.

2.2. Gene identification

The *N. vitripennis* genome contains only a single calreticulin gene. Expression had been detected across the entire lifespan by tiling microarray for five developmental stages (male and female) as well as in the female reproductive tract and the venom apparatus (Werren et al., 2010). This indicates that endogenous and secreted venom calreticulin are expressed from the same primary transcript. The venom calreticulin gene **(*v-crc*)** model was taken from version 2.0 of the official gene set (Gilbert et al., 2013) with location identified as the peri-centromeric region of chromosome 1 (NV19007/Nasvi2EG031929). One parolog, calnexin, has not been identified as a venom component in bioinformatics screens of the *N. vitripennis* venom transcriptome (de Graaf et al., 2010). PCR product of the calreticulin primer set (Methods, Section 2.4) yields only a single gene product.

2.3. Venom gene knockdown

Genomic DNA extracted from a *N. vitripennis* male of the lab strain AsymCx was used to amplify a single copy of the *v-crc* gene. *N. vitripennis* calreticulin dsRNA was synthesized from genomic DNA using the MEGAScript RNAi Kit [Life Technologies, Grand Island NY]. All kits were used according to the manufacturer protocol. One paralog – calnexin – is present in the 2.0 *N. vitripennis* genome assembly (Nasvi2EG001077+) (Gilbert et al., 2013). Sequence comparison between calreticulin and calnexin indicates nucleotide sequence identity of 79% (50/63). Micro-RNA target prediction software used to query the *N. vitripennis* transcriptome [Wasp Atlas, RNAi target identification tool; University of Leicester] identified calreticulin as the sole target of double stranded RNA (**dsRNA**) synthesized from the calreticulin amplicon with 100% matching 19-mers. We are therefore confident that *v-crc* KD is specific to calreticulin expressed in *N. vitripennis* and does not target any other venom proteins. Complimentary dsRNA to the *v-crc* transcript was injected into the dorsal posterior end of 6-day instar *N. vitripennis* larvae at a final concentration of 0.36 μg/μl in ddH_2O with 0.5 μl of green food coloring per 10 μl dsRNA solution as a visual marker for microinjection (Werren et al., 2009).

Control wasps were injected with dsRNA complimentary to the *E. coli* LacZ gene (The *N. vitripennis* genome does not contain a Lac operon) to control for possible gene expression changes related to initiation of the RNAi machinery. To compare efficiency of later stage dsRNA KD, pupae were also injected according to the previously published protocol (Lynch and Desplan, 2006) (Supplementary material, siRNA KD of venom calreticulin). All KD *N. vitripennis* were separated by sex at the late pupal stage, prior to eclosion. Adult females were retained for subsequent KD venom experiments. *V-crc* KD females showed a reduction in host stinging and egg laying. Therefore, for host RNA-seq quantification we only utilized hosts that were clearly envenomated based on the presence of a sting site. These hosts showed developmental arrest typical of wild-type envenomated hosts.

2.4. PCR amplification and gel quantification

To confirm *v-crc* KD, RNA was extracted from whole abdomen of dsRNA injected *N. vitripennis* adult females using TRIzol Reagent [Life Technologies, Grand Island NY], followed by chloroform phase extraction, and isopropanol RNA precipitation as per the manufacturer's protocol. Primers were designed to amplify a 245 base pair segment (positions 695–939) of the *v-crc* gene [Forward primer *v-crc* 5′-ATTCGGGATTATCAATCATAGG-3′, Reverse primer *v-crc* 5′-CGAATCTGGTCAGCTAGAG-3'] and housekeeping gene RP-49 [Forward primer RP-49 5-'CTTCCGCAAAGTCCTTGTTC-3′, Reverse