ATP/ADP Translocases: a Common Feature of Obligate Intracellular Amoebal Symbionts Related to Chlamydiae and Rickettsiae

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Received 14 September 2003/Accepted 8 October 2003

ATP/ADP translocases catalyze the highly specific transport of ATP across a membrane in an exchange mode with ADP. Such unique transport proteins are employed by plant plastids and have among the prokaryotes so far only been identified in few obligate intracellular bacteria belonging to the *Chlamydiales* and the *Rickettsiales*. In this study, 12 phylogenetically diverse bacterial endosymbionts of free-living amoebae and paramecia were screened for the presence of genes encoding ATP/ADP transport proteins. The occurrence of ATP/ADP translocase genes was found to be restricted to endosymbionts related to rickettsiae and chlamydiae. We showed that the ATP/ADP transport protein of the *Parachlamydia*-related endosymbiont of *Acanthamoeba* sp. strain UWE25, a recently identified relative of the important human pathogens *Chlamydia trachomatis* and *Chlamydia pneumoniae*, is functional when expressed in the heterologous host *Escherichia coli* and demonstrated the presence of transcripts during the chlamydial developmental cycle. These findings indicate that the interaction between *Parachlamydia*-related endosymbionts and their amoeba hosts concerns energy parasitism similar to the interaction between pathogenic chlamydiae and their human host cells. Phylogenetic analysis of all known ATP/ADP translocases indicated that the genes encoding ATP/ADP translocases originated from a chlamydial ancestor and were, after an ancient gene duplication, transferred horizontally to rickettsiae and plants.

Obligate intracellular bacteria live within a highly specialized niche, the eukaryotic cell. This lifestyle gave rise to unique adaptations, for example, the reduction of bacterial metabolism (37) and the exploitation of host metabolites (36). Chlamydiae and rickettsiae (comprising major bacterial pathogens of humans and animals) have evolved a remarkable adaptation which enables them to utilize ATP generated by their eukaryotic hosts by making use of specific carrier proteins (49). These transmembrane proteins catalyze the import of host-derived ATP into the prokaryotic cell across the bacterial cell membrane, which is otherwise impermeable for the relatively large and charged nucleotides. In an exchange mode, these transport proteins export bacterial ADP back into the host cytosol.

In total, four bacterial ATP/ADP transport proteins have been functionally characterized to date, including those of *Chlamydia trachomatis* (43), *Rickettsia prowazekii* (11, 48), and the *Rickettsia*-related paramecium parasites *Holospora obtusa* and *Caedibacter caryophilus* (28). Bacterial ATP/ADP transport proteins are highly specific for their substrates (ATP and ADP). They belong to the family of solute transporters exhibiting 12 predicted transmembrane helices and display several highly conserved motifs while sharing only moderate amino acid sequence similarity (34, 49). Interestingly, transport proteins exhibiting structural and functional features similar to bacterial ATP/ADP transport proteins have been identified

and characterized from chloroplasts and heterotrophic plastids of various plants and algae, which provide the organelles with ATP necessary for anabolic reactions like starch production and degradation or fatty acid biosynthesis (28, 31, 32). In terms of sequence homology and structural similarity, bacterial and plastidic ATP/ADP transport proteins differ fundamentally from mitochondrial ADP/ATP carrier transport proteins, which catalyze the reverse transport direction, exporting ATP from mitochondria into the eukaryotic cytosol (49).

The aim of this study was to investigate whether the presence of genes coding for ATP/ADP transport proteins, (i.e., the ability to thrive as energy parasites within their eukaryotic hosts) is a common feature among endosymbionts of free-living amoebae and paramecia. Therefore, a number of recently identified, phylogenetically diverse, obligate intracellular symbionts of *Acanthamoeba* spp. and *Paramecium tetraurelia* were investigated, including (i) the betaproteobacterial “*Candidatus* Procabacter acanthamoebae” (21), (ii) “*Candidatus* Amoebophilus asiaticus” belonging to the *Bacteroidetes* (22), (iii) a *Francisella*-related endosymbiont (4), (iv) three *Rickettsia*-related bacteria (15, 20), and (v) four chlamydia-related bacteria (16, 24) (Fig. 1).

Special focus was given to the latter group of obligate intracellular symbionts due to their affiliation to the chlamydiae, which cause a wide variety of diseases and are among the most common human pathogens (30, 35). The recent finding of chlamydia-related bacteria thriving as obligate intracellular symbionts of ubiquitous, free-living amoebae significantly changed our view of chlamydial diversity and their occurrence in the environment (2, 16, 23, 24). The so-called environmental

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FIG. 1. 16S rRNA-based neighbor-joining (with the Jukes-Cantor correction) phylogenetic tree showing the relationship of bacterial endosymbionts of amoebae or paramecia investigated in this study. Superscript numbers indicate protozoan hosts: 1, *H. vermiformis*; 2, *Acanthamoeba* sp. strain TUME1; 3, *Acanthamoeba* sp.; 4, *Acanthamoeba* sp. strain UWE25; 5, *Acanthamoeba* sp. strain UWC12; 6, *Acanthamoeba* sp. strain UWC6; 7, *Acanthamoeba* sp. strain UWE2; 8, *P. tetraurelia*; 9, *Acanthamoeba* sp. strain UWC36; 10, *Acanthamoeba polyphaga* HN-3; 11, *Acanthamoeba* sp. strain UWE39; *Acanthamoeba* sp. strain TUMSJ-321. Arrow, to outgroup; bar, 10% estimated evolutionary distance.

chlamydiae, classified within the new genera *Neochlamydia* (24) and *Parachlamydia* (12), also possess the unique biphasic chlamydial developmental cycle and might be considered new emerging pathogens, since several studies indicated a possible role for these novel chlamydiae in respiratory infection of humans (5, 7, 8).

Here we show that the presence of genes encoding ATP/ADP transport proteins is a common feature of all investigated bacterial endosymbionts belonging to the rickettsial or chlamydial evolutionary lineage, and the respective gene could not be detected in any other endosymbiont analyzed in this study. Comprehensive phylogenetic analysis of deduced ATP/ADP transport protein sequences demonstrated that these genes originated from a chlamydial ancestor and were subject of multiple horizontal gene transfer events. The functional characterization of the corresponding proteins is crucial since sequence homology per se does not allow one to get unambiguous insight into the physiological functions. This is clearly demonstrated by the analysis of two isoforms of nucleotide transport proteins identified in the genome sequence of *C. trachomatis*, which function as a ATP/ADP antiporter and a proton-driven nucleotide importer, respectively (43). Therefore, the putative ATP/ADP transport protein of the

Parachlamydia-related endosymbiont of *Acanthamoeba* sp. strain UWE25 identified in this study was selected for a more detailed biochemical and transcriptional analysis. We showed that the gene encoding this transport protein is transcribed during the developmental cycle of the environmental chlamydia strain UWE25 and that it functions as highly specific ATP/ADP translocase when expressed in the heterologous host *Escherichia coli*.

MATERIALS AND METHODS

Maintenance of protozoa and their bacterial endosymbionts. Bacterial endosymbionts and their eukaryotic host strains used in this study are shown in Fig. 1. *Acanthamoeba* spp. and *Hartmannella vermiformis* harboring obligate intracellular symbionts were maintained in Trypticase soy-yeast extract broth (containing 30 g of Trypticase soy broth [Oxoid, Basingstoke, England]/liter; 10 g of yeast extract/liter) (46) and fluid SCGYE medium (containing 10 g of casein/liter, 2.5 g of glucose/liter, 5 g of yeast extract/liter, 1/10 fetal bovine serum, 1.325 g of Na_2HPO_4 /liter, 0.8 g of KH_2PO_4 /liter) (10), respectively. Cultures were incubated at 20 and 30°C, respectively, and fresh medium was applied every 5 to 10 days. Paramecia were maintained in lettuce medium or in a decoction of cereal leaves supplemented with living *Enterobacter aerogenes* cells at 23°C as described elsewhere (38).

Identification, cloning, and sequencing of genes coding for ATP/ADP transport proteins. Simultaneous isolation of DNA from protozoan hosts and their bacterial endosymbionts was performed with either the FastDNA kit (Bio 101,

Carlsbad, Calif.) or the DNeasy tissue kit (Qiagen, Hilden, Germany) according to the protocols recommended by the manufacturers. A set of four different degenerate primers targeting an internal fragment of known ATP/ADP translocase genes (expected length between 750 and 850 bp) was used to screen for the presence of homologous genes in whole-DNA preparations of the investigated endosymbionts (28). Primer sequences were as follows: PFL63 (forward primer), 5'-TTYTAYRYXHTXDSXGARYTNTGGGG-3' (X, inosine); PFL64 (forward primer), 5'-TTYTGGGGNTTYGCNAAYSARATHAC-3'; PFL66 (reverse primer), 5'-RTCNARNGGDATRTANGCCAT-3'; PFL67 (reverse primer), 5'-GCXCCNCCNSWYTTNCC-3' (X, inosine). PCRs were performed with a temperature gradient thermocycler with a standard PCR cycling program, varying the annealing temperature from 45 to 65°C. A typical PCR mixture contained 100 mmol of MgCl₂, 10 mmol of deoxynucleoside triphosphates, 1.5 U of *Taq* DNA polymerase (Promega, Mannheim, Germany), 50 pmol (each) of forward and reverse primer, and 100 ng of template DNA in a total volume of 50 µl. Negative controls (no DNA added) were included in all PCRs.

Based on the obtained nucleotide sequence of the internal fragment of the ATP/ADP translocase gene of the *Parachlamydia*-related endosymbiont UWE25, two arbitrary PCR approaches were applied to determine the complete gene sequence. The 5' rapid amplification of cDNA ends system (Invitrogen Life Technologies, Karlsruhe, Germany) was used under conditions recommended by the manufacturer to obtain the 5'-terminal part of the gene with a gene-specific primer and the primer provided by the manufacturer. In addition, an asymmetric arbitrary PCR with only a single primer (5'-CTCCACTATTCTTAGCTGTCA TCITTTGGTGCGGCTC-3') targeting the known internal fragment was used to obtain the 3'-terminal part of the ATP/ADP translocase gene. Briefly, the reaction mixture described above was used, and amplification conditions were as follows: (i) an initial denaturation step at 94°C for 2 min, (ii) 30 stringent cycles of denaturation at 94°C for 15 s, annealing at 62°C for 30 s, and elongation at 72°C for 30 s, (iii) one nonstringent cycle of denaturation at 94°C for 30 s, annealing at 52°C for 60 s, and elongation at 72°C for 90 s, (iv) 30 stringent cycles of denaturation at 94°C for 15 s, annealing at 62°C for 30 s, elongation at 72°C for 30 s, and (v) a final elongation step at 72°C for 7 min. Negative controls (no DNA added) were included in all PCRs. The presence and size of amplification products were checked with agarose gel electrophoresis and ethidium bromide or SYBR Green staining (Biozym, Hess. Oldendorf, Germany).

If amplification products yielded only a single band after agarose gel electrophoresis, they were used directly for cloning. Otherwise, a band of the expected size was cut out of the agarose gel and the gel plug was digested with GELase (Epicentre, Madison, Wis.) prior to cloning. The TOPO TA cloning kit containing the cloning vector pCR2.1 (Invitrogen Life Technologies) was used for all cloning reactions. Nucleotide sequences of cloned DNA fragments were determined by cycle sequencing of purified plasmid DNA with the Thermo Sequenase cycle sequencing kit (Amersham Life Science, Little Chalfont, United Kingdom), dye-labeled vector-specific primers, and an automated DNA sequencer (LI-COR 4200; LI-COR, Inc., Lincoln, Neb.) under conditions recommended by the manufacturers.

Phylogenetic analysis. A database containing all ATP/ADP translocase gene sequences available from public databases (EMBL, GenBank, and DDBJ) was established by using the ARB software package (29) (<http://www.arb-home.de>), and partial and full-length ATP/ADP transporter gene sequences obtained in this study were added to this database. Deduced amino acid sequences were aligned automatically with ClustalW (41) implemented in the ARB software, and the resulting alignment was refined manually. Phylogenetic amino acid sequence trees were constructed by applying the PHYLIP distance matrix (Fitch) and maximum-parsimony methods (14) and a maximum-likelihood approach using PROTml 2.3 (and the JTT- or Dayhoff-F-amino-acid replacement model) implemented in ARB. Bootstrap analysis was performed by using the PHYLIP parsimony tool protpars (resampling 100 times). A filter considering only those alignment positions that were conserved in at least 10% of all sequences was used for all treeing calculations. Initially, trees were calculated with full-length sequences only, and partial sequences were added subsequently to the respective trees without changing their topology by use of the ARB parsimony interactive method.

Transcriptional analysis. All reagents used for RNA manipulations were treated with 0.1% (vol) diethylpyrocarbonate before use. Amoebae harboring the *Parachlamydia*-related endosymbiont UWE25 were harvested by centrifugation (2,350 × g, 5 min, 4°C), resuspended in TRIzol (Invitrogen Life Technologies), and immediately homogenized with the BeadBeater Fast-Prep FP120 instrument (Bio 101). Whole-RNA purification was performed according to the recommendations of the manufacturer, followed by a DNase treatment with DNase I (Invitrogen Life Technologies). Reverse transcriptase PCR (RT-PCR) was performed with the Titan One-Tube RT-PCR system (Roche, Mannheim,

Germany) according to the manufacturer's instructions with primers targeting an 844-bp fragment of the identified ATP/ADP transporter gene of the *Parachlamydia*-related endosymbiont UWE25 (forward primer, 5'-TTTGGGGATTGCT AACC-3'; reverse primer, 5'-AGATTTTCCTAAACGAGC-3'). Total RNA concentrations of 100 ng were used in the reaction mixture together with 50 pmol of each primer. The annealing and reverse transcription temperature was 52°C. Each RT-PCR was accompanied by a negative control (no RNA added) and a control PCR with *Taq* DNA polymerase instead of RT to demonstrate the absence of DNA. To ensure that the obtained RT-PCR amplification products were of endosymbiotic origin, RT-PCR was performed with whole RNA from infected and noninfected amoebae.

Heterologous expression of genes coding for putative ATP/ADP transport proteins in *E. coli* and characterization of adenine nucleotide transport. For heterologous expression, the gene coding for the putative ATP/ADP transport protein was amplified from whole-DNA preparations of amoebae containing the *Parachlamydia*-related endosymbiont UWE25 by using the Extensor Hi-Fidelity PCR enzyme mix (ABgene, Epsom, United Kingdom) and the following primers: forward primer, 5'-CAGGGATCCATCGCAAGATGCGAAACAAGAC-3' (introducing a *Bam*HI restriction site instead of the start codon); reverse primer, 5'-CGGGGATCCTTAGCTAGTAGCTATTTCGATGT-3' (containing a *Bam*HI restriction site after the stop codon). The resulting amplification products were digested with the restriction endonuclease *Bam*HI, purified, and inserted in frame into the IPTG-inducible expression vector pET16b containing a promoter site for the T7 RNA polymerase (Novagen, Heidelberg, Germany). The newly constructed plasmid containing the putative ATP/ADP translocase gene was transformed into and maintained in *E. coli* TOP10 cells (Invitrogen). The size of the cloned gene was checked by sequencing.

For uptake experiments, isopropyl-β-D-thiogalactopyranoside (IPTG)-induced intact *E. coli* BL21(DE3) cells (Stratagene, Amsterdam, The Netherlands) transformed with the newly constructed plasmid were used. The time dependency of [α -³²P]ATP and [α -³²P]ADP uptake by *E. coli* BL21(DE3) expressing the putative ATP/ADP transporter of the *Parachlamydia*-related endosymbiont UWE25 was investigated in the presence of 50 µM radioactively labeled [α -³²P]ATP or [α -³²P]ADP, respectively. Controls were performed with BL21(DE3) cells transformed with pET16b vector without insert. *K_m* values were determined by applying the Eadie-Hofstee equation and the Hanes equation. Effector studies were conducted with 500 µM radioactively labeled substrate ([α -³²P]ATP) in the presence of 2.5 mM concentrations of each effector.

Nucleotide sequence accession numbers. Nucleotide sequences of ATP/ADP translocase genes determined in this study were deposited with EMBL, GenBank, and DDBJ under accession numbers AJ582021 (*ntt1*, UWE25 of the *Parachlamydia*-related endosymbiont UWE25), AJ582020 (partial *ntt* gene of the endosymbiont of *Acanthamoeba* sp. strain TUME1), AJ582022 (partial *ntt* gene of *Neochlamydia hartmannellae*), AJ582023 (partial *ntt* gene of *Parachlamydia* sp. strain PL9), AJ582017 (partial *ntt* gene of the endosymbiont of *Acanthamoeba* sp. strain UWC36), AJ582018 (partial *ntt* gene of "*Candidatus* Paracaeidibacter symbiosus"), and AJ582019 (partial *ntt* gene of "*Candidatus* Caedibacter acanthamoebae").

RESULTS

Identification of genes encoding putative nucleotide transport proteins in bacterial endosymbionts of protozoa. A broad-range degenerate PCR targeting an internal fragment of known ATP/ADP translocase genes was employed to screen 12 phylogenetically diverse bacterial endosymbionts of amoebae and paramecia for the presence of genes coding for nucleotide transport proteins. The investigated endosymbionts included bacteria belonging to the *Bacteroidetes* ("*Candidatus* Amoebohilus asiaticus") (22), the *Betaproteobacteria* ("*Candidatus* Procabacter spp.") (21), the *Gammaproteobacteria* (4), the *Alphaproteobacteria* (two evolutionary lineages most closely related to *Rickettsia* and to *Caedibacter/Holospora*, respectively) (15, 20), and the *Chlamydiales* (16, 24) (Fig. 1). While all DNA preparations were positive in a control PCR with a 16S rRNA gene-targeted primer set (demonstrating the presence of sufficient bacterial DNA and the absence of PCR inhibitors), only the DNA of endosymbionts affiliated with the *Rickettsiales* or

the *Chlamydiales* produced an amplicon of the expected size in the ATP/ADP transporter gene assay (approximately 800 bp) (data not shown). Amplification products were subsequently cloned and sequenced. Homology searches in the public databases EMBL, GenBank, and DDBJ revealed significant similarities of the obtained gene fragments to published nucleotide transport proteins.

Determination of the complete gene sequence coding for a putative ATP/ADP transport protein of the *Parachlamydia*-related endosymbiont UWE25. The only recently identified chlamydia-related endosymbionts of free-living amoebae, also referred to as environmental chlamydiae, are of special interest due to their possible role in respiratory disease of humans (5, 7, 8, 33). Therefore, the *Parachlamydia*-related endosymbiont UWE25 was selected for a more-detailed analysis. Based on the sequenced internal gene fragment of its putative ATP/ADP transport protein, the complete open reading frame was determined by using arbitrary PCR approaches to amplify its 5'- and 3'-terminal parts. Reamplification and sequencing of the complete gene with a primer targeting the 5' and 3' ends of the assembled full-length sequence demonstrated that the recovered sequence was not chimeric. The amplified gene, hereafter referred to as *ntt1* of UWE25 (according to the nomenclature suggested by Linka et al. (28), had a length of 1,542 bp, resulting in a 513-amino-acid protein with a predicted mass of approximately 57 kDa.

Comparative sequence analysis and phylogeny of nucleotide transport proteins. Partial and full-length nucleotide transport protein sequences obtained from the chlamydia- and rickettsia-related endosymbionts of amoebae were added to a data set containing all publicly available homologues. Deduced amino acid sequences were aligned and subjected to a detailed comparative sequence analysis. The complete data set contained 57 bacterial nucleotide transport proteins, 6 plastidic ATP/ADP transport proteins, and 4 open reading frames detected in the genome of the microsporidium *Encephalitozoon cuniculi* (27), which had only weak amino acid sequence identities with all other nucleotide transport proteins (17 to 25%). The newly identified transport protein of the *Rickettsia*-related *Acanthamoeba* endosymbiont UWC36 showed 42% amino acid sequence identity to the ATP/ADP transport protein of *R. prowazekii* (RpNTT1) and 44% amino acid sequence identity to the ATP/ADP transport protein of the *Paramecium caudatum* endosymbiont *C. caryophilus* (CcNTT1). The nucleotide transport protein of the *Acanthamoeba* endosymbiont "*Candidatus* Caedibacter acanthamoebae" showed 46% amino acid sequence identity to the ATP/ADP transport protein of *H. obtusa* (HoNTT1), and the nucleotide transport protein of the *Acanthamoeba* endosymbiont "*Candidatus* Paracaedibacter symbiosus" showed only 36% amino acid sequence identity with HoNTT1. As expected, the ATP/ADP transport protein sequences of the *Parachlamydia*-related *Acanthamoeba* endosymbionts (UWE25, PL9, TUME1, and *N. hartmannellae*) showed the highest amino acid sequence identity to chlamydial ATP/ADP transport protein sequences (58 to 66%).

The application of distance matrix, maximum-parsimony, and maximum-likelihood treeing methods with a filter including 485 amino acid alignment columns that were conserved in at least 10% of all analyzed sequences demonstrated that the nucleotide transport proteins of *Rickettsia*-related endosymbi-

onts of amoebae form a monophyletic group together with rickettsial ATP/ADP transport proteins (Fig. 2). The nucleotide transport proteins of *Parachlamydia*-related endosymbionts of amoebae clustered together with ATP/ADP transport proteins of the *Chlamydiaceae* (Fig. 2). All plastidic ATP/ADP translocases formed a monophyletic group and were most closely related to chlamydial ATP/ADP transport proteins.

The aligned data set containing all recognized bacterial and plastidic nucleotide transport protein sequences is available as an ARB database (including phylogenetic trees) or as a FastA flat file at our website <http://www.microbial-ecology.net/ntt>.

Characterization of an ATP/ADP transport protein (NTT1 of UWE25) of the *Parachlamydia*-related endosymbiont UWE25. A common feature of membrane-bound solute transport proteins is the presence of 12 predicted membrane-spanning hydrophobic α -helical domains (34, 45, 49). The determined putative ATP/ADP transport protein of the *Parachlamydia*-related endosymbiont UWE25 was therefore analyzed by using different transmembrane topology prediction methods (DAS [9], MEMSAT2 [26], TMHMM 2.0 [39], TOPPED 2 [47], HMMTOP [44], SOSUI [19], and ConPred [25]) and compared with the functionally characterized ATP/ADP transport proteins of *R. prowazekii* (RpNTT1) and *C. trachomatis* (CtNTT1) (Fig. 3). While the applied prediction algorithms were able to detect the 12 transmembrane domains of RpNTT1 (1), all but one (HMMTOP) failed to resolve the two C-terminal transmembrane domains of CtNTT1 and NTT1 of UWE25. Taking into account that the accuracy of available transmembrane topology prediction methods is generally only around 73% (25), the striking congruency of the hydrophobicity plots of all three ATP/ADP transport protein sequences analyzed demonstrated that the highly conserved secondary structure of solute transport proteins is also found in the ATP/ADP transport protein of the *Parachlamydia*-related endosymbiont UWE25.

To analyze expression of the identified putative ATP/ADP transport protein of the *Parachlamydia*-related endosymbiont UWE25, RT-PCR was performed with total RNA purified from amoebae harboring bacterial endosymbionts by using a primer targeting an 844-bp fragment of *ntt1* of UWE25. The presence of an amplicon of the expected size in triplicate experiments clearly demonstrated that *ntt1* of UWE25 is transcribed during intracellular multiplication of the *Parachlamydia*-related endosymbiont UWE25 within its *Acanthamoeba* host (Fig. 4). The absence of an RT-PCR product in the simultaneous analysis of the same amoeba isolate without chlamydial endosymbionts provided additional evidence for the bacterial origin of *ntt1* of UWE25 (Fig. 4).

Previous studies have shown that *C. trachomatis* possesses two isoforms of nucleotide transport proteins (43). Despite their high sequence similarity with previously characterized ATP/ADP transport proteins, the two transport proteins of *C. trachomatis* exhibit fundamental differences in substrate specificity and mode of transport. While CtNTT1 is a highly specific ATP/ADP transport protein, CtNTT2 is a nucleoside triphosphate transport protein functioning as an H^+ symporter responsible for the net uptake of various ribonucleoside triphosphates (43). Consequently, a detailed biochemical characterization of nucleotide transport proteins newly identified on the basis of sequence homology is essential to allow unambig-

FIG. 2. Phylogenetic relationships of bacterial and plastidic ATP/ADP transport proteins. A distance matrix tree (Fitch) containing partial and full-length amino acid sequences of ATP/ADP transport proteins is shown. Black circles indicate parsimony bootstrap values greater than 95%; grey circles indicate bootstrap values between 90 and 95%. Empty circles indicate bootstrap values between 85 and 90%; bootstrap values below 85% are not shown. Triangles represent groups of six respective rickettsial sequences. The square indicates the location of the root (determined by using homologous protein sequences identified in the genome sequence of *E. cuniculi*) (27). Bar, 10% estimated evolutionary distance.

FIG. 3. Hydropathy analysis of the predicted amino acid sequences of the ATP/ADP translocases of the *Parachlamydia*-related endosymbiont UWE25 (NTT1 of UWE25), *C. trachomatis* (CtNTT1), and *R. prowazekii* (RpNTT1). Hydropathy analysis was performed with TOPPED2 (47). The locations of the 12 transmembrane domains of RpNTT1 are indicated (1).

vious conclusions on their function in situ. Therefore, the putative ATP/ADP transport protein of the *Parachlamydia*-related endosymbiont UWE25 was subcloned into expression vector pET16b and expressed in the heterologous host *E. coli*, allowing the performance of uptake experiments with radioactively labeled nucleotides.

In *E. coli*, the expression of *ntt1* of UWE25 allowed uptake of ATP and ADP (Fig. 5) while negative controls (expression

FIG. 4. Transcription of the ATP/ADP translocase gene of the *Parachlamydia*-related endosymbiont UWE25 (*ntt1* of UWE25) during multiplication in its *Acanthamoeba* host demonstrated by RT-PCR. Lane 1, RT-PCR with RNA from amoebae without the *Parachlamydia*-related endosymbiont UWE25; lane 2, RT-PCR negative control (no RNA added); lane 3, RT-PCR with RNA from amoebae harboring the *Parachlamydia*-related endosymbiont UWE25; lane 4, RT-PCR negative control (no RNA added); lane 5, PCR with RNA from amoebae containing UWE25 (control for the absence of DNA in the RNA preparation); lane 6, PCR positive control with DNA from purified UWE25 elementary bodies; lane 7, PCR negative control (no RNA added); lanes m, molecular size markers.

FIG. 5. Time dependency and substrate saturation of α -³²P-labeled nucleotide uptake into *E. coli* BL21(DE3) cells. IPTG-induced *E. coli* BL21(DE3) cells harboring pET16b with or without insert (control) were incubated with the indicated concentrations of α -³²P-labeled nucleotides. Data are means of the results from three independent experiments. (A) Time dependency of [α -³²P]ADP and [α -³²P]ATP uptake; (B) substrate saturation of [α -³²P]ATP, [α -³²P]ADP, and [α -³²P]GTP uptake.

vector without insert) showed no significant translocation of ATP or ADP (Fig. 5). The kinetics of ATP and ADP uptake were similar to those of the previously characterized ATP/ADP transport protein of *C. trachomatis* (CtNTT1), as were the substrate affinities for ATP (K_m = 95 μ M, maximum reaction velocity [V_{max}] = 384 nmol mg of protein⁻¹ h⁻¹) and ADP (K_m = 55 μ M, V_{max} = 384 nmol mg of protein⁻¹ h⁻¹) (Table 1). Substrate specificity was further analyzed by uptake experiments with labeled GTP, which demonstrated that NTT1 of UWE25 translocates only negligible amounts of GTP (Fig. 5) (K_m = 128 μ M, V_{max} = 12 nmol mg of protein⁻¹ h⁻¹), resembling the reported characteristics of CtNTT1. Moreover, the substrate specificity was examined by analyzing the effect of structurally related putative inhibitors on ATP influx. With the exception of ADP, none of the tested compounds (GTP, UTP, and CTP) substantially inhibited ATP uptake by NTT1 of UWE25 (Table 2).

DISCUSSION

Diversity and phylogeny of ATP/ADP transport proteins. Despite being phylogenetically not closely related, the parasitic lifestyle of the *Rickettsiales* and *Chlamydiales* is characterized by the use of ATP/ADP translocases to gain host ATP. The evolutionary history of genes encoding these transport proteins, which also occur in plastids of algae and higher plants, has recently received considerable attention (3, 17, 28, 50) because it should contribute to the revelation of how the ancestors of the respective bacteria managed to survive and multiply in eukaryotic hosts. Such knowledge is important to understand basic features of important human pathogens as well as a milestone during plant evolution. However, published scenarios for the evolution of ATP/ADP transporters differ dramatically (3, 17, 28, 50), which might also reflect the fact that several evolutionary lineages within the *Rickettsiales* and

TABLE 1. Comparison of K_m and V_{max} values of the ATP/ADP translocase of the *Parachlamydia*-related endosymbiont UWE25 and other functionally characterized ATP/ADP transport proteins

Protein ^a	Organism	Result for:						Reference
		ATP		ADP		GTP		
		<i>K_m</i> (μM)	<i>V_{max}</i> (nmol mg of protein ⁻¹ h ⁻¹)	<i>K_m</i> (μM)	<i>V_{max}</i> (nmol mg of protein ⁻¹ h ⁻¹)	<i>K_m</i> (μM)	<i>V_{max}</i> (nmol mg of protein ⁻¹ h ⁻¹)	
NTT1	Endosymbiont UWE25	95	384	55	384	128	12	This study
NTT1	<i>C. trachomatis</i>	48	370	39	625			43
NTT2	<i>C. trachomatis</i>	1,158	128	1,758	18	30	108	43
NTT1	<i>Solanum tuberosum</i>	25	100	20	200			42
NTT1	<i>Arabidopsis thaliana</i>	17	29	12	80			32
NTT2	<i>Arabidopsis thaliana</i>	22	7	20	24			42
NTT1	<i>H. obtusa</i>	320		344				28
NTT1	<i>C. caryophilus</i>	182		252				28
NTT1	<i>Galdieria sulphuraria</i>	65		18				28
NTT1	<i>R. prowazekii</i>	114	6.0					28
		104						11

^a Nomenclature of ATP/ADP transport protein is as suggested by Linka et al. (28).

TABLE 2. Effect of various nucleotides on ATP transport activity of the ATP/ADP translocase of the *Parachlamydia*-related endosymbiont UWE25 (NTT1 of UWE25) expressed in the heterologous host *E. coli*

Effector	Rate of ATP uptake ^a (nmol mg of protein ⁻¹ h ⁻¹) (% of control)
None	371 (100)
ATP	103 (27.76)
ADP	31.8 (8.57)
GTP	273.3 (73.67)
UTP	278.8 (75.15)
CTP	280.4 (75.58)

^a ATP uptake was measured at a substrate concentration of 500 μ M; the effectors were always present at concentrations of 2.5 mM. Data are means of the results from three independent experiments.

Chlamydiales are not adequately represented in current ATP/ADP translocase gene databases. Furthermore, knowledge about the presence or absence of these transport proteins in other obligate intracellular bacteria is scarce. An additional level of complexity in studies on the phylogeny of bacterial and plastidic ATP/ADP translocases is introduced by the fact that at least *C. trachomatis* possesses an additional paralogous gene which exhibits high sequence similarity but functions as a nucleotide-H⁺ symporter (and not as an ATP/ADP antiporter) (43) (Table 1). Thus, an unambiguous functional assignment of these transporters cannot be inferred from the gene sequence alone but clearly requires biochemical characterization of the protein in suitable expression systems (32, 43). Furthermore, characterization of ATP/ADP translocases from phylogenetically different organisms by heterologous gene expression might also assist in the development of antibacterial drugs or herbicides targeting this unique transport system, which is absent in human and mammalian cells.

Recent studies have demonstrated that free-living amoebae and paramecia represent an important reservoir for novel deep-branching members of the *Rickettsiales* (15, 20, 40) and the *Chlamydiales* (2, 16, 24). In this study, we demonstrated by PCR with degenerate primers that all seven strains of those obligate intracellular bacteria investigated possess genes with significant sequence homology to known ATP/ADP translocase genes. Consequently, energy parasitism appears to be an essential and thus widely distributed mechanism in members of both groups independent from the host cells they exploit. In contrast, we failed to detect homologous genes in several protozoan endosymbionts belonging to the *Betaproteobacteria*, the *Gammaproteobacteria*, and the *Bacteroidetes*, indicating that ATP/ADP transporter-based energy parasitism might not be a general feature of all (obligate) intracellular bacteria thriving in protozoa. This is consistent with the lack of a homologue in the genome sequence of *Legionella pneumophila* (Columbia Genome Center *Legionella* Project [http://genome3.cpmc.columbia.edu/~legion/]), which is also able to thrive in free-living amoebae. However, lack of detectable ATP/ADP translocase genes in some endosymbionts analyzed in this study must be interpreted with caution, because the applied degenerate PCR primers might not be suitable for amplification of

all ATP/ADP translocase genes. Only whole-genome sequences of the investigated endosymbionts would unequivocally prove the absence of those genes.

Protein phylogeny inference based on the extended data set (Fig. 2) showed that, independent from the treeing method applied, all putative ATP/ADP transporters form a monophyletic grouping, to the exclusion of the chlamydial nucleotide transport proteins and some hypothetical proteins with unknown function from the eukaryotic parasite *E. cuniculi*. Within the ATP/ADP translocase group, all treeing methods support monophyletic clustering of all transporters from the *Rickettsiales*, *Chlamydiales*, and plastids, respectively. Furthermore, the *Chlamydiales* and plastid group share a common ancestor in all trees analyzed. This overall tree topology is consistent with recently published trees by Greub and Raoult (17) and Amiri and coworkers (3) but differs from those trees presented by Linka et al. (28) and Wolf et al. (50) because, in the latter studies, the proteins of *E. cuniculi* were not used as an out-group.

The most parsimonious evolutionary scenario inferred from the obtained ATP/ADP translocase tree topology is that a nucleotide transport protein was invented by a chlamydial ancestor as tool to support its intracellular lifestyle. Before the split of the *Chlamydiales* into *Parachlamydiaceae* and *Chlamydiaceae*, which occurred about 0.7 billion years ago (17), this gene was duplicated and the newly obtained gene evolved into an ATP/ADP translocase gene. This ATP/ADP transporter gene was subsequently transferred via lateral gene transfer from this chlamydial ancestor to an ancestral member of the *Rickettsiales*—a transfer route which has obviously been used also for other genes during evolution (50). After this event, the chlamydial ancestor transferred the ATP/ADP translocase gene to the nucleus of plants. Whether this transfer occurred directly between chlamydia and plants or via lateral gene transfer to the plastidic ancestor cannot be resolved. In this context it is interesting that chlamydial genomes harbor a surprisingly high number of genes with similarities to plant genes which were derived from *Cyanobacteria* and thus function in chloroplasts. It is tempting to speculate that the chlamydia-like genes in plants either reflect a direct involvement of an ancestor of these bacteria in endosymbiosis or a common ancestry of *Chlamydiales* with *Cyanobacteria*. The latter hypothesis has received some support from recent comparative group I intron sequence and whole-genome sequence analyses (6, 13).

The evolutionary scenario suggested here is fully consistent with the data presented by Greub and Raoult (17) but differs from what has been postulated by Amiri and coworkers (3). The latter study suggested that ATP/ADP translocases were invented in the ancestor of the *Rickettsiales* and mitochondria and was subsequently transferred into the nuclear genome of the early mitochondrial cell. This scenario requires one to postulate that after these events the ATP/ADP translocase genes were laterally transferred from early eukaryotes to the chlamydial ancestor, in which it was duplicated. The tree topology of ATP/ADP and nucleotide transport proteins (Fig. 2) conflicts with this scenario because one would then expect the chlamydial nucleotide transport proteins to deeply branch off from the chlamydial ATP/ADP translocase group. The fact that chlamydial ATP/ADP translocases and other chlamydial nucleotide transporters do not form a monophyletic grouping

to the exclusion of plastidic ATP/ADP translocases indicates that a transfer of the respective gene from an early eukaryote or plant to the ancestor of the *Chlamydiales* is rather unlikely (but cannot be completely excluded because the different substrate specificity of the ATP/ADP and nucleotide transport proteins might have led to different selective forces during evolution, which in turn might bias phylogeny inference of the branching point of the nucleotide transporters).

Chlamydia-related endosymbionts possess a functional ATP/ADP transporter. Here we showed, for the first time, that a bacterial endosymbiont of free-living amoebae belonging to the *Parachlamydiaceae* possesses a functional transport protein that imports ATP in exchange for ADP in a highly specific manner (Fig. 5; Tables 1 and 2). The presence of ATP/ADP translocase mRNA demonstrated that this transport protein is transcribed and of importance for intracellular multiplication of *Parachlamydiaceae* (Fig. 4). Biochemical characterization of the heterologously expressed transporter demonstrated that the parachlamydial transporter has the highest $V_{\max}$ for ATP transport of all characterized prokaryotic and eukaryotic ATP/ADP transporters (Table 1). Consequently, similar to the medically important chlamydiae *C. trachomatis* and *Chlamydia pneumoniae*, environmental chlamydiae are able to live as energy parasites within their eukaryotic host cells. The demonstrated presence of a functional ATP/ADP translocase in environmental chlamydiae, which live within free-living amoeba, suggests an important role of protozoa in the evolution of chlamydiae and provides evidence that the last common ancestor of the *Parachlamydiaceae* and *Chlamydiaceae* was also characterized by an intracellular lifestyle.

However, chlamydiae do not reside directly within the host cytosol but live inside a specialized vacuole termed inclusion. Using fluorescently labeled tracer molecules, Heinzen and Hackstadt (18) have shown that the inclusion membrane does not contain pores allowing passive diffusion of metabolites (including highly charged molecules like ATP) between the host cytosol and the inclusion. Assuming that chlamydial ATP/ADP transport proteins are located in the bacterial membrane, ATP and ADP have to be translocated across the inclusion membrane by a second, yet unknown, functional transport mechanism.

The data presented in this study show that the analysis of the interaction between chlamydial endosymbionts and their amoeba host cells will contribute to a better understanding of the evolution of chlamydiae and their virulence mechanisms. In this context, the ongoing whole-genome sequence analysis of a representative of the environmental chlamydiae (<http://www.microbial-ecology.net/edge>) will provide novel insights into the lifestyle of environmental chlamydiae and into the evolution of chlamydiae from endosymbionts of unicellular eukaryotes to major human pathogens.

ACKNOWLEDGMENTS

This work was funded by Deutsche Forschungsgemeinschaft grant WA 1027/2-2 and bmb+f (German ministry for education and science) grants 01KI0104 and PTJ-BIO/03U213B to M.W. The work in the lab of E.N. was supported by the Deutsche Forschungsgemeinschaft.

We greatly acknowledge Hans-Dieter Görtz for providing paramoecia cultures, Lothar Richter for allowing us access to an unpublished manuscript, and Frank Maixner and Sibylle Schadhauer for technical help.

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