Trans-species transfer of Wolbachia: microinjection of *Wolbachia* from *Litomosoides sigmodontis* into *Acanthocheilonema viteae*

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SUMMARY

Intracellular bacteria of the genus *Wolbachia* are found in most filarial nematodes, but are lacking in some species like *Acanthocheilonema viteae*. Due to their symbiotic nature and their role in the pathology of filarial infections they are considered to be potential targets for intervention against filarial infections in man. Infection of *A. viteae* (a species which does not naturally carry *Wolbachia*) with *Wolbachia* bacteria could allow comparative studies on the effect of the endobacterium on the parasite and on the host's immune systems. As a step towards such studies we microinjected adult female *A. viteae* with *Wolbachia* obtained from *Litomosoides sigmodontis*. The bacteria were isolated from *L. sigmodontis* by density-gradient centrifugation, microinjected into *A. viteae* worms and bacterial DNA detected by PCR with *Wolbachia* specific primers (ftsZ gene). Microinjected worms were cultured *in vitro*, and 81% survived for 10 days. Implantation of microinjected worms into *Meriones unguiculatus*, the rodent host of *A. viteae* resulted in 38% survival. The DNA of the microinjected worms recovered from jirds 8 weeks after implantation contained *Wolbachia* DNA as shown by PCR, suggesting that *Wolbachia* of *L. sigmodontis* can be horizontally transmitted to *A. viteae*.

Key words: *Wolbachia*, microinjection, filariasis, *Acanthocheilonema viteae*, *Litomosoides sigmodontis*, trans-species transfer.

INTRODUCTION

Filarial nematodes are parasitic worms that cause a variety of diseases in man including lymphatic filariasis or river blindness. Globally 140 million people are infected with filarial nematodes (WHO, 2000), mainly in tropical countries. To date there is no chemotherapy with direct effects against adult worms and no vaccine available. Drugs which act against the microfilariae are available, however. Since adult worms can live and produce microfilariae for up to 15 years, patients have to be treated for long periods of time (Addiss, 1998; Richards *et al*. 1998), which is impractical and expensive in most affected nations. Therefore, there is a need for new chemotherapies with direct effects against adult worms.

Since the seventies it has been known that most filarial nematodes harbour intracellular bacteria of the order Rickettsiales. Using electron microscopy the bacteria were detected in the hypodermis and the ovaries of adult worms as well as in all larval stages (McLaren *et al*. 1975; Kozek & Figueroa, 1977). There is evidence that these bacteria are vertically transmitted, most likely transovarially, from the female through the eggs to the offspring (Taylor & Hoerauf, 1999). Only recently it was shown that these bacteria are related to *Wolbachia*, a group of intracellular bacteria that are widespread in diverse anthropod groups such as acarids, isopods and insects (Sironi *et al*. 1995); it is estimated that 10–15% of all insect species harbour *Wolbachia* (Werren, 1997). Today the genus *Wolbachia* includes the intracellular bacteria from filarial nematodes and from arthropods although no formal redescription of *Wolbachia* has been published in recent years and a classification has only been done to the genus level (Bandi *et al*. 1998).

The *Wolbachia* from filarial nematodes are probably endosymbionts in the sense that they are necessary for the normal growth and reproduction of their hosts. Studies revealed that worms which were treated with antibiotics lost the bacteria and showed growth disorders and infertility (Hoerauf *et al*. 1999). In addition, the endosymbionts are thought to contribute to the pathology of filarial infections by increasing inflammatory reactions (Saint André *et al*. 2002). Because all major human pathogenic filarial nematodes harbour *Wolbachia*, the use of antibiotics targeting the endosymbiontic bacteria

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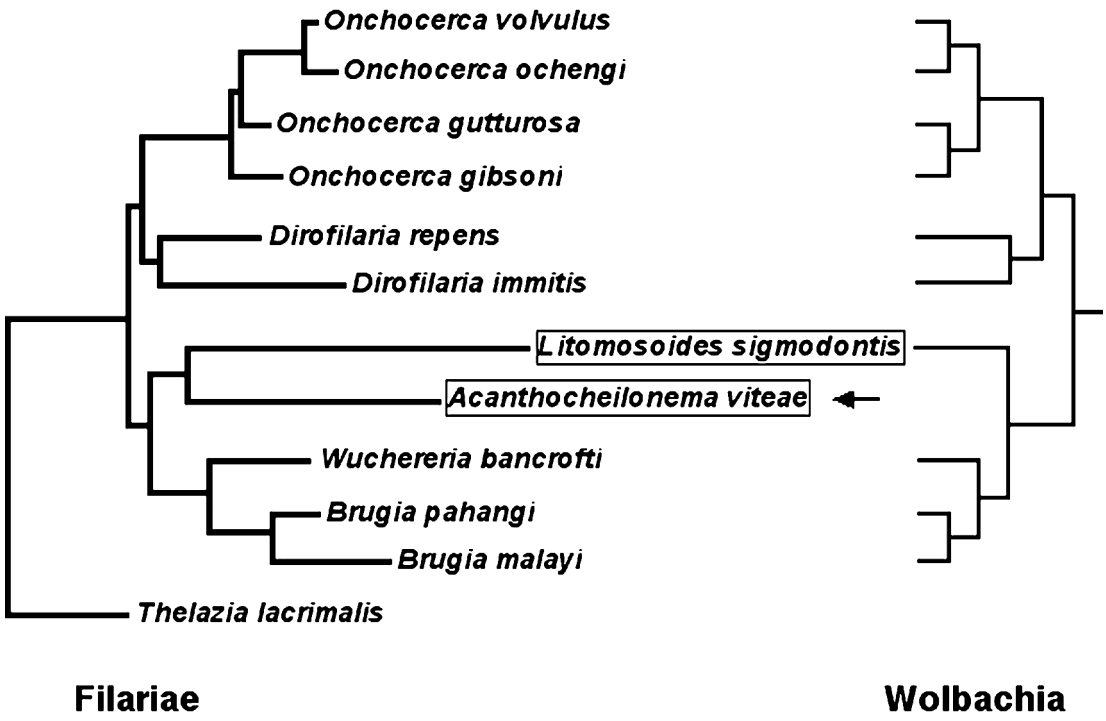

Fig. 1. Phylogenetic tree of filarial worms, based on the sequence of cytochrome C oxidase I from mitochondria. *Thelazia lacrimalis* was used as an outgroup. On the right the phylogenetic tree of the corresponding *Wolbachia* bacteria (trees adapted from Casiraghi *et al*. 2001). Note that *A. viteae* does not harbour *Wolbachia* bacteria (arrow), thus there is no corresponding branch on the Wolbachia tree. The species that are boxed (*A. viteae* and *L. sigmodontis*) were used in our experiments.

is considered as an alternative chemotherapeutic strategy against filarial infections in man (Taylor *et al*. 2000*a*).

Only a few species of filarial nematodes do not harbour any *Wolbachia*. One of these is the rodent filarial nematode *Acanthocheilonema viteae* (Fig. 1). In order to further characterize the association and the interactions between *Wolbachia* and filarial nematodes, we examined the possibility to transfer *Wolbachia* from the closely related filaria *L. sigmodontis* into *A. viteae*. Several studies have demonstrated horizontal transfer of *Wolbachia* between different insect species (Grenier *et al*. 1998; Heath *et al*. 1999; van Meer & Stouthammer, 1999), but to our knowledge there are no reports describing the horizontal transfer of *Wolbachia* between filarial nematodes.

MATERIALS AND METHODS

Cultivation of the filarial nematode A. viteae

The life-cycle of *A. viteae* was maintained in the natural host, the jird *Meriones unguiculatus* and the soft tick *Ornithodoros moubata* as previously described (Lucius & Textor, 1995). The adult worms were cultivated for up to 3 weeks in RPMI 1640 (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS, 4 mM L-glutamine, 100 U/ml penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) at 37 °C and 5% CO_2. For longer periods of culture it was necessary to implant worms into recipient jirds. Jirds were anaesthetized by inhalation with Metofane (Abbott GmbH, Wiesbaden, Germany) and injection of 0·3 ml of Ketamin-Xylazin anaesthetic. The area for surgery on the back of the animals was shaved and disinfected with ethanol. Adult worms were implanted between skin and muscles through a small incision (~5 mm). In order to reduce immune reactions of the host against the implanted worms, the natural course of infection was mimicked by infecting the jirds with 80 L_3 irradiated by exposure to a gamma-ray source (STS, Braunschweig, Germany; energy dose 3000–4000 Rad.) three weeks prior to implantation. As described by Storey & Al-Mukhtar (1983) worms infected with irradiated L_3 prior to transplantation accept transferred worms whereas uninfected jirds soon encapsulate and destroy the transferred worms.

Determination of microfilarial density

Blood was taken from the retroorbital venus plexus of infected jirds and 44 μl was mixed with 100 μl of 10% (v/v) Teepol 610 (Serva, Heidelberg, Germany). Microfilariae were then counted under the microscope using a Fuchs-Rosenthal counting chamber (Roth, Karlsruhe, Germany) and the filarial density in blood was calculated.

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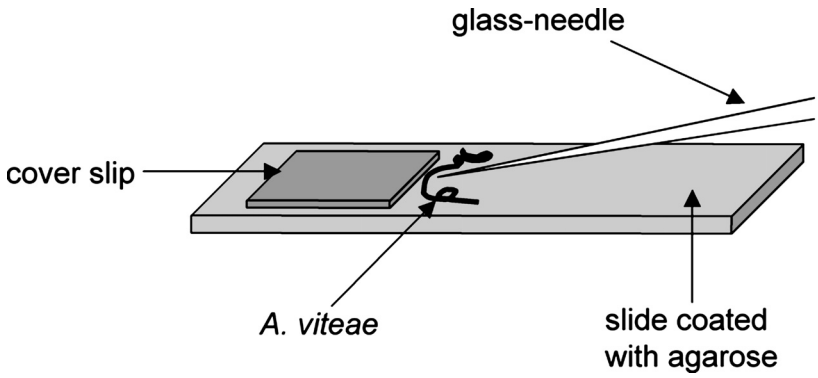

Fig. 2. Sketch illustrating the technique used to microinject *Acanthocheilonema viteae* adult worms with *Wolbachia*.

Isolation of Wolbachia *from the filarial nematode* L. sigmodontis

The isolation protocol was based on a protocol for the purification of the intracellular bacterium *Chlamydia pneumoniae* from host cells (Al-Younes, 2001). All steps were carried out under sterile conditions and on ice or with refrigerated materials. A total of 50–250 adult *L. sigmodontis* were cut into small sections, homogenized with a Dounce glass-homogenizer and vortexed with glass-beads for 10 min. The glass-beads and remaining large particles were separated by centrifugation at 800 ***g*** at 4 °C for 10 min. The supernatant, including the bacteria, was collected and centrifuged again (225 000 ***g***, 4 °C, 30 min; ultracentrifuge L7-55 with rotor 70.1-TI, Beckman, Munich, Germany). The supernatant was discarded and the pellet was re-suspended in Hepes buffer containing 30% Percoll. After another centrifugation (225 000 ***g***, 4 °C, 30 min) a small white zone containing the *Wolbachia* formed within the Percoll gradient. The band was aspirated with a needle and the material washed with SPG-buffer (220 mM sucrose, 4 mM KH_2PO_4, 9 mM Na_2HPO_4, 5 mM L-glutamate, pH 7·4) by centrifugation. The bacteria were resuspended in SPG-buffer and aliquoted. The aliquots were incubated on ice for 2 h and then frozen at −80 °C for storage (Maass & Dalhoff, 1995).

Transfer of Wolbachia *suspension into* A. viteae *by microinjection*

For immobilization of *A. viteae* during microinjection, microscope slides were coated with a thin layer of agarose. A few drops of agarose solution (2% in H_2O; >50 °C) were placed on a slide and spread out by pressing a second slide on top of it. After the agarose had solidified (5–10 sec) the second slide was removed. The agarose was dried for 15 min at 80 °C (Mello & Fire, 1995). Microinjection needles were made from glass capillaries (inner diameter 0·58 mm, outer diameter 1 mm; Clark Electromedical Instruments, England) using a needle puller (Kopf Instruments, Tujunga, USA). The pulled needles had a diameter of 1–2 μm. The microinjection was carried out using the inverted microscope Axiovert 135 (Zeiss; Jena, Germany) with the microinjection needle connected to a micromanipulator (Leitz; Wetzlar, Germany) and a microinjector (Eppendorf; Hamburg, Germany).

Juvenile female *A. viteae* were isolated with thin forceps as described by Lucius & Textor (1995), 5 weeks after infection of jirds with L_3. They had not yet started to produce microfilariae and were kept under standard tissue culture conditions in RPMI 1640 (5% CO_2, 37 °C) until transfer to the agarose slides. Half of the slide was covered with a cover-slip in a way that the worm could be held against this edge during microinjection (Fig. 2). The worms were oriented parallel to the edge. As soon as the movements of the worms were slowed down by the agarose, the injection needle was adjusted next to the worm and injection was performed quickly to prevent drying out of the worm. In order to penetrate the cuticle of the worms it was necessary to use glass-needles with a sharp point. During microinjection the point of the injection needle always broke off which led to an increase of the diameter of the needle. Breaking of the needle tip did not obviously harm the worms. The *Wolbachia* suspension was infected with 28 MPa into the pseudocoele of *A. viteae*. The worms were transferred back into RPMI medium and analysed for the presence of *Wolbachia* or implanted into jirds for long-term transfer of *Wolbachia*.

Detection of Wolbachia *by PCR*

The presence of *Wolbachia* in filarial tissue, in preparations and in microinjected *A. viteae* was analysed using nested PCR. DNA of each microinjected worm was isolated with a DNeasy Tissue Kit (Qiagen; Hilden, Germany) according to the manufacturer's recommendations. Two pairs of primers, specific for the cell division protein (ftsZ) gene of *Wolbachia* from *L. sigmodontis* (GenBank accession number

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AF081199), were synthesized: ftsZ fw (5′TAAAAGATAGTCATATGC3′) and ftsZ rv (5′CTTCACGCACTCTATTCG3′), as well as the nested primers ftsZ fwn (5′GCCATTTGACTTTGAAGG3′) and ftsZ rvn (5′CCAATCATTGCTTTACCC3′).

One μl of the reaction product from the first PCR, producing a 615 bp fragment, was used for the second PCR with the nested primers generating a 279 bp fragment. The cycling conditions were as follows: denaturation (5 min at 94 °C), 30 cycles, 1 min at 94 °C, 1 min at 52 °C, 2 min at 72 °C) and a final extension step (7 min at 72 °C). A negative control (no template DNA) and a positive control (*L. sigmodontis* DNA) were always included. After PCR, the presence and size of the reaction product was analysed by running 5 μl of the reaction mix on a 1% agarose gel.

Electron microscopy

L. sigmodontis. Male *L. sigmodontis* were fixed with 2·5% glutaraldehyde in 0·1 M Na-cacodylate buffer, pH 7·4, for 24 h at 4 °C. The primary fixative was removed and the worms were washed 3 times for 30 min each with 0·1 M Na-cacodylate buffer at 4 °C. For secondary fixation 2% osmium tetroxide in Na-cacodylate buffer was used for 4 h at 4 °C. After washing in cold buffer solution the worms were stained overnight at 4 °C with 0·5% uranyl acetate in 70% ethanol and then dehydrated in a graded ethanol series and embedded in Spurr's epoxy resin. Sections were cut with a diamond knife using the Ultracut S (Leica, Vienna, Austria). The grids were post-stained with uranyl acetate and Reynold's lead citrate and viewed in a Zeiss EM 900 electron microscope.

L. sigmodontis *extracts*. Pellets from *L. sigmodontis* extracts were resuspended in the primary fixative (2·5% glutaraldehyde in 0·1 M Na-cacodylate buffer) and fixed overnight at 4 °C. After centrifugation at 1500 ***g*** for 5 min the supernatant (fixative) was discarded. The pellet was mixed with a small volume of melted 2·5% agarose at 45 °C and immediately chilled on ice. The solidified agarose was cut into blocks (about 1 mm^3). These agarose blocks were prepared further as described above for *L. sigmodontis*.

RESULTS AND DISCUSSION

Our comparative ultrastructural studies of *L. sigmodontis* and of *A. viteae* confirmed that both male and female *L. sigmodontis* harboured *Wolbachia* mainly in the hypodermal tissues and lateral chords, but also within the microfilariae in the uterus (Fig. 3A and B), whereas *A. viteae* worms were free of *Wolbachia*. PCR with primers specific for the *Wolbachia* ftsZ gene supported the presence of *Wolbachia* in *L. sigmodontis* and their absence in *A. viteae*. This confirms earlier results by Bandi *et al*. (1998) who also used *Wolbachia*-specific ftsZ primers to construct a phylogenetic tree of the genus *Wolbachia*.

A comparison of various microinjection methods revealed that the worms were difficult to hold with an aspiration needle and very sensitive to contact with chemicals or drying out. Thus, a combination of techniques used for microinjection of *Caenorhabditis elegans* and microinjection of murine oocytes was developed (see Materials and Methods section). With this technique, safranine dye was injected into adult *A. viteae* with the help of a glass needle which had an inner diameter of about 1 μm. It was possible to inject a volume of about 0·5 μl into the nematodes. The dye stained the pseudocoele (Fig. 4) and remained in the worm when the needle was withdrawn. Usually, the tip of the glass needle broke off inside the worms, but this did not seem to have any negative effects on the worms. To demonstrate the survival of *A. viteae* after microinjection, the worms were subsequently kept in culture. Out of 26 microinjected worms, 21 (81%) were viable after 10 days *in vitro*, as compared to 23 out of 24 (96%) non-microinjected worms. The microinjected worms did not show differences in motility or any morphological alterations as compared to the control worms.

Wolbachia were isolated from *L. sigmodontis* by density-gradient centrifugation. Electron microscopical (EM) analysis of the suspension (Fig. 3C and D) revealed that endobacteria as well as membrane structures and organelles of nematode origin were present in the suspension. Because the EM studies showed that the structure of the isolated *Wolbachia* was intact, we assumed that a large proportion of the isolated *Wolbachia* were alive and potentially infective. To determine whether *Wolbachia* passed the glass needle in sufficient amounts to be detectable, *Wolbachia* suspension was pipetted through the microinjection assembly, collected in a tube and the sample was analysed by PCR. The tests revealed that the bacteria did not pass through intact needles, probably due to clogging of the lumen by cell debris. However, breaking of the tip, as it occurred in the worms, allowed the passage of *Wolbachia* in amounts that allowed detection by PCR (data not shown).

The presence of *Wolbachia* in the microinjected worms was determined by PCR. Experiments with DNA extracted from whole microinjected worms did not yield a PCR signal, probably due to a massive over-representation of nematode DNA in the sample. To alter the proportions between nematode and *Wolbachia* DNA, we microinjected the anterior portion of the worms, cut it off and then isolated the DNA immediately after injection. Subsequent PCR with DNA isolated from these worm fractions

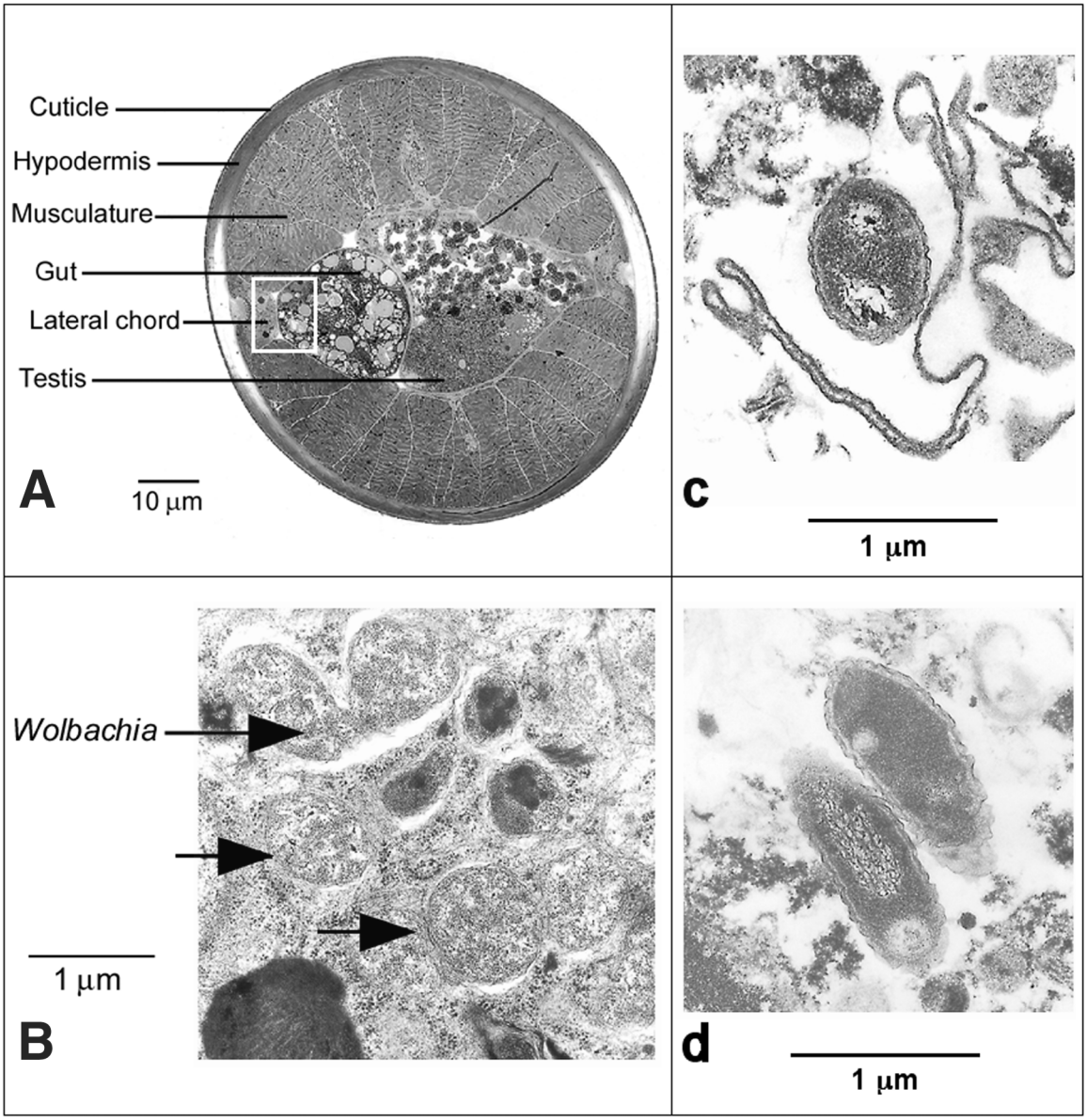

Fig. 3. (A) Electron micrograph of a cross-section of a *Litomosoides sigmodontis* male adult worm (overview). (B) Higher magnification of the area indicated in (A) with the white square. Arrows show *Wolbachia* in the lateral chord. (C and D) Electron micrograph of 2 separate extracts of *Wolbachia* from *L. sigmodontis* showing apparently intact bacteria.

confirmed that the *Wolbachia* had been introduced into the worms (data not shown).

After these preliminary experiments, 6-week-old *A. viteae* were subjected to microinjection of *Wolbachia* and subsequently implanted into *M. unguiculatus*, the rodent host of *A. viteae*. We choose worms of 6 weeks of age isolated from jirds based on earlier observations that the female worms had already been inseminated but did not yet produce microfilariae. An inoculation with *Wolbachia* at this stage could thus allow the infection of uterine embryonic stages by the endobacteria, and thus theoretically result in the vertical transmission of *Wolbachia*. A total of 16 microinjected worms were subcutaneously implanted into *M. unguiculatus*. These jirds had been tolerized to *A. viteae* by pre-treatment with irradiated infective larvae, as experiments reported by Storey & Al-Mukhtar (1983) suggest that this pre-treatment increases the survival rate of transplanted worms. In this experiment 38% of the microinjected worms survived and their production of microfilariae showed that they were functionally intact (Table 1). However, the higher survival rate and higher production of microfilariae by control worms showed that the microinjection reduced the chances of survival in the host animals. It is unclear whether the higher mortality was due to damage of the worms by the microinjection, preferential killing of microinjected worms by the immune system of the host, or due to damage caused by the bacteria.

PCR with DNA isolated from single microinjected *A. viteae* yielded positive signals, demonstrating that

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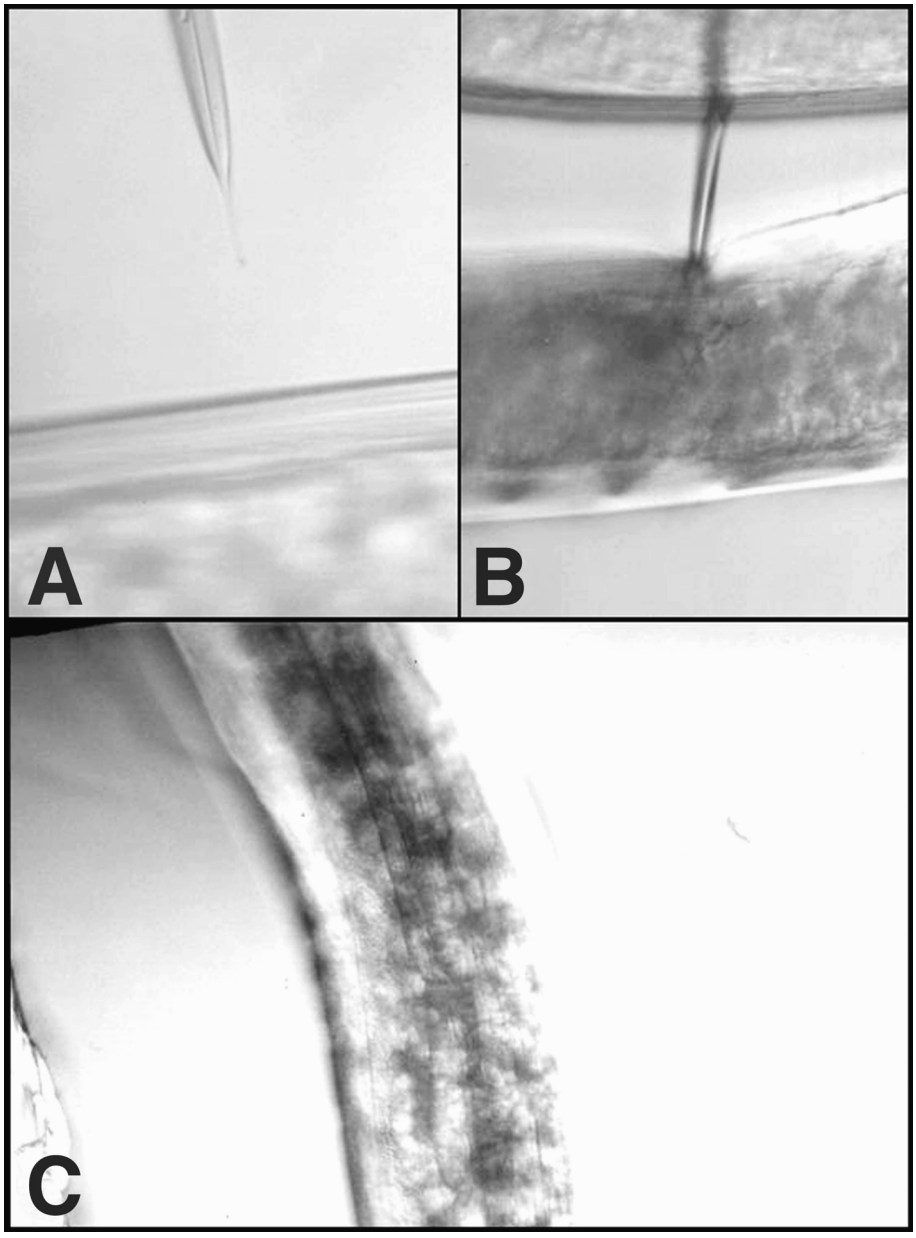

Fig. 4. (A) *Acanthocheilonema viteae* (bottom) and injection needle (top) prior to microinjection. (B) Microinjection of *A. viteae* with safranine. (C) *A. viteae* after microinjection. Note that there is no leakage of the dye after injection, indicating complete transfer of fluid into the worm.

Wolbachia DNA was detectable in the worms 8 weeks after implantation (Fig. 5). In this experiment, DNA extracted from total worms was analysed since we assumed that the bacteria were no longer concentrated in the region of the microinjection. The fact that we obtained positive PCR signals in spite of the presence of large amounts of nematode DNA suggests, but does not prove, that the bacteria had increased in number during the 8 weeks of worm implantation. Since it was not possible to detect bacterial DNA from whole worms directly after microinjection (see above), we assume that positive PCR signals with DNA from whole worms at 8 weeks after the microinjection are due to the growth of the bacteria. PCR performed with DNA extracted from blood microfilariae of jirds implanted with microinjected *A. viteae* did not reveal the presence of *Wolbachia* in the microfilariae, indicating that a vertical transmission of *Wolbachia* had not occurred under the circumstances of our experiment.

There may be several reasons why microfilariae derived from microinjected worms did not harbour *Wolbachia*. It is possible that the period of 8 weeks between microinjection and analysis might be too short for the bacteria to disseminate through the tissue of the female worm into the oocysts. It is also possible that the embryos were already too advanced at the time of microinjection. An infection of such a late embryonic stage could be difficult. It might also be possible that in spite of the growth of *Wolbachia* in *A. viteae*, the passage to the next generation is

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Table 1. Survival and production of microfilariae by *Acanthocheilonema viteae* microinjected with *Wolbachia* and implanted into *Meriones unguiculatus*

No. of implanted worms per jird	Density of microfilariae/ μl blood	No. of surviving worms	Survival rate (%)
8 microinjected worms	5·9	3	38
8 microinjected worms	2·1	3	38
8 non-microinjected worms	32·8	7	88
7 non-microinjected worms	22·7	7	100
0 worms	0	0	—
0 worms	0	1*	—

* This worm is derived from the injection of 80 irradiated L_3.

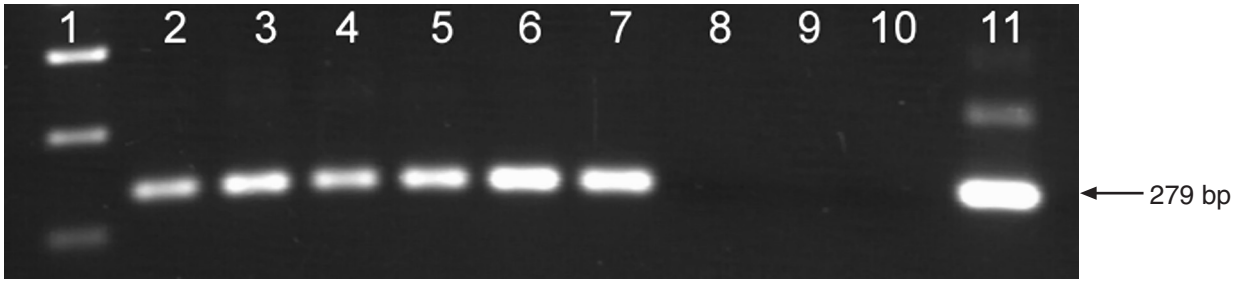

Fig. 5. PCR amplification of the Wolbachia ftsZ gene from *Acanthocheilonema viteae* DNA after microinjection and 8 weeks after implantation. Lane 1: marker; Lanes 2–7: DNA from individual *A. viteae* worms as template; Lanes 8 and 9: DNA from microfilariae produced by microinjected *A. viteae* as template; Lane 10: negative control (DNA from *A. viteae* worms not microinjected); Lane 11: positive control (DNA from. *L. sigmodontis* worms). Note that due to the small sizes of the PCR bands only the relevant bottom half of the gel is depicted.

an intricate process resulting from a co-evolution between filariae and their endosymbionts. Such a process might not function between *Wolbachia* from *L. sigmodontis* and *A. viteae*.

Our data show for the first time that filarial nematodes can be successfully microinjected, survive in their host animals for a prolonged period of time and produce microfilariae. Beyond the application for studies on *Wolbachia*, the technique developed could also be used for introduction of RNA and DNA into the parasites, particularly in the context of RNAi studies or the development of transgenic filariae. In most of the studies on transgenic helminths so far published particle bombardment was used as the transfection method (Davis *et al*. 1999; Jackstadt *et al*. 1999; Wippersteg *et al*. 2001; Higazi *et al*. 2002). We used microinjection, which has been successfully used as a transfection technique in *Caenorhabditis elegans* (Mello & Fire, 1995) to inject the *Wolbachia*, because particle bombardment is not applicable for transferring living bacteria.

Our experiments suggest, but do not prove, the growth and thus the establishment of *Wolbachia* isolated from *L. sigmodontis* in *A. viteae*. Definitive proof could be achieved by EM studies. We did not perform such EM studies in our current experiments, since these require fixation of material, which prevents DNA extraction for PCR studies.

Successful horizontal transfer of *Wolbachia* between different host species has been reported from diverse groups of arthropods. *Wolbachia* have been transferred between different strains of the flour beetle *Tribolium confusum* (Chang & Wade, 1994), between different *Drosophila* species (Poinsot *et al*. 1998) and between the parasitoid wasp *Muscidifurax uniraptor* (order Hymenoptera) and *Drosophila simulans*, order Diptera (Van Meer & Stouthamer, 1999). A natural horizontal transmission was observed from *Drosophila simulans* to the parasitic wasp *Leptopilina boulardi* (Heath *et al*. 1999). This occurrence of horizontal transmission between species is also reflected by the fact that the phylogenetic tree of *Wolbachia* from arthropods does not match the phylogenetic tree of their host species. It means that closely related *Wolbachia* can be found in phylogenetic diverse arthropod host species (Werren, Zhang & Guo, 1995; Zhou, Rousset & O'Neill, 1998). In contrast, the evolutionary trees of *Wolbachia* from filariae and their nematode hosts are found to be congruent (Bandi *et al*. 1998), which indicates the existence of a long and stable evolutionary association between *Wolbachia* and filarial nematodes leading to a mutualistic relationship. Such a long relationship is expected to increase the probability of developing host specificity and thus decrease the chances of horizontal transmission. The

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results from our experiments suggest that such a horizontal transmission might occur, in spite of the long-standing association between filariae and their endobacteria.

If it were possible to further improve the horizontal transfer of *Wolbachia* to achieve a vertical transfer of endosymbionts it would be possible to compare *Wolbachia*-containing and *Wolbachia*-free *A. viteae* lines. Such comparisons could shed light on the role of the endosymbionts for worm development (Hoerauf *et al*. 1999), the proposed influence on the antifilarial immune response (Taylor, Cross & Bilo, 2000*b*) and the recently reported importance for the pathogenesis of filarial infections (Saint André *et al*. 2002).

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