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Mitochondrial genes for heme-dependent respiratory chain complexes are up-regulated after depletion of *Wolbachia* from filarial nematodes ☆

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ABSTRACT

The filarial nematodes *Brugia malayi*, *Wuchereria bancrofti* and *Onchocerca volvulus* cause elephantiasis or dermatitis and blindness resulting in severe morbidity. Annually, 1.3 billion people are at risk of infection. Targeting the essential *Wolbachia* endobacteria of filarial nematodes with doxycycline has proven to be an effective therapy resulting in a block in embryogenesis, worm development and macrofilaricidal effects. However, doxycycline is contraindicated for a large portion of the at risk population. To identify new targets for anti-wolbachial therapy, understanding the molecular basis of the *Wolbachia*–filaria symbiosis is required. Using the *B. malayi* microarray we identified differentially expressed genes in the rodent filaria *Litomosoides sigmodontis* after depletion of *Wolbachia* which might have a role in symbiosis. The microarray data were filtered for regulated genes with a false discovery rate <5% and a ⩾2-fold-change. Most of the genes were differentially expressed at day 36 of tetracycline treatment, when 99.8% of *Wolbachia* were depleted. Several classes of genes were affected, including genes for translation, transcription, folding/sorting of proteins, motility, structure and metabolic and signalling pathways. Quantitative PCR validated 60% of the genes found to be regulated in the microarray. A nuclear encoded heme-binding protein of the globin family was up-regulated upon loss of *Wolbachia*. Interestingly, mitochondrial encoded subunits of respiratory chain complexes containing heme and riboflavin were also up-regulated. No change in the expression of these genes was seen in tetracycline treated *Wolbachia*-free *Acanthocheilonema viteae*. As *Wolbachia* synthesise heme and filaria do not, we hypothesise that without the endosymbionts no functional heme-containing enzymes can be formed, leading to loss of energy metabolism which then results in up-regulation of the mitochondrial encoded subunits in an attempt to correct the deviation from homeostasis. Our results support targeting the *Wolbachia* heme synthesis pathway for the discovery of new anti-filarial drugs.

1. Introduction

Filarial infections are a health problem, causing clinical symptoms including lymphedema, hydrocele, elephantiasis, dermatitis and blindness predominantly in developing countries. More than 1 billion people are at risk of either lymphatic filariasis or onchocerciasis caused by *Wuchereria bancrofti* and *Brugia* spp. or *Onchocerca volvulus*, respectively (WHO, 2010). Current drug treatment programs to eliminate these diseases are based on diethylcarbamazine or ivermectin in combination with albendazole, which kill the microfilaria but are only weakly macrofilaricidal (Ottesen et al., 1997; Hussein et al., 2004; Dreyer et al., 2006). Repeated treatments over many years are needed to break the transmission cycle. Furthermore, evidence for drug resistance is appearing (Esterre et al., 2001; Osei-Atweneboana et al., 2007), strengthening the need for other drugs that effectively sterilise and/or kill adult worms. A new chemotherapeutic approach uses doxycycline targeting endosymbiotic *Wolbachia* bacteria of the order Rickettsiales, which are found in the hypodermis of female and male adult worms as well as in the embryos and larval stages (McLaren et al., 1975; Kozek and Marroquin, 1977; Kozek, 1977; Pfarr et al., 2008). *Wolbachia* have been shown to be essential for worm development and survival and therefore have gained great interest in filarial research (Hoerauf et al., 2000, 2001a; Taylor et al., 2005). Although therapy with doxycycline is effective in blocking transmission, killing adult worms and improving disease symptoms (Debrah et al., 2006, 2007; Hoerauf et al., 2008; Mand et al.,

☆ Nucleotide sequence data reported in this paper are available in the GenBank™ database under the Accession No.: GU971360, GU971361, GU971362, GU971363, GU971364, GU971365, GU971366, GU971367, GU971368, GU971369, GU971370, GU971371, GU971372, GU971373. MIAME compliant microarray data are available at the Gene Expression Omnibus database (Accession No. GSE20976).

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2009), it is not suitable for mass drug administration (MDA), as it has to be given for 3–6 weeks and cannot be given to children under 9 years, nor pregnant or breastfeeding women. However, in foci with co-endemicity with *Loa loa*, where ivermectin is contraindicated, it has been successfully delivered as part of a Community Directed Treatment program (Wanji et al., 2009). Doxycycline is also suitable for individual therapy and is being considered for "end game" control efforts in foci where transmission has stopped, but where a few individuals are still infected (Hoerauf, 2008; Taylor et al., in press). Nevertheless, because of its limitations in use for MDA new drugs to eliminate *Wolbachia* are required.

Understanding the molecular basis and identification of important pathways and proteins involved in the filaria–*Wolbachia* relationship is required for development of drugs which disrupt the symbiosis. Sequencing and annotation of the genomes of *Brugia malayi* and *Wolbachia* of *B. malayi* (*w*Bm) revealed metabolic gaps in both genomes, suggesting likely dependencies between filaria and *Wolbachia*, including provisioning of purines from *Wolbachia* to *B. malayi* and conversely provisioning of amino acids from *B. malayi* to *Wolbachia* (Foster et al., 2005; Ghedin et al., 2007). One experimental approach to identify genes important in symbiosis used differential display and identified a phosphate permease that is up-regulated after depletion of *Wolbachia* (Heider et al., 2006). However, the exact molecular mechanisms underlying the symbiosis remain unclear. Filarial sequence information led to the development of a *B. malayi* microarray, which is now another tool for analyzing the filaria–*Wolbachia* symbiosis, as previously shown with *B. malayi* (Ghedin et al., 2009).

Litomosoides sigmodontis is a murine filaria, closely related to *Brugia* spp., *O. volvulus* and *W. bancrofti* that is used to study filariasis (Allen et al., 2000, 2008; Hoerauf et al., 2001b). The worms harbour *Wolbachia* bacteria, making them a suitable model for research into symbiosis. The filarial life cycle can be maintained in BALB/c mice. BALB/c mice with an IL-5 knock-out (KO) have an additional advantage as the mice have higher worm burdens due to the dampening of the Th2 response (Saeftel et al., 2003; Volkmann et al., 2003). Depletion of bacteria in *L. sigmodontis* can be achieved by tetracycline treatment for 4 weeks, resulting in a reduction of the bacterial DNA level by >90% and a block in embryogenesis. In contrast, tetracycline (Tet) treatment of *Acanthocheilonema viteae*, a *Wolbachia*-free filarial nematode, does not show any effects on the worm (Hoerauf et al., 1999).

In this study, we used the *B. malayi* microarray chip to discover genes that are differentially expressed in *L. sigmodontis* after depletion of their *Wolbachia* endosymbionts. Our aim was to identify genes that indicate biochemical or physiological dependencies in the *Wolbachia*–filaria relationship that could be targets for the development of drugs that interrupt/block symbiosis.

2. Materials and methods

2.1. Animal model and Tet treatment

Ethical clearance for animal handling was approved by the regional authority in Cologne, Germany (AZ 50.203.2-BN15, 40/04). Animals were maintained in the Institute for Medical Microbiology, Immunology and Parasitology, University Clinic Bonn, Germany.

Fully permissive IL-5 KO BALB/c mice (6–8 weeks old) were infected with *L. sigmodontis* by mites (*Ornithonyssus bacoti*) carrying infective L3s as previously described (Al-Qaoud et al., 1997). Tet treatment of mice started around day 60, when worms were patent and microfilariae were seen in blood of infected mice. Mice were treated daily for 36 days with i.p. injections of 1 mg Tet in 200 µl PBS (50 mg Tet/kg). At days 6, 15 and 36 after the start of Tet treatment, worms from untreated control and Tet-treated mice were recovered from the thoracic cavity. Female worms were snap frozen in liquid nitrogen and stored at −80 °C for subsequent RNA and DNA isolation.

Acanthocheilonema viteae L3s were isolated from *Ornithodoros moubata* ticks and *Meriones unguiculatus* (jirds) were infected by injection of 80 L3s as previously described (Lucius and Textor, 1995). When the animals had a patent infection, they were orally treated with Tet (0.5% w/v) in the drinking water, which was changed daily. After 6 weeks of treatment, jirds were euthanised and *A. viteae* worms were recovered from skin, muscles and visceral cavity. Six weeks of oral Tet treatment of *L. sigmodontis*-infected jirds has been shown to lead to a persistent depletion of *Wolbachia*, demonstrating sufficient Tet delivery with this treatment (Arumugam et al., 2008). *Acanthocheilonema viteae* worms were snap frozen in liquid nitrogen and stored at −80 °C until RNA and DNA extraction was carried out.

2.2. RNA and DNA extraction

Total RNA was isolated from three (quantitative PCR) or five to 10 (microarray) female *L. sigmodontis* per tube using the Trizol (Invitrogen GmbH, Karlsruhe, Germany) extraction method, following the manufacturer's protocol with some modifications. Briefly, nematodes in 800 µl Trizol reagent were transferred to 2 ml tubes containing 0.5 mm glass beads and homogenised in a Precellys 24 homogeniser (Peqlab, Erlangen, Germany) 4 × 10 s at 5000 rpm. Instead of chloroform, 80 µl of 1-brom-3-chloro-propane (Sigma–Aldrich, Steinheim, Germany) was used to separate the homogenate into RNA-containing aqueous and DNA-containing organic phases. For precipitation of RNA, linear acrylamide (Applied Biosystems, Foster City, USA) was used as co-precipitant with a final concentration of 9 µg/ml and 1 vol. isopropanol (Merck, Darmstadt, Germany). RNA was treated with RNAse-free DNAse I (Applied Biosystems) for 1 h at 37 °C, followed by clean-up and concentration of the RNA with the RNeasy MinElute kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol. Concentration and integrity of RNA was determined with an automated gel electrophoresis system (Experion, Bio-Rad, München, Germany).

Extraction of total RNA from two female *A. viteae* worms was carried out as described above, but with the modification that 1 ml Trizol, 100 µl 1-brom-3-chloro-propane and a homogenisation program of 2 × 30 s at 6800 rpm were used, as *A. viteae* are bigger than *L. sigmodontis* worms.

DNA extraction from the organic phase was carried out following the protocol for Trizol reagent (Invitrogen).

2.3. Microarray experimental procedures and analyses

Our studies used the second-generation filarial microarray (BmV2array) developed by the Filarial Microarray Consortium (http://www.filariasiscenter.org/molecular-resources/research-materials), of which our laboratory is a partner. The array contains 65 mer oligonucleotides corresponding to 15,455 expressed sequence tags (ESTs) and open reading frames (ORFs) from *B. malayi*, 1016 from *O. volvulus*, 878 from *W. bancrofti* and 804 *w*Bm. The number of different oligonucleotides corresponding to one gene varies from one to more than 20. Information about the number of protein domain matches is reported by Li et al. (2009) in a supplementary file (summary of protein domain matches for BmV2array elements). Expression profiles from 6, 15 or 36 days Tet-treated female *L. sigmodontis* were compared with the expression of age-matched untreated control worms. RNA quality control, cDNA synthesis, hybridisation of cDNA to the *B. malayi* microarray chip, image analysis and normalisation of data were performed at the core microarray facility of Washington University School of Medicine, St. Louis, MO, USA.