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Rhodnius prolixus: Identification of immune-related genes up-regulated in response to pathogens and parasites using suppressive subtractive hybridization

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Abstract

We report the identification of immune-related molecules from the fat body, and intestine of *Rhodnius prolixus*, an important vector of Chagas disease. Insects were challenged by introducing pathogens or *Trypanosoma cruzi*, the parasite that causes Chagas disease, into the hemocoel. RNA from intestines, or fat body were isolated 24 h after stimulation. We used suppressive subtractive hybridization to identify immune-related genes, generated three subtracted libraries, sequenced the clones and assembled the sequences. The functional annotation revealed expressed sequence tags (ESTs) generated in response to various stimuli in all tissues, and included pathogen recognition molecules, regulatory molecules, and effector molecules.
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Keywords: *Rhodnius prolixus*; Parasitic infection; Innate immunity; Suppressive subtractive hybridization; Expressed sequence tags

1. Introduction

Rhodnius prolixus (family: Reduviidae) is an important vector of *Trypanosoma cruzi*, a protozoan parasite and etiological agent of American trypanosomiasis (Chagas disease) in Northern-South and Central America. Chagas disease affects an estimated 13 million people in the Americas causing significant morbidity; most acute infections are asymptomatic, yet 25–30% of these become chronic, leading to approximately 14,000 deaths annually [1]. Currently, there is neither a preventive vaccine nor an effective treatment to cure chronic Chagas disease as the drugs used, based on nitro heterocyclic compounds, have a very limited efficacy in the chronic stage and toxic side effects often lead to treatment cessation.

Transmission of *T. cruzi* is atypical and shares very little with other major insect-borne diseases in which the parasites invade the salivary glands and are injected into the vertebrate as it takes a blood meal. *T. cruzi*, resides in the intestine/rectum of triatome insects. As the insect engorges, the insect defecates and droplets containing the parasites are deposited on the host's skin and may enter via the bite site or a mucosal membrane. This transmission strategy is inefficient, and we have hypothesized previously that by remaining exclusively in the gut,

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T. cruzi is not exposed directly to the hemolymph which contains the most potent components of the insects' immune response [2]. The immune response of insects is innate, lacks the acquired component of vertebrates yet still is very efficient in eliminating pathogens using a combination of humoral and/or cellular defense responses.

The first step in the immune response requires the recognition of parasites as non-self. Insects recognize unique pathogen-associated molecular patterns (PAMPs) that are characteristic of microbial organisms [3] using host pattern recognition receptors (PRRs) [4]. The two major PRRs in insects are the peptidoglycan recognition proteins (PGRPs) and the Gram-negative bacteria-binding proteins (GNBPs) [5]. Once specific PRRs are activated by the appropriate PAMP, signaling cascades are initiated. Surface molecules present on Gram-negative bacteria are PAMPs recognized by the receptors in the IMD pathway which results in the nuclear translocation of Relish (an NF-κB-like transcription factor), and the induction of antimicrobial peptides (AMPs) such as Cecropin, Drosocin, defensin and Diptericin [6,7]. In *Drosophila melanogaster*, challenge with fungi and Gram-positive bacteria activates the Toll pathway, which results in the NF-κB-like transcription factor, *Dif*, being translocated to induce expression of Drosomycin. This activation process also triggers various other proteolytic cascades, including melanization and coagulation, in which serine proteases and serpins are involved [5] and cellular-mediated mechanisms including phagocytosis, nodulation, and encapsulation by hemocytes [8]. This insect immune system is very efficient and large numbers of bacteria can be removed within minutes of entry into the hemocoel [9]. In addition, the humoral response can contribute to the release of reactive intermediates of nitrogen or oxygen [10] all of which can contribute to the removal of parasites.

Insect innate immunity against larger parasites, has been studied mostly in mosquitoes given their importance as vectors of major human diseases [11]. Approximately 2 weeks after acquisition of an infected blood meal, *Plasmodium* sporozoites are released into the hemocoel and face both humoral and cellular immune responses. Despite massive parasite mortality, malaria parasites infect the salivary glands and subsequently are transmitted to the vertebrate host during a blood meal. Parasite mortality in mosquitoes is mediated by phagocytosis and the anti-plasmodial activity of AMPs has been shown in vitro [12,13]. The exact molecular mechanisms by which eukaryotic parasites are recognized and killed are not well characterized and are an active research area.

Studies on the molecular interactions between *T. cruzi* and triatome vectors are scarce compared with other insect/parasite combinations. Ultra-structural studies have revealed potential and probable ultra-structural interactions occurring in vivo between *T. cruzi* and the intestine of the vectors [14], but because different regions of the intestine vary in their nutritional potential and surface characteristics, we do not know how these differences affect local gene expression that may affect *T. cruzi* development. If the parasite is injected into the hemolymph of *R. prolixus*, lysozyme, prophenoloxidase (proPO), and agglutination are activated [15], and the parasite is killed and cannot be recovered [16]. However, *T. cruzi* normally does not enter the hemocoel. In vitro studies have demonstrated the susceptibility of *T. cruzi* to insect immue peptides [17,18], and in vivo studies have generated insects refractory to the parasite by engineering the bacterial gut symbionts to express a potent AMP in the midgut [19]. Studies on a closely related organism, *Trypanosoma rangeli,* which crosses the midgut epithelia and survives in the hemolymph, suggest that this parasite avoids the humoral immune system by infecting hemocytes and has the capacity to disable the proPO pathway that normally leads to melanization [20,21]. Subsequent studies [22] have demonstrated host immune responses in which lectins bind to carbohydrate moieties on the surface of *T. rangeli,* preventing their attachment to midgut and salivary glands. Identifying the specific pool of genes involved in host–parasite interactions could provide an insight into molecular mechanisms involved in parasite development and the specificity of these interactions.

The expression of these immune factors is pathogen specific; insects such as *D. melanogaster* discriminate between fungal and bacterial infections and use two main pathways, the Toll and the IMD pathways, to express specific molecules involved in their defense [23]. We have identified similar pathogen-specific responses in *R. prolixus* to bacteria and *T. cruzi* using suppressive subtractive hybridization (SSH). This technique selectively identifies differentially expressed genes in response to a particular stimulus rather than a general transcriptome analysis. We report here the generation

and functional annotation of pathogen-specific expressed sequence tags (ESTs) from three subtracted libraries constructed from fat body and intestinal tissues of *R. prolixus* after exposure to bacterial pathogens and the parasite *T. cruzi*.

2. Materials and methods

2.1. Insect colony maintenance

A *R. prolixus* colony has been maintained at Simon Fraser University at room temperature with a 12 h light/dark cycle. The colony is blood fed approximately every 3 weeks on guinea pigs.

2.2. Immune activation and tissue dissection

Bacteria (*E. coli* and *M. luteus*) were grown in liquid LB culture over night at 37 °C with vigorous shaking and 0.75 ml of each bacterial culture were mixed together and pelleted by centrifugation for 5 min at 5000*g* in a tabletop centrifuge. A sterile minuten pin was dipped in the bacterial pellet and injected into *R. prolixus* adults or fifth instar nymphs thoraxes [2]. Naïve (non-challenged) insects were used as controls. *T. cruzi* was obtained from the feces of infected *Triatoma infestans*. The parasites were washed with PBS, and centrifuged at 4 °C for 5 min at 5000*g*, and re-suspended in liver infusion tryptose (LIT) media and counted. Five microliters containing approximately 2500 parasites were inoculated into adult insects with a sterile syringe. Control insects were inoculated with 5 μl of sterile LIT media. Twenty-four hours after immune challenge (bacteria, *T. cruzi* or LIT), fat bodies and intestinal tissues were dissected and thoroughly rinsed in ice cold PBS to wash any contaminating feces and/or blood meal. Tissues were stored in RNAlater (Ambion, Austin, TX, USA) or directly used for subsequent RNA isolation.

2.3. Total RNA and mRNA isolation

Tissues stored in RNAlater were centrifuged at 14,000*g* for 5 min at 4 °C. The supernatant was removed and tissues were washed with 1 ml of DEPC-treated water and pelleted once again to remove the liquid supernatant. Total RNA extraction was performed using Triazol (Invitrogen, Burlington ON) according to manufacturer's specifications. mRNA was isolated using Purist poly-A micro-spin columns (Ambion, Austin, TX, USA); 1 μg of poly-A RNA was used in the construction of each subtracted library.

2.4. Subtractive library construction

We generated three subtractive libraries: a midgut library in response to bacterial (*E. coli* and *M. luteus*) injection; a fat body library in response to bacterial injection and a fat body library in response to *T. cruzi* injection. All three subtracted libraries were built using PCR-Select cDNA Subtraction kit according to manufacturers' recommendations (Clontech, Palo Alto, CA, USA). SSH permitted the enrichment of differentially expressed sequences by hybridizing a *TESTER* (pool of cDNAs from which differentially expressed genes were identified) to a *DRIVER* (control cDNAs used to remove common sequences) [24,25]. Ligation of specific adapters to both ends of the cDNAs was performed prior to subtraction hybridization, followed by PCR amplification with specific primers to the adapters. Amplification of hybrids corresponding to common sequences was suppressed, yielding a library enriched for differentially expressed sequences. For gut and fat body subtracted libraries in response to bacterial challenge, *TESTERS* cDNAs were constructed with mRNA from bacteria inoculated samples and *DRIVERS* with mRNA from naïve (non-inoculated) insects.

The fat body—*T. cruzi* subtracted library (forward) was built using mRNA from immune activated fat bodies as *TESTER* and mRNA from sterile media (LIT) inoculated insects as *DRIVER*. Reverse subtracted libraries were built for fat body tissue libraries for subsequent differential screening, where TESTER and DRIVER designations are inversed. Forward subtracted libraries were ligated overnight at 4 °C into 2 μg of pGemT Easy plasmid vector (Promega, Madison, WI, USA) using 3 μl of the secondary PCR products from each library, and transformed by heat shock into *E. coli* JM109 ultra-competent cells (Promega, Madison, WI, USA). The resulting EST library was plated on LB agar supplemented with 100 μg/ml ampicillin, 80 μg/ml Xgal, 0.5 mM IPTG and incubated overnight at 37 °C.

2.5. Subtractive efficiency analysis and differential screening

The efficiency of the subtraction of all three libraries was estimated using PCR by comparing the abundance of known cDNAs before and after

subtraction. β-actin was selected as a non-differentially expressed gene. Internal primers were used to amplify a portion of this gene (qActF:5′AATCAA-GATCATTGCTCCACCAG3′; ActR:5′TTAGAA-GCATTTGCGGTGGAC3′) under the following conditions: 94 °C for 1 min followed by 33 cycles of 94 °C for 20 s, 60 °C for 20 s and 72 °C for 30 s. Five microliters aliquots were removed from each reaction after 18, 23, 28 and 33 cycles and examined by electrophoresis on a 2% agarose gel and stained with ethidium bromide to confirm subtraction success.

Fat body subtracted libraries were screened for differentially expressed ESTs following manufacturer's instructions using the PCR-select cDNA subtraction screening kit (Clontech, Palo Alto, CA, USA). Clones from bacteria and *T. cruzi* libraries (95 and 194, respectively) were selected randomly and grown in 50 μl of LB-ampicilin (100 μg/ml) for 6 h at 37 °C with moderate shaking in 96-well plates. Two microliters of bacterial culture were spotted in duplicate on Hybond + membranes (Amersham Biosciences, Baie d'Urfé QC); allowed to grow for 2 h at 37 °C on a LB agar plate, denatured in 0.5 M NaOH; 1.5 M NaCl for 4 min, neutralized in 1.5 M NaCl; 0.5 M Tris/HCl pH 7.5 for another 4 min and allowed to dry for 30 min at room temperature. Nucleic acids were fixed to the membrane by using a UV crosslinker XL 1000 (Spectronics corporation, Westbury, NY, USA).

One hundred and fifty nanograms from the forward and reverse subtracted libraries were used to create a ^{32}P-labeled probe by random priming using PCR-Select differential screening kit (Clontech, Palo Alto, CA, USA) following manufacturer's instructions. Forward and reverse subtracted probes were hybridized in individual tubes with the DNA membrane at 65 °C for 2.5 h in a rotatory oven using Rapid–Hyb buffer (Amersham Biosciences, Baie d'Urfé QC). Following hybridization the membranes were washed with low stringency (2 × SSC, 0.5% SDS; 3 × , 20 min each) and high stringency (0.2 × SSC, 0.5% SDS; 3 × , 20 min each) buffers at 65 °C to eliminate non-specific binding due to excess probe. Membranes were exposed to a Kodak BioMax MS film (Eastman Kodak, Rochester, NY, USA) overnight at room temperature.

2.6. Plasmid isolation, DNA sequencing and database search

Selected colonies (strong signal with the forward and low signal with the reverse subtracted probe) were grown overnight in 5 ml of LB medium with 5 μl of Ampicillin (100 μg/μl) and purified using the Wizard Plus Miniprep DNA Purification System (Promega, Madison, WI, USA). Sequencing reactions were performed using Big Dye v3.1 chemistry and run on an ABI PRISM 377 (Applied Biosystems, Foster city, CA, USA) at the DNA sequencing facility of the University of British Columbia. Analysis of the sequence data, detection of open reading frames and sequence alignment, were performed using DNAstar modules Seqman, Megalign and Editseq (DNAstar, Madison, WI, USA). Database search was performed using BLAST-X against non-redundant database at NCBI with default parameters. The best annotated hit from the similarity search was retained. For functional prediction of ESTs found in the database, we used an online gene ontology annotation tool termed GoFigure [26] and clustered the ESTs based on the biological process annotation when available. Novel ESTs were submitted to dbEST at the National Center for Biotechnology Information (NCBI) and assigned accession nos. 37906674–37906768 (GeneBank accession EB084319–EB084413).

3. Results

3.1. Midgut subtracted library in response to bacteria

In this study, we assessed the presence of immune-related transcripts in midgut tissues in response to bacterial invasion of the hemocoel of *R. prolixus*. These genes represent components of a systemic immune response; genes induced in the midgut after a stimulation of the fat body. We randomly isolated and sequenced 90 independent clones from the midgut subtracted library in response to bacterial injection into the hemocoel. After sequencing, we precluded from our analysis redundant clones, sequences with inserts under 60bp in length and clones providing poor quality sequence.

Although, we attempted to minimize the presence of bacterial sequences by using polyA RNA in the construction of the subtracted library, four clones contained bacterial DNA, possibly originating from one or more of the midgut bacterial symbionts that triatome insects naturally harbor and these were excluded from subsequent analysis.

In total, 66 clones (73%) corresponded to different EST sequences (Table 1). Similarity search

by comparison to public database at NCBI using BLAST-X resulted in 16 clones with no significant match, and five to hypothetical proteins deduced in silico from genome sequencing and annotation projects.

Housekeeping genes (ribosomal, mitochondrial), whose amplification during the SSH is normally repressed, also were found in the library. This was likely because we isolated tissues (gut) from regions distant from those directly stimulated (fat body)

Table 1
Bacteria inoculated *R. prolixus* midgut subtraction library

Clone	NCBI gi	Length (bp)	Blast-X match	Accession	*E*	Putative gene function
2.59	37906735	325	Formin like protein	NP_035841.1	4e−23	Cytoskeleton
1.48	37906690	333	Kinesin like	gi41688591	4e−9	Cytoskeleton
2.61	37906737	325	WW domain binding protein 3	XP_235648.2	5e−26	Cytoskeleton
2.1	37906693	252	Alpha actinin	gi7441362	5e−36	Cytoskeleton
2.34	37906719	502	Lysozyme	AAN87265.1	1e−7	Defence
2.35	29335960	469	Defensin B	AAO74625.1	2e−50	Defence
2.22	37906708	317	Transferrin	AAW70172.1	1e−17	Defense
1.4	4204973	587	Nitrophorin 3	U61143.1	1e−84	Defense
2.2	37906694	278	Transferrin	AAA27820.1	5e−18	Defense
1.42	37906685	603	Hypothetical protein	CAH93767.1	0.057	Hypothetical protein
2.9	37906699	189	Hypothetical protein	XP_761391.1	0.064	Hypothetical protein
2.32	37906717	301	Hypothetical protein	XP_729786.1	0.4	Hypothetical protein
2.47	37906725	389	Hypothetical protein	CAD52327.1	0.69	Hypothetical protein
2.60	37906736	466	Hypothetical protein	BAB29490.1	2e−7	Hypothetical protein
2.50	37906728	241	Phosphomannose isomerase	AAK69388.1	1e−12	Metabolism
2.3	37906695	563	ATPase subunit 6	AAG31613.1	1e−37	Metabolism
1.3	37906676	291	polyamine oxidase	XP_508137.1	1e−8	Metabolism
2.29	37906714	460	ATP synthase β subunit	AAT06139.1	2e−65	Metabolism
2.15	37906702	238	Poly A binding protein	CAA40721.1	3e−29	Metabolism
2.36	37906720	1036	Sugar transporters	NP_568494.1	4e−16	Metabolism
2.49	37906727	687	Maltase precursor	CAA93821.1	6e−28	Metabolism
2.52	37906730	287	Polyamine oxidase	NP_997011.1	7e−14	Metabolism
1.16	20378665	468	Cytochrome oxydase 1	AAM20928.1	2e−60	Mitochondrial
2.40	37906721	439	mitochondrial thioredoxin	BAA13447.1	6e−28	Mitochondrial
1.45	37906688	486	Cytochrome P450	BAA28946.1	9e−17	Mitochondrial
2.7	37906697	346	Mitochondrial peptidase	AB163419.1	1e−8	Peptidase
2.58	37906734	456	Aminopeptidase	Gi25311909	2e−6	Peptidase
1.52	37906692	336	Cathepsin B	AAT48985.1	5e−46	Peptidase
2.16	37906703	337	Leucine aminopeptidase	XP_786205.1	8e−8	Peptidase
2.33	37906718	552	Dipetalogastin	CAA10384.1	5e−45	Peptidase inhibitor
2.31	37906716	510	Thrombin inhibitor	AAK57342.1	6e−35	Peptidase inhibitor
1.14	37906682	154	CSP	CAB65177.1	1e−12	Receptor
2.28	37906713	484	growth hormone inducible transmembrane protein	AAD44495.1	5e−21	Receptor
1.13	37906681	99	membrane-associated ring finger	NP_005876.2	7e−12	Receptor
2.10	37906700	365	Veph-A	XP_342257.1	8e−5	Receptor
2.27	37906712	635	Mucin subunit	AAA85523.1	9e−4	Receptor
2.46	37906724	592	NADH dehydrogenase	AAG31614.1	1e−62	Ribosomal
2.17	37906704	370	S24 ribosomal protein	AAS91555.1	2e−41	Ribosomal
2.48	37906726	245	Ribosomal protein L26	AAK92162.1	5e−17	Ribosomal
1.46	37906689	173	PRKA1	XP_790232.1	4e−9	Rna binding
2.23	37906709	394	GASZ	ABA90396.1	0.91	Signalling
1.5	37906677	328	Nin one	NP_001016830.1	1e−10	Stress response
1.44	37906687	618	UNR	CAD52327.1	1e−54	Stress response
1.6	37906678	322	Nin one	AAQ16153.1	4e−12	Stress response
2.19	37906706	140	HSP 70	AAP57537.3	4e−18	Stress response
2.55	37906731	377	HSP70	BAB92074.1	4e−42	Stress response
2.18	37906705	150	Chaperonin	NP_741154.1	5e−15	Stress response

Table 1 (*continued*)

Clone	NCBI gi	Length (bp)	Blast-X match	Accession	*E*	Putative gene function
1.43	37906686	140	HSP70	AAP57537.3	6e−18	Stress response
2.44	37906723	398	Zinc finger containing protein	CAD52327.1	0.69	Transcriptional control
1.7	37906679	618	UNR	gi137045	3e−54	Transcriptional control
1.1	37906674	149	NSM			Unknown
1.15	37906683	180	NSM			Unknown
1.2	37906675	185	NSM			Unknown
1.40	37906684	297	NSM			Unknown
1.49	37906691	161	NSM			Unknown
1.8	37906680	197	NSM			Unknown
2.11	37906701	522	NSM			Unknown
2.21	37906707	171	NSM			Unknown
2.25	37906710	182	NSM			Unknown
2.30	37906715	304	NSM			Unknown
2.42	37906722	440	NSM			Unknown
2.5	37906696	276	NSM			Unknown
2.51	37906729	313	NSM			Unknown
2.56	37906732	190	NSM			Unknown
2.57	37906733	337	NSM			Unknown
2.8	37906698	449	NSM			Unknown

ESTs classified based on BLAST-X analysis against non-redundant database at NCBI. NSM: No significant match.

which allowed for fewer overall differentially expressed genes and therefore more ESTs that corresponded to non-differentially expressed genes. The efficiency of the subtraction depends on the number of genes differentially expressed; larger numbers of these mRNAs are found in areas of lowest background.

What is most interesting is the identification of seven ESTs corresponding to genes that have been shown to participate in different immune response mechanisms. Among this category, we found lysozyme, nitrophorin, transferrin, defensin and a mucin subunit, corresponding to effector, signaling and possibly recognition mechanisms. Other ESTs included putative transcriptional regulators such as transcription factors identified by the presence of DNA binding domains as well as peptidases (cathepsin B and an aminopeptidase) whose enzymatic activity can have an effect in the development and establishment of *T. cruzi*.

3.2. Fat body subtracted library in response to bacteria

Ninety-five clones from a fat body bacteria inoculated subtractive library were spotted and differentially screened. Clones producing a strong hybridization signal with the forward library probe and simultaneously producing a low hybridization signal with the reverse library probe have over a 95% probability of being differentially expressed transcripts in response to the immune challenge. Twenty randomly picked clones and seven highly up-regulated clones were sequenced, compared to NCBI database, and their putative function determined with GoFigure [26] (Table 2). Subtraction efficiency analysis by PCR, and the results obtained after sequencing, demonstrated the high quality of this library as housekeeping transcripts were barely detected (data not shown) and three out of seven up-regulated genes corresponded to all three defensin isoforms we had previously identified by an independent HPLC analysis of immune hemolymph [2].

3.3. Fat body subtracted library in response to T. cruzi

One hundred and ninety clones randomly picked were spotted on a membrane and differentially screened by hybridization to either a forward or a reverse ^{32}P-labeled probe. Ten clones producing a strong hybridization signal with the forward library and simultaneously producing a low hybridization signal with the reverse library probe were identified and subsequently sequenced and compared to public databases (Table 3). Subtraction efficiency

Table 2
Bacteria inoculated *R. prolixus* fat body subtraction library

Clone	NCBI gi	Length (bp)	BLAST-X hit	Accession	*E*	Putative gene function
Rdm 17	37906753	681	Kinesin like	gi41688591	9e−62	ATP binding
B10	29335958	453	*R. prolixus* Defensin A	AAO74624.1	1e−45	Defence
H8	29335960	350	*R. prolixus* Defensin B	AAO74625.1	2e−42	Defence
H2	29335960	514	*R. prolixus* Defensin B	AAO74625.1	2e−51	Defence
Rdm 2	29335960	628	*R. prolixus* Defensin B	AAO74625.1	9e−51	Defence
Rdm 5	37906741	884	*P. yoeli* Hypothetical protein	EEA15590.1	0.062	Hypothetical protein
C11	37906760	465	Unknown protein	XP_379325.2	0.29	Hypothetical protein
F1	37906758	552	Hypothetical protein	CAG05504.1	0.82	Hypothetical protein
Rdm 20	37906756	600	*H. sapiens* hypothetical protein	EAL24336.1	3e−7	Hypothetical protein
Rdm 15	37906751	1012	*A. mellifera* ubiquitin ligase	XP_394362.2	6e−46	Ligase
Rdm 19	37906755	348	*C.elegans* pyruvate dehydrogenase	NP_500340.1	2e−38	Metabolism
Rdm 10	37906746	573	Dihydropteridine reductase	gi442830	2e−49	Metabolism
B9	37906759	438	*M. sexta* Hemolymph proteinase	AAV91014.1	9e−30	Peptidase
Rdm 6	37906742	1033	*G. gallus* metalloprotease	XP_4185641.1	9e−54	Peptidase
Rdm 4	37906740	505	*A. mellifera* Metaxin like	XP_624291.1	1e−24	Protein transport
Rdm 18	37906754	458	*B. clausii* ABC transporter	BAD64657.1	0.8	Receptor
Rdm 8	37906744	735	β1-3 GRP	gi52782700	1e−22	Receptor
Rdm 16	37906752	332	*M.musculus* proteasome 26S	AAH19112.1	4e−7	Ribosomal
Rdm 14	37906750	1022	*H. sapiens* ubiquitin	NP_066289.2	5e−160	Ribosomal
D5	37906757	278	Hypothetical transcription factor	AAX26421.1	0.36	Transcriptional control
Rdm 1	37906738	424	NSM			Unknown
Rdm 11	37906747	678	NSM			Unknown
Rdm 13	37906749	423	NSM			Unknown
Rdm 3	37906739	1001	NSM			Unknown
Rdm 9	37906745	248	NSM			Unknown
Rdm 7	37906743	320	*A. gambiae* genomic clone	XP_312744.2	0.12	Unknown
Rdm 12	37906748	278	*A. mellifera* genomic clone	XP_394116.1	1e−21	Unknown

ESTs classified based on BLAST-X analysis against non-redundant database at NCBI. The first four clones were randomly picked whereas the rest were selected after differential screening. Rdm: Randomly picked clone. NSM: No significant match.

Table 3
Trypanosoma cruzi inoculated *R. prolixus* fat body subtracted library

Mb	Clone	Length (bp)	NCBI gi	BLAST-X hit	Accession	*E*	Putative gene function
1–95	C2	1133	37906764	*S. scrofa* Flotillin	BAD08436.1	9e−96	Cytoskeleton
97–191	F7	507	37906767	*B. Taurus* Hypothetical protein	XP_583059.1	7e−14	Hypothetical protein
1–95	C10	536	37906765	*C. felis* Mucin-Peritrophin	AAM21357.1	6e−6	Receptor
1–95	C7	551	16117393	*R. prolixus* 16S ribosomal	AF324519.1	0.0	Ribosomal
97–191	B3	1022	2895883	*R. prolixus* Ribosomal RNA	AF045707.1	7e−180	Ribosomal
1–95	A8	459	37906762	*A. mellifera* genomic clone	XP_394615.2	5e−49	Stress response
1–95	H9	364	37906768	*A. mellifera* Dorsal B	AAP23056.1	1e−12	Transcriptional control
1–95	A4	481	37906761	*P. troglodytes* Formin-like	XP_522563.1	0.001	Transcriptional control
1–95	A9	488	37906763	NSM			Unknown
1–95	D5	1123	37906766	NSM			Unknown

ESTs classified based on BLAST-X analysis against non-redundant database at NCBI. NSM: no significant match.

analysis by PCR showed that the efficiency of the subtraction was lower than the other two libraries. This probably was due to the use of cDNAs obtained from LIT inoculated insects as the DRIVER (control). All genes up-regulated in response to wounding in both DRIVER and TESTER would have been removed leaving only the genes expressed specifically in response to the presence of *T. cruzi* in the hemocoel. Two genes, despite having a differential hybridization profile corresponded to false positives, encoding for ribosomal genes. Two clones isolated from this

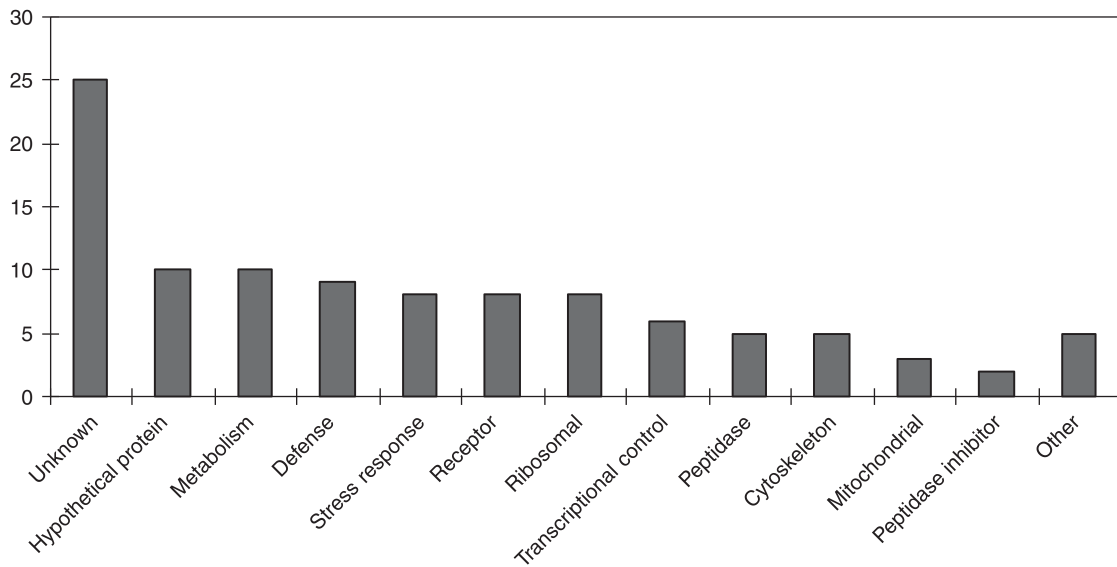

Fig. 1. Functional prediction and classification of the generated ESTs based on gene ontology using GoFigure [26]. Novel ESTs were submitted to dbEST at the National Center for Biotechnology Information and assigned accession numbers 37906674–37906768 (GeneBank accession EB084319–EB084413).

library were of particular interest. Clone C10 with a high similarity to a mucin/peritrophin receptor molecule and clone H9 corresponding to a partial sequence of a Dorsal/Rel homolog.

When combined (Fig. 1) all three subtracted libraries contain 103 EST sequences (94 are novel) from the hemipteran *R. prolixus*. A large majority of the sequences here reported do not have any similarities with other sequences in the databases or have similarities with sequences encoding for hypothetical proteins obtained from genome sequencing projects whose functional role is yet unknown.

4. Discussion

Insect immunty has received a great deal of attention in the past 20 years both from a basic research as well as applied perspective. However, these studies have focused mainly on the higher orders of insects: Diptera, Hymenoptera and Lepidoptera. Studies in more ancient insects such as hemipterans are scarce. Given the heterogeneity of invertebrates and their immune systems [27], identifiying components of the innate immune response in hemipteran insects provides an invaluable evolutionary view of immunity. These insects and their trypanosomatid parasites provide, despite the limited genetic tools available, an excellent case study because of the fundamental differences in the host life cycles (incomplete versus complete metamorphosis of higher insects) and the impact of the parasite on the immune system of the insect. Two very closely related flagellate parasites, have very different life cycles in the same insect: *T. rangeli* circumvents the immune system and survives in the hemolymph whereas *T. cruzi* is limited to the intestines of the insect, removed from most immune effector molecules. We do not know if this is a function of differential recognition or activation processes of the insect or evasion techniques by the parasite. Prior to testing these different possibilities we first must identify and characterize the different components of this vector's immune system. Lysozyme, pro-PO, and agglutination have been detected after natural infection with *T. rangeli* and artificial injection of *T. cruzi* into the hemolymph of *R. prolixus* [15]. Despite these initial findings, relatively little is known concerning the molecular mechanisms involved in the recognition, activation and effector molecules of the hemipteran immune response to parasites.

Many different approaches have been used to identify immune-related molecules involved in vector–parasite interactions. Complete transcriptome studies have sequenced large EST libraries from the tsetse fly midgut [28] and mosquito hemocytes [29], and microarrays were used to identify transcripts of *D. melanogaster* expressed in

response to viruses, bacteria, fungi and a protozoan parasite [30]. We used SSH and differential screening to identify novel and known *R. prolixus* ESTs up-regulated in different tissues after two different challenges. Many studies have described the role of the fatbody in producing potent immune molecules. Our data indicate that the midgut also is immunocompetent and produces AMPs such as defensin and lysozyme and likely second messenger molecules (transferrin, nitric oxide). Our results are consistent with other studies and advocate for the presence of a systemic immune response in which communication molecules induce the expression of immune factors in cells/tissues distant from the initial point of infection [31].

We describe here, in more detail, the identifictaion of six molecules belonging to the three fundamental pillars of immunity: recognition, activation and effector mechanisms involved in the immune response of *R. prolixus*.

Transferrin: A protein involved in iron metabolism in both vertebrates and invertebrates and responsive to juvenile hormone [32]. Transferrin is also believed to be a component of the innate immune system by sequestering iron away from bacterial pathogens [33]. Transferrin has been shown to be up-regulated in vitro in *A. aegypti* cells treated with heat-killed bacterial cells and in termites following exposure to an entomopathogenic fungus [34,35]. The transferrin gene was recently cloned and characterized in *A. aegypti*; its promoter region is rich in putative NF-$\kappa\beta$ binding sites, which is consistent with its postulated role in insect innate immunity [36]. Although the exact role of transferrin in insect innate immunity has not yet been clearly elucidated a hint of its possible role comes from studies in the goldfish *C. auratus* where it functions as an immune stimulatory signal, when enzymatically cleaved, by activating macrophages [37–39].

Nitrophorins: Nitric oxide is a multifunctional molecule; its role in innate immunity has been reported extensively against bacteria and eukaryotic parasites [10,40,41]. Six nitrophorins have been identified in *R. prolixus* [42,43] mainly from salivary gland tissue after blood feeding where NO is transported to function as a vasodilator and facilitates the blood meal acquisition. Upon *T. rangeli* infection of the hemolymph, nitric oxide activity has been detected and high levels of superoxide seem to limit the H14 strain of *T. rangeli*, which fails to complete its life cycle in *R. prolixus* [44]. We isolated an EST with high sequence similarity to nitrophorin 3 in midgut tissue after bacterial infection (Table 1) and we can only hypothesize that the presence of NO in the gut may be used to regulate the growth of bacterial flora but also may have a negative effect of *T. cruzi* development.

β1-3 glucan recognition protein (GRP): The innate immune system recognizes microorganisms through a series of PRRs that are highly conserved in evolution. β-GRPs are pattern recognition molecules that are conserved from insects to mammals and recognize foreign organisms and their unique cell wall components. The first GRP was isolated from the hemolymph of *B. mori* [45], and subsequently GRPs were identified as pathogen recognition molecules in *M. sexta*, *A. subalbatus* and *D. melanogaster* cells. These molecules activate the phenoloxidase (PO) cascade leading to pathogen encapsulation [46–48].

Hemolymph proteinase (HP): We identified an EST with high homology to a *M. sexta* serine proteinase found in the hemolymph [49]. Serine proteinase pathways play a pivotal role in controlling immune processes in insects. HPs, secreted into the hemolymph from the fat body or hemocytes, are responsible for initiating the complex biochemical cascade involved in proPO cleavage and activation. PO, activated from proPO through proteolysis by proPO-activating proteinase (PAP), is a key enzyme implicated in several defense mechanisms in invertebrates. Other proteinases were identified (cathepsin B and a leucine aminopeptidase), albeit from midgut tissue where these molecules aid in blood meal digestion. Cathepsin D, however, has been linked to the cleavage of immune-related molecules in fish [50] and in *R. prolixus* infected with *T. cruzi,* its activity is decreased [51]. Initial data on Cathepsin B transcript levels indicate its gut specificity but no differential expression when compared to non-infected controls (data not shown).

Rel/Dorsal: This molecule belongs to a super family of nuclear factors. In *D. melanogaster* Dorsal plays a central role in the establishment of dorsoventral polarity during early embryogenesis, whereas relish plays a main role in the IMD pathway by activating the transcription of AMPs [52,53]. Recently, Raikhel and colleagues identified its homolog in *A. aegypti*. Relish 1 (REL1) selectively binds to different NF-$\kappa\beta$ motifs from insect immune gene promoters and mediates a

specific antifungal immune response against *B. bassiana* [54]. Using a transgenic approach, in combination with RNAi technique, they elucidated its role as a key downstream regulator of Toll immune pathway in *A. aegypti* [55]. The dual role of this molecule in such important, yet very different, processes such as development and immunity makes it a very interesting case study for its recruitment by one or the other process from an evolutionary perspective. Expression and functional studies of this molecule should shed light on its role in *R. prolixus* as a developmental and/or immune-related transcription factor.

Mucin/Peritrophin like: Mucins are surface or free glycoproteins known to bind lectins (another group of surface glycoproteins). *T. cruzi*'s genome encodes for large families of surface molecules, which include *trans*-sialidases, mucins, gp63 s, and a large novel family (>1300 copies) of mucin-associated surface protein (MASP) genes [56–58]. Specific *R. prolixus* lectins interact selectively with *T. cruzi* [59], including a hemolymph galactoside-binding lectin, which could play an important role in the development of *T. rangeli* in the hemocoel of the insect vector. This lectin markedly enhanced the activation of clump formation by *T. rangeli* in *R. prolixus* hemocyte monolayers, with an increase in clump size and hemocyte aggregation [60]. More recently, gp150 an ecdysone-regulated mucin found in *D. melanogaster* hemocytes, midgut, and salivary glands is released from larval hemocytes to become a component of the clot and participates in the entrapment of bacteria [61].

Our study did not identify any AMPs other than defensin and lysozyme despite the fact that more than 250 different AMPs have been described from different insect orders. This suggests that *R. prolixus* may have a different arsenal of AMPs (possibly comprising molecules we have designated as having no known function), that this insect has not developed a wide variety of defense molecules, or that the production of these molecules is not transcriptionally regulated. Interestingly, we found little overlap among the genes up-regulated in our three subtracted libraries. Defensin was the only AMP found in both fat body and midgut in response to bacterial injection. Different pathogens elicit specific immune responses. In *D. melanogaster* for instance, Gram-positive bacteria and fungi trigger the Toll pathway, whereas Gram-negative bacteria trigger the IMD pathway (recently reviewed in [62]). The immune response in insects to large foreign organisms such as parasites is mediated by nodulation and encapsulation and not solely by AMPs. Therefore, it is not surprising that genes found in response to bacterial challenge differ from the genes found in response to a protozoan parasite such as *T. cruzi*. It is worthwhile to note that lysozyme was found only in response to bacterial challenge and not to *T. cruzi*, in contrast to a recent microarray-based study in *D. melanogaster* where lysozymes were found to be the main response to the protozoan parasite *O. muscaedomesticae* [30].

Our results indicate the activation of several pathogen-specific genes in response to bacterial or parasitic invasion of the hemocoel. Some of these are homologous to genes described in other insect–parasite systems but the large number of unidentified genes suggests the possibility of unique immune genes in hemimetabolous insects. Future studies will characterize these novel immune-related genes in terms of biological activity and their effects on parasite development and transmission.

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