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Lawsonia intracellularis Contains a Gene Encoding a Functional Rickettsia-Like ATP/ADP Translocase for Host Exploitation[▽]

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ATP/ADP translocases are a hallmark of obligate intracellular pathogens related to chlamydiae and rickettsiae. These proteins catalyze the highly specific exchange of bacterial ADP against host ATP and thus allow bacteria to exploit their hosts' energy pool, a process also referred to as energy parasitism. The genome sequence of the obligate intracellular pathogen *Lawsonia intracellularis* (*Deltaproteobacteria*), responsible for one of the most economically important diseases in the swine industry worldwide, revealed the presence of a putative ATP/ADP translocase most similar to known ATP/ADP translocases of chlamydiae and rickettsiae (around 47% amino acid sequence identity). The gene coding for the putative ATP/ADP translocase of *L. intracellularis* (*L. intracellularis* nucleotide transporter 1 [NTT1$_{Li}$]) was cloned and expressed in the heterologous host *Escherichia coli*. The transport properties of NTT1$_{Li}$ were determined by measuring the uptake of radioactively labeled substrates by *E. coli*. NTT1$_{Li}$ transported ATP in a counterexchange mode with ADP in a highly specific manner; the substrate affinities determined were 236.3 (± 36.5) μM for ATP and 275.2 (± 28.1) μM for ADP, identifying this protein as a functional ATP/ADP translocase. NTT1$_{Li}$ is the first ATP/ADP translocase from a bacterium not related to *Chlamydiae* or *Rickettsiales*, showing that energy parasitism by ATP/ADP translocases is more widespread than previously recognized. The occurrence of an ATP/ADP translocase in *L. intracellularis* is explained by a relatively recent horizontal gene transfer event with rickettsiae as donors.

Some of the most important bacterial pathogens of humans can only replicate within eukaryotic cells. These obligate intracellular bacteria have developed sophisticated mechanisms to interact with and exploit their hosts. Prime examples of obligate intracellular bacterial pathogens are members of the orders *Chlamydiales* and *Rickettsiales* (hereafter referred to as chlamydiae and rickettsiae, respectively). These phylogenetically largely unrelated groups of microorganisms employ nucleotide transport (NTT) proteins, which import nucleotides or allow parasitization of their hosts' energy pool by exchanging bacterial ADP for host ATP (6, 10, 16, 17, 26, 30, 45, 51). Among bacteria, NTT proteins are unique to chlamydiae and rickettsiae and were, in addition, only found in plastids of plants and algae (30, 39, 50, 57).

NTT proteins have been classified into the ATP/ADP antiporter family AAA by Saier and coworkers (TC number 2.A.12 in the Transport Classification Database [44]). Yet, recent studies showed that NTT proteins comprise transporters with highly dissimilar transport modes and substrate affinities. An alternative classification of NTT proteins according to transport mode was therefore proposed, subdividing the NTT protein family into three classes; class I contains nucleotide antiporters, class II contains proton-driven nucleotide symporters, and class III contains NAD^+/ADP antiporters (17). Bacterial and plastidic NTT proteins are fundamentally different from the analogous ADP/ATP carriers of the mitochondrial carrier family with respect to structure and transport characteristics (25, 41–43, 57). In contrast to ATP/ADP translocases of NTT protein family class I, which enable bacterial energy parasitism, mitochondrial ADP/ATP carriers function in the reverse direction, exporting newly synthesized ATP from the mitochondrial matrix to the host cytosol in exchange for ADP.

Using BlastP (2) against the nonredundant protein sequences at GenBank/EMBL/DDBJ in order to find as-yet-unrecognized NTT proteins, we recently identified a gene coding for an NTT protein most similar to known chlamydial and rickettsial ATP/ADP translocases in the genome sequence of *Lawsonia intracellularis* PHE/MN1-00. *L. intracellularis* is a gram-negative, microaerophilic, obligate intracellular bacterium belonging to the *Deltaproteobacteria*. *L. intracellularis* enters the host cell via induced phagocytosis; the phagosome is quickly degraded, and *Lawsonia* resides directly in the cytoplasm (27). *L. intracellularis* is an important veterinary pathogen causing proliferative enteropathy (ileitis) in many mammals but mostly in pigs (27, 37, 46). Proliferative enteropathy is characterized by a progressive proliferation of immature intestinal epithelial cells (enterocytes) following infection with *L. intracellularis*. The disease, which can persist for several weeks, leads to anorexia, diarrhea, reduced growth of infected animals, and decreased reproductive performance (27, 34, 46). Although proliferative enteropathy is considered one of the

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most economically important diseases in the swine industry worldwide, causing hundreds of millions of U.S. dollars in extra costs annually (27, 29, 36), data on the molecular mechanisms important for the pathogenicity and interaction of *L. intracellularis* with its host cells are surprisingly scarce.

In this study, we characterized the predicted ATP/ADP translocase from *L. intracellularis* by using heterologous expression in *Escherichia coli*. We identified its biochemical transport properties and showed that it functions as an ATP/ADP antiporter, importing host ATP in exchange for bacterial ADP. Pathway reconstruction based on the *L. intracellularis* genome sequence, however, suggested that *L. intracellularis* is still able to regenerate ATP on its own. Phylogenetic analysis suggested that the ATP/ADP translocase from *L. intracellularis* was acquired from a rickettsial or a chlamydial donor by lateral gene transfer, possibly during infection of the same eukaryotic host cell.

MATERIALS AND METHODS

PCR and cloning. *L. intracellularis* NCTC 12656 cells were used for DNA isolation with the DNeasy tissue kit (Qiagen, Vienna, Austria) according to the recommendations of the manufacturer. The gene Li0007, coding for the putative ATP/ADP transport protein, was amplified by using the High Fidelity PCR enzyme mix (MBI-Fermentas, St. Leon-Rot, Germany) according to the instructions of the manufacturer. A forward primer (5′-GAG AAC CTC GAG ATG AGT GAT AAA GGC AAG-3′) introducing an XhoI restriction site before the start codon and a reverse primer (5′-GAG AAC CTC GAG TTA GTT TGT GCA GAG CTC-3′) containing an XhoI restriction site after the stop codon were used. PCR conditions were as follows: denaturation at 94°C for 3 min, followed by 35 cycles of (i) denaturation at 94°C for 30 s, (ii) annealing at 56°C for 40 s, and (iii) elongation at 68°C for 90 s and a final elongation step at 68°C for 10 min. The resulting amplification products were gel purified and cloned into the cloning vector pCR-XL-TOPO by using the TOPO XL cloning kit (Invitrogen Life Technologies, Lofer, Austria). The resulting plasmid was digested with restriction endonuclease XhoI, gel purified, and inserted in frame into isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression vector pET16b containing a promoter site for the T7 RNA polymerase (Novagen, Heidelberg, Germany). The newly constructed plasmid (p*Li*NTT1) was transformed into and maintained in *E. coli* XL1-Blue cells (Stratagene, Heidelberg, Germany). The integrity of the cloned gene was confirmed by sequencing on an ABI 3130 XL Genetic Analyzer by using the BigDye terminator kit v3.1 (ABI, Vienna, Austria).

Heterologous expression in *E. coli*. For heterologous expression of p*Li*NTT1, *E. coli* strain BLR(DE3) was used. Synthesis of recombinant nucleotide transporters was conducted as previously described (16, 17). Briefly, *E. coli* cells harboring p*Li*NTT1 were induced with 1 mM IPTG at an optical density at 600 nm of 0.5. After 1 h, cells were pelleted (3,000 × *g*, 5 min, 8°C) and resuspended in 50 mM potassium phosphate buffer medium (pH 7.0) to an optical density at 600 nm of 5.0, kept at room temperature, and subsequently used for uptake experiments.

Analysis of substrate specificity and uptake kinetics. To analyze the transport properties of $NTT1_{Li}$, 100 μl of either induced *E. coli* cells harboring p*Li*NTT1 or noninduced cells (control) was added to 100 μl of phosphate buffer containing the indicated concentrations of α-^{32}P-labeled substrates. Cells without p*Li*NTT1 but also induced by the addition of IPTG exhibited the same uptake as the control cells (data not shown). Uptake was allowed at 30°C for the indicated time spans and terminated by removal of the external substrate. For the latter purpose, cells were applied to nitrocellulose filters (0.45-μm pore size), prewetted with phosphate buffer medium, and set under a vacuum. The cells were subsequently washed three times with 4 ml phosphate buffer, and filters were transferred into 10-ml scintillation vessels containing 4 ml of water. Radioactivity in the samples was quantified in a scintillation counter (Tricarb 2500; Canberra-Packard, Heidelberg, Germany).

Back-exchange analysis and thin-layer chromatography. To characterize the transport mode of recombinant $NTT1_{Li}$, back-exchange studies were conducted. For preloading, pellets of 2 ml of IPTG-induced *E. coli* cells synthesizing $NTT1_{Li}$ were incubated in phosphate buffer containing 50 μM radioactively labeled [α-^{32}P]ATP for 5 min. Subsequently, cells were harvested by centrifugation, washed two times, and resuspended in phosphate buffer medium containing 500 μM (10-fold excess) unlabeled nucleotides. Back exchange was carried out at 30°C for 2.5 min and terminated by rapid centrifugation. The remaining internal radioactivity in the pellet and the exported label in the supernatant were quantified in a scintillation counter. Additionally, radioactively labeled compounds exported by *E. coli* cells expressing $NTT1_{Li}$ were identified by thin-layer chromatography (32). For this, a 10-μl aliquot of the supernatant was loaded onto a 0.5-mm poly(ethylene amine) cellulose thin-layer chromatography plate and dried with a fan. Retardation factor values of radioactively labeled nucleotides and phosphate were determined after radioautography and corresponded to values of unlabeled nucleotides visualized under UV light and to radioactively labeled standards. Corresponding radioactively labeled positions were marked on the thin-layer plate, cut out, and quantified in a scintillation counter.

Phylogenetic analysis. A database containing all of the nucleotide transport protein sequences available in the EMBL/GenBank/DDBJ public databases was established by using the ARB software package (31). For this, amino acid sequences were aligned automatically with MAFFT (24) and the alignment was imported into ARB. Phylogenetic trees were constructed with the PHYLIP distance matrix (Fitch) and maximum-parsimony methods (12), TREE-PUZZLE (using the VT model of amino acid substitution), and PROTml 2.3 (using the JTT amino acid replacement model) implemented in ARB. Bootstrap analysis was performed with 1,000 resamplings. A filter considering only those alignment positions that were conserved in at least 30% of all sequences (resulting in a total of 441 alignment columns) was used for all treeing calculations.

Comparative genome analysis. Comparative sequence analysis of the publicly available *L. intracellularis* PHE-MN1-00 genome sequence was performed by using Entrez Genome at the National Center for Biotechnology Information website (56) and Integrated Microbial Genomes at the Joint Genome Institute website (33). Analysis of metabolic pathways was performed by using the Kyoto Encyclopedia of Genes and Genomes website (23). GC skew analysis was done with the GenSkew tool available at the Munich Information Center of Protein Sequences website (38).

Nucleotide sequence accession number. The gene sequence of the ATP/ADP translocase from *L. intracellularis* NCTC 12656 was submitted to the EMBL/GenBank/DDBJ databases under accession number AM941722.

RESULTS

Identification and comparative sequence analysis of $NTT1_{Li}$. The biochemically well-characterized ATP/ADP translocase $NTT1_{Pam}$ (pc0250) of the amoeba symbiont *Protochlamydia amoebophila* UWE25 (9, 45, 53) was used as a query for BlastP searches (2) against the nonredundant protein sequence data set (nr) at the National Center for Biotechnology Information website (56). Using an E value cutoff of $1e^{-15}$, a putative nucleotide transport protein was identified in the genome sequence of *L. intracellularis* PHE/MN1-00. The identified protein (Li0007, GenBank accession number YP_594385) comprises 532 amino acids and shows a predicted molecular mass of 59 kDa and a predicted pI of 9.14, which corresponds well to known nucleotide transporters. Li0007 showed a match with PFAM family PF03219 ("TLC ATP/ADP transporter") and was noted as a "nucleotide transport protein" in the automatically derived annotation.

The cloned gene encoding the putative ATP/ADP translocase from *L. intracellularis* NCTC 12656 was identical to Li0007 from *L. intracellularis* PHE/MN1-00. According to the nucleotide transporter nomenclature used by Haferkamp and coworkers (16, 17), we refer to this protein as $NTT1_{Li}$. The amino acid sequence of $NTT1_{Li}$ was added to a data set containing all publicly available NTT proteins. Sequences were aligned and subjected to a detailed comparative analysis. $NTT1_{Li}$ showed the highest amino acid sequence identity to a nearly full-length sequence (423 amino acids) of a nucleotide transport protein from the rickettsial amoeba symbiont "*Candidatus* Paracaedibacter symbiosus" ($NTT1_{Ps}$, 53.1%) and 47.6

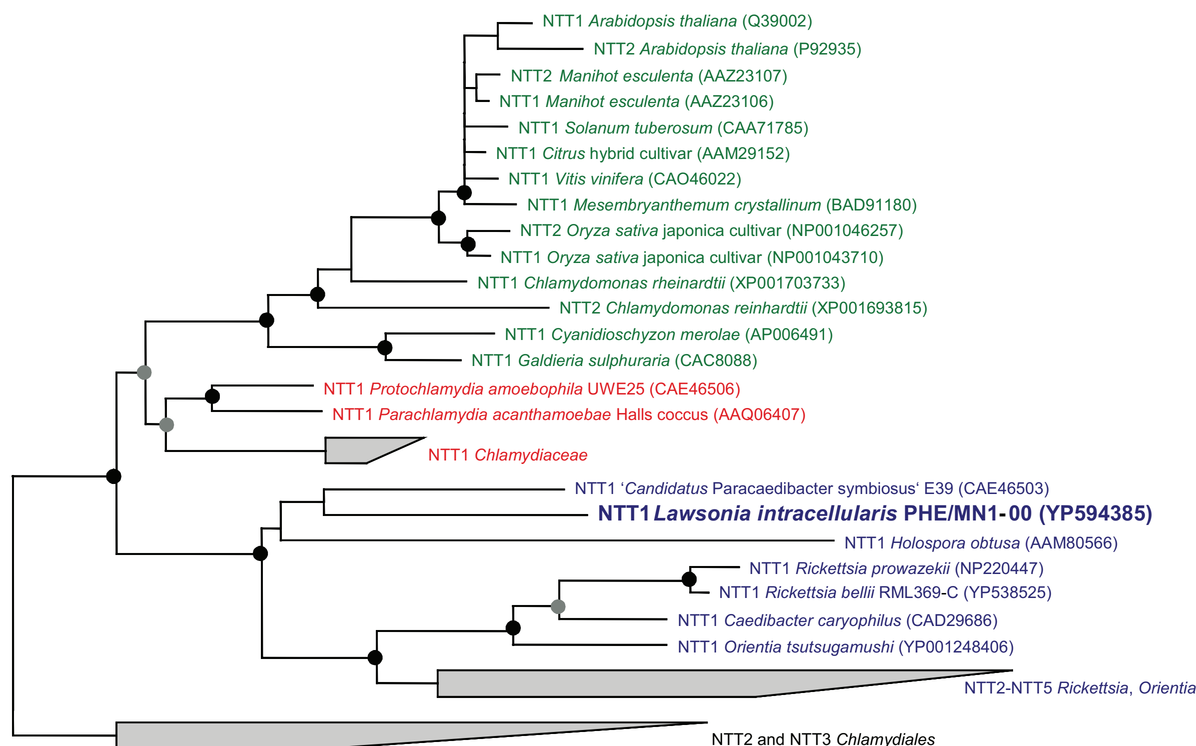

FIG. 1. Phylogenetic relationships of bacterial and plastidic ATP/ADP translocases and other nucleotide transport proteins. The amino acid-based tree was calculated by the TREE-PUZZLE method. Black circles indicate well-supported nodes showing greater than 90% TREE-PUZZLE support and parsimony bootstrap values (1,000 resamplings). Nodes that are supported by TREE-PUZZLE support values above 75% but which show only low-parsimony bootstrap values (less than 90%) are indicated by gray circles. TREE-PUZZLE support values below 75% and parsimony bootstrap values below 90% are not shown. Plastidic ATP/ADP translocases from plants and algae are in green, ATP/ADP translocases from chlamydiae are in red, and rickettsial ATP/ADP translocases and nucleotide transport proteins are in blue. GenBank accession numbers are in parentheses. The bar indicates a 10% estimated evolutionary distance.

and 46.5% sequence identity to $NTT1_{Pam}$, the ATP/ADP transporter of *Protochlamydia amoebophila* UWE25, and $NTT1_{Rp}$, the ATP/ADP translocase from *Rickettsia prowazekii*, respectively. Amino acid sequence identity to plastidic ATP/ADP transporters from plants and algae was 39 to 44%; sequence identity to functionally characterized NTT class II proteins was below 39%.

A number of highly conserved (charged) amino acid residues that are essential for the function of the ATP/ADP translocase of *Arabidopsis thaliana* ($NTT1_{At}$) (52), i.e., K155, E245, E385, and K527 (referring to the $NTT1_{At}$ numbering), are present in $NTT1_{Li}$. Using ConPred II (5), $NTT1_{Li}$ is predicted to contain 12 transmembrane alpha helices. This is consistent with the secondary-structure analysis of known ATP/ADP translocases and experimental evidence suggesting the presence of 12 transmembrane helices in $NTT1_{Rp}$ (1). Phylogenetic analysis using distance matrix, maximum-parsimony, and maximum-likelihood treeing methods demonstrated that $NTT1_{Li}$ consistently clustered together with rickettsial ATP/ADP translocases and their paralogues with high confidence (Fig. 1). The overall topology of the trees obtained was supported by all of the treeing methods applied and was similar to previously published analyses (3, 15, 45, 54).

Biochemical characterization of $NTT1_{Li}$ in *E. coli*. Based on the results of our comparative sequence analysis, we hypothesized that $NTT1_{Li}$ functions as an ATP/ADP translocase. We thus decided to investigate whether ATP and ADP are indeed substrates of $NTT1_{Li}$ by determining the time dependency of radioactively labeled ATP and ADP uptake by $NTT1_{Li}$ expressed in *E. coli*. These experiments also served to identify the linear phase of import, which is a prerequisite for the calculation of affinities and reaction velocities. In all cases, noninduced (control) cells showed no significant uptake of radioactivity (Fig. 2). ATP import by $NTT1_{Li}$ was linear for about the first 10 min (Fig. 2A); ADP import was linear for the first 10 to 15 min (Fig. 2B). Transport measurements with rising concentrations of labeled substrates (5 to 1,000 μM) allowed the calculation of V_{max} values for ATP and ADP import by $NTT1_{Li}$. ATP was imported at a rate of 139.2 (± 8.3) nmol mg protein^{-1} h^{-1}, and ADP was imported at a rate of 149.2 (± 13.7) nmol mg protein^{-1} h^{-1}. In addition, substrate affinities of $NTT1_{Li}$ for ATP and ADP were calculated. $NTT1_{Li}$ had an apparent substrate affinity of 236.3 (± 36.5) μM for ATP and 275.2 (± 28.1) μM for ADP.

In order to verify that ATP and ADP are the preferred substrates of $NTT1_{Li}$, effector studies measuring the uptake of [α-^{32}P]ATP in the presence of unlabeled putative effectors (in 10-fold excess) were performed. Of the various putative substrates tested, only ATP (25.8% ± 1.7% residual activity) and ADP (39.9% ± 1.1% residual activity) significantly inhibited

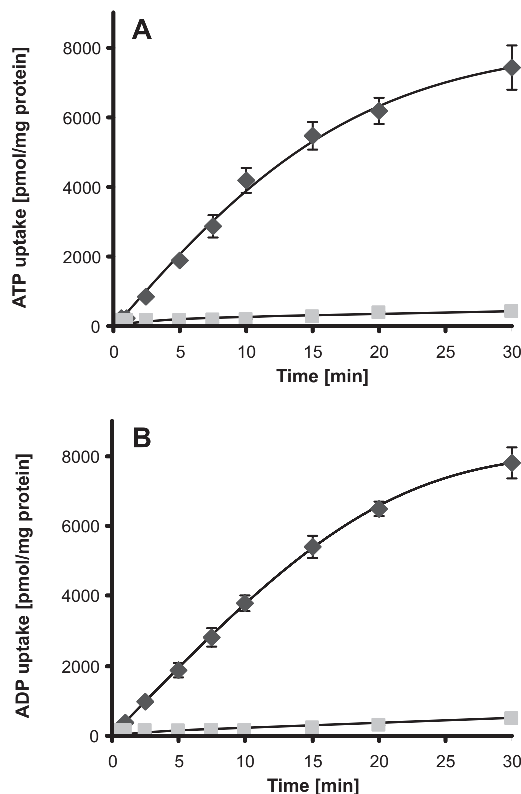

FIG. 2. Time dependency of ATP and ADP uptake into *E. coli* cells expressing the ATP/ADP translocase NTT1$_{Li}$ from *L. intracellularis*. Shown is the time-dependent uptake of [α-^{32}P]ATP (A) and [α-^{32}P]ADP (B) mediated by *E. coli* expressing NTT1$_{Li}$ (black diamonds) or into noninduced cells harboring p*Li*NTT1 (control, gray squares). Cells were incubated in phosphate buffer medium containing 50 μM labeled nucleotide for the indicated time periods. Data are the mean of at least three independent experiments. Standard errors are given.

the uptake of [α-^{32}P]ATP when compared to the transport without effector, indicating that ATP and ADP are indeed the only transported substrates for NTT1$_{Li}$ (Fig. 3). To analyze whether NTT1$_{Li}$ acts in a counterexchange mode of transport or as a proton-driven net import protein, we determined nucleotide uptake in the presence of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone. Addition of 100 μM carbonyl cyanide *m*-chlorophenylhydrazone reduced ATP import only to 73% (± 6.8%) residual activity (Fig. 3), indicating that NTT1$_{Li}$-mediated ATP uptake is not driven by the proton gradient, a feature which is characteristic of known ATP/ADP translocases (53).

In order to further characterize NTT1$_{Li}$ as an ATP/ADP antiporter, back-exchange studies were performed. *E. coli* cells expressing NTT1$_{Li}$ were preloaded with labeled ATP, external radioactivity was removed, and the cells were resuspended in phosphate buffer (control) or transport buffer (containing putative counterexchange substrates). Back exchange was carried out at 30°C for 2.5 min. After centrifugation, the radioactivity in the cells and in the supernatant was quantified. Quantification of exported radioactivity allows differentiation between counterexchange (antiport) and unidirectional transport (symport) (17). *E. coli* cells expressing NTT1$_{Li}$ and preloaded with [α-^{32}P]ATP released significant amounts of internal label (36 to 40% of the initial amount) only after resuspension in buffer medium supplemented with unlabeled ADP or ATP compared with the control (Fig. 4A). We verified by thin-layer chromatography that radioactive nucleotides exported in the presence of unlabeled substrates are mainly ADP, but also minor amounts of AMP and ATP were exported, whereas inorganic phosphate export was independent of the presence of counterexchange nucleotides (Fig. 4B).

DISCUSSION

Physiological significance of NTT1$_{Li}$. Biochemical characterization of the ATP/ADP translocase NTT1$_{Li}$ from *L. intracellularis* suggested that this pathogen, similar to chlamydiae and rickettsiae, is able to exploit its host cell by importing ATP in exchange for bacterial ADP. The determined transport properties of NTT1$_{Li}$ are in the range of reported K_m and V_{max} values of other functionally characterized ATP/ADP translocases from chlamydiae and rickettsiae (for an overview, see references 17 and 45). The affinity of NTT1$_{Li}$ should be sufficient to transport ATP at in vivo concentrations (see reference 17 for details).

Both rickettsiae and chlamydiae are capable of generating their own energy (4, 14, 18, 19, 21, 35). In order to survive as long as possible within a host cell, a general strategy of intracellular bacteria seems to be to limit the import of nutrients essential for the host in a way that the host is not impaired too severely because death of the host would lead to loss of their protective niche (22). By supplementing—but not replacing—bacterial energy production, ATP/ADP translocases might thus represent a fine-tuned and essential adaptation facilitating long-term survival within eukaryotic host cells. However, rickettsiae and chlamydiae are unable to synthesize nucleotides de novo (14, 19, 55), which is compensated for by two to five paralogous NTT proteins that are used for the net uptake of nucleotides, respectively (6, 16, 17, 51).

The genome sequence of *L. intracellularis* revealed that this pathogen, similar to chlamydiae and rickettsiae, should be able to generate its own energy by using a basic, probably microaerophilic, respiratory chain. The genome also encodes a complete glycolytic pathway; the ATP-dependent phosphofructokinase is notably absent but is probably complemented by the pyrophosphate-dependent phosphofructokinase (Li0052), allowing the glycolytic pathway to be functional. However, unlike chlamydiae and rickettsiae, *L. intracellularis* should be able to synthesize both purine and pyrimidine nucleotides de novo, which is consistent with the absence of additional nucleotide transporters for the net uptake of nucleotides in *L. intracellularis*. Like other obligate intracellular bacteria, *L. intracellularis* possesses a small, streamlined genome (1.76 Mb, including three plasmids). It is thus tempting to speculate that the genes that are retained are essential and fulfill a similar function in *L. intracellularis* as in rickettsiae and chlamydiae.

Phylogeny of nucleotide transport proteins and evolutionary implications. The identification of a functional ATP/ADP translocase in *L. intracellularis* reveals a surprising conservation of energy parasitism among largely unrelated groups of bacterial pathogens belonging to the chlamydiae, rickettsiae,

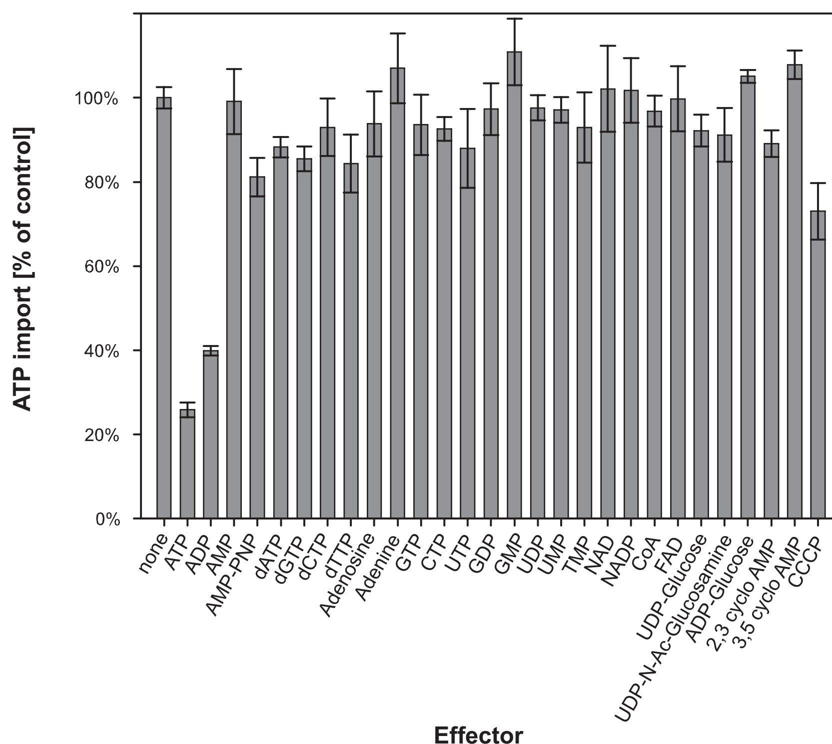

FIG. 3. Effects of various metabolites on [α-^{32}P]ATP uptake into *E. coli* cells expressing the ATP/ADP translocase $NTT1_{Li}$ from *L. intracellularis*. Uptake of [α-^{32}P]ATP mediated by recombinant $NTT1_{Li}$ was measured at a substrate concentration of 50 μM and stopped after 3 min. Unlabeled effectors were present in 10-fold excess. Rates of nucleotide uptake are given as percentages of control rates (nonaffected transport = 100%). Data are the mean of three independent experiments. Error bars indicate the standard error. CoA, coenzyme A; FAD, flavin adenine dinucleotide; Ac, acetyl; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

and *Deltaproteobacteria*. Protein phylogeny of nucleotide transport proteins revealed that $NTT1_{Li}$ consistently formed a deep branch with ATP/ADP translocases from "*Ca*. Paracaedibacter symbiosus" ($NTT1_{Ps}$) and *Holospora obtusa* ($NTT1_{Ho}$) and clustered together with other rickettsial nucleotide transport proteins (Fig. 1).

The evolution of nucleotide transport proteins, particularly of ATP/ADP translocases, has received considerable attention as phylogenetic analysis allowed insights into early events during the origin of the plant cell (3, 15, 20, 30, 45, 54, 58). In general, two evolutionary scenarios were postulated: First, ATP/ADP translocases may have been invented in an ancestor of mitochondria and *Rickettsia*. ATP/ADP translocase-encoding genes were subsequently transferred to chlamydiae and the nuclear genome of an early mitochondriate cell, respectively, and were retained in plants to facilitate plastid function (3). A rickettsial origin of ATP/ADP translocases, however, would not explain the distinct phylogenetic relationship between chlamydial and plastidic ATP/ADP translocases.

The alternative scenario therefore assumes that ATP/ADP translocases originated in a chlamydial ancestor and were then transferred to plants and rickettsiae, respectively (15, 45). This hypothesis is consistent with the presence of sequences encoding (putative) ATP/ADP translocases in representatives of all major evolutionary lineages within the chlamydiae, including *Chlamydiaceae*, *Parachlamydiaceae*, *Waddliaceae* (GenBank accession number AAX45329), *Simkaniaceae* (AAX45330), and *Criblamydiaceae* (8, 15, 19, 45, 47–49, 55). A chlamydial origin of ATP/ADP translocases also gained recent support from two independent phylogenomic studies which, in addition, suggested a contribution of chlamydiae to the origin of the primary photosynthetic eukaryote (20, 54).

Our analysis comprising a larger data set of ATP/ADP translocases than previous studies, including novel chlamydial, plant and algal sequences and the first deltaproteobacterial ATP/ADP translocase, provides strong evidence for a chlamydial origin of ATP/ADP translocases. The tree topology obtained, which is well supported and in general agreement with previously published results (15, 45), can be best explained by ancient horizontal transfer events from a chlamydial ancestor to plants and rickettsiae and a third transfer from a rickettsia-like organism to deltaproteobacterial *L. intracellularis*.

The genome of *L. intracellularis*, however, does not show clear signs of recent lateral acquisition of foreign DNA. G+C skew analysis indicated no obvious regions with unusual G+C content indicative of genomic islands (data not shown). Consistently, the G+C content of the gene encoding $NTT1_{Li}$ is, at 33%, not different from the overall G+C content of the *L. intracellularis* genome, which contains only a few transposases (n = <10) and phage-like genes (n = 5). This indicates either that signposts of lateral gene transfer of $NTT1_{Li}$ were blurred due to amelioration (28), i.e., that the gene transfer event occurred a long time ago, or that the donor genome had a G+C content similar to that of the *L. intracellularis* genome. Interestingly, *Rickettsia* species show a genomic G+C content of between 29 and 32.5%, while chlamydiae have a generally

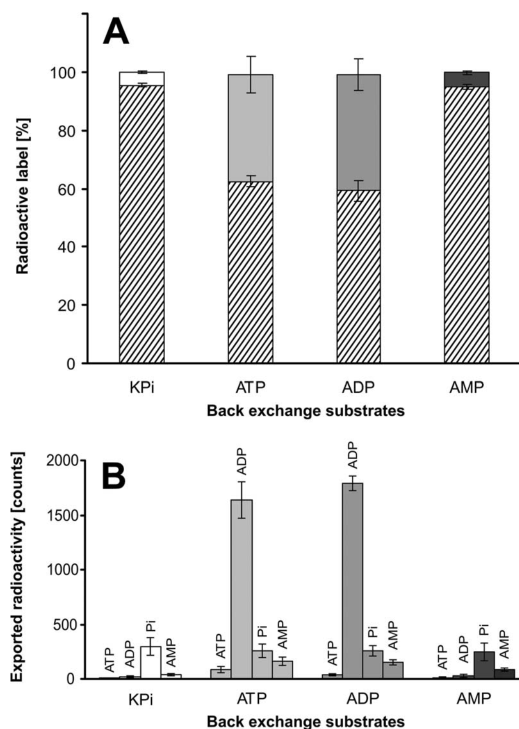

FIG. 4. Quantification and characterization of exported radioactively labeled nucleotides by *E. coli* cells expressing the ATP/ADP translocase $NTT1_{Li}$ from *L. intracellularis*. Preloading with radioactivity was achieved by incubation of induced *E. coli* cells in phosphate buffer containing 50 μM [α-^{32}P]ATP for 5 min at 30°C. Washed cells were incubated in 500 μM nonlabeled adenine nucleotides or phosphate buffer (control) for 2.5 min at 30°C. Back-exchange substrates are indicated on the *x* axis, i.e., potassium phosphate buffer (KPi; white bars), ATP (light gray bars), ADP (dark gray bars), and AMP (black bars). (A) Fraction of exported label (white, gray, and black bars) and remaining radioactivity in the cells (hatched bars). (B) Nature of the exported label (indicated above the bars) as determined by thin-layer chromatography and quantification of the radioactive spots by scintillation counting. Data are the mean of three independent replicates.

higher genomic G+C content (34.7 to 41.3%). Taken together, these findings and the well-supported grouping of $NTT1_{Li}$ with rickettsial ATP/ADP translocases in our phylogenetic analysis (Fig. 1) point to rickettsiae as putative donors of the *L. intracellularis* ATP/ADP translocase.

In contrast to chlamydiae and rickettsiae, *L. intracellularis* is not related to a deeply branching evolutionary lineage of exclusively intracellular bacteria but belongs to the *Desulfovibrionaceae* (37, 46), most of which are free-living, sulfate-reducing microorganisms (11); its closest relatives are *Bilophila wadsworthia* and *Desulfovibrio desulfuricans*, from which *L. intracellularis* split about 200 million years ago (if a divergence rate for the 16S rRNA gene of 1%/50 million years is assumed) (40). Compared to rickettsiae and chlamydiae, which form deep branches in the *Alphaproteobacteria* and the *Bacteria*, respectively, and which evolved far more than a billion years ago (7, 13), *L. intracellularis* has thus only relatively recently adapted to an obligate intracellular life style. The acquisition of a gene encoding an ATP/ADP translocase (from a rickettsia-like donor) might have facilitated this process.

Conclusion. In this study, we have analyzed the first ATP/ADP translocase from a deltaproteobacterial pathogen and could show that this ancient and important mechanism for host cell interaction is conserved among major human and animal pathogens. Biochemical characterization of this ATP/ADP translocase allowed first insights into the molecular basis of the intracellular life style of *L. intracellularis*.

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