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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 (SnNTT1 to SnNTT4), 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 SnNTT1 functions as an ATP/ADP antiporter, SnNTT2 as a guanine nucleotide/ATP/H+ symporter driven by the membrane potential, and SnNTT3 as a nucleotide triphosphate antiporter. In addition, SnNTT3 is able to transport dCTP, which has not been shown for a prokaryotic transport protein before. No substrate could be identified for SnNTT4. 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 Lawsonia 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+ (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), among these novel Chlamydiae 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 (PamNTT1) to parasitize the host energy pool, a GTP/ATP/H+ symporter (PamNTT5), a UTP/H+ symporter (PamNTT3), a nucleoside triphosphate antiporter balancing the bacterial nucleotide pool (PamNTT2), and a transporter providing NAD+ in exchange for ADP (PamNTT4). 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
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 (SnNTT1 to SnNTT4), and all except one showed the highest sequence similarity to chlamydial NTTs. Nucleotide transport measurements performed with all four *SnNTT* 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 ABB software package (49). Prediction of transmembrane regions was done using ConPred II (4), the PHDhtm program (61) at the PredictProtein search (J. Craig Venter Institute [JCVI]), automatically annotated using the VR-1471 genome sequence was obtained from The Institute for Genomic Research (26).

**Cultivation, DNA isolation, PCR, and cloning.** The *S. negevensis* strain used for this study was kindly provided by Klaus Henning (Institut für Epidemiologie, Friedrich-Ludolf-Institut, Würzburg, Germany) (30). *S. negevensis* was grown in *Acanthamoeba* sp. strain UWC1 (21) at 28°C in peptone-yeast-glucose (PYG) broth containing 20 g peptone, 18 g glucose, 2 g yeast extract, 1 g sodium citrate, 980 mg MgSO₄, 7H₂O, 355 mg Na₂HPO₄, 7H₂O, 340 mg KH₂PO₄, 20 mg Fe(NH₄)₂(SO₄)₂, 0.5 mg FeCl₃, 1 mg EDTA per liter of distilled water at pH 6.5. Supplemental RNA extractions from amoeba 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 (16, 38, 41).

**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-Buster Fast Prep FP120 Instrument (Bio 101). For RNA precipitation—in addition to the isopropl 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 pSnNTT1 to pSnNTT4, the *E. coli* strain BLR(DE3) was used, and for heterologous expression of pSnNTT4, BLR(DE3) and Rosetta2 cells were used. Synthesis of recombinant NT Ts was conducted according to previously described methods (26, 27). Briefly, *E. coli* cells harboring pSnNTTs were induced with 1 mM IPTG at an optical density at 600 nm (OD₆₀₀) 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 OD₆₀₀ 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 NT Ts (i.e., activities of proton/nucleotide symporters and Km and Vₘₐₓ values of nucleotide counterexchangers).

**Analysis of substrate specificity and uptake kinetics.** To analyze the transport properties of pSnNTTs, 100 μl of either induced *E. coli* cells harboring pSnNTTs or noninduced cells (control) were added to 100 μl phosphate buffer containing the indicated concentrations of 32P-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 14C-labeled compounds, 4 ml scintillation cocktail. Radioactivity in the samples was quantified in a scintillation counter (Tri Carb 2500; Canberra-Packard).

**Back-exchange analysis and thin-layer chromatography.** To characterize the transport mode of recombinant SnNTT1, an efflux study was conducted. For this purpose, a pellet of 2 ml *S. negevensis* strain Z (16, 38, 41) from which the SnNTT1 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 SnNTT1 to SnNTT4 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 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 (*SnNTT1* to *SnNTT4*) were detected in the draft genome se-
quency of *S. negevensis* strain Z (SnNTT1 to SnNTT4). All but one (SnNTT4) 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. SnNTT1 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 PamNTT1, the ATP/ADP translocase from *P. amoebophila* (see Table S2 in the supplemental material). SnNTT3 also exhibits the highest, but less pronounced, amino acid sequence identity (45%) to PamNTT1. SnNTT2 shows the highest amino acid sequence identity (52%) to PamNTT2 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), SnNTT4 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 SnNTT4 is more distantly related to the known NTT proteins than SnNTT1 to -3. Among biochemically characterized nucleotide transporters, SnNTT4 exhibits the highest amino acid sequence identity (22%) with PamNTT3, 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 SnNTT1 to SnNTT4 (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 SnNTT1 to -4 are also in agreement with those of known nucleotide transporters: the SnNTT protein sizes range from 428 to 528 amino acids, with molecular masses of 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 SnNTT proteins contain the conserved domain PF03219 of the PFAM TLC family, which comprises all known NTT proteins (17). SnNTT1 to SnNTT3 possess all of the charged amino acid residues reported to be critical for the function of the plastidic ATP/ADP transporter AtNTT1 from *Arabidopsis thaliana* (70); the residues K155, E245, E385, and K527 (referring to AtNTT1 numbering), of which only K527 is conserved in SnNTT4.

All SnNTT 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 SnNTT genes. For each gene examined, a negative control without template and a control to prove the presence 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).

**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 SnNTT1 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 SnNTTs were able to import ATP, which is accepted as a substrate of several other bacterial NTTs. Because SnNTT1 to -3 showed measurable import of [α-32P]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-32P]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. Favorable substrates cause a high reduction of [α-32P]ATP import, whereas no substrates or nucleotides that are not preferentially transported result in no or a lower reduction.

Uptake of [α-32P]ATP by SnNTT1 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-32P]ATP uptake mediated by SnNTT2 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 SnNTT2. Thus, we could speculate that guanine nucleotides might be the main substrates of SnNTT2, imported with higher preference than ATP. The strongest inhibition of SnNTT3-mediated [α-32P]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-32P]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, SnNTT3.
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 [$\alpha$-$^32$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 relative to ATP import (Km/H9251 by ATP (10% residual activity) indicates that these transmembrane carriers exhibit a lower Km value for ATP uptake than for ATP (Km/H9251 = 179 μM, followed by GDP (Km/H9251 = 461 μM) and finally ATP (Km/H9251 = 654 μM) (Table 1). The highest Vmax 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, SnNTT2 might be able to transport GMP, in addition to GTP and GDP, indicating that SnNTT2 acts mainly as a guanine nucleotide transporter (Fig. 2B). To further test the biochemical properties of SnNTT2, 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 SnNTT2 was significant but weaker than that of GTP and GDP (Fig. 2B and 4). This further supports the observation that ATP and GDP are the main substrates of SnNTT2.

E. coli expressing SnNTT3 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 F, respectively), whereas UTP, GTP, and ATP import rates were linear for a longer time (Fig. 3G, 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). SnNTT3 displayed a high apparent affinity (Km/H9251 = 9 μM) and a moderate maximal velocity (19 nmol mg$^{-1}$ protein h$^{-1}$) for CTP (Table 1). High-affinity transport mediated by SnNTT3 could also be observed for UTP (Km/H9251 = 34 μM) and dCTP (Km/H9251 = 42 μM), but maximal velocities were lower than those of CTP uptake (14 and 16 nmol mg$^{-1}$ protein h$^{-1}$, respectively) (Table 1). SnNTT3 exhibits low affinities for purine nucleoside triphosphate uptake (Km/H9251 = 375 and 407 μM).
Compared to other substrates of SnNTT3, 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 SnNTT1 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 SnNTT1 to -3, we analyzed whether transport activity can be inhibited by the addition of the protonophore m-chlorophenylhydrazine (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 SnNTT1-mediated ATP import (Fig. 5). Furthermore, E. coli cells expressing SnNTT1 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 SnNTT1 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, SnNTT1 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 SnNTT1-mediated ATP uptake, GTP imp-

FIG. 3. Time dependency of $\alpha^{32}$P-labeled nucleotide uptake into IPTG-induced and noninduced E. coli cells. Induced cells harboring the plasmids pSNNT1 (filled circles), pSNNT2 (filled triangles), and pSNNT3 (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 $[\alpha^{32}$P]ATP (A) and $[\alpha^{32}$P]ADP (B) uptake mediated by recombinant SnNTT1. (C and D) Time dependency of $[\alpha^{32}$P]GTP uptake (C) and $[\alpha^{32}$P]GDP uptake (D) mediated by recombinant SnNTT2. (E to I) Time dependency of $[\alpha^{32}$P]CTP uptake (E), $[\alpha^{32}$P]UTP uptake (F), $[\alpha^{32}$P]dCTP uptake (G), $[\alpha^{32}$P]GTP uptake (H), and $[\alpha^{32}$P]ATP uptake (I) mediated by recombinant SnNTT3.
port by SnNTT2 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 SnNTT2 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). SnNTT2 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 SnNTT3-than on SnNTT1-mediated nucleotide uptake, but this effect was less pronounced than for SnNTT2 (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, SnNTT3-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 SnNTT3 belongs to the class I nucleotide transporters catalyzing a counterexchange transport of nucleoside triphosphates (26). The fact that SnNTT3 accepts triphosphorylated nucleosides as import and most likely also as

### Table 1. Apparent $K_m$ and $V_{\text{max}}$ values of E. coli cells expressing recombinant SnNTT proteins compared with the characteristics of nucleotide transport proteins from other chlamydiae

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<thead>
<tr>
<th>Effector</th>
<th>ATP/ADP translocase</th>
<th>GTP/ATP/H$^+$ symporter</th>
<th>RNA nucleotide transporter</th>
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<tr>
<td></td>
<td>SnNTT1</td>
<td>PamNTT1</td>
<td>CNTT1</td>
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<tr>
<td>ATP</td>
<td>138 (344)</td>
<td>95 (384)</td>
<td>48 (370)</td>
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<td>ADP</td>
<td>75 (316)</td>
<td>55 (384)</td>
<td>39 (625)</td>
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<tr>
<td>GTP</td>
<td>128 (12)</td>
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$K_m$ values are given in µM, and $V_{\text{max}}$ values (in parentheses) are given in nmol mg protein$^{-1}$·h$^{-1}$. For the determination of the $K_m$, 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 $V_{\text{max}}$ 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 $V_{\text{max}}$ values for PamNTT1, PamNTT2, PamNTT5, CNTT1, and CNTT2 were taken from Schmitz-Esser et al. (64), Haferkamp et al. (26), and Tjaden et al. (67).
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 the membrane potential by the protonophore would lead to a decreased cellular energy state, resulting in a lower content

FIG. 6. Back-exchange properties of SnNTT1. Cells harboring pSnNTT1 were induced with IPTG and preloaded with 50 µM [α-32P]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.

FIG. 7. Thin-layer chromatography analysis of exported nucleotides. E. coli cells expressing SnNTT1 to SnNTT3 were preloaded with [α-32P]ATP for SnNTT1 (A), [α-32P]GTP for SnNTT2 (B), and [γ-32P]CTP for SnNTT3 (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 SnNTT2 (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.

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 (SnNTT1) 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 SnNTT1 were similar to those of heterologously expressed PamNTT1 and CtNTT1, with slightly lower affinities for ATP and ADP and a lower transport velocity of ADP observed for SnNTT1 and PamNTT1 than for CtNTT1 (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 to reduced recombinant protein synthesis (see Fig. S1 in the supplemental material). Because P. amoebophila possesses a transporter for NADP+, 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 SnNTT4.
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 SnNTT1 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 (SnNTT2) 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 SnNTT2. The transport mode and the substrate specificity of SnNTT2 are similar to those of PamNTT5 from P. amoebophila (26), which also mediates proton-coupled import of GTP and ATP. In contrast to SnNTT2, PamNTT5 did not accept GDP as a main import substrate but showed slight ADP uptake with very low maximal velocity (26). Interestingly, SnNTT2 exhibits the highest amino acid sequence identity to PamNTT5 (52%), a nucleotide antiporter, and the amino acid sequence identity of SnNTT2 to PamNTT5 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).

SnNTT3 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 PamNTT2 (26) and for ChtNTT2 (67). Like PamNTT2, but in contrast to ChtNTT2, SnNTT3 belongs to the class I NTTs, comprising NTTs with an antiport transport mode (26). Interestingly, SnNTT3 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, SnNTT3 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 SnNTT3 for dCTP ($K_a = 0.042$ 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 SnNTT3-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 (SnNTT4) 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, SnNTT4 shows the highest amino acid sequence similarity (22%) to PamNTT3, 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 SnNTT4 are more similar to those of the NAD$^+$ transporter of P. amoebophila (PamNTT4) (see Fig. S2 and Table S3 in the supplemental material). Similar to that transporter, SnNTT4 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 SnNTT4 does not act as a typical nucleotide transporter but, similar to PamNTT4, 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 SnNTT4. Possible explanations are (i) that the appropriate substrate was not tested, (ii) that SnNTT4 is not functional in the heterologous host E. coli (although the protein is present in the E. coli membrane), (iii) that SnNTT4 is driven by a gradient that was not applied and investigated, or (iv) that SnNTT4 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 SnNTT4 could be that, unlike other nucleotide transporters characterized in E. coli, SnNTT4 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-
Parasitizing 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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