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Nucleotide Parasitism by *Simkania negevensis* (*Chlamydiae*)▽†

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Intracellular bacteria live in an environment rich in most essential metabolites but need special mechanisms to access these substrates. Nucleotide transport proteins (NTTs) catalyze the import of ATP and other nucleotides from the eukaryotic host into the bacterial cell and render *de novo* synthesis of these compounds dispensable. The draft genome sequence of *Simkania negevensis* strain Z, a chlamydial organism considered a newly emerging pathogen, revealed four genes encoding putative nucleotide transport proteins (*Sn*NTT1 to *Sn*NTT4), all of which are transcribed during growth of *S. negevensis* in *Acanthamoeba* host cells, as confirmed by reverse transcription-PCR. Using heterologous expression in *Escherichia coli*, we could show that *Sn*NTT1 functions as an ATP/ADP antiporter, *Sn*NTT2 as a guanine nucleotide/ATP/H^+ symporter driven by the membrane potential, and *Sn*NTT3 as a nucleotide triphosphate antiporter. In addition, *Sn*NTT3 is able to transport dCTP, which has not been shown for a prokaryotic transport protein before. No substrate could be identified for *Sn*NTT4. Taking these data together, *S. negevensis* employs a set of nucleotide transport proteins to efficiently tap its host's energy and nucleotide pools. Although similar to other chlamydiae, these transporters show distinct and unique adaptations with respect to substrate specificities and mode of transport.

Nucleotide transport proteins (NTTs) are commonly linked to the term "energy parasitism" (55) because they enable obligate intracellular bacteria to harvest ATP and other high-energy compounds from eukaryotic host cells. Among bacteria, NTT proteins catalyzing ATP/ADP exchange have been found in *Chlamydiae* and *Rickettsiales*, comprising major intracellular pathogens of humans (15, 45, 48, 62, 64, 67), and recently also in the obligate intracellular veterinary pathogen *Lawsonsia intracellularis* belonging to the *Deltaproteobacteria* (63), in the plant pathogen "*Candidatus* Liberibacter asiaticus" belonging to the *Rhizobiales* (75), and in the obligate intracellular amoeba symbiont "*Candidatus* Amoebophilus asiaticus" belonging to the *Bacteroidetes* (65). In addition to ATP/ADP translocases, some obligate intracellular bacteria encode nucleotide transport isoforms for the import of nucleotides other than ATP or the cofactor NAD^+ (7, 22, 26, 27), thereby compensating for their inability to synthesize these metabolites *de novo* (22, 33, 66, 76). Nucleotide transporters are thus important proteins for host cell interaction of obligate intracellular bacteria. Interestingly, nucleotide transport proteins were also found in eukaryotes: in plant and algal plastids (5, 48, 54, 68, 79) and in the microsporidian parasite *Encephalitozoon cuniculi* (73).

In *Chlamydiae*, ATP uptake activity was first described for the avian pathogen *Chlamydophila psittaci* (28). Nearly 2 decades later, two nucleotide transport proteins of the human pathogen *Chlamydia trachomatis* were identified on the molecular level; one transporter catalyzes ATP/ADP exchange, and the second transporter mediates net uptake of RNA nucleotides (67). The diversity of the *Chlamydiae* is, however, not limited to the well-known human and animal pathogens of the genus *Chlamydia* (or *Chlamydophila*) but is more complex than previously recognized (for reviews, see references 13, 14, 32, and 46). Several new chlamydial families have been described recently, all of which comprise obligate intracellular bacteria that share the characteristic chlamydial developmental cycle. With respect to nucleotide transport proteins, the amoeba symbiont *Protochlamydia amoebophila* UWE25 *(Parachlamydiaceae)* is particularly well characterized among these novel *Chlamydiae* (12, 26, 27, 33, 64, 71, 72). *P. amoebophila* encodes an ATP/ADP antiporter (*Pam*NTT1) to parasitize the host energy pool, a GTP/ATP/H^+ symporter (*Pam*NTT5), a UTP/H^+ symporter (*Pam*NTT3), a nucleoside triphosphate antiporter balancing the bacterial nucleotide pool (*Pam*NTT2), and a transporter providing NAD^+ in exchange for ADP (*Pam*NTT4). Using these five NTTs, *P. amoebophila*, which is devoid of pathways for *de novo* synthesis of nucleotides and NAD^+, is able to import all RNA nucleotides, ATP, and NAD^+ from its amoeba host.

Another recently discovered chlamydial organism is *Simkania negevensis* (38, 41, 42). The natural host of *S. negevensis* is unknown, as the bacterium was isolated originally as a contaminant of human and simian cell cultures (38, 41). Because *S. negevensis* is able to establish a stable symbiosis with different amoeba hosts (37, 53), amoebae are assumed to serve as a reservoir for *S. negevensis* in the environment. A worldwide seroprevalence in healthy and diseased individuals has been reported for *S. negevensis*, and these bacteria might be associated with respiratory diseases, such as bronchiolitis and community-acquired pneumonia (18, 19, 23, 29, 40, 47). *S. negevensis* has been suggested to represent a newly emerging pathogen (18, 39), and the versatile infection capability and worldwide

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prevalence of *S. negevensis* are being studied. However, very little is known about the molecular basis of the intracellular life style of *S. negevensis*.

To investigate metabolic host cell interaction of *S. negevensis* with respect to nucleotides, we analyzed draft genome sequence data for *S. negevensis* and searched for putative nucleotide transport proteins. We identified genes encoding four putative NTT-type proteins in the *S. negevensis* genome (*Sn*NTT1 to *Sn*NTT4), and all except one showed the highest sequence similarity to chlamydial NTTs. Nucleotide transport measurements performed with all four *Sn*NTT proteins heterologously expressed in *Escherichia coli* suggested that *S. negevensis* is capable of importing ATP and other nucleotides from its host cell. In contrast to *P. amoebophila*, an NAD^+ transporter is apparently not required, as *S. negevensis* should be able to synthesize this essential cofactor *de novo*.

MATERIALS AND METHODS

Sequence analysis. The unfinished *S. negevensis* strain Z (ATCC number VR-1471) genome sequence was obtained from The Institute for Genomic Research (J. Craig Venter Institute [JCVI]), automatically annotated using the PEDANT annotation system for genome sequence analysis (20), and searched using BLAST (3). Amino acid sequence identities were determined using a comprehensive data set of nucleotide transport proteins aligned with MAFFT (44) and the *ARB* software package (49). Prediction of transmembrane regions was done using ConPred II (4), the PHDhtm program (61) at the PredictProtein server, PRED-TMR (57), and Split 4.0 (36).

Cultivation, DNA isolation, PCR, and cloning. The *S. negevensis* strain used for this study was kindly provided by Klaus Henning (Institut für Epidemiologie, Friedrich-Loeffler-Institut, Wusterhausen, Germany) (30). *S. negevensis* was grown in *Acanthamoeba* sp. strain UWC1 (21) at 28°C in peptone-yeast-glucose (PYG) broth containing 20 g protease peptone, 18 g glucose, 2 g yeast extract, 1 g sodium citrate, 980 mg $MgSO_4 \cdot 7H_2O$, 355 mg $Na_2HPO_4 \cdot 7H_2O$, 340 mg KH_2PO_4, 20 mg $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ per liter of distilled water at a pH of 6.5. Simultaneous DNA extractions from amoebae and *S. negevensis* were performed using the DNeasy Blood and Tissue Kit (Qiagen) according to the protocol recommended by the manufacturer. For amplification of the 16S rRNA gene of the *S. negevensis* strain, the following primers were used at an annealing temperature of 56°C: PCf (forward primer [34]) and 16S2 (reverse primer [58]). The PCR product was purified using the QIAquick PCR purification kit (Qiagen) and sequenced directly on an ABI 3130 XL Genetic Analyzer using the Big Dye Terminator Kit v3.1 (ABI). The full-length *S. negevensis* 16S rRNA nucleotide sequence obtained showed a 16S rRNA similarity of 99.3% to *S. negevensis* strain Z, indicating that the *S. negevensis* strain used in this study is not identical but is highly similar to *S. negevensis* strain Z (16, 38, 41).

The *Sn*NTT1 to *Sn*NTT4 genes were amplified with the Extensor Hi-Fidelity PCR Enzyme Mix (ABgene), and the primers used for the amplification of the genes were designed based on draft genome sequence data for *S. negevensis* strain Z (see Table S1 in the supplemental material). The primers included an additional restriction site at the start codon and downstream of the stop codon and were used at an annealing temperature of 56°C (see Table S1 in the supplemental material). The resulting amplicons were gel purified and cloned into the cloning vector pCR-XL-TOPO using the TOPO XL PCR Cloning Kit (Invitrogen Life Technologies); the plasmids produced were cut using the restriction enzymes listed in Table S1 in the supplemental material. The desired gene fragments were gel purified and ligated in frame into the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression vector pET16b containing an N-terminal 10-histidine tag and a promoter for the T7 RNA polymerase (Novagen). The plasmids obtained (p*Sn*NTT1 to p*Sn*NTT4) were transformed into and maintained in *E. coli* XL1-Blue cells (Stratagene).

The identities of the cloned genes (*Sn*NTT1 to *Sn*NTT4) were verified by sequencing. *Sn*NTT1 and *Sn*NTT4 were identical to the genes from the draft genome sequence of *S. negevensis* strain Z. *Sn*NTT2 and *Sn*NTT3 contained a single nucleotide mismatch compared to the corresponding genes of *S. negevensis* strain Z, resulting in a single amino acid substitution.

RNA isolation and reverse transcription (RT)-PCR. Purification of total RNA of *Acanthamoeba* sp. UWC1 harboring *S. negevensis* was conducted using the TRIzol reagent (Invitrogen Life Technologies) as described by the manufacturer. Briefly, amoeba cells containing *S. negevensis* were pelleted by short centrifugation (5,204 × *g*; 5 min; 20°C), resuspended in TRIzol reagent, and homogenized using the Bead-Beater Fast Prep FP120 Instrument (BIO 101). For RNA precipitation—in addition to the isopropyl alcohol recommended by the supplier of the TRIzol reagent—0.5 M ammonium acetate (Roth) and 20 μg of glycogen (Ambion, Inc.) were added, and precipitation of RNA was allowed for 15 min at −20°C. Residual DNA was removed by a DNase treatment using DNase I. Five micrograms of RNA was transcribed into cDNA with the RevertAid First Strand cDNA Synthesis Kit (MBI-Fermentas) according to the protocol given by the manufacturer using gene-specific primers for reverse transcription (see Table S1 in the supplemental material). Subsequently, first-strand cDNA was subjected to a standard PCR with a cycling program using 40 cycles and an annealing temperature of 56°C (see Table S1 in the supplemental material). The absence of DNA in the RNA preparation was verified by PCR, and negative controls (no cDNA added) were included in each PCR mixture. The identities of the amplification products were confirmed by sequencing, and all experiments were conducted independently in duplicate.

Heterologous expression in *E. coli*. For heterologous expression of p*Sn*NTT1 to p*Sn*NTT3, the *E. coli* strain BLR(DE3) was used, and for heterologous expression of p*Sn*NTT4, BLR(DE3) and Rosetta2 cells were used. Synthesis of recombinant NTTs was conducted according to previously described methods (26, 27). Briefly, *E. coli* cells harboring p*Sn*NTTs were induced with 1 mM IPTG at an optical density at 600 nm (OD_{600}) of 0.5. After 1 h, the cells were pelleted (3,000 × *g*; 5 min; 8°C), resuspended in phosphate buffer (50 mM potassium phosphate, pH 7.0) to an OD_{600} of 5.0, and immediately used for uptake experiments. Furthermore, the time for the conduct of the transport measurements was kept to a minimum to minimize changes in the bacterial metabolite pool and energy state that might influence the biochemical features of the investigated *Sn*NTTs (i.e., activities of proton/nucleotide symporters and K_m and V_{max} values of nucleotide counterexchangers).

Analysis of substrate specificity and uptake kinetics. To analyze the transport properties of *Sn*NTTs, 100 μl of either induced *E. coli* cells harboring p*Sn*NTTs or noninduced cells (control) were added to 100 μl phosphate buffer containing the indicated concentrations of α-^{32}P-labeled nucleotides. Nucleotide uptake into control cells was negligible and comparable to import into *E. coli* cells harboring the empty pET16b vector (data not shown). However, certain nicotinic acid derivatives were imported into control and empty pET16b vector-containing cells. Uptake was allowed at 30°C for the indicated times and was terminated by removal of the external substrate. For this purpose, cells were applied to nitrocellulose filters (0.45-μm pore size), prewetted with phosphate buffer medium, and set under vacuum. The cells were subsequently washed three times with 4 ml phosphate buffer, and the filters were transferred into 5-ml scintillation vessels containing 4 ml water or, in the case of ^{14}C-labeled compounds, 4 ml scintillation cocktail. Radioactivity in the samples was quantified in a scintillation counter (Tricarb 2500; Canberra-Packard).

Back-exchange analysis and thin-layer chromatography. To characterize the transport mode of recombinant *Sn*NTT1, an efflux study was conducted. For preloading, a pellet of 2 ml of IPTG-induced *E. coli* cells synthesizing *Sn*NTT1 was incubated for 5 min in phosphate buffer containing 50 μM radioactively labeled ATP. Subsequently, cells were harvested by centrifugation, washed two times, resuspended in phosphate buffer medium containing 500 μM (10-fold excess) unlabeled nucleotides, and incubated at 30°C for the given time spans. Efflux was terminated by rapid centrifugation, and residual intracellular radioactivity was measured in a scintillation counter.

Molecular characteristics of the radioactively labeled compounds exported by *E. coli* cells expressing *Sn*NTT1 to *Sn*NTT3 were identified by thin-layer chromatography (50). For this, a 10-μl aliquot of the supernatant was loaded onto a 0.5-mm polyethyleneimine cellulose thin-layer chromatography plate and dried with a fan. The chromatograms were developed in closed glass tanks with 0.5 M sodium formate buffer (pH 3.4) for 30 s, followed by 2 M sodium formate buffer (pH 3.4) for 2 min and finally with 4.0 M sodium formate buffer (pH 3.4) until the front nearly reached the top. Retardation factor values of radioactively labeled nucleotides and phosphate were determined after autoradiography and corresponded to the values of unlabeled nucleotides visualized under UV light and to radioactively labeled standards. Radioactively labeled positions were marked on the thin-layer plate, cut out, and quantified in a scintillation counter.

RESULTS

Four putative nucleotide transport proteins in *S. negevensis*. Four genes encoding putative nucleotide transport proteins (*Sn*NTT1 to *Sn*NTT4) were detected in the draft genome se-

quence of *S. negevensis* strain Z (*Sn*NTT1 to *Sn*NTT4). All but one (*Sn*NTT4) of these transporters showed the highest sequence similarities with nucleotide transport proteins from other members of the *Chlamydiae*. Among biochemically characterized NTT proteins, they were most similar to transporters from *P. amoebophila* UWE25. *Sn*NTT1 is identical to a partial sequence of a putative ATP/ADP translocase from *S. negevensis* strain Z (GenBank accession number AAX45330) and exhibits the highest amino acid sequence identity (65%) to *Pam*NTT1, the ATP/ADP translocase from *P. amoebophila* (see Table S2 in the supplemental material). *Sn*NTT3 also exhibits the highest, but less pronounced, amino acid sequence identity (45%) to *Pam*NTT1. *Sn*NTT2 shows the highest amino acid sequence identity (52%) to *Pam*NTT2 from *P. amoebophila*, a nucleotide antiporter. According to a BLASTp search against the nonredundant protein database at the National Center for Biotechnology Information (NCBI) website (3, 78), *Sn*NTT4 is most similar to a number of hypothetical membrane proteins from diverse bacterial species, with amino acid sequence identity values up to 27% (best BLASTp hit, a hypothetical protein of *Croceibacter atlanticus*; NCBI accession no. YP_003716609), indicating that *Sn*NTT4 is more distantly related to the known NTT proteins than *Sn*NTT1 to -3. Among biochemically characterized nucleotide proteins, *Sn*NTT4 exhibits the highest amino acid sequence identity (22%) with *Pam*NTT3, the UTP/H^+ transporter from *P. amoebophila* (see Table S2 in the supplemental material).

Prediction of transmembrane regions (using four different algorithms) indicated the presence of 10 to 12 transmembrane alpha helices for *Sn*NTT1 to *Sn*NTT4 (see Table S3 in the supplemental material). This is consistent with secondary-structure predictions of biochemically characterized nucleotide transport proteins, for which between 11 and 12 transmembrane domains are recognized (7, 26, 43, 63, 67, 75). The only nucleotide transporter whose topology has been investigated experimentally in some detail is the ATP/ADP translocase from *Rickettsia prowazekii*, showing 12 transmembrane domains (1, 2, 6).

In addition, some general characteristics of *Sn*NTT1 to -4 are also in agreement with those of known nucleotide transporters: the *Sn*NTT protein sizes range from 428 to 528 amino acids, with molecular masses from 48.7 to 58.7 kDa, and the predicted pI values vary between 9.4 and 9.6 (see Table S3 in the supplemental material). All four *Sn*NTT proteins contain the conserved domain PF03219 of the PFAM TLC family, which comprises all known NTT proteins (17). *Sn*NTT1 to *Sn*NTT3 possess all of the charged amino acid residues reported to be critical for the function of the plastidic ATP/ADP transporter *At*NTT1 from *Arabidopsis thaliana* (70): the residues K155, E245, E385, and K527 (referring to *At*NTT1 numbering), of which only K527 is conserved in *Sn*NTT4.

All *Sn*NTT genes are transcribed during intracellular growth. To investigate whether the predicted nucleotide transport proteins are expressed during intracellular multiplication of *S. negevensis* in *Acanthamoeba* host cells, we performed reverse transcription-PCR analysis using primer sets targeting fragments of the four *Sn*NTT genes. For each gene examined, a negative control without template and a control to prove the absence of DNA in the RNA extraction (RNA after DNase digestion) were included. This experiment demonstrated that all investigated putative NTT genes are transcribed during growth of *S. negevensis* in *Acanthamoeba* sp. UWC1 (Fig. 1).

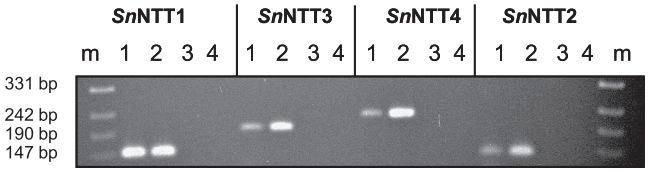

FIG. 1. Transcription of *Sn*NTT1 to *Sn*NTT4 during multiplication of *S. negevensis* within *Acanthamoeba castellani* UWC1. Lanes 1, amplification of cDNA synthesized from whole RNA from amoebae harboring *S. negevensis*; lanes 2, PCR positive control using DNA isolated from *S. negevensis* growing in amoebae; lanes 3, PCR negative control (no cDNA added); lanes 4, PCR using whole RNA from amoebae containing *S. negevensis* (control for the absence of DNA in the purified and DNase-digested RNA); m, molecular size marker.

SnNTT1 to -3 catalyze the import of different nucleotides into *E. coli* cells. In order to elucidate the biochemical properties of the putative nucleotide transporters from *S. negevensis*, we performed import studies with *E. coli* cells expressing the recombinant *Sn*NTT1 to -4, an approach that often has been successfully applied to study the substrate specificities and transport modes of bacterial nucleotide transporters (7, 15, 26, 27, 45, 63, 64, 67, 75). Here, we initially tested whether the four *Sn*NTTs were able to import ATP, which is accepted as a substrate of several other bacterial NTTs. Because *Sn*NTT1 to -3 showed measurable import of [α-^{32}P]ATP, we applied this substrate in a competition experiment (Fig. 2) to get an initial idea about the substrate specificities of the corresponding transporters. For this, [α-^{32}P]ATP uptake was measured in the presence of a 10-fold excess of unlabeled potential substrates and calculated in respect to the unaffected ATP import (set to 100%). Effective competition with ATP import indicates a potential substrate. Favored substrates cause a high reduction of [α-^{32}P]ATP import, whereas no substrates or nucleotides that are not preferentially transported result in no or a lower reduction.

Uptake of [α-^{32}P]ATP by *Sn*NTT1 was significantly reduced (to 9% or 32%) solely by an excess of unlabeled ADP or ATP (Fig. 2A), suggesting that this protein catalyzes the transport of ATP and ADP in a highly specific manner. A substantial inhibitory effect on [α-^{32}P]ATP uptake mediated by *Sn*NTT2 was observed in the presence of unlabeled GTP or GDP (22% or 15.4% of the control, respectively) (Fig. 2B). Furthermore, a pronounced reduction was also obtained with unlabeled GMP (44.5% residual activity) (Fig. 2B), whereas all other tested molecules, including ATP, had less (more than 60% residual activity) or no effect on the import rates of *Sn*NTT2. Thus, we could speculate that guanine nucleotides might be the main substrates of *Sn*NTT2, imported with higher preference than ATP. The strongest inhibition of *Sn*NTT3-mediated [α-^{32}P]ATP uptake was obtained by application of unlabeled UTP and CTP (2.3% and 3.3% residual activity, respectively) (Fig. 2C). Addition of unlabeled GTP and ATP lowered [α-^{32}P]ATP import to a lesser extent (20.9% and 26.9% residual activity, respectively) (Fig. 2C). Interestingly, dCTP greatly reduced ATP import (to 8.8%), whereas the presence of other deoxynucleoside triphosphates (dNTPs) caused only a slight decrease (more than 50% residual activity). Thus, *Sn*NTT3

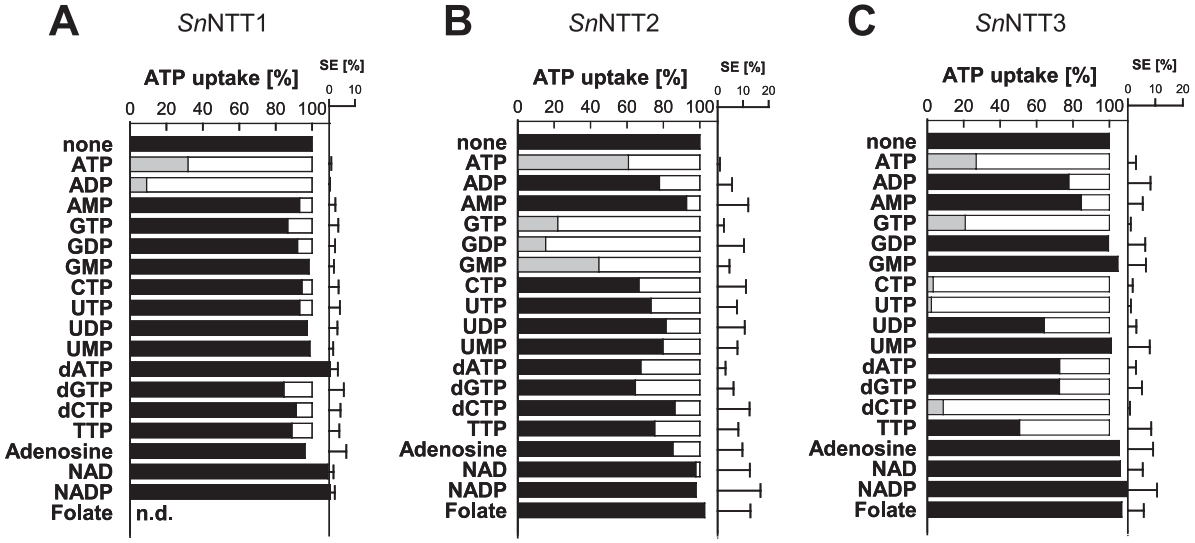

FIG. 2. Effects of various metabolites on [α-^{32}P]ATP import mediated by recombinant *Sn*NTT1 (A), *Sn*NTT2 (B), and *Sn*NTT3 (C). Import of [α-^{32}P]ATP was measured at a concentration of 50 μM, and nucleotides or nucleotide derivatives were applied in 10-fold excess. Rates of [α-^{32}P]ATP uptake are given as percentage of control rates (unaffected net ATP import = 100%). Uptake measurements were carried out at 30°C, and import by *Sn*NTT1, *Sn*NTT2, and *Sn*NTT3 was allowed for 2 min, 5 min, and 10 min, respectively. The data are the means of at least four independent experiments. Standard errors (SE) were below 10% for *Sn*NTT1 (A) and below 20% for *Sn*NTT2 (B) and *Sn*NTT3 (C). n.d., not done.

apparently exhibits a relatively broad substrate spectrum and prefers pyrimidine rather than purine nucleoside triphosphates.

It is important to mention that the addition of unlabeled ATP has multiple effects on [α^{32}P]ATP import: it increases the substrate concentration and thus decreases the specific radioactivity in the sample. However, this approach provides insights into the biochemical properties of the carriers with respect to ATP import. The observed degree of import reduction by ATP (>10% residual activity) indicates that these transporters exhibit a K_m for ATP higher than 50 μM (otherwise, values of ≤10% would be expected). Furthermore, because of the marginal impact of ATP addition on *Sn*NTT2-mediated [α^{32}P]ATP uptake, this carrier apparently exhibits a lower K_m than *Sn*NTT1 or *Sn*NTT3 for ATP import.

To investigate whether the nucleotides that caused the greatest reduction of ATP import act as inhibitors or are in fact substrates of *Sn*NTT1 to -3, we carried out transport measurements with the corresponding labeled isotopes. We performed time course experiments and determined the transport kinetics of each substrate. Import studies with rising concentrations of the respective substrates were applied to calculate affinities (K_m) and maximal reaction velocities (V_{max}).

Time kinetics performed with recombinant *Sn*NTT1 showed that ADP not only is an additional substrate, but also is imported at about 2-fold-higher rates than ATP (Fig. 3A and B, respectively). ATP and ADP uptake was linear for the first 5 min and reached a plateau phase after 15 and 10 min, respectively. The maximal velocities of ATP and ADP uptake were in the same range (344 nmol mg^{-1} protein h^{-1} and 316 nmol mg^{-1} protein h^{-1}, respectively), but the apparent substrate affinities were nearly two times higher for ADP (K_m = 75 μM) than for ATP (K_m = 138 μM) (Table 1).

E. coli cells expressing *Sn*NTT2 showed significant linear GTP and GDP uptake for about the first 10 min (Fig. 3C and D, respectively), whereas ATP import was rather weak (about 1/10 of GTP import) (data not shown). Transport measurements with rising substrate concentrations revealed that *Sn*NTT2 exhibits the highest apparent substrate affinities for GTP (K_m = 179 μM), followed by GDP (K_m = 461 μM) and finally ATP (K_m = 654 μM) (Table 1). The highest V_{max} values were determined for GDP import (219 nmol mg^{-1} protein h^{-1}), whereas the values for GTP and ATP were comparably low (88 nmol mg^{-1} protein h^{-1} and 40 nmol mg^{-1} protein h^{-1}, respectively) (Table 1). According to the effector experiments, *Sn*NTT2 might be able to transport GMP, in addition to GTP and GDP, indicating that *Sn*NTT2 acts mainly as a guanine nucleotide transporter (Fig. 2B). To further test the biochemical properties of *Sn*NTT2, concentration-dependent inhibition of ATP uptake was analyzed (Fig. 4). Consistent with the initial effector analysis, the influence of GMP on the uptake of ATP by *Sn*NTT2 was significant but weaker than that of GTP and GDP (Fig. 2B and 4). This further supports the observation that GTP and GDP are the main substrates of *Sn*NTT2.

E. coli expressing *Sn*NTT3 took up all RNA nucleotides and also dCTP. The rates of CTP and dCTP uptake showed a linear increase during the first 5 to 10 min (Fig. 3E and G, respectively), whereas UTP, GTP, and ATP import rates were linear for a longer time (Fig. 3F, H, and I, respectively). Within 30 min, the highest import rates were measured for UTP and CTP (Fig. 3F and E), followed by dCTP and GTP uptake (Fig. 3G and H), whereas ATP import was comparatively low (Fig. 3I). *Sn*NTT3 displayed a high apparent affinity (K_m = 9 μM) and a moderate maximal velocity (19 nmol mg^{-1} protein h^{-1}) for CTP (Table 1). High-affinity transport mediated by *Sn*NTT3 could also be observed for UTP (K_m = 34 μM) and dCTP (K_m = 42 μM), but maximal velocities were lower than those of CTP uptake (14 and 16 nmol mg^{-1} protein h^{-1}, respectively) (Table 1). *Sn*NTT3 exhibits low affinities for purine nucleoside triphosphate uptake (K_m = 375 and 407 μM).

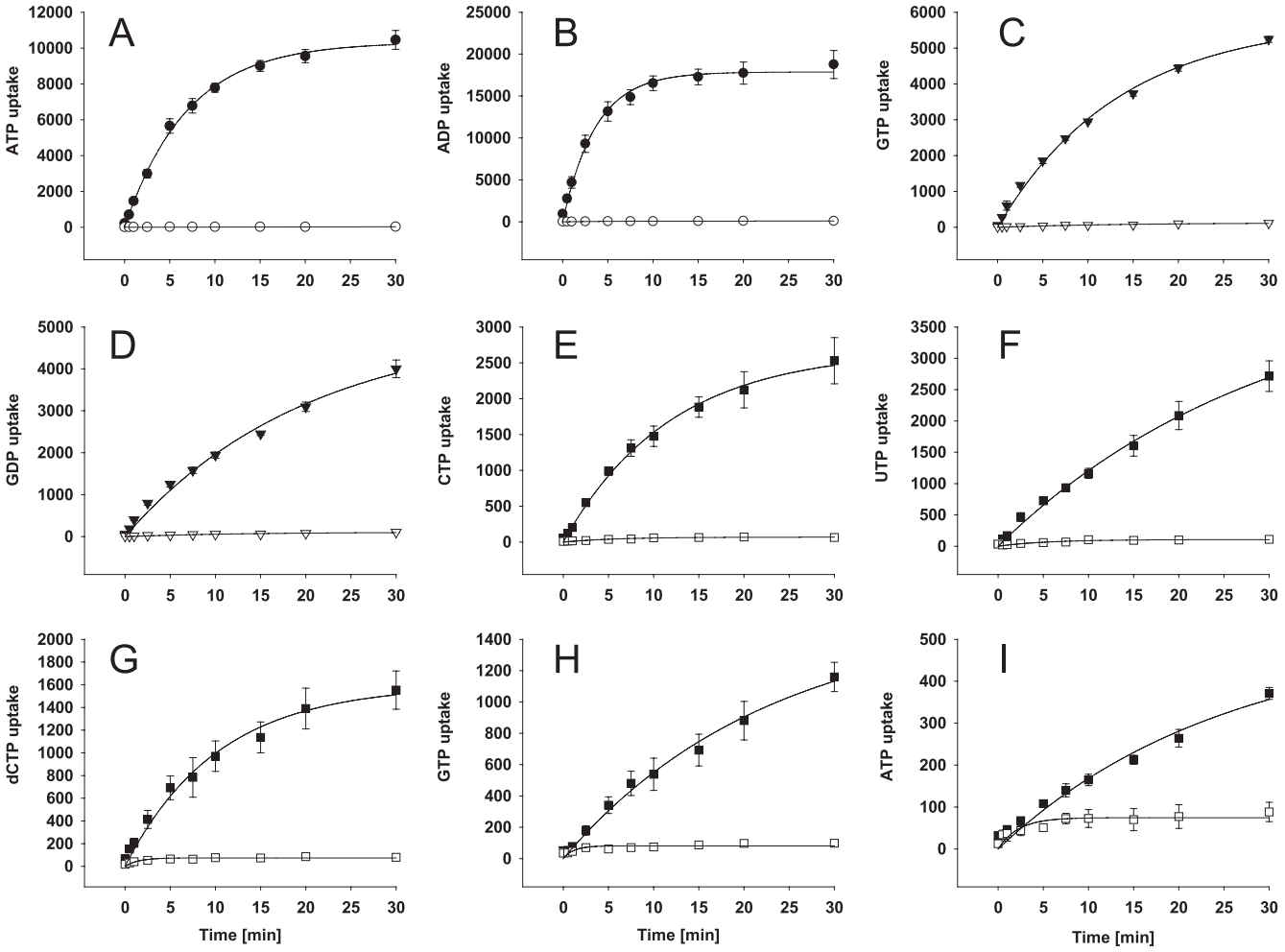

FIG. 3. Time dependency of α-^{32}P-labeled nucleotide uptake into IPTG-induced and noninduced *E. coli* cells. Induced cells harboring the plasmids p*Sn*NTT1 (filled circles), p*Sn*NTT2 (filled triangles), and p*Sn*NTT3 (filled squares) or the corresponding noninduced cells (control; empty symbols) were incubated in phosphate-buffered medium containing 50 μM labeled nucleotide for the indicated times. Nucleotide uptake is given in pmol mg protein^{-1}. The data points and error bars represent the means and standard errors of three independent experiments, respectively. (A and B) Time dependency of [α-^{32}P]ATP (A) and [α-^{32}P]ADP (B) uptake mediated by recombinant *Sn*NTT1. (C and D) Time dependency of [α-^{32}P]GTP uptake (C) and [α-^{32}P]GDP uptake (D) mediated by recombinant *Sn*NTT2. (E to I) Time dependency of [α-^{32}P]CTP uptake (E), [α-^{32}P]UTP uptake (F), [α-^{32}P]dCTP uptake (G), [α-^{32}P]GTP uptake (H), and [α-^{32}P]ATP uptake (I) mediated by recombinant *Sn*NTT3.

Compared to other substrates of *Sn*NTT3, ATP is imported with the lowest and GTP with the highest maximal velocity (10 and 28 nmol mg^{-1} protein h^{-1}, respectively) (Table 1).

Transport modes of *Sn*NTT1 to -3. All NTTs biochemically known so far either catalyze a proton-driven net uptake of nucleotides or act in a counterexchange mode of transport. To get a first impression of the transport modes of *Sn*NTT1 to -3, we analyzed whether transport activity can be inhibited by the addition of the protonophore *m*-chlorophenylhydrazone (CCCP), which would suggest that the nucleotide transport is driven by the proton gradient across the cytoplasmic membrane. Furthermore, efflux studies and back-exchange experiments help to identify a possible counterexchange mode and the nature of exported substrates (26, 63).

Addition of 10 to 500 μM CCCP only slightly reduced *Sn*NTT1-mediated ATP import (Fig. 5). Furthermore, *E. coli* cells expressing *Sn*NTT1 and preloaded with ATP showed a measurable efflux of radioactivity over 30 min when resuspended in buffer medium containing unlabeled ATP or ADP (Fig. 6). In contrast, negligible amounts of exported nucleotides were detected when cells were incubated in buffer medium containing unlabeled AMP or no substrate (Fig. 6). Notably, efflux of labeled nucleotides was higher in the presence of unlabeled ADP than with unlabeled ATP (Fig. 6). This is in agreement with the higher affinity of *Sn*NTT1 for ADP than for ATP (Table 1). Thin-layer chromatography analysis identified ADP as the favored adenine nucleotide exported in the presence of unlabeled substrates (Fig. 7A), followed by ATP and AMP (compared to cells incubated in phosphate buffer alone). Due to its independence of a proton gradient and due to the capacity to export adenine nucleotides only in the presence of suitable import substrates, *Sn*NTT1 can be categorized as an ATP/ADP translocase (with a possible slight export capacity for AMP) belonging to class I nucleotide transporters according to the classification by Haferkamp et al. (26).

In contrast to the *Sn*NTT1-mediated ATP uptake, GTP im-

TABLE 1. Apparent K_m and V_{max} values of *E. coli* cells expressing recombinant *Sn*NTT proteins compared with the characteristics of nucleotide transport proteins from other chlamydiae

Effector	K_m (V_{max}) value[a]							
	ATP/ADP translocase			GTP/ATP/H^+ symporter		RNA nucleotide transporter		
	*Sn*NTT1	*Pam*NTT1	*Ct*NTT1	*Sn*NTT2	*Pam*NTT5	*Sn*NTT3	*Pam*NTT2	*Ct*NTT2
ATP	138 (344)	95 (384)	48 (370)	654 (40)	360 (58)	375 (10)	437 (450)	1,158 (128)
ADP	75 (316)	55 (384)	39 (625)		273 (4)			1,759 (18)
GTP		128 (12)		179 (88)	22 (98)	407 (28)	156 (412)	31 (109)
GDP				461 (219)				
GMP								
CTP						9 (19)	570 (1,920)	528 (164)
UTP						34 (14)	676 (501)	302 (133)
dCTP						42 (16)		

[a] K_m values are given in μM, and V_{max} values (in parentheses) are given in nmol mg protein^{-1} h^{-1}. For the determination of the K_m values, 16 different substrate concentrations (2 μM to 1,500 μM) were used. Because measurements of the different concentrations were performed successively, one-third of the bacteria had to be stored for approximately 10 min. To avoid effects of storage time (possible changes of the internal nucleotide composition) on the biochemical characteristics, measurements were performed two times in parallel, once with increasing and once with decreasing concentrations. The mean of these technical replicates was calculated and represented one biological replicate. Determination of the K_m and V_{max} is based on at least four independent experiments (biological replicates) and was repeated if necessary (until the standard errors were below 10%; standard errors are not shown). K_m and V_{max} values for *Pam*NTT1, *Pam*NTT2, *Pam*NTT5, *Ct*NTT1, and *Ct*NTT2 were taken from Schmitz-Esser et al. (64), Haferkamp et al. (26), and Tjaden et al. (67).

port by *Sn*NTT2 was greatly decreased after addition of the protonophore CCCP (down to 37% and 18% residual activity, respectively) (Fig. 5). This finding suggests a proton-coupled symport mechanism of *Sn*NTT2 and is additionally supported by the thin-layer chromatography of the efflux, showing that negligible amounts of radioactively labeled nucleotides are exported in both the presence and absence of unlabeled substrates (Fig. 7B). *Sn*NTT2 can thus be classified as a class II nucleotide transporter catalyzing a proton-driven unidirectional transport of nucleotides.

The protonophore CCCP had a greater impact on *Sn*NTT3- than on *Sn*NTT1-mediated nucleotide uptake, but this effect was less pronounced than for *Sn*NTT2 (Fig. 5). Therefore, this analysis does not clearly argue for or against proton dependency of the transport. However, back-exchange analysis combined with thin-layer chromatography showed that in the presence of CTP, dCTP, and ATP, *Sn*NTT3-expressing *E. coli* cells preloaded with CTP preferentially exported radioactively labeled CTP (Fig. 7C). The export of CMP and CDP is comparatively low, in particular when release in the absence of substrates is taken into account (Fig. 7C). The back-exchange analysis suggests that *Sn*NTT3 belongs to the class I nucleotide transporters catalyzing a counterexchange transport of nucleoside triphosphates (26). The fact that *Sn*NTT3 accepts triphosphorylated nucleosides as import and most likely also as

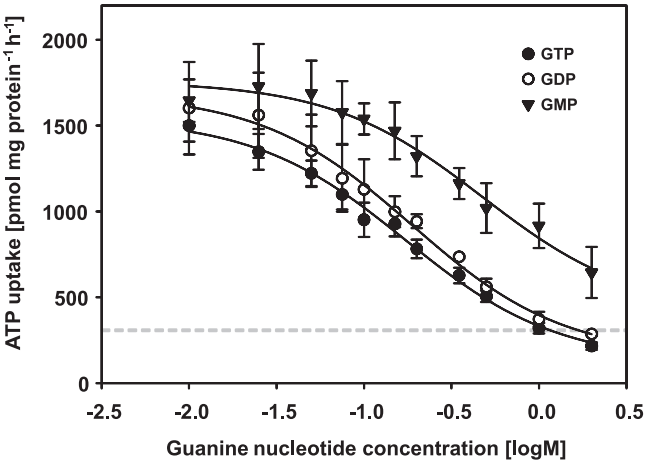

FIG. 4. Influence of increasing guanine nucleotide concentrations on the uptake of [α-^{32}P]ATP by *E. coli* cells expressing *Sn*NTT2. Uptake of 50 μM [α-^{32}P]ATP in the presence of GTP, GDP, and GMP lasted 8 min into the expression of *Sn*NTT2 by *E. coli* cells. The background level uptake of 50 μM [α-^{32}P]ATP into noninduced cells is indicated (gray line). The data points and error bars represent the means and standard errors of three independent experiments, respectively. The data were fitted ($r^2 > 0.99$) by nonlinear regression using the one-site competition equation implemented in the Ligand Binding Module of SigmaPlot software: $f = \min + (\max - \min)/(1 + 10^{x - \log EC50})$.

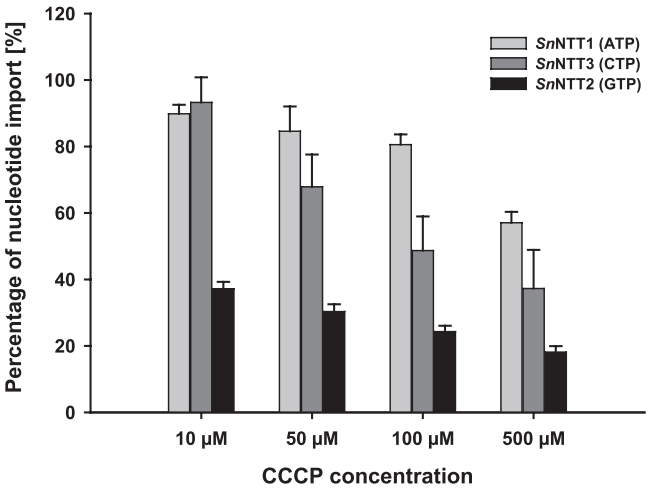

FIG. 5. Effect of the protonophore CCCP on uptake of [α-^{32}P]ATP (*Sn*NTT1), [α-^{32}P]GTP (*Sn*NTT2), and [α-^{32}P]CTP (*Sn*NTT3) into IPTG-induced *E. coli* cells. Uptake of [α-^{32}P]NTP mediated by recombinant *Sn*NTTs was measured at a substrate concentration of 50 μM. The rates of nucleotide uptake are given as percentages of control rates (unaffected transport = 100%). The data are the means of at least three independent experiments. The error bars indicate standard errors.

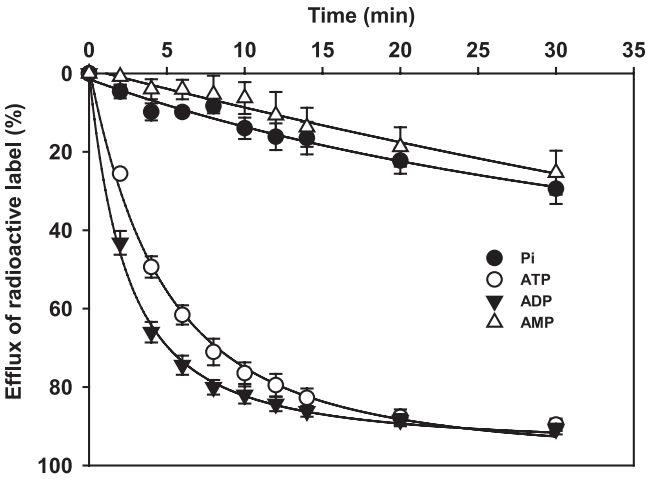

FIG. 6. Back-exchange properties of *Sn*NTT1. Cells harboring p*Sn*NTT1 were induced with IPTG and preloaded with 50 μM [α-^{32}P]ATP. Subsequently, the cells were washed and suspended in phosphate buffer containing either no substrate (control) or 500 μM unlabeled ATP, ADP, or AMP. At the indicated time intervals, efflux was terminated, the residual internal radioactivity was measured, and the fraction of exported radioactive label was calculated as a percentage of the internal label at starting time (0 min). Data points and error bars represent the means and standard errors of three independent experiments, respectively. Pi, inorganic phosphate.

export substrates (Fig. 7C) might be the reason for the slight inhibitory effect of CCCP on nucleotide transport. Destruction of the membrane potential by the protonophore would lead to a decreased cellular energy state, resulting in a lower content of nucleoside triphosphates and thus in reduced availability of counterexchange substrates of *Sn*NTT3.

No transport is detectable for *Sn*NTT4. None of the nucleotides (Fig. 2) was taken up by *E. coli* expressing *Sn*NTT4, although we could confirm by SDS-PAGE and autoradiography that suitable amounts of protein were produced and inserted into the *E. coli* membrane. The highest expression of *Sn*NTT4 was observed for Rosetta cells and cultivation in YT medium (2× yeast extract and tryptone), whereas application of BLR cells and cultivation in Terrific broth (TB) medium led to reduced recombinant protein synthesis (see Fig. S1 in the supplemental material). Because *P. amoebophila* possesses a transporter for NAD^+, we also checked this cofactor as a substrate, as well as nicotinic acid, nicotine amide, and the corresponding mononucleotides as potential substrates, in import studies. No differences in the uptake rates of these substances were measurable between induced and noninduced cells. However, we cannot rule out the possibility that *E. coli* possesses endogenous import systems for certain nicotinic acid derivatives that masked potential additional transport caused by recombinant *Sn*NTT4.

DISCUSSION

Nucleotide transport proteins are key proteins for exploitation of the host cell metabolism. Their evolution dates back several hundred million years, and it has been hypothesized that these transporters (among other proteins) contributed to the emergence of the primary photosynthetic cell (5, 8, 25, 35, 56, 64, 74). Today, they are employed mainly by plastids and two extremely successful groups of intracellular bacterial pathogens and symbionts, rickettsiae and chlamydiae. The chlamydial organism *S. negevensis* encodes four nucleotide transport proteins, all of which are expressed during intracellular growth (Fig. 1), suggesting that they are both functional and important for the intracellular lifestyle of *S. negevensis*.

ATP/ADP translocases are a hallmark of obligate intracellular bacteria belonging to the *Chlamydiae* and *Rickettsiales* and are used to parasitize energy in the form of ATP from the host cell cytosol (15, 25, 45, 48, 64, 67, 79). *S. negevensis* employs an ATP/ADP translocase (*Sn*NTT1) with high amino acid sequence identity to the ATP/ADP translocases from *P. amoebophila* (65%) and *C. trachomatis* (63%) (see Table S2 in the supplemental material). The kinetic uptake parameters of *Sn*NTT1 were similar to those of heterologously expressed *Pam*NTT1 and *Ct*NTT1, with slightly lower affinities for ATP and ADP and a lower transport velocity of ADP observed for *Sn*NTT1 and *Pam*NTT1 than for *Ct*NTT1 (Table 1) (64, 67). Previous studies have shown that characterization of nucleotide transport proteins by reconstitution into proteoliposomes can reveal different values for substrate affinity and transport

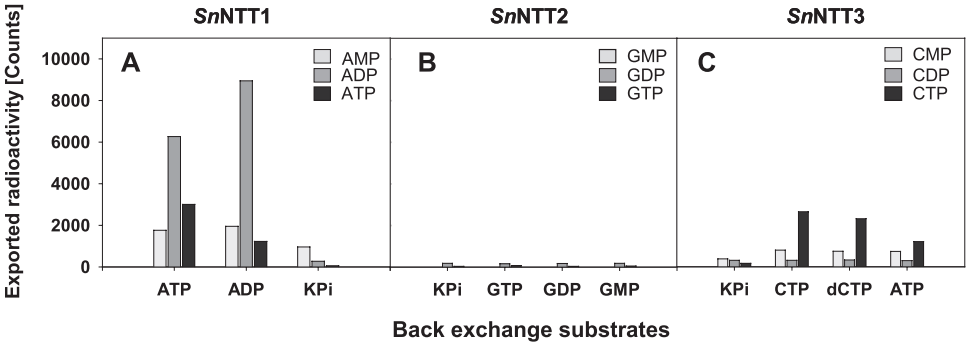

FIG. 7. Thin-layer chromatography analysis of exported nucleotides. *E. coli* cells expressing *Sn*NTT1 to *Sn*NTT3 were preloaded with [α-^{32}P]ATP for *Sn*NTT1 (A), [α-^{32}P]GTP for *Sn*NTT2 (B), and [α-^{32}P]CTP for *Sn*NTT3 (C). Back exchange was initiated with unlabeled substrates, as indicated on the *x* axis. Thin-layer chromatography spots of exported radioactively labeled nucleotides were excised, and radioactivity was quantified with a scintillation counter (indicated on the *y* axis). For the symporter *Sn*NTT2 (B), only negligible amounts of exported nucleotides were detected. The experiment was performed three times for each transporter, and the result of one representative thin-layer chromatography experiment is displayed. KPi, inorganic potassium phosphate.

velocity. The general characteristics, such as the transport mode and substrate specificities, however, were similar in the two experimental approaches (48, 54, 64, 71, 72). Analysis of the draft genome sequence of *S. negevensis* strain Z indicated that it contains a gene set for a functional respiratory chain and ATP synthesis. Like other chlamydiae, *S. negevensis* is thus most likely not auxotrophic for ATP (32, 33, 76). The ATP/ADP translocase activity of *Sn*NTT1 might rather supplement the bacterial energy pool only under certain conditions or at certain stages of the *S. negevensis* developmental cycle.

S. negevensis seems to be unable to synthesize nucleotides *de novo*, a commonly known impairment of nearly all chlamydiae analyzed so far (32, 33, 59, 76). It is thus crucial for *S. negevensis* to import nucleotides from the host cell for the biosynthesis of RNA and DNA. The second nucleotide transporter of *S. negevensis* (*Sn*NTT2) mainly accepts GTP and GDP (Fig. 3C and D) and, to a minor extent, ATP and probably also GMP (Fig. 2B and 4) as substrates. Another nucleotide transporter specific for GTP and GDP has been characterized recently in *R. prowazekii* (7); however, the transport mode of this protein has not been determined. Using the protonophore CCCP (Fig. 5) and back-exchange experiments (Fig. 7B), we could show a proton-coupled uniport transport mechanism for *Sn*NTT2. The transport mode and the substrate specificity of *Sn*NTT2 are similar to those of *Pam*NTT5 from *P. amoebophila* (26), which also mediates proton-coupled import of GTP and ATP. In contrast to *Sn*NTT2, *Pam*NTT5 did not accept GDP as a main import substrate but showed slight ADP uptake with very low maximal velocity (26). Interestingly, *Sn*NTT2 exhibits the highest amino acid sequence identity to *Pam*NTT2 (52%), a nucleotide antiporter, and the amino acid sequence identity of *Sn*NTT2 to *Pam*NTT5 is significantly lower at 37% (see Table S2 in the supplemental material). Sequence similarity alone thus cannot be used to infer the substrate specificities and transport modes of nucleotide transport proteins (5, 26).

*Sn*NTT3 transports all RNA nucleotides via a proton-independent exchange mode, with the highest affinities for pyrimidine nucleotides, suggesting a transport preference for the latter (Fig. 2C, 3E to I, 5, and 7C and Table 1). Transport of all RNA nucleotides was also shown for *Pam*NTT2 (26) and for *Ct*NTT2 (67). Like *Pam*NTT2, but in contrast to *Ct*NTT2, *Sn*NTT3 belongs to the class I NTTs, comprising NTTs with an antiport transport mode (26). Interestingly, *Sn*NTT3 also transports dCTP with high affinity, albeit with a low transport velocity (Fig. 3G and Table 1). So far, transport of dCTP has not been observed for bacterial nucleotide transport proteins. Thus, *Sn*NTT3 is the first prokaryotic transport protein for which import of dCTP has been reported, and indeed, measured dCTP concentrations in eukaryotes (0.008 to 0.05 mM intracellular concentration [52, 69]) and prokaryotes (0.06 to 0.2 mM intracellular concentration [10, 11, 51]) fit the apparent affinity of *Sn*NTT3 for dCTP (K_m = 0.042 mM), suggesting that dCTP transport is possible under *in vivo* conditions. dCTP can serve as a precursor for the synthesis of thymidine nucleotides (77). The *Sn*NTT3-mediated import of dCTP should thus provide an advantage for *S. negevensis*, although these bacteria might also be able to generate their own dCTP: *S. negevensis* encodes a class I ribonucleotide reductase (RNR) (EC 1.17.4.1) that is highly similar to the characterized *C. trachomatis* RNR (31, 60). Functional characterization of the *C. trachomatis* RNR revealed the specific reduction of CDP to dCDP, while CMP and CTP were not used as substrates (31, 60). Similarly, *S. negevensis* could catalyze the deoxygenation of CDP, followed by the phosphorylation of dCDP to obtain dCTP, which is catalyzed by the nucleoside diphosphate kinase (NDK) (EC 2.7.4.6).

No transport substrate could be identified for the fourth putative nucleotide transporter (*Sn*NTT4) of *S. negevensis*, in spite of testing several nucleotides as possible substrates and despite evidence that this transporter was produced and inserted into the *E. coli* membrane in sufficient amounts (see Fig. S1 in the supplemental material). Among characterized nucleotide proteins, *Sn*NTT4 shows the highest amino acid sequence similarity (22%) to *Pam*NTT3, a UTP/H^+ symporter (see Table S2 in the supplemental material). However, the protein length and molecular weight, the number of predicted transmembrane helices, and the hydrophobicity plot profiles of *Sn*NTT4 are more similar to those of the NAD^+ transporter of *P. amoebophila* (*Pam*NTT4) (see Fig. S2 and Table S3 in the supplemental material). Similar to that transporter, *Sn*NTT4 lacks three of the conserved amino acids known to be critical for nucleotide transport in ATP/ADP translocases (K155, E245, and E385 in *A. thaliana* NTT1 numbering [70]). This might indicate that *Sn*NTT4 does not act as a typical nucleotide transporter but, similar to *Pam*NTT4, as an NAD^+ transporter. However, we were not able to measure the transport of NAD^+ or its precursors, and preliminary analysis of the draft genome sequence of *S. negevensis* indicated the presence of a complete *de novo* synthesis pathway for NAD^+. *S. negevensis* thus should not be dependent on the import of this essential cofactor from its host. It remains unclear why no substrate could be identified for *Sn*NTT4. Possible explanations are (i) that the appropriate substrate was not tested, (ii) that *Sn*NTT4 is not functional in the heterologous host *E. coli* (although the protein is present in the *E. coli* membrane), (iii) that *Sn*NTT4 is driven by a gradient that was not applied and investigated, or (iv) that *Sn*NTT4 requires a counterexchange substrate that is missing or that exists in insufficient amounts in *E. coli*. Previous investigations of diverse nucleotide transport proteins heterologously expressed in *E. coli* demonstrated that (at least) all DNA and RNA nucleotides, as well as NAD^+, exist in *E. coli* cells in sufficient amounts to act as counterexchange substrates (5, 26). Although cultivation of the *E. coli* cells, protein expression, and import measurements were optimized to minimize changes of the energy state and metabolite composition during uptake experiments, we cannot fully exclude the possibility that these effects played a role. Another possible explanation for why no substrate could be identified for *Sn*NTT4 could be that, unlike other nucleotide transporters characterized in *E. coli*, *Sn*NTT4 requires posttranslational modifications that the heterologous host cannot perform. It should be noted that two of the five putative nucleotide transporters from *R. prowazekii* also failed to take up any of the nucleoside mono-, di-, and triphosphates tested, although the presence of mRNA of those transporters suggested that they are required during intracellular growth (7).

S. negevensis is the third chlamydial species whose set of nucleotide transport proteins has been characterized biochemically. The conservation of these transporters among four major chlamydial lineages, the *Chlamydiaceae*, the *Parachlamydi-*

aceae, the *Waddliaceae*, and the *Simkaniaceae*, is consistent with the notion that nucleotide transporter proteins were already present in the chlamydial ancestor (9, 25, 63, 64). The most likely explanation for the occurrence of multiple nucleotide transporter isoforms is that during evolution gene duplication events led to the emergence of the gene copies observed in extant chlamydiae. Interestingly, the proliferation of nucleotide transporters differs among the chlamydial families. The pathogenic *Chlamydiaceae* employ two nucleotide transporter isoforms (67, 76); the *Parachlamydiaceae*, represented by *P. amoebophila* and *Parachlamydia acanthamoebae* strain Hall's coccus (24, 33), together with *Waddliaceae* (9), encode five isoforms; and the *Simkaniaceae* encode four isoforms. These differences most likely reflect the different functional constraints during the evolution of the different chlamydial lineages in their phylogenetically highly diverse hosts.

All extant chlamydiae encode a functional ATP/ADP translocase and thus exploit their host cell's energy pool. Additionally, they employ a nucleotide transporter accepting all RNA nucleotides, although the transport modes of these proteins differ. NTT2 from *C. trachomatis* (*Ct*NTT2) is driven by a proton gradient and thus directly allows net uptake of all RNA nucleotides. The RNA nucleotide transporters of *S. negevensis* (*Sn*NTT3) and *P. amoebophila* (*Pam*NTT2), however, act in a counterexchange mode of transport, and therefore, nucleotide import is always coupled with the simultaneous release of nucleotides. Accordingly, *P. amoebophila* and *S. negevensis* rely on net uptake of at least one type of nucleotide that can act as an export substrate of the RNA nucleotide counterexchanger and that drives the import of other required nucleotides. A GTP/ATP/H^+ symporter (*Pam*NTT5) and a uridine nucleotide/H^+ symporter (*Pam*NTT3) are present in *P. amoebophila*, and the imported GTP, ATP, and UTP can be exported by *Pam*NTT2 to gain CTP and, if required, also other nucleoside triphosphates. Because of the moderate affinity and the high transport velocity, CTP might be the favored import substrate of *Pam*NTT2, whereas the high affinity for GTP might also allow significant GTP uptake (Table 1) (26). *S. negevensis* possesses only one proton-driven transporter that is specific for GTP, GDP, and ATP (*Sn*NTT2). Imported GTP and ATP can be used as counterexchange substrates to allow uptake of pyrimidine nucleotides by *Sn*NTT3. This physiological function might be supported by the fact that *Sn*NTT3 imports all nucleoside triphosphates with similar velocities but exhibits remarkably high affinities ($K_m < 50$ μM) for CTP, UTP, and dCTP uptake compared to other substrates of *Sn*NTT3, to *Pam*NTT2, or to the proton-driven RNA nucleotide importer *Ct*NTT2 (Table 1).

So far, only *P. amoebophila* employs a transporter specific for NAD^+ (*Pam*NTT4), and the transport of dCTP is restricted to *S. negevensis* (*Sn*NTT3). With respect to nucleotide import, the *Chlamydiaceae* thus encode the smallest and functionally least redundant set of nucleotide transport proteins, while *P. amoebophila* and *S. negevensis*, consistent with their larger genome sizes, maintained or established a larger set of these transporters.

In conclusion, we identified four new nucleotide transport proteins in *S. negevensis* and successfully determined the transport characteristics of three of them. The characterized nucleotide transport proteins allow *S. negevensis* to efficiently parasitize its host's energy and nucleotide pools, thereby compensating for its reduced biosynthetic capabilities. The availability of a larger set of biochemically well-characterized and phylogenetically related nucleotide transport proteins with different specific transport properties will help to better investigate and understand the structure-function relationships of this unique class of proteins.

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