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Differential display of genes expressed in the filarial nematode *Litomosoides sigmodontis* reveals a putative phosphate permease up-regulated after depletion of *Wolbachia* endobacteria

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Abstract

Mutualist symbiotic *Wolbachia* endobacteria are found in most filarial nematodes. *Wolbachia* are essential for embryogenesis and for larval development into adults, and thus represent a new target for anti-filarial drug development. Tetracycline antibiotics deplete *Wolbachia* in animal model filaria *Litomosoides sigmodontis* and *Brugia pahangi*, as well as in the human parasites *Brugia malayi*, *Onchocerca volvulus* and *Wuchereria bancrofti*. Very little is known about the molecular details of the symbiotic interaction between *Wolbachia* and filarial nematodes. Nematode genes that respond to anti-*Wolbachia* antibiotic treatment may play important roles in the symbiosis. Differential display PCR was used to detect several candidate genes that are up-regulated after 3, 6, 15, 30 and 36 days of tetracycline treatment. One of these genes, *Ls-ppe-1*, was similar to a family of phosphate permeases, and had putative orthologues in *O. volvulus* and *B. malayi*. *Ls-ppe-1* steady-state mRNA levels were elevated by day 3–6 of treatment, and remained elevated through to 70 days post-treatment. In *Caenorhabditis elegans*, the knockdown of a homologous phosphate permease results in embryonic lethality, with the production of degenerating embryos, a phenotype also seen in filarial nematodes after depletion of *Wolbachia* with tetracycline. The potential role of *Ls-ppe-1* in the nematode-bacterial symbiosis is discussed.

Keywords: Phosphate transporter; Filarial nematode; *Wolbachia*; Differential display; Real-time PCR; Chemotherapy; Symbiosis

Introduction

Filarial infections are a worldwide health problem in developing countries (WHO, 2000a, b). Current drugs such as ivermectin or diethylcarbamazine kill filarial larvae in the human host, but may not be sufficient to stop transmission (Abiose et al., 2000; Ottesen, 2000; Richards et al., 2000) and have the disadvantage that mass treatments have to be repeated annually for several years (WHO, 2000b). New chemotherapeutic approaches that either lead to long-term sterilization or macrofilaricidal activity are required (Dadzie et al., 2003; Molyneux et al., 2003).

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Almost 30 years ago, intracellular bacteria were discovered in filarial nematodes (Kozek and Marroquin, 1977; McLaren et al., 1975). In the last decade, interest in the endosymbiotic bacteria in filarial nematodes has rekindled, and the bacteria were positively assigned to the alpha-proteobacterial genus *Wolbachia* (Genchi et al., 1998; Hoerauf et al., 1999). *Wolbachia* are present in all developmental stages and in nearly all species of filarial nematodes, and exist in a mutualistic symbiosis with their hosts (Bandi et al., 2001; Fenn and Blaxter, 2004; Taylor and Hoerauf, 2001).

Depletion of *Wolbachia* by tetracycline antibiotics leads to problems with larval moulting and growth, sterility in females and a block in embryogenesis (Hoerauf et al., 1999; Taylor and Hoerauf, 2001). Studies in animal models and in human filarial infections support these findings and further show that the block of embryogenesis persists for at least 18 months in human onchocerciasis caused by *Onchocerca volvulus* (Hoerauf et al., 2003a, c). Doxycycline treatment of human bancroftian filariasis resulted in a loss of *Wolbachia* and an almost complete absence of microfilaremia (Hoerauf et al., 2003b), and importantly, yielded evidence for killing of adult *Wuchereria bancrofti* by doxycycline therapy (Taylor et al., 2005). In contrast, tetracycline treatment of the *Wolbachia*-free filarial nematode *Acanthocheilonema viteae* has no nematicidal effects. Despite the excellent prospects for chemotherapy by antibiotics as a treatment for onchocerciasis, and possibly also for lymphatic filariasis, the molecular basis of the symbiosis between *Wolbachia* and its filarial host remains unknown. For this reason the expression pattern of nematode genes that are up-regulated after treatment with tetracycline, and thus are potentially involved in the symbiotic interaction, was investigated using the murine filarial model *Litomosoides sigmodontis*.

Materials and methods

Animal model

In most experiments, fully permissive IL-5-deficient BALB/c mice (6–8 weeks old) were infected with *L. sigmodontis*. In contrast to wild type mice, IL-5-deficient mice have up to 200-fold higher parasite load and prolonged patency (Volkmann et al., 2001, 2003) which permits prolonged therapeutic regimes (>2 weeks), not possible in wild-type BALB/c mice. Another benefit in using IL-5-deficient BALB/c mice rather than wild type mice is the reduction in inflammatory nodule formation around the adult nematodes, facilitating parasite recovery for further analyses.

Treatment was started at day 58 post-infection (the time of patency, when released L1 larvae are detected in the blood) with intraperitoneal injections of 50 mg tetracycline per kg body weight per day (Hoerauf et al., 1999). In separate experiments, gentamicin, an antibiotic known to be ineffective in eliminating Rickettsiales, was applied at 15 mg per kg body weight per day (Hoerauf et al., 1999). Nematodes were collected on days 3, 6, 15, 30 and 36 of the treatment trial. The nematodes were separated by sex, snap frozen in liquid nitrogen and stored at −80 °C for RNA isolation. In additional experiments, nematodes were also collected on days 50 and 70 to analyze the expression pattern of *Ls-ppe-1* 2 weeks and 1 month after the treatment was stopped.

Ornithodoros moubata ticks infected with *A. viteae* were kindly provided by Prof. Richard Lucius, Humboldt University, Berlin. L3 larvae were isolated and naïve *Meriones unguiculatus* were infected with 80 L3 larvae as described (Lucius and Textor, 1995). Infected animals carrying adult nematodes were treated orally for 6 weeks with tetracycline in drinking water at a concentration of 0.5% (w/v). The water was changed daily. Treated animals were sacrificed at 3, 6, 15, and 36 days post infection and the nematodes isolated. Nematodes were snap frozen in liquid nitrogen and stored at −80 °C for later RNA isolation.

RNA extraction

Total RNA was isolated from 10 female nematodes using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To eliminate genomic DNA contamination, an additional DNA digest with RNase-free DNase I (Ambion, Austin, TX, USA) was performed for 30 min at 37 °C. RNA was extracted with phenol chloroform and then precipitated with lithium chloride at a final concentration of 2.5 M (Ambion) and 10 μg/ml linear acrylamide (Ambion) as co-precipitant.

For better isolation of small quantities of RNA and to reduce genomic DNA contamination, a modified Trizol extraction was used. Ten female nematodes in 800 μl Trizol reagent (Invitrogen GmbH, Karlsruhe, Germany) were finely minced with scissors and then homogenized with a glass–glass homogenizer at 1200 rpm (Sartorius BBI Systems, Melsungen, Germany). Instead of chloroform, 80 μl 1-bromo-3-chloro-propane (Sigma-Aldrich, Steinheim, Germany) was added to separate the homogenate into RNA-containing aqueous and DNA and protein-containing organic phases. DNase treatment was performed as above and then removed following the RNeasy clean up protocol (Qiagen). RNA concentration was determined at 260 nm using an Eppendorf BioPhotometer (Eppendorf Inc., Wesseling, Germany).