Chlamydia trachomatis Transports NAD via the Npt1 ATP/ADP Translocase

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Obligate intracellular bacteria comprising the order *Chlamydiales* lack the ability to synthesize nucleotides *de novo* and must acquire these essential compounds from the cytosol of the host cell. The environmental protozoan endosymbiont *Protochlamydia amoebophila* UWE25 encodes five nucleotide transporters with specificities for different nucleotide substrates, including ATP, GTP, CTP, UTP, and NAD. In contrast, the human pathogen *Chlamydia trachomatis* encodes only two nucleotide transporters, the ATP/ADP translocase *C. trachomatis* Npt1 (Npt1_{Ct}) and the nucleotide uniporter Npt2_{Ct}, which transports GTP, UTP, CTP, and ATP. The notable absence of a NAD transporter, coupled with the lack of alternative nucleotide transporters on the basis of bioinformatic analysis of multiple *C. trachomatis* genomes, led us to re-evaluate the previously characterized transport properties of Npt1_{Ct}. Using [adenylate-³²P]NAD, we demonstrate that Npt1_{Ct} expressed in *Escherichia coli* enables the transport of NAD with an apparent K_m and V_{max} of 1.7 μ M and 5.8 nM $mg^{-1} h^{-1}$, respectively. The K_m for NAD transport is comparable to the K_m for ATP transport of 2.2 μ M, as evaluated in this study. Efflux and substrate competition assays demonstrate that NAD is a preferred substrate of Npt1_{Ct} compared to ATP. These results suggest that during reductive evolution, the pathogenic chlamydiae lost individual nucleotide transporters, in contrast to their environmental endosymbiont relatives, without compromising their ability to obtain nucleotides from the host cytosol through relaxation of transport specificity. The novel properties of Npt1_{Ct} and its conservation in chlamydiae make it a potential target for the development of antimicrobial compounds and a model for studying the evolution of transport specificity.

Obligate intracellular bacteria reside within a unique niche that provides access to numerous essential compounds. This environment allows bacteria to evolve different transport mechanisms to obtain select essential nutrients from their host while subsequently reducing their genomes by eliminating more taxing and redundant biosynthetic pathways (1). One striking example is the loss of the ability to synthesize nucleotides *de novo* (with the exception of CTP, which may be synthesized from UTP) by the Gram-negative bacteria comprising the order *Chlamydiales* (2). This order includes the highly relevant human pathogens *Chlamydia trachomatis* and *Chlamydia pneumoniae*, as well as a variety of pathogens causing disease in domestically important animal species (3). These pathogens (and their environmental relatives) undergo a unique development cycle transitioning between the metabolically limited, infectious elementary body (EB) and the replicative intracellular reticulate body (RB) (4). RBs reside within a host-derived membrane vacuole termed an inclusion. Essential compounds for which chlamydiae are auxotrophic must move from the host cell cytoplasm through the inclusion and subsequently into the bacterium. Accordingly, chlamydiae have been shown to alter host cell vesicle trafficking patterns and may be able to subvert host cell transporters for their own benefit (5, 6).

Studies characterizing the nucleotide transporter properties of the chlamydial ancestor *Protochlamydia amoebophila* UWE25, an environmental amoeboid symbiont, have revealed the presence of five different proteins, PamNTT1 through PamNTT5, belonging to the nucleotide transporter (NTT) family (7). NTTs may be divided into three different classes, nucleotide antiporters (class I), proton-driven nucleotide symporters (class II), and NAD/ADP antiporters (class III) (7). NTTs appear to be restricted to a limited number of obligate intracellular bacteria other than the members of the order *Chlamydiales*, including members of the order *Rickettsiales*, *Lawsonia* spp., and “*Candidatus* Liberbacter spp.,” as well

as plastids, which all possess ATP/ADP translocases (class I NTTs), presumably to acquire energy from their host (8–12). To date, class II and III NTTs have been characterized only in *Chlamydia trachomatis* and *P. amoebophila* (7, 10, 13). While NTTs may be identified in genomes because of their amino acid conservation and the presence of 9 to 12 transmembrane regions, nucleotide transport specificity and class type cannot be predicted through sequence analysis and require experimental validation.

Chlamydia spp. possess two characterized NTTs, designated Npt1_{Ct} and Npt2_{Ct} in the case of the *C. trachomatis* homologs, which act as an ATP/ADP translocase (class I) and as a nucleotide symporter (GTP, UTP, CTP, and ATP, class II), respectively (10). These transporters presumably compensate for the inability of chlamydiae to synthesize these compounds *de novo* (14). Similar to *P. amoebophila*, *Chlamydia* spp. also lack the ability to synthesize NAD *de novo* from L-aspartate or through salvage pathways. NAD is an essential universal electron carrier and coenzyme involved in metabolic redox reactions (when in its NADP form). *P. amoebophila* possesses a class III NTT, PamNTT4, to obtain NAD from the host cytoplasm (13). The absence of NAD synthetic pathways, coupled with the paucity of NTTs in chlamydiae compared

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to its environmental ancestor, led us to hypothesize that Npt1_{Ct}, which, like PamNTT4, functions as a nucleotide antiporter and can transport ADP, developed relaxed specificity allowing it to transport both ATP and NAD in exchange for ADP.

Using a recombinant *Escherichia coli* strain producing Npt1_{Ct} (CTL0321, *C. trachomatis* 434/Bu), we demonstrate the ability of this NTT to transport both ATP and NAD in exchange for ADP. On the basis of competition assay results and transport affinity, Npt1_{Ct} appears to have a moderately higher affinity for NAD than for ATP (although physiologically both may serve as substrates). Our findings report the first NTT capable of transporting both ATP and NAD and further illustrate the unpredictable transport properties of NTTs. The novel transport properties of Npt1_{Ct} and its conservation across the pathogenic *Chlamydia* spp. make it a unique target for the development of antimicrobial compounds.

MATERIALS AND METHODS

Bacterial strains. *C. trachomatis* 434/Bu was grown in L2 mouse fibroblasts as previously described (15). Genomic DNA used in subsequent PCRs was then extracted from EBs with the DNeasy Tissue kit (Qiagen, Valencia, CA) as directed by the manufacturer. *E. coli* DH5 α was used for cloning procedures and cultured in Luria-Bertani (LB) medium with shaking or on LB agar plates at 37°C with 100 μ g/ml ampicillin for plasmid selection. *E. coli* OverExpress C43(DE3) (Lucigen, Middleton, WI), used for protein expression and transport experiments, was routinely cultured in 2 $\times$ YT medium (8 g tryptone, 5 g yeast extract, and 5 g NaCl per liter adjusted to pH 7.0) with shaking or on LB agar plates at 37°C. The growth medium used for C43(DE3) was supplemented with 100 μ g/ml ampicillin for the selection of plasmids and 1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG) for protein production experiments.

Vector and recombinant strain construction. CTL0321 (encoding Npt1_{Ct}) was PCR amplified from purified *C. trachomatis* 434/Bu genomic DNA with Platinum Taq DNA polymerase (Invitrogen, Life Technologies, Grand Island, NY) and reaction components as directed. Deoxynucleoside triphosphates were obtained from Fermentas, Thermo Fisher Scientific (Glen Burnie, MD). Primers 321F (5'-CAAGCATATGACTCA AACC GCGG AAAAACC-3' [NdeI site underlined]) and 321R (5'-GCCG GATCCTTAAGAAACACCTTCT ATAGC-3' [BamHI site underlined]) were used to amplify CTL0321 from template DNA under the following thermal cycler conditions: 94°C for 2 min; 25 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 2 min; and a final extension at 68°C for 5 min. PCR products were then ligated into pGEM-T as directed (Promega, Madison, WI), and ligation reactions were used to transform *E. coli* DH5 α via electroporation. Transformants were selected on LB agar with 100 μ g/ml ampicillin. Clones were PCR screened for inserts with primers 321F/321R, and positive clones were grown for plasmid isolation with the QIAprep Miniprep kit (Qiagen). Gene inserts were removed from the pGEM-T backbone by digestion with NdeI and BamHI (New England BioLabs, Beverly, MA) and ligated into the similarly digested pET-19b expression vector (EMD4Biosciences, Gibbstown, NJ) with T4 DNA ligase (New England BioLabs) to create pET-19bNpt1_{Ct}, which encodes T7 promoter-regulated, N-terminally decahistidine-tagged CTL0321. DH5 α was transformed with the ligation products via electroporation, and transformants were selected on LB agar plates with ampicillin. Clones were PCR screened for the presence of CTL0321 with primers 321F/321R, and a single clone was selected for plasmid isolation and sequence verification with primers T7proseq (5'-GATATAGGCGCCAGCAACCG-3') and T7terseq (5'-CT TGGTTATGCCGGTACTGCC-3'). *E. coli* C43(DE3) was then transformed via electroporation with either pET-19bNpt1_{Ct} (creating ATM1173) or pET-19b to create a control strain (ATM1172).

NAD transport assays with ATM1173. (i) **Expression of His-Npt1_{Ct}.** Protein expression under transport growth conditions was verified by Western blotting. ATM1173 was grown in 2 $\times$ YT medium to mid-log phase in the presence of ampicillin (100 μ g/ml) and IPTG (1 mM). Bac-

teria were then pelleted, lysed in Laemmli buffer, and subjected to 10% SDS-PAGE. Gels were then either stained with Coomassie brilliant blue for detection of total protein or transferred to nitrocellulose for Western blotting. Blots were blocked with 5% milk-Tris-buffered saline (MTBS), probed with mouse anti-His antibodies (GE Healthcare, Pittsburgh, PA) diluted 1:1,000 in MTBS, washed in 0.5% Tween 20-Tris-buffered saline (TTBS), and then probed with a horseradish peroxidase-conjugated rabbit anti-mouse antibody (GE Healthcare) diluted 1:1,000 in MTBS. Blots were then washed with TTBS and incubated with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific Pierce, Rockford, IL). Bands were detected with an ImageQuant LAS 4000 digital imager (GE Healthcare).

(ii) **NAD import.** ATM1172 and ATM1173 were grown in 2 $\times$ YT medium in the presence of ampicillin (100 μ g/ml) and IPTG (1 mM) to mid-log phase, pelleted, washed in M9 salts, and then suspended in M9 salts for transport assays (yielding $\sim 1 \times 10^8$ bacteria per assay). For time course experiments, bacteria were incubated with 50 nM [α -³²P]ATP or [adenylate-³²P]NAD at 37°C for various time periods. [α -³²P]ATP and [adenylate-³²P]NAD at 800 Ci/mmol were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO), or PerkinElmer (Boston, MA). For determination of the NAD K_m , NAD with a specific activity of 150 Ci/mmol was custom ordered from PerkinElmer. To determine the kinetics of ATP and NAD transport by Npt1_{Ct}, bacteria were incubated with different concentrations of [α -³²P]ATP or [adenylate-³²P]NAD at 30°C for 30 s. Reactions were halted by dilution in 20 ml of ice-cold phosphate-buffered saline (PBS). Bacteria were then trapped on a 0.45- μ m filter and washed with 20 ml ice-cold PBS, and the radioactivity present in the bacteria was determined by adding the filter to 5 ml Ready-Safe liquid scintillation cocktail (Beckman Coulter, Brea, CA) and measuring the counts per minute with a scintillation counter. Radioactivity was normalized by converting the counts per minute to the activity of [³²P]ATP or [adenylate-³²P]NAD per mg of sample (determined by the Lowry assay). GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA) was then used to determine the apparent K_m and V_{max} values by plotting the data in the Michaelis-Menten enzyme kinetics template by nonlinear regression analysis. Kinetic parameter experiments were performed in triplicate with three replicates per trial, while others were repeated in duplicate with three replicates per trial.

(iii) **Npt1_{Ct} transport specificity.** ATM1173 was prepared as described for transport assays. Samples were then incubated for 30 s at 30°C with 100 nM [adenylate-³²P]NAD and 10 μ M unlabeled compound (GTP, ATP, NAD, ADP, NADH, or NADP obtained from Sigma-Aldrich, St. Louis, MO) added simultaneously. Assays were halted by dilution and filtration, followed by washing in PBS. Levels of [adenylate-³²P]NAD in the bacteria were measured with a scintillation counter.

(iv) **NAD efflux.** ATM1173 prepared as described for transport assays was preloaded with [adenylate-³²P]NAD (100 nM) for 30 s at 30°C. The reaction was stopped by dilution in 1 ml of ice-cold M9 salts, and the bacteria were pelleted via centrifugation at 4°C, washed with 1 ml of M9 salts, and repelleted via centrifugation. After centrifugation, samples were suspended in M9 salts with or without 10 μ M NAD, ADP, or ATP. The samples were then incubated at 30°C for 5, 10, or 30 min. After dilution, filtering, and washing, the levels of [adenylate-³²P]NAD remaining in the bacteria were measured with a scintillation counter and efflux rates were standardized per minute.

Bioinformatics. NTT amino acid sequences were obtained from NCBI GenBank and aligned with Clustal Omega at default settings to obtain sequence alignments and percent amino acid identities. Alignments were then modified with BioEdit (16). Neighbor-joining tree analysis of NTTs was performed with ClustalW at the default settings.

RESULTS

Identification of Npt1_{Ct} as the NAD transporter in chlamydiae. *Chlamydia* spp. possess two NTTs on the basis of genome annotation and experimental validation, Npt1_{Ct} and Npt2_{Ct} (10). Nei-

FIG 1 ATP and NAD uptake by *E. coli* ATM1173 (Npt1_{Ct}) as a function of time. The uptake of $[^{32}\text{P}]\text{ATP}$ and $[\text{adenylate-}^{32}\text{P}]\text{NAD}$ by ATM1172 (empty-vector control strain) and ATM1173 versus time is shown in panels A and B, respectively. ^{32}P -labeled compounds were used at a final concentration of 50 nM. Reactions were halted by dilution in PBS. Levels of $[^{32}\text{P}]\text{ATP}$ and $[\text{adenylate-}^{32}\text{P}]\text{NAD}$ (reported in counts per minute) present in bacteria at the assay endpoints were measured with a scintillation counter.

ther transporter shows strong homology with the *P. amoebophila* NAD/ADP translocase PamNTT4 (see Fig. S1 in the supplemental material). BLASTP analysis with PamNTT4 (the NAD/ADP translocase) and PamNTT1 (the ATP/ADP translocase) amino acid sequences (from *P. amoebophila*) failed to identify new NTT candidates in chlamydial genomes. Thus, we hypothesized that one of the existing chlamydial NTTs had evolved a relaxed substrate specificity allowing NAD transport in addition to their previously assigned function.

Despite the greater similarity between Npt2_{Ct} and PamNTT4 than between Npt1_{Ct} and PamNTT4 (see Fig. S1 and S2 in the supplemental material), we targeted Npt1_{Ct} for our study because of its antiporter activity and ability to transport ADP. These transport properties make Npt1_{Ct} more functionally similar to PamNTT4 than to Npt2_{Ct}. Npt1_{Ct} (CTL0321 from *C. trachomatis* 434/Bu, as used previously [10]) was cloned into pET-19b to create an N-terminally histidine-tagged fusion protein (expression was verified with anti-His Western blotting [data not shown]). NTT studies have shown that an N-terminal histidine tag does not interfere with NTT transport function (10, 17). Similar to the prior study, Npt1_{Ct} was expressed in *E. coli* C43(DE3) for use in

nucleotide transport studies (*E. coli* cannot transport intact nucleotides) (13). To ensure that the Npt1_{Ct} construct possessed the previously characterized ATP transport function, we initially tested for $[^{32}\text{P}]\text{ATP}$ uptake versus time. As expected, ATM1173 (expressing Npt1_{Ct}), but not ATM1172 (carrying the empty pET-19b vector), was able to transport ATP, as observed by the accumulation of intracellular $[^{32}\text{P}]\text{ATP}$ (Fig. 1A). Accumulation of $[^{32}\text{P}]\text{ATP}$ was saturable, consistent with a protein-mediated carrier transport mechanism. We next tested for uptake of $[\text{adenylate-}^{32}\text{P}]\text{NAD}$. Similar to the ATP results, NAD uptake by ATM1173, but not by ATM1172, was observed and this uptake was saturated over time (Fig. 1B).

Npt1_{Ct} transport specificity. To determine whether Npt1_{Ct} prefers NAD or ATP as a transport substrate, we carried out experiments to determine the transport kinetics for these substrates. The apparent K_m and V_{max} for NAD were 1.7 μM and 5.8 $\text{nmol mg}^{-1} \text{h}^{-1}$, while the apparent K_m and V_{max} for ATP were 2.2 μM and 155 $\text{nmol mg}^{-1} \text{h}^{-1}$ (Fig. 2). Previously, the apparent K_m and V_{max} of Npt1_{Ct} for ATP transport were determined to be 48 μM and 370 $\text{nmol mg}^{-1} \text{h}^{-1}$ in an *E. coli* expression background identical to that used in our study (albeit under different growth con-

FIG 2 Kinetics of NAD and ATP transport in *E. coli* ATM1173 (Npt1_{Ct}). The transport of $[\text{adenylate-}^{32}\text{P}]\text{NAD}$ (A) or $[^{32}\text{P}]\text{ATP}$ (B) by Npt1_{Ct}-expressing bacteria incubated with various concentrations of $[^{32}\text{P}]\text{ATP}$ or $[\text{adenylate-}^{32}\text{P}]\text{NAD}$ was determined by scintillation counting and standardized to the amount of protein per sample. Nonlinear regression analysis with GraphPad Prism software was used to determine the apparent K_m and V_{max} values for NAD and ATP transport (shown in panels A and B, respectively).

FIG 3 Effects of putative competitors on [adenylate- ^{32}P]NAD uptake by ATM1173 (Npt1_{Ct}). [Adenylate- ^{32}P]NAD uptake assays were performed in the presence of various unlabeled competitors. Levels of [adenylate- ^{32}P]NAD present in bacterial pellets were measured with a scintillation counter. Values are reported as percentages of [adenylate- ^{32}P]NAD taken up in the presence of competitors compared to those in a no-competitor control uptake assay. All assays contained [adenylate- ^{32}P]NAD at 100 nM and the unlabeled competitor at 10 μM .

ditions; see Discussion) (10). In closer agreement with our study, Hatch et al. determined the K_m and $V_{\max}$ for ATP transport by *C. psittaci* RBs to be 5.4 μM and 72 $\text{nmol mg}^{-1} \text{h}^{-1}$, respectively (18). In comparison to the Npt1_{Ct} NAD transport kinetics, PamNTT4 expressed in an *E. coli* C43(DE3) background was previously determined to have an apparent K_m of 15 μM and a $V_{\max}$ of 80 $\text{nmol mg}^{-1} \text{h}^{-1}$ for NAD (13).

To further analyze transport specificity and substrate preference, we measured [adenylate- ^{32}P]NAD transport by ATM1173 in the presence of different putative competitor compounds. Unlabeled ADP, NAD, and NADH all inhibited [adenylate- ^{32}P]NAD transport by greater than 70% compared to the no-competitor control reaction (Fig. 3). While ATP inhibited [adenylate- ^{32}P]NAD transport, it did so by only ~50% compared to the control. This is consistent with the differing K_m s of Npt1_{Ct} for ATP and NAD, which indicate a preference for NAD over ATP as a transport substrate. As expected on the basis of previous reports, GTP did not inhibit the transport of [adenylate- ^{32}P]NAD. Since *Chlamydia* spp. lack an NAD kinase (required to convert NAD to NADP) on the basis of genome annotations, we evaluated whether NADP could compete with [adenylate- ^{32}P]NAD transport. NADP reduced the transport of [adenylate- ^{32}P]NAD by only ~20%.

Mode of NAD transport. Npt1_{Ct} was tested for functionality as an NAD/ADP translocase as described for the class III PamNTT4 transporter. For these experiments, ATM1173 was preloaded with [adenylate- ^{32}P]NAD and then incubated with unlabeled NAD, ATP, or ADP as a potential exchange substrate. The level of efflux of [adenylate- ^{32}P]NAD induced by the substrates was compared to that of a no-substrate control reaction. In contrast to PamNTT4, in which ADP acts as a poor substrate for driving NAD efflux, ADP was the best efflux substrate, resulting in the loss of ~60% of the internal [adenylate- ^{32}P]NAD (Fig. 4).

DISCUSSION

The pathogenic and environmental chlamydiae reside in a unique, membrane-bound intracellular niche that provides access, by way of the host cell's cytoplasm, to a rich source of nutrients essential

FIG 4 [Adenylate- ^{32}P]NAD efflux from ATM1173 (Npt1_{Ct}) in the presence of different efflux substrates. ATM1173 was preloaded with [adenylate- ^{32}P]NAD (100 nM) and then incubated with different substrates (M9 salts with or without 10 μM unlabeled NAD, ADP, or ATP). The level of [adenylate- ^{32}P]NAD retained in bacterial pellets after incubation was measured with a scintillation counter and is reported as a percentage of [adenylate- ^{32}P]NAD retained versus that retained in the M9 salts control sample (set at 100%).

for bacterial growth. Perhaps not surprisingly, chlamydiae have evolved to acquire a number of essential compounds from the host cell by using various bacterial solute transporters and can subvert host cell vesicle trafficking to deliver lipids and useful proteins both to and within the growing inclusion (6, 10, 19–21). The presence of solute transporters in addition to *de novo* metabolic pathways in chlamydial ancestors likely served as a driving force behind the reductive evolution exhibited by these bacteria as transporters allowed for the loss of energetically costly pathways in exchange for an obligate intracellular lifestyle due to their subsequent auxotrophy for various compounds, including amino acids, vitamins, and nucleotides. Elucidation of this metabolic interplay between chlamydiae and their host provides opportunities to reveal an Achilles' heel that can then be targeted for the development of antimicrobial compounds while simultaneously revealing nutritional requirements needed for the development of axenic growth conditions.

The members of the order *Chlamydiales*, which includes the genus *Chlamydia*, to which the human and animal pathogens belong, are deficient to various degrees in *de novo* nucleotide synthesis and salvage pathways. As such, all of the members possess nucleotide transporters, with the best characterized being the PamNTT1 to PamNTT5 transporters from *P. amoebophila* and Npt1-2_{Ct} from *C. trachomatis* (transport properties are summarized in Fig. 5) (2, 7, 10, 13, 22). However, unlike the more metabolically diverse members of the *Waddliaceae*, *Simkaniaceae*, and *Parachlamydiaceae* (with the exception of the genus *Protochlamydia*) families, the chlamydiae lack both the *de novo* biosynthetic pathways and salvage pathways for NAD and NADP, suggestive of a need to acquire NAD (and potentially NADP) from the host cell. While *P. amoebophila* possesses a dedicated NAD/ADP translocase for this purpose, the chlamydiae possess only two nucleotide transporters belonging to the class I and II NTT families (10, 13). While all of the class I and II NTTs previously tested were negative for NAD transport (7, 9, 11, 12, 23), the need for NAD transport by chlamydiae led us to examine whether Npt1_{Ct}, the ATP/ADP translocase, could also transport NAD.

FIG 5 Model of nucleotide transport in *Chlamydia* spp. and *P. amoebophila*. The use of Npt1_{Ct} to transport ATP and NAD allowed for the loss of a PamNTT4 class III NTT homolog in *Chlamydia* spp. For simplicity, bacteria are drawn with a single membrane. Npt1_{Ct} is predicted to be an inner membrane protein with nucleotides passing freely across the bacterial outer membrane. The inclusion membrane is illustrated as a dashed line.

Results from our transport kinetic experiments, competition assays, and efflux experiments indicated that Npt1_{Ct} could transport both NAD (and NADH) and ATP in exchange for ADP. This novel specificity would classify Npt1_{Ct} as a hybrid class I/III NTT. The “gain of function” demonstrated by Npt1_{Ct} further stresses the inability to predict transport specificity and mode of NTT transport on the basis of sequence identity analysis alone. In regard to the reductive evolution of the chlamydiae compared to their environmental endosymbiont ancestors, the relaxed specificity of Npt1_{Ct} would have allowed for the loss of a PamNTT4 homolog. Why chlamydiae do not require homologs to PamNTT2, -3, and -5 found in *P. amoebophila* is unclear, but perhaps either reduced nucleotide nutritional requirements or Npt2_{Ct} transport efficiency (which shows substrate overlap with PamNTT2, -3, and -5) made maintaining extra transporters an energetic liability. In light of our findings on Npt1_{Ct} substrate preference, it would be more accurate to define Npt1_{Ct} as a nucleotide translocase rather than as an ATP/ADP translocase, as it is currently listed in NCBI databases.

On the basis of the apparent K_m values and competition assay results, a slight preference of Npt1_{Ct} for NAD over ATP was observed. However, we speculate that under physiological conditions, both substrates are utilized. The K_m values for NAD and ATP (1.7 and 2.2 μ M) are both well below the intracellular concentrations of NAD and ATP ($\sim$ 1.07 mM for NAD and 1.05 to 4.6 mM for ATP [7]), which would allow the acquisition of these compounds from the host. In specific regard to NAD, the K_m is $\sim$ 50-fold lower than the K_m for mitochondrial NAD transporters (which belong to the mitochondrial carrier family, a six-transmembrane transport family unrelated to the NTTs), emphasizing its utility for NAD transport *in vivo* (24–26). Substrate choice should therefore be driven by the extracellular concentrations of ATP and NAD. The slightly lower K_m for NAD may reflect the need to compensate for the higher extracellular concentration of

ATP than of NAD, thus allowing NAD transport requirements to be met for the growing bacterium in the presence of excess ATP.

As our study sought to address transporter preference for ATP versus NAD, we re-evaluated Npt1_{Ct} ATP transport kinetics. While the V_{max} value for ATP did not vary substantially ($\sim$ 2-fold lower), the K_m was $\sim$ 20-fold lower than previously reported (10). Our study used the same expression background and recombinant protein construction as the prior study but under slightly modified expression conditions. Limited experiments performed as previously described resulted in ATP transport values $\sim$ 6-fold lower per concentration tested. Ultimately, the use of a surrogate bacterial expression host precludes the definitive determination of kinetic parameters in regard to the true values for chlamydiae. However, the re-evaluated K_m for ATP is in close agreement with the K_m determined for ATP transport by *C. psittaci* RBs (5.4 μ M) (18).

Npt1_{Ct} has been found in both EB and RB forms of *C. trachomatis* 434/Bu by mass spectrometry analysis, and Npt1_{Ct} transcripts are present throughout development (27, 28). These findings are consistent with the use of Npt1_{Ct} during each stage of development to acquire NAD and ATP. We posit that chlamydiae require NAD throughout growth while exogenous ATP would be needed to supplement ATP generated through substrate level phosphorylation in glycolysis and during immediate early infection to acquire ATP before the activation of the glycolytic pathway (29). Whether bacteria modify NTTs to regulate substrate preference during growth to fulfill specific nucleotide requirements is unknown.

Identification of Npt1_{Ct} as a hybrid class I/III NTT makes it an intriguing model for studying how the amino acid context impacts transport specificity. Future efforts will focus on determining which residues are required for nucleotide transport and whether the class I and III NTT properties of Npt1_{Ct} can be “separated.” Identification of key residues in Npt1_{Ct} required for ATP or NAD

transport specificity could help clarify why PamNTT4 is specific for NAD and why other class I NTTs exclude NAD as a substrate. As NTTs have been identified only in intracellular pathogens of both plants and animals (and plastids), they make ideal drug targets. Insights into substrate recognition would subsequently prove useful for designing NTT transport inhibitors.

While NAD transport by Npt1_{Ct} “solves” the need of chlamydiae for exogenous NAD, it remains unclear how these bacteria obtain NADP, as competition studies showed NADP to be a poor substrate for Npt1_{Ct} and chlamydiae lack an annotated NAD kinase. Furthermore, as the inclusion membrane’s permeability to molecules between 45 and 520 Da is unknown, how nucleotides cross the inclusion membrane, whereupon they would be accessible to the bacteria, is unclear (30, 31). Recent work by Saka et al. suggests that Npt1_{Ct} is present in both the bacterial and inclusion membranes (28). The presence of a functional bacterial transport protein in both bacterial and host lipid membranes would be a novel finding. Ultimately, our study further clarifies an important aspect of chlamydial biology by demonstrating how these pathogens overcome their *de novo* nucleotide synthesis deficiencies and illustrates the need for continued study of NTT function.

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