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ORIGINAL PAPER

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An aspartate aminotransferase of *Wolbachia* endobacteria from *Onchocerca volvulus* is recognized by IgG1 antibodies from residents of endemic areas

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Abstract *Wolbachia* are intracellular α-proteobacteria, closely related to *Rickettsia*, that infect various arthropods and filarial parasites. In the present study, the cDNA encoding the aspartate aminotransferase (AspAT) of *Wolbachia* from the human pathogenic filarial parasite *Onchocerca volvulus* (Ov-WolAspAT) was identified. At the amino acid level, the identity of the Ov-WolAspAT was 56% to *Rickettsia prowazekii* AspAT and 54% to the AspAT of the nitrogen-fixing bacterium *Sinorhizobium meliloti*, but the highest degree of identity was found to the putative AspAT of *Wolbachi*a from *Brugia malayi* and *Drosophila melanogaster* (85%). All of these bacterial AspATs are members of the AspAT subclass Ib. A 35 kDa fragment of the Ov-WolAspAT was expressed in *Escherichia coli*, and immunolocalization using polyclonal antibodies against this antigen revealed that Ov-WolAspAT is present in a considerable proportion of the *Wolbachia* from *O. volvulus*, as well as in the endobacteria of several other filarial parasites. Western blot analysis using recombinant Ov-WolAspAT as antigen showed that IgG1 antibodies were present in 70 (51%) individuals living in areas endemic for *O. volvulus*, *B. malayi* or *Wuchereria bancrofti* and no IgG4 or IgE antibodies were found. Among 40 sera of persons from Uganda and Liberia who were putatively not infected with human filarial parasites, 11 (28%) individuals presented IgG1 antibodies, while none of the 33 sera from healthy Europeans and none of the 14 sera from patients with proven *Rickettsia* or *Brucella* infections reacted with the antigen. These results also show that an intracellular protein of *Wolbachia* endobacteria (WolAspAT) acts as antigen in human filariasis.

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Introduction

Filarial parasites infect more than 150 million people in tropical and subtropical countries and are responsible for various diseases including river blindness and lymphatic filariasis. It has been known for decades that many filarial species harbor intracellular bacteria, but only the recent discovery that these *Wolbachia* bacteria may be essential for the survival and reproduction of their host has fostered research interest on them. Tetracycline treatment eliminates *Wolbachia* from the rodent filaria *Litomosoides sigmodontis* and subsequently leads to reduced growth and sterility of the worms (Hoerauf et al. 1999). Similar observations were made with *Brugia pahangi* and *Dirofilaria immitis* (Bandi et al. 1999). Extensive antibiotic treatment was successful in killing the cattle parasite *Onchocerca ochengi* (Langworthy et al. 2000). Furthermore, studies on the human parasite *Onchocerca volvulus* showed a sterilizing effect of doxycycline due to the elimination of *Wolbachia* (Hoerauf et al. 2000a, 2001).

These bacteria were not only selected as a target for anti-filarial chemotherapy, they were also suggested to be involved in the development of pathology in lymphatic filariasis (Taylor et al. 2000) and onchocerciasis (Brattig et al. 2000; Keiser et al. 2002; Saint Andre et al. 2002). In contrast to the *Wolbachia* endobacteria of insects, the relationship between the filarial *Wolbachia* appears to resemble the phylogeny of their helminth hosts (Casiraghi et al. 2001a). A number of *Wolbachia* proteins have been discovered as side products of the *Brugia malayi* and the *O. volvulus* genome projects (Williams et al. 2000) or accidentally while searching for

certain worm proteins (Henkle-Dührsen et al. 1998; Koszarski 1999). In addition, the *Wolbachia* surface protein (WSP) of some filarial parasites was cloned and used to detect antibodies against *Wolbachia* in animals infected with filarial parasites (Bazzocchi et al. 2000a, 2000b; Punkosdy et al. 2001a). Nonetheless, the role of *Wolbachia* as antigens in human filariasis is to date unclear.

Individuals infected with filarial parasites may come into contact with *Wolbachia* antigens when parasites die and release their bacteria. In persons with high parasite loads, thousands of microfilariae die each day and produce antigens (Schulz-Key and Karam 1986). However, the amount of *Wolbachia*-derived antigens is very low in comparison to filarial antigens. Exported or surface proteins can act as antigens while *Wolbachia* are still intact, for example WSP. In addition, during the clearance of microfilariae and their endobacteria intracellular proteins of *Wolbachia* also have the chance to come into contact with the host's immune response.

In the present study, we identified the gene for the aspartate aminotransferase (AspAT) of *Wolbachia* from *O. volvulus* (Ov-WolAspAT). AspATs are variously involved with nitrogen metabolism, amino acid synthesis and degradation, and carbon utilization and serve as an important link for several metabolic pathways to the tricarboxylic cycle (Cooper and Meister 1985). Therefore, they may be expressed at significant levels and could be released during the clearance of parasites. We investigated the reactivity of sera from individuals from filariasis endemic areas with recombinant Ov-WolAspAT antigen and showed that this *Wolbachia* antigen is frequently recognized by human IgG1 antibodies, but never by IgG4 or IgE antibodies, which is typical for filarial antigens.

Materials and methods

Parasite material

Adult *O. volvulus* were obtained by nodulectomy and collagenase digestion from onchocerciasis patients from western Uganda (Fischer et al. 1993). In the course of a related project, a cDNA library (SAW98PF-OvAF) was constructed in the λZap II vector (Stratagene, La Jolla, Calif., USA) using adult *O. volvulus* from a patient treated with the drug ivermectin. Additional genomic and cDNA libraries were obtained from the Filarial Genome Project Resource Center at Smith College, Northampton, Mass., USA (Genome@smith.edu).

For immunohistology, onchocercal nodules from untreated patients in Ghana ($n=21$), Liberia ($n=6$), Burkina Faso ($n=1$), and Uganda ($n=8$) were used. In total, these nodules contained more than 100 adult *O. volvulus*. In addition, 30 nodules were studied that had been extirpated from patients who had been treated for 6 weeks with 100 mg doxycycline per day to eliminate *Wolbachia* (Hoerauf et al. 2000a). These nodules contained about 100 *Wolbachia*-free *O. volvulus* and a few young, newly acquired worms with endobacteria. Nodulectomies for research purposes and the doxycycline drug trial were approved by the Ethics Committees of the Medical Board in Hamburg and of the School of Medical Sciences in Kumasi, Ghana. Nodules containing other *Onchocerca* species, isolated filarial parasites or other helminths had also been collected during previous studies (Table 1).

Blood samples

Samples of *O. volvulus* microfilaria positive and negative individuals were collected from an hyperendemic onchocerciasis focus in western Uganda (Fischer et al. 1993). The microfilaria positive individuals had microfilarial loads of more than 100 mf/mg skin, while the others had become microfilaria-negative due to repeated treatment with ivermectin. For comparison, additional sera were obtained from a nearby village at Lake Albert in Uganda that was not endemic for *O. volvulus* but was endemic for *Mansonella perstans*. Selected samples were collected from microfilaria negative individuals. Another set of African sera was collected in the village Kenema in Grand Cape County in south-western Liberia. This area was hypoendemic for *O. volvulus* and not endemic for any other human filariae. None of the persons from whom the sera were examined showed *O. volvulus* microfilariae in two skin biopsies using the collagenase technique or any reaction on a Mazzotti test 24 h after 100 mg diethylcarbamazine. These sera were negative for antibodies against *O. volvulus* using the specific Ov20 or Ov16 recombinant proteins. However dogs, monkeys, antelopes and hornbills were known to be infected by filariae in this area. Sera from patients with hyperreactive onchocerciasis (sowda) were collected in Liberia during a longitudinal study on this form of infection by the Bernhard Nocht Institute. The patients showed the typical signs of hyperreactive onchocerciasis as described by Albiez et al. (1985) and had a positive Mazzotti test.

Additional samples of microfilaria positive and negative individuals were collected in an area highly endemic for *B. malayi* in central Sulawesi, Indonesia (Fischer et al. 2000) and in a coastal area from Alor Island, Indonesia, endemic for *Wuchereria bancrofti*

Table 1 Immunohistological observations of *Wolbachia* endobacteria in various filarial species using antibodies against recombinant *Onchocerca volvulus* WolAspAT. For references to source see Taylor and Hoerauf (1999)

Filarial parasites	*Wolbachia*	Host	Source
Acanthocheilonema viteae	Neg.	*Mastomys*	Laboratory strain
Brugia malayi	Pos.	*Mastomys*	Laboratory strain
Dirofilaria repens	Pos.	Human	Mediterranean area
Litomosoides sigmodontis	Pos.	*Mastomys*	Laboratory strain
Onchocerca armillata	Pos.	Cattle	Cameroon
Onchocerca dukei	Pos.	Cattle	Cameroon
Onchocerca fasciata	Pos.	Camel	Saudi Arabia
Onchocerca flexuosa	Neg.	Red deer	Germany
Onchocerca gibsoni	Pos.	Cattle	Australia
Onchocerca gutturosa	Pos.	Cattle	Uganda
Onchocerca jakutensis	Pos.	Red deer	Germany
Onchocerca ochengi	Pos.	Cattle	Cameroon
O. volvulus, untreated	Pos.	Human	Ghana, Liberia, Uganda
O. volvulus, doxycycline-treated	Neg.	Human	Ghana

(Supali et al. 2002). As non-endemic controls, plasma samples were chosen from 17 adults from Germany who had never traveled to filariasis endemic areas outside of Europe, and from 16 school children from eastern Europe who were infected with *Ascaris lumbricoides*. The sera positive for *Rickettsia* or *Brucella* were obtained by the Diagnostics Department of the Bernhard Nocht Institute.

Isolation of Ov-WolAspAT DNA

An expressed sequence tag (EST) analysis of the SAW98PF-OvAF cDNA library revealed a DNA sequence which encoded a protein that was very similar to a bacterial AspAT. The identified clone (SWOvAFCB153SK, GenBank gi|5430846|) was 381 bp long. The available Ov-WolAspAT sequence information was sufficient to search the TIGR database from the unfinished *Drosophila melanogaster-Wolbachia* genome (http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi) to identify putative AspAT from the *Wolbachia* of *D. melanogaster* (Dm-WolAspAT). A sequence with a high level of identity and an open reading frame of 1,191 bp was identified. Based on this sequence, several sets of primers were designed. Using a Dm-WolAspAT forward (Dm1F 5′ATGTCAGACCTTG CAAAAAG3′) and an Ov-WolAspAT reverse primer (Ov1R 5′CTTGCATTCTGCTATAACTG3′) and an Ov-WolAspAT forward (Ov1F 5′AGTCAAAGGAAAAGAGATCTG3′) and a Dm-WolAspAT reverse primer (Dm1R 5′TCATTTTAACTC CTGGCACG3′), two fragments of the Ov-WolAspAT sequence were obtained that reveal an assembled sequence of about 860 bp. Since no PCR product was obtained using the Dm1F and Dm1R primers, a new pair of primers based on the Ov-WolAspAT sequence was used for protein expression.

DNA sequencing was performed according to standard protocols using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, Calif., USA) and an ABI Prism 377 automated DNA sequencer. Analysis of nucleotide and amino acid sequence for special sequence motifs and structures were performed using PROSITE tools (http://www.expasy.ch./tools).

Expression and purification of Ov-WolAspAT

Two in-frame primers (Ov2F 5′ATGTCTTTATAAAACTATC3′ and Ov2R 5′ATTTAATG CTTCAACCGCAGC3′) were selected to amplify a 825 bp fragment of the Ov-WolAspAT as mentioned above. The PCR product was directly cloned in the pCRT7 TOPO TA vector (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. One clone with the correct orientation was selected and used to transform BL21(DE3)pLysS *Escherichia coli* cells (Invitrogen). To induce expression of the recombinant protein, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM and incubation was continued at 37°C for about 3 h until cells were harvested. The recombinant His-tagged Ov-WolAspAT protein was purified by affinity chromatography using nickel-nitrilotriacetic acid agarose (Qiagen, Hilden, Germany) under denaturing conditions according to the description of the manufacturer. To raise polyclonal antibodies against the rOv-WolAspAT, a rabbit was immunized and boosted three times with 100 µg of the antigen using Freund's adjuvant (Eurogentec, Seraing, Belgium). The antiserum was obtained 3 months following initial immunization.

Immunohistology

Nodules and filariae had been fixed in 80% ethanol, 4% buffered formaldehyde or in Karnovsky fixative containing 1% paraformaldehyde and 2% glutaraldehyde in cacodylate buffer. The specimens were embedded in paraffin using standard methods. For immunostaining, the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method was used according to the recommendations of the manufacturer (Dako Diagnostika, Hamburg, Germany). The polyclonal rabbit antibody against rOv-WolAspAT was used as the primary antibody at a dilution of 1:60 after dilutions between 1:10 and 1:400 had been tried. As secondary antibody, anti-rabbit mouse immunoglobulins (clone MR12/53, Dako Diagnostika) were applied. Fast red TR salt (Sigma, Deisenhofen, Germany) was used as the chromogen, and hematoxylin (Merck, Darmstadt, Germany) functioned as the counterstain.

To make sure that the rabbit had no other antifilarial or antibacterial antibodies, the preimmune serum collected before immunization with the rOv-WolAspAT was used. To check for the presence or absence of endobacteria in the worms, sections from the same specimens that were stained for WolAspAT were used. These had been stained for endobacteria using rabbit antisera against recombinant heat shock protein 60 (hsp60) of *Wolbachia* from *O. volvulus* (Koszarski 1999; Hoerauf et al. 1999) or from *Yersinia enterocolitica* (Hoerauf et al. 2000a). Both antibodies had been shown to label filarial *Wolbachia* using transmission electron microscopy (Hoerauf et al. 2000b; Koszarski 1999). In addition, sections from these specimens that had been stained with polyclonal antibodies against recombinant catalase from *O. volvulus Wolbachia* were used for comparison (Henkle-Dührsen et al. 1998).

Western blot analysis

For SDS page gel electrophoresis and Western blot, 5–15% (w/v) polyacrylamide linear-gradient slab gels were run with rOv-WolAspAT as loading protein at equal concentrations of 4 µg/cm gel width. Protein was then transferred electrophoretically to nitrocellulose paper with a semi-dry transfer apparatus. The protein band was reversibly stained using Ponceau and the nitrocellulose was cut into 3- to 5-mm strips. The strips were blocked for 1 h with 3% (w/v) non-fat milk powder in PBS and incubated overnight at 4°C with patients' sera at dilutions 1:20 (IgG subclasses, IgE) and 1:50 total IgG. For a control, soluble total *O. volvulus* extract was used as antigen. Peroxidase-conjugated anti-IgG or anti-IgG1, anti-IgG2, anti-IgG3, anti-IgG4 (Calbiochem, La Jolla, Calif., USA) and anti-IgE (Dako) in combination with peroxidase-conjugated goat anti-mouse antibodies (Calbiochem) were used at a dilution of 1:1,000 and incubated for 1 h at RT. Nitrocellulose strips were then developed with 4-chloro-1-naphthol (Sigma) and the enzymatic reaction was stopped with distilled water. All incubation steps were separated by washing the strips with alkaline phosphatase buffer. The sera were not absorbed with IgG4 for the detection of specific IgE antibodies against WolAspAT because no IgG4 antibodies were detected. The rOv-WolAspAT was also detected by the serum of a rabbit immunized with the recombinant protein. The rabbit antiserum was used at a dilution of 1:100 and as secondary antibody an alkaline phosphatase-conjugated donkey anti-rabbit IgG antibody (Dianova, Hamburg, Germany) was used at a dilution of 1:10,000 and for the color reaction NBT/BCIP (Sigma) was applied as substrate. Alternatively, the rOv-WolAspAT was detected by an anti-His antibody (Anti-HisG-HRP, Invitrogen), which was used at a dilution of 1:5,000 and reacted with 4-chloro-1-naphtol as substrate. To check for cross-reaction of the rabbit antiserum with AspAT of the class Ia, commercially available cytosolic AspAT from porcine heart (Sigma) was used as antigen and probed with the antiserum in a Western blot. A complement binding reaction assay using antigens of *Rickettsia prowazekii, Coxiella burnettii, Chlamydia* and *Brucella abortus* was used to test the reactivity of the rabbit antiserum with α-proteobacteria other than *Wolbachia*.

Results

Sequence analysis and classification of WolAspAT

Based on PCR primers derived from the Dm-WolAspAT gene, a fragment of 825 bp of the *O. volvulus*

WolAspAT was isolated. Ov-WolAspAT is encoded by a gene with an A/T content of 64%. At the nucleotide level, the WolAspAT of *O. volvulus*, *B. malayi* and *D. melanogaster* showed a high degree of identity (>82%), and the few nucleotide exchanges were scattered over the entire sequence (data not shown). At the amino acid level, the identity varied among the three WolAspAT between 84 and 86% (Fig. 1). The highest level of

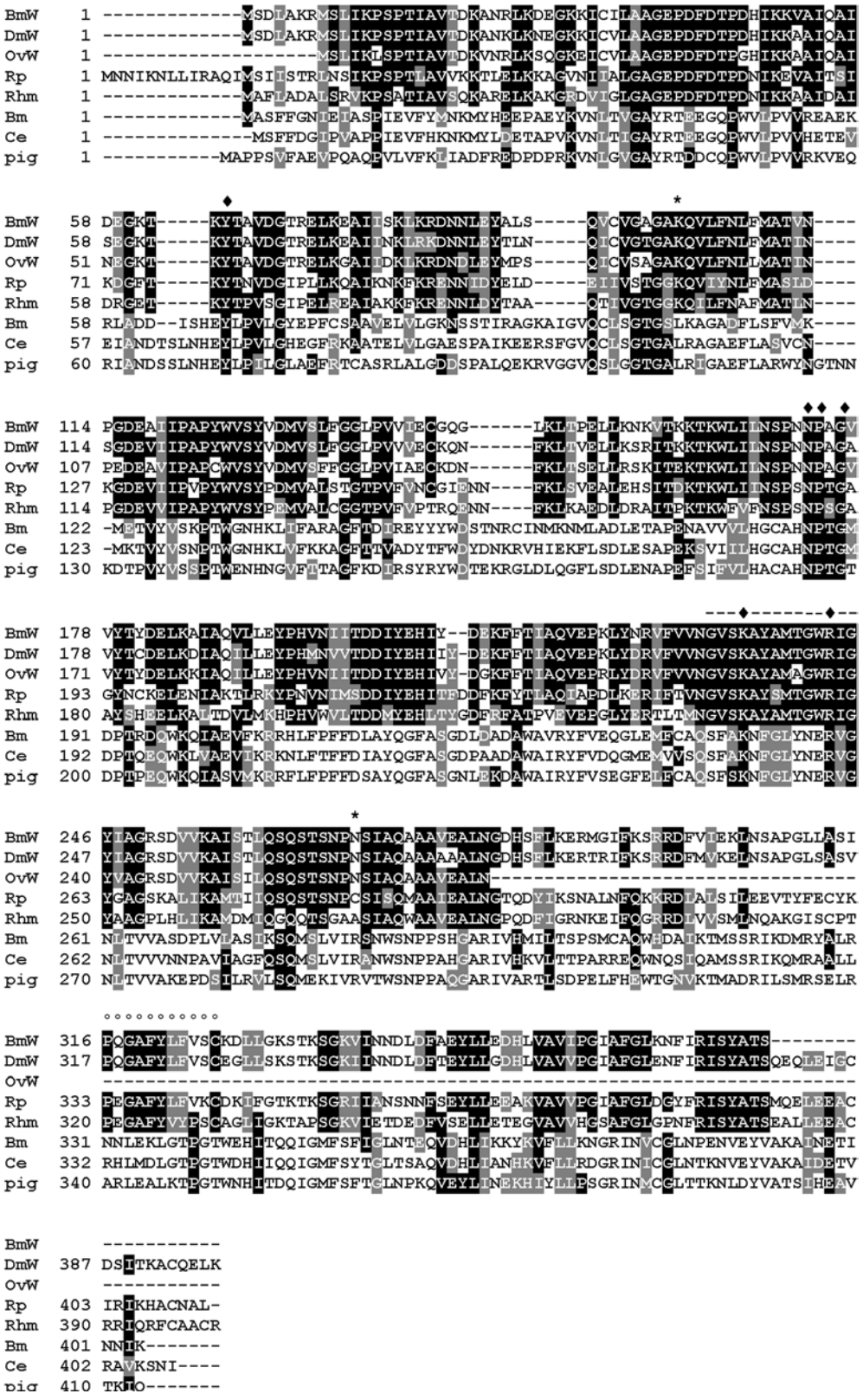


Fig. 1 Alignment of the deduced amino acid sequences of AspAT of different subgroups. AspAT Ib: *Wolbachia* of *Brugia malayi* (*BmW*), *Drosophila melanogaster* (*DmW*) and *Onchocerca volvulus* (*OvW*); *Rickettsia prowazekii* (*Rp*), *Sinorhizobium meliloti* (*Rhm*). AspAT Ia: *B. malayi* (*Bm*), *Caenorhabditis elegans* (*Ce*), and pig cytosolic. The pyridoxal phosphate binding site is indicated by *dashes*, and functional residues conserved in both subclasses are indicated by a rhombus. The residues corresponding to the reactive Arg-292 (subgroup Ia) and Lys-109 (subgroup Ib) are marked by an *asterisk*

identity was found to the AspAT of subclass Ib of the other α-proteobacteria *R. prowazekii* (56%, Andersson et al. 1998) and *Sinorhizobium meliloti* (54%, aatA, Watson and Rastogi 1993). The WolAspAT and the *R. prowazekii* and *S. meliloti* AspAT have a pyridoxal phosphate binding site (Fig. 1, Bm-WolAspAT, position 232) in a conserved region with one amino acid exchange in the Ov-WolAspAT and in the *R. prowazekii* AspAT. The WolAspAT has a conserved Lys residue at position 101 (Bm-WolAspAT) that corresponds to the Lys-109 residue which is suspected to be a functional group in the subclass Ib AspAT of the α-proteobacterium *Thermus thermophilus* (Nobe et al. 1998).

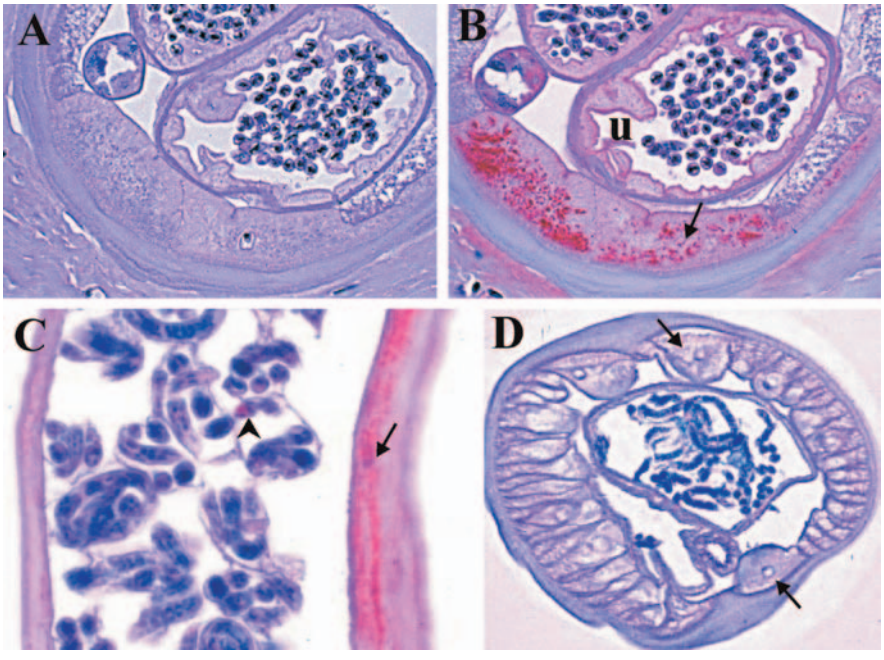
Expression of the Ov-WolAspAT and characterization of the antiserum

The fragment of the rOv-WolAspAT encoding a protein of a theoretical molecular mass of 30 kDa was cloned in a His-tag expression vector. The resulting His-tagged rOv-WolAspAT had a theoretical molecular mass of about 35 kDa, which agrees well with the experimental molecular mass of 34.5 kDa as determined by polyacrylamide electrophoresis. The rOv-WolAspAT was expressed independently of the presence of pyridoxal phosphate and could be detected by an anti-His antibody. Attempts to purify the native rOv-WolAspAT from *E. coli* failed; it was therefore purified from the pellet under denaturing conditions. The antiserum raised against rOv-WolAspAT was tested for cross-reactivity with other bacteria or with isolated AspAT of class Ia. It did not react with either commercial *Rickettsia, Coxiella, Chlamydia* or *Brucella* antigens, or with pig cytosolic AspAT as antigen, indicating a high specificity of the antiserum.

Immunolocalization of WolAspAT

The polyclonal rabbit antiserum raised against rOv-WolAspAT was used for the localization of AspAT by immunohistochemistry. The pre-immune serum did not stain endobacteria or any filarial structures in sections of adult *O. volvulus* (Fig. 2A), whereas the immune serum clearly stained endobacteria (Fig. 2B, C). In addition, weak to moderate staining in the epithelia of the worms was occasionally observed (Fig. 2B, C). Endobacteria were stained as granules using dilutions of 1:10–1:200 with decreasing intensity at higher dilutions. The degree of staining of the *Wolbachia* was dependent on the fixation and subsequent preparation of the specimens. Some sections showed that not all endobacteria were well labeled, independently of fixation and subsequent processing. *Wolbachia* that were labeled clearly by the serum against rOv-WolAspAT were observed in the median layer of the hypodermis of the female worms (Fig. 2B, C) and in all embryonic stages from morulae to coiled and stretched microfilariae (Fig. 2B, C). Their density in the hypodermis varied considerably from zero

Fig. 2A–D Immunohistochemical staining of native AspAT in *Wolbachia* endobacteria using a polyclonal rabbit anti serum raised against rOv-WolAspAT. **A** No staining is seen in a section of a female *O. volvulus* when pre-immune serum was used. **B** Strong red labeling of many endobacteria in the median layer of the lateral chords (*arrow*) and the interchordal hypodermis in a serial section to Fig. 3A. Weak staining of the uterus epithelium (*u*) is seen when compared with **A**. **C** Longitudinal section of a female *B. malayi* with labeling of endobacteria in the hypodermis (*arrow*) and in coiled microfilariae (*arrowhead*) in the uterus. **D** No staining is found in the lateral chords (*arrows*) of a female *A. vitae*, a species of filaria which lacks endobacteria. Ethanol (**A**, **B**) or formaldehyde (**C**, **D**) fixation, APAAP staining using pre-immune and immune serum raised against rOv-WolAspAT; magnification, **A**–**C** ×350, **D** ×410)



to many bacteria in different sections, as is known from immunohistochemistry with other antibodies and from electron microscopy.

The antiserum against rOv-WolAspAT also labeled the endobacteria of all other filariae available to us and known to possess *Wolbachia* (Table 1). To examine the specificity of the antiserum against rOv-WolAspAT further adult *O. volvulus* were examined that had been depleted of their endobacteria by doxycycline treatment of patients. In more than 100 worms, no granular endobacteria were detected. Also, sections of the filarial parasites *Acanthocheilonema viteae* (Fig. 2D) and *Onchocerca flexuosa* did not show any granular endobacteria in the hypodermis or embryos. These species do not possess endobacteria (Bandi et al. 1998; Hoerauf et al. 1999; Brattig et al. 2001). No stained granular structures were found in other nematodes that do not harbor endobacteria, such as *Ascaris suum*, *A. lumbricoides* and *Parastrongylus costaricensis*. We conclude that AspAT is localized in the filarial *Wolbachia*, since granular staining is independent of the reaction observed in all filarial species examined, including those without endobacteria and doxycycline-treated *O. volvulus*.

Human antibody response against WolAspAT

The antibody response against WolAspAT was studied by Western blot using rOv-WolAspAT as antigen. At first, 12 serum pools, each containing three sera from individuals infected with *O. volvulus*, were examined. Total IgG antibodies were found in all serum pools. Individual testing of 32 sera from these pools showed that 17 were positive for IgG1 antibodies (Table 2). In total, IgG1 antibodies were detected in 30 (52%) individuals infected with *O. volvulus*, but with different statuses of infection. Microfilaria positive and negative individuals, as well as patients with sowda, showed similar rates of antibody reactivity. In addition, 40 (51%) sera from individuals from areas highly endemic for lymphatic filariasis in Indonesia reacted with the antigen (Table 2). These results indicate a serological cross-reaction of sera from individuals from areas highly endemic for lymphatic filariasis using the rOv-WolAspAT antigen.

As non-endemic controls from tropical Africa, we examined 16 sera from Uganda. The donors lived in an area not endemic for *O. volvulus* and endemic for *M. perstans*, but were found to be amicrofilaremic (Table 2). Of these, three samples were positive for rOv-WolAspAT antibodies, one of which also reacted with the specific Ov16 recombinant antigen and was excluded. The two remaining individuals may have had an amicrofilaremic *M. perstans* infection. In addition, we examined 25 sera of persons from an area in Liberia which is hypoendemic for onchocerciasis. These sera were negative for the *O. volvulus* antigen Ov20/S1. The Liberian area was not endemic for any other human filaria species but animal filariae were abundant. Nine of these samples were positive, but five of them showed only a very faint band in the Western blot. These reactions may have been due to exposure to animal filariae. In all, 11 (28%) of the sera seronegative for recombinant *O. volvulus* antigens from Uganda and Liberia, and without any known human filarial infection, were reactive with the rOv-WolAspAT antigen. No reactivity with the rOv-WolAspAT antigen was found in the sera of 17 individuals from Germany never exposed to human pathogenic filariae and in 16 sera of school children from eastern Europe who were infected with *A. lumbricoides* (Table 2). Furthermore, we found no reactivity of the rOv-WolAspAT with 14 sera from patients with antibodies against *Rickettsia* or *Brucella* antigen extract (Table 2). These findings suggest that the rOv-WolAspAT antigen does not cross-react with antibodies against ubiquitous bacteria or against the related α-proteobacteria of the genera *Rickettsia* and *Brucella*.

IgG isotyping revealed that the 12 serum pools from onchocerciasis patients contained exclusively IgG1 antibodies with no IgG2, IgG3 or IgG4 antibodies being

Table 2 Detection of IgG1 antibodies against WolAspAT in sera from individuals with different degrees of exposition to filarial parasites. Sera from healthy Germans, school children from eastern Europe with *Ascaris lumbricoides* infections or from patients serologically positive for *Rickettsia* or *Brucella* infection functioned as non-endemic control sera. African control sera were either from microfilariae (*mf*) negative individuals, from an hypoendemic onchocerciasis area in Liberia or from an area in Uganda non-endemic for *O. volvulus*. All African control sera were serologically negative for *O. volvulus*. *O. volvulus* sera were collected in hyperendemic areas in Uganda or in Liberia (hyerreactive onchocerciasis, sowda). *Wuchereria bancrofti* and *Brugia malayi* sera were obtained from two highly endemic areas in Indonesia. Antibodies were detected by Western blot using rOv-WolAspAT as antigen

Source of sera		No. examined	rOvWol-AspAT reactive	
			No.	%
Filariasis endemic areas				
Onchocerca volvulus	mf positive	32	17	53
	mf negative	10	6	60
	sowda	16	7	44
Wuchereria bancrofti		41	13	32
B. malayi		38	27	71
Subtotal		137	70	51
African controls				
Uganda		15	2	13
Liberia		25	9	36
Subtotal		40	11	28
European controls				
German		17	0	0
Eastern Europe		16	0	0
Rickettsiosis		8	0	0
Brucellosis, salmonellosis		6	0	0
Subtotal		47	0	0

found (compare Fig. 3). An IgG1 antibody response is characteristic for bacterial antigens, whereas an IgG4 antibody response is typical for filarial antigens (Ottesen et al. 1985). IgG4 and IgE antibodies share epitopes and the antigen recognition of both occurs in parallel (Hussain and Ottesen 1986). It is known that patients with sowda exhibit an especially strong IgG4 and IgE antibody response to onchocercal antigens (Brattig et al. 1994; Mpagi et al. 2000). Therefore, we tested sera from patients with sowda and generalized onchocerciasis for IgE and IgG4 antibodies reactive with rOv-WolAspAT (Table 3). None of the sera showed an IgG4 (48 sera tested) or IgE (32 sera tested) response, while in 23 samples IgG1 antibodies against rOv-WolAspAT were detected. Corresponding results were obtained when the IgG1 positive sera from eight individuals with *W. bancrofti* and eight with *B. malayi* were tested for IgG4 and IgE antibodies. In contrast to these results, specific IgG4 and IgE antibodies were detected in almost all sera examined from onchocerciasis patients when tested with the recombinant *O. volvulus* antigen OvS1/Ov20 (Table 3). These results indicate that the humoral immune response to the *Wolbachia* AspAT antigen is due to IgG1 antibodies, although IgG4 and IgE antibodies may be present in response to filarial antigens.

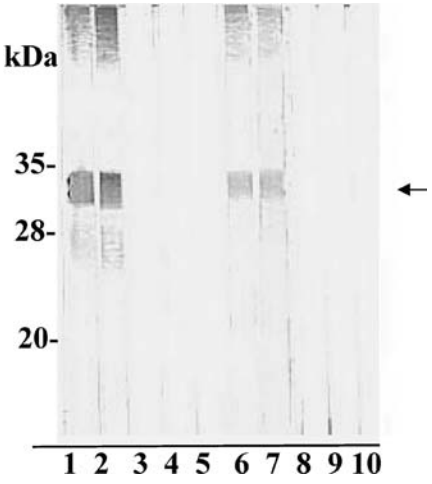


Fig. 3 IgG isotyping by Western blot using rOv-WolAspAT as antigen and patients' sera as primary antibodies. As secondary antibodies anti-human total IgG (*lanes 1*, *6*), IgG1 (*lanes 2*, *7*), IgG2 (*lanes 3*, *8*), IgG3 (*lanes 4*, *9*) or IgG4 (*lanes 5*, *10*) were used. A specific band at about 35 kDa (*arrow*) was observed in *lanes 1* and *2* and in *6* and *7*. Sera were obtained from a patient infected with *O. volvulus* with a microfilaria density of 253 mf/mg skin (*lanes 1–5*) and from a microfilaria negative individual from Indonesia living in an area highly endemic for *B. malayi* (*lanes 6–10*)

Discussion

In the present study, the AspAT of *Wolbachia* endobacteria from *O. volvulus* was identified. The fragment of the *Wolbachia* gene was found in a cDNA library of adult *O. volvulus*. Recently, a gene encoding for a catalase of *Wolbachia* was also identified from an *O. volvulus* cDNA library (Henkle-Dührsen et al. 1998). The WolAspAT genes from the *Wolbachia* of *O. volvulus*, *B. malayi* and *D. melanogaster* showed no introns and were very similar. This finding, together with the high degree of identity to the AspAT of other α-proteobacteria, the classification to the subclass Ib and the immunolocalization in the filarial endobacteria, clearly demonstrate that the AspAT identified from the filarial nematode *O. volvulus* is of bacterial origin.

AspAT catalyse the reversible transfer of an amino group to an α-keto acid. Their structure, function and localization in prokaryotic and eukaryotic organisms differ considerably. In mammalian GABAergic neurons, α-ketoglutarate can be converted by AspAT to the neurotransmitter glutamate (Kugler 1993). *Wolbachia* species and the closely related *R. prowazekii* have a relatively small genome of roughly 1 MB, which is about four times smaller than that of similar, free-living bacteria (Andersson et al. 1998; Sun et al. 2001). In *Rickettsia*, many genes have been lost during the evolution of the intracellular way of life and only six genes involved in amino acid metabolism are left, whereas free-living bacteria have five times as many (Andersson et al. 1998). One of the remaining genes codes for the AspAT. Animal AspATs of the subclass Ia have cytosolic and mitochondrial forms and are functionally active as dimers. It is assumed that the mitochondrial form is more involved in energy metabolism, and its identity to the plasma membrane fatty acid-binding protein has been reported (Isola et al. 1995). The cytoplasmatic AspATs of subclass Ia from *B. malayi, Caenorhabditis elegans*

Table 3 Detection of IgG4 and IgE antibodies against *Wolbachia* AspAT or against an *O. volvulus* protein in individuals with generalized or hyperreactive onchocerciasis. Antibodies were detected by Western blot using a fragment of the recombinant *Wolbachia* AspAT (rOv-WolAspAT) or the recombinant OvS1/Ov20 protein as antigen. *n.d.* Not done

	Wolbachia antigen		*O. volvulus* antigen	
	IgG4 no. examined/positive	IgE no. examined/positive	IgG4 no. examined/positive	IgE no. examined/positive
Generalized, mf positive	32[a]/0	16[a]/0	32/32	n.d.
Hyperreactive	16[b]/0	16[b]/0	16/15	16/16

[a]16 sera were IgG1 positive
[b]7 sera were IgG1 positive

and the pig were compared to the WolAspAT and GenBank. The similarity to the WolAspAT is only about 13%, and identical amino acids are scattered over the entire protein with the exception of one region, where 9 out of 18 amino acids are identical (Fig. 1, Bm-WolAspAT position 173). Different amino acids are even used in the pyridoxal phosphate attachment sites. Only a few conserved residues are found, but these functional groups are known to be conserved even in the two different subclasses of AspAT. So far, no mitochondrial AspAT from filarial parasites has been identified in about 22,441 *B. malayi* EST and 14,608 *O. volvulus* EST (Parkinson et al. 2001). However, we showed that the *Wolbachia* of filarial parasites possess another type of AspAT that may contribute to the AspAT activity in the worm. The function of the different types of AspAT still has to be investigated. In filarial parasites, amino acids can be efficiently used in energy generating pathways in which AspAT is involved (Davies and Köhler 1990) and homogenates of filarial parasites have considerable AspAT activity (Beg et al. 1996).

Our immunohistological results clearly show that WolAspAT is localized in the *Wolbachia*. These were clearly stained as distinct granulae. Although the number of stained endobacteria varied somewhat according to the antibody used, the staining with WolAspAT antibody was similar to that observed when antibodies against the Hsp60 or the catalase of *Wolbachia* were used (Henkle-Dührsen et al. 1998; Hoerauf et al. 1999; Koszarski 1999). There was a strong cross-reaction among *Wolbachia* endobacteria from different species of filaria , indicating that WolAspAT is conserved among filarial *Wolbachia*. However, even filaria species without *Wolbachia* showed some non-granular staining, and, therefore, it has to be postulated that they possess an antigen which shares epitopes with the WolAspAT. In some worm sections we also found some staining of spermatozoa and the nerve ring away from the vicinity of *Wolbachia* (data not shown). This would also be in agreement with the localization of AspAT in vertebrates (Papadimitriou and van Dujin 1970; Kugler 1993).

Our study provides strong evidence that an intracellular *Wolbachia* protein acts as an antigen in human filariasis. Using the *B. malayi* WSP as antigen, two out of 12 rhesus monkeys infected with *B. malayi* developed a specific IgG1 response to this surface antigen, while two uninfected monkeys did not react (Punkosdy et al. 2001a). In another study, total IgG antibodies against *D. immitis* WSP were detected in three cats experimentally infected with *D. immitis* and in 20 cats held as pets and seropositive for excretory/secretory heartworm antigens (Bazzocchi et al. 2000a). In cats seronegative for the filarial antigens, no antibodies against WSP were detected, but no information about the specificity of the WSP was provided. The same study showed that the cell-cycle protein FTSZ of *Wolbachia* is not specific for these endobacteria and antibodies could be detected in both cats assumed to be infected with filarial parasites and those assumed to be uninfected. This observation indicates that *Wolbachia* proteins cross-react with antigens from bacteria other than *Wolbachia*. In contrast, our data show that the antibodies reactive to the WolAspAT were found only in individuals living in tropical or subtropical countries where filarial parasites, including animal filariae, are abundant.

Recently, antibodies against the WSP were detected in residents of an area endemic for *W. bancrofti* on Haiti using a recombinant *B. malayi* WSP as antigen (Punkosdy et al. 2001b). In this study, WSP antibodies were also detected in 17% of asymptomatic and amicrofilaremic persons. This observation is in agreement with our results on the WolAspAT and also suggests that individuals with prepatent or occult filarial infection have antibodies against *Wolbachia*. So far, an antibody response to *Wolbachia* antigens has only been demonstrated for the structural protein WSP. From our results, we can conclude that there is also an antibody reaction to internal *Wolbachia* proteins which are involved in their metabolism.

The characterization of the human antibody reactivity to rOv-WolAspAT as an IgG1 response in the absence of IgG4 and IgE antibodies indicates that the antibodies detected in humans are not directed against a filarial protein, but against WolAspAT. The finding that no reaction was found with sera from Europe or from patients with *Rickettsia* or *Brucella* infections together with the observation that the antibody raised in rabbits did not cross-react with *Rickettsia* antigens makes it unlikely that the antibody reaction detected in residents of filariasis endemic areas is due to cross-reaction with antibodies directed against other gram-negative bacteria. However, our results and those of Punkosdy and co-workers (2001b) provide evidence that immunological cross-reactivity between *Wolbachia* from different species of filaria does occur. Since *Wolbachia* are also frequently found in blood sucking arthropods (Jeyaprakash and Hoy 2000), it remains to be seen whether *Wolbachia*-derived proteins from arthropods act as antigens and cross-react with antibodies against filarial *Wolbachia*. Studies on the sandflea, *Tunga penetrans,* from Ghana showed that they harbor *Wolbachia*, and a fragment of the Tp-WolAspAT gene was isolated that had a high degree of similarity to the WolAspAT reported in this study (P. Fischer and co-workers unpublished data). Our results indicate that the WolAspAT is relatively specific for *Wolbachia* because it showed no cross-reaction with sera from patients with other α-proteobacterial infections, but the high degree of similarity of *Wolbachia* proteins from arthropods and filarial parasites may impede the identification of specific immune reactions to *Wolbachia* in filariasis patients.

The definition of the role of immune responses to bacterial antigens in filariasis is not easy. *Wolbachia* endobacteria may play a certain role as the source for the bacterial antigens. However, it is known that, especially in lymphatic filariasis, bacterial superinfection is

an important cause of the progression of pathology (Dreyer and Piessens 2000). In onchocerciasis, the severity of acute onchodermatitis may also be influenced by external bacterial infections. In elephantiasis patients, there is a tendency for IgG1 responses to total worm extracts to be high compared to microfilaremic individuals without disease, who usually have a stronger IgG4 response (Kurniawan-Atmadja et al. 1998). It cannot be ruled out that bacteria invading the damaged lymph system of elephantiasis patients induce IgG1 antibodies which may cross-react with the *Wolbachia* antigens present in the extracts of whole filarial worms. Therefore, in order to elucidate the role of *Wolbachia* endobacteria in the pathogenesis of filariasis, it is important to characterize their specific antigens.

The *Wolbachia* of filarial parasites are closely related and similarity of their antigens can be expected (Bandi et al. 1998; Casiraghi et al. 2001a). The broad range of clinical symptoms observed in the different human filarial infections has to be explained to a large part by features of the worm linked with environmental factors and not by the endobacteria. For example, *Mansonella ozzardi* is generally accepted as a filarial species with low pathogenicity, but it has *Wolbachia* endobacteria closely related to those of other filarial parasites (Casiraghi et al. 2001b). If molecules like the *Wolbachia*-derived lipopolysaccharide play a major role in inflammatory pathogenesis, as proposed for lymphatic filariasis (Taylor et al. 2000), major differences in these molecules between different filarial *Wolbachia* species have to be postulated. Therefore, the characterization of antigens of *Wolbachia* is crucial. The results of our work show that an anti-serum against the Ov-WolAspAT can be used to localize the enzyme in the *Wolbachia* of probably all filarial species. This may be due to a high degree of conservation of the protein. On the other hand, immunohistological observations show that, to date, no antibody against a recombinant *Wolbachia* antigen is available that selectively detects the *Wolbachia* of only one species of filaria (D.W. Büttner and co-workers unpublished data).

It has been reported that parts of the cellular immune response to filarial parasites are not directed against the filarial parasites, but against their *Wolbachia*. For example, the reaction of neutrophil granulocytes was shown to be influenced by *Wolbachia* or molecules derived from them (Brattig et al. 2001; Saint Andre et al. 2002). In this report we provide some evidence that the same applies for the humoral immune response. The observation that no IgG4 and IgE antibodies against WolAspAT could be detected in individuals infected with filarial parasites, which are characteristic for filarial infections (Ottesen et al. 1985; Hussain and Ottesen 1986), indicates differences in immune responses to *Wolbachia* and filarial antigens.

Taken together, a non-surface protein of *Wolbachia* endobacteria of filarial parasites has been identified which was recognized by the humoral immune system of individuals living in areas endemic for filariasis.

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