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Genes of the bovine lungworm *Dictyocaulus viviparus* associated with transition from pasture to parasitism

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ABSTRACT

Genes necessary to enable nematode parasitic life after free-living larval life are of substantial interest to understand parasitism. We investigated transcriptional changes during transition to parasitism in the bovine lungworm *Dictyocaulus viviparus*, one of the most important parasites in cattle farming due to substantial economic losses. Upregulated transcripts in either free-living, developmentally arrested L3 or parasitic immature L5 were identified by suppression subtractive hybridization (SSH) followed by differential screening and subsequent virtual Northern blot verification. From 400 sequenced clones of parasitic L5, 372 (93.0%) upregulated high quality ESTs were obtained clustering into 30 contigs and 38 singletons. Most conceptual translated peptides were SCP/TAPS "family" members also known as pathogenesis-related protein (PRP) superfamily (28.5% of total ESTs), cysteine proteases (24.5%), and H-gal-GP orthologues (9.9%). These proteins are predicted to play key roles in fundamental biological processes such as nutrition and development but also parasite–host interactions and immune defense mechanisms. Increased energy requirement of the rapidly developing L5 lungworm stage was obvious in a proportion of 12.2% upregulated ESTs being components of the respiratory chain. From the developmentally arrested L3 stage sequencing of 200 clones resulted in 195 high quality ESTs (97.0%) clustering into 7 contigs and 3 singletons only. Besides a hypothetical protein (70.1% of total ESTs) most transcripts encoded the cleavage stimulation factor subunit 2 (17.5%), which is a component of the poly(A^+) machinery and found to be involved in gene silencing. Obtained data provide the basis for future fundamental research into genes associated with parasitic lifestyle but also applied research like vaccine and/or drug development.

1. Introduction

The life cycle of the bovine lungworm *Dictyocaulus viviparus*, one of the most important parasitic nematodes in cattle on pasture in temperate zones, comprises two major phases: (a) The free-living phase with development from first to infective third larval stage (L3) and (b) the parasitic phase after ingestion by the cattle host with development into reproductive adult parasites. The parasitic phase is generally accompanied by bronchopneumonia so that lungworm infections in cattle cause considerable economic losses or even death of infected animals.

When entering the cattle host, third stage lungworm larvae as well as further developmental stages are exposed to the host's immune system with its various defense mechanisms. At the same time, larvae have to penetrate the host's tissues to reach their settlement site, which are the lung airways. Furthermore, anytime after transition to parasitic organisms, lungworms feed for the first time of their lives. These fundamental changes in the parasite's way of life require basic changes in gene transcription and subsequent expression. Some studies have been conducted to analyze differential gene transcription in the bovine lungworm, but these studies have focused on the phenomenon of hypobiosis (Strube et al., 2007; Laabs et al., 2012a). Recently, an EST in silico subtraction approach was used to identify genes uniquely transcribed in L3 and adult lungworms, respectively (Cantacessi et al., 2011). However, this approach was restricted to exclusive transcripts rather than transcriptional up- and down-regulations during transition to parasitic lifestyle. Studies exploring transcriptional changes associated with the transition process were conducted with *Ancylostoma caninum* and *Haemonchus contortus* by comparison of free-living L3 with serum stimulated L3 in case of the canine hookworm *A. caninum* (Moser et al., 2005; Datu et al., 2008, 2009) or CO_2-activated L3 in case of the ruminant gastrointestinal nematode *H. contortus* (Cantacessi et al., 2010). Numerous activation-associated genes in hookworms were members of the pathogenesis-related protein (PRP) superfamily including *Ancylostoma* secreted proteins (ASPs) and, furthermore, catalytic proteases belonging to the classes of cysteine-, aspartic- and metalloproteases (Moser et al., 2005; Datu et al., 2008). In activated *H. contortus* L3 a similar spectrum of proteases and other molecules linked to catalytic activity as in activated hookworms was observed (Cantacessi

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et al., 2010). But even if both, *H. contortus* and *A. caninum*, are haematophagous bursate nematodes, they have different biologies. Thus, Cantacessi et al. (2010) suppose that those molecules found in activated L3 of both parasite species may play key roles in host tissue invasion, degradation and/or digestion.

In contrast to other studies with in vitro activated larvae, in the present study free-living L3 of *D. viviparus* were compared with ex vivo isolated parasitic lungworm larvae (immature L5) to identify unique or upregulated transcripts associated with one or the other way of life. Suppression subtractive hybridization (SSH) followed by differential screening and bioinformatic analysis provided information about transcriptional changes accompanying transition to parasitism in a non-haematophagous, bursate nematode with a special area of settlement, which is the aerobic environment of the cattle lung.

2. Materials and methods

2.1. Parasite material

For *D. viviparus* third stage larvae (L3) production (field strain HannoverDv2000), first stage larvae were isolated from the faeces of experimentally infected calves and stored 14 days at room temperature in 20–50 ml tap water to ensure L3 development. To exsheath L3 and eliminate bacteria and protozoans from the tap water, larvae were incubated at room temperature for 15 min in 0.6% v/v sodium hypochlorite (NaClO) with continuous shaking followed by three washes with diethyl pyrocarbonate (DEPC)-treated water.

Parasitic fifth stage lungworm larvae (L5, also known as immatures or preadults) were obtained from a calf experimentally infected with 20,000 L3 by perfusion of the lungs over a 36 μm mesh sieve on day 15 post infection. Recovered L5 were washed extensively in large volumes of 0.9% sodium chloride to eliminate possible contaminating bovine cell material. Both larval populations were stored at −75 °C in an extraction buffer (pH 7.0) containing 5.5 M guanidinium isothiocyanate (GIT) and 25 mM sodium citrate until mRNA isolation.

Animal experiments were permitted by the ethics commission of the Lower Saxony State Office for Consumer Protection and Food Safety under reference number AZ 33-42502-06/1160.

2.2. Poly(A)$^+$ RNA isolation

Parasite material (about 55,000 L3 and 1000 L5, respectively) was homogenized using the TissueRuptor (Qiagen) to destroy the cuticle and cell walls. Poly(A)$^+$ RNA was extracted using oligo (dT) cellulose beads (illustra QuickPrep™ *Micro* mRNA Purification Kit, GE Healthcare) according to the manufacturer's instructions. To determine the Poly(A)$^+$ RNA yield and purity the absorbance at 260 nm was measured using the Nanodrop® ND-1000 UV–Vis Spectrophotometer (PEQLAB Biotechnologie GmbH).

2.3. Suppression subtractive hybridization (SSH)

The SSH technique enables identification of genes that are upregulated or unique in one population but not in the other. Its principle is based primarily on (1) two subtractive hybridization steps to exclude common sequences between the two populations and (2) a suppression PCR that equalizes the abundance of cDNAs and thus enriches rare differential transcripts within the target population. First, L3- and L5-poly(A)$^+$ RNA was transcribed into cDNA by using the SMART™ PCR cDNA Synthesis Kit (Clontech Laboratories). Subsequent SSH was performed with the PCR-Select™ cDNA Subtraction Kit (Clontech Laboratories) following the manufacturer's instructions except that for efficient subtraction the 4-fold amount of driver cDNA was utilized in the second hybridization round as described previously (Strube et al., 2007). Before performing subtractive hybridization, adaptor ligation efficiency was tested to verify that, as recommended by the manufacturer, at least 25% of the cDNAs had adaptors on both ends. These experiments were performed as recommended by the manufacturer whereby two control experiments for each adaptor ligated cDNA were included. One reaction set-up was conducted amplifying an *elongation factor 1α* (*ef-1α*) sequence stretch, the other amplified gene fragment was *β-tubulin*. Used gene specific primers were *ef-1α* for 5′-TGCTCGCCCAAACTCTG-3′ and *ef-1α* rev 5′-CC ATGGCATATTAGGTGAT-3′ as well as *β-tubulin* for 5′-GTCCCGCGT GCTGTTCTTGTT-3′ and *β-tubulin* rev 5′-CCTTCAGCCTCTTTACGCAC-TACG-3′.

2.4. Control of subtraction efficiency

Two control reactions, namely PCR amplification and Southern blot analyses were carried out to determine cDNA subtraction efficiency. For PCR subtraction efficiency tests *ef-1α* and *β-tubulin* in both, subtracted and unsubtracted cDNAs, were amplified using primers listed in the above section as these genes were known to be transcribed in both larval populations (Strube et al., 2008). PCR cycling (18–38 cycles, steps of five cycles) was performed using the following temperature profile: initial denaturation at 94 °C for 30 s, followed by denaturation at 94 °C for 10 s, annealing at 56 °C for 30 s and extension at 68 °C for 30 s.

For SSH efficiency testing by Southern blot, two times equal amounts (1 μg) of subtracted and unsubtracted cDNAs from both larval populations were resolved in an 1% agarose gel, then denatured in the gel and transferred to two positively charged nylon membranes (Roche Applied Science) and cross-linked by baking for 30 min.

Prehybridization was done with DIG Easy Hyb (Roche Applied Science) containing 10 mg denatured herring sperm DNA/ml for 45 min at 42 °C, which is recommended as the optimal but stringent hybridization temperature for mammalian DNA containing 40% GC sequences and probes that are 80–100% identical to the target. Probing was done for 20 h at 42 °C with fresh DIG Easy Hyb (Roche Applied Science) containing 100 ng subtracted L3 and L5 cDNA, respectively, that was labeled with DIG-dUTP (Roche Applied Science). After hybridization the blots were washed two times at room temperature for 5 min with 2× SSC containing 0.1% SDS followed by two washes at 68 °C for 15 min with preheated 0.5× SSC containing 0.1% SDS. Chemiluminescence was detected with the Bio-Imaging system MF-ChemiBIS 3.2 (Biostep) during 5 min exposition.

2.5. Subtracted libraries and differential screening

Subtracted L3 and L5 cDNAs were ligated into pCR™ 4-TOPO™ vector (Invitrogen) followed by transformation into *Escherichia coli* strain One Shot® Mach1™ (Invitrogen). PCR was used to check randomly picked clones for a single insert to represent the L3 and L5 subtracted library, each containing 2016 clones. These libraries were subjected to differential screening to verify differential transcription of contained L3 and L5 ESTs, respectively. In this method, the subtracted libraries are hybridized with subtracted and unsubtracted cDNA probes of both, the corresponding and contrary population. Clones representing mRNAs that are truly differentially transcribed will hybridize only or at least much more intense with the corresponding probes. In brief, high density arrays including quadruplicates of each clone as well positive and negative controls were custom-spotted by Source BioScience LifeSciences and fixed by UV cross-linking (120 μJ × 100, 40 s). Hybridization probes were generated by PCR digoxigenin (DIG)-dUTP labeling using a