 doi:10.1111/j.1365-2958.2006.05193.x

Tapping the nucleotide pool of the host: novel nucleotide carrier proteins of *Protochlamydia amoebophila*

Ilka Haferkamp,[1] Stephan Schmitz-Esser,[2] Michael Wagner,[2] Nadjeschka Neigel,[1] Matthias Horn[2]* and H. Ekkehard Neuhaus[1]

[1]*Pflanzenphysiologie, Technische Universität Kaiserslautern, Erwin Schrödinger Str., D-67653 Kaiserslautern, Germany.*

[2]*Department für Mikrobielle Ökologie, Universität Wien, A-1090 Vienna, Austria.*

Summary

***Protochlamydia amoebophila* UWE25 is related to the *Chlamydiaceae* comprising major pathogens of humans, but thrives as obligate intracellular symbiont in the protozoan host *Acanthamoeba* sp. The genome of *P. amoebophila* encodes five paralogous carrier proteins belonging to the nucleotide transporter (NTT) family. Here we report on three *P. amoebophila* NTT isoforms, *Pam*NTT2, *Pam*NTT3 and *Pam*NTT5, which possess several conserved amino acid residues known to be critical for nucleotide transport. We demonstrated that these carrier proteins are able to transport nucleotides, although substrate specificities and mode of transport differ in an unexpected manner and are unique among known NTTs. *Pam*NTT2 is a counter exchange transporter exhibiting submillimolar apparent affinities for all four RNA nucleotides, *Pam*NTT3 catalyses an unidirectional proton-coupled transport confined to UTP, whereas *Pam*NTT5 mediates a proton-energized GTP and ATP import. All NTT genes of *P. amoebophila* are transcribed during intracellular multiplication in acanthamoebae. The biochemical characterization of all five NTT proteins from *P. amoebophila* in this and previous studies uncovered that these metabolically impaired bacteria are intimately connected with their host cell's metabolism in a surprisingly complex manner.**

Introduction

Survival and reproduction of obligate intracellular bacteria take place within eukaryotic host cells. This cellular association depends upon metabolic interactions between both organisms, which may also be crucial for reproduction, survival and pathogenicity of the host (Görtz and Brigge, 1998; Taylor and Hoerauf, 2001; Zientz *et al*., 2004). In general the obligate intracellular lifestyle correlates with a drastically reduced genome of the bacteria, typically showing defects in essential biosynthetic pathways (Moran, 2002) and compensatory transport mechanisms for the import of key metabolites from the host cytosol (Moulder, 1991).

For example, members of the family *Chlamydiaceae,* comprising exclusively major obligate intracellular pathogens of humans like *Chlamydia trachomatis*, lost the ability to synthesize nucleotides *de novo*, and it has been shown that these essential building blocks are instead imported from the host cell's cytosol. This metabolic interaction, commonly referred to as energy parasitism, is mediated by nucleotide transport proteins (NTT), which have so far only been identified in few obligate intracellular bacteria and plastids of plants (Krause *et al*., 1985; Winkler and Neuhaus, 1999; Linka *et al*., 2003; Schmitz-Esser *et al*., 2004), and which have been grouped into the ATP:ADP antiporter (AAA) family (Saier, 2000; Ren *et al*., 2004). *C. trachomatis* possesses two NTT isoforms: *Ct*NTT1 catalyses the import of host-derived ATP in strict counter exchange with bacterial ADP, whereas *Ct*NTT2 catalyses an unidirectional, proton-driven uptake of all four RNA nucleoside triphosphates (Stephens *et al*., 1998; Tjaden *et al*., 1999).

The recent identification of bacteria related to the *Chlamydiaceae* in the environment revealed a previously underestimated diversity and an unexpected distribution of chlamydiae in nature (Birtles *et al*., 1997; Ossewaarde and Meijer, 1999; Fritsche *et al*., 2000; Horn *et al*., 2000; Horn and Wagner, 2001). For example, the newly identified environmental chlamydia strain *Protochlamydia amoebophila* UWE25 resides as an endosymbiont in the ubiquitous protozoan host *Acanthamoeba* sp. (Fritsche *et al*., 2000; Collingro *et al*., 2005a). Like their pathogenic counterparts, environmental chlamydiae are obligate intracellular bacteria, and possess an unique developmental cycle alternating between an infectious, metabolically inert form (the elementary body) and a replicative, metabolically active form (the reticulate body). Genome

Accepted 6 April, 2006. *For correspondence. E-mail horn@microbial-ecology.net; Tel. (+43) 1 4277 54393; Fax (+43) 1 4277 54389.

sequencing of *P. amoebophila* revealed that it contains, in contrast to members of the *Chlamydiaceae*, in total five genes with sequence similarity to known NTT genes (Horn *et al*., 2004). These analyses suggested that the presence of NTTs is an ancient chlamydial trait and it has been hypothesized that other bacterial human pathogens and plants received *ntt* genes from a chlamydial ancestor via horizontal gene transfer (Greub and Raoult, 2003; Horn *et al*., 2004; Schmitz-Esser *et al*., 2004).

Three of the five NTT proteins from *P. amoebophila* have so far been analysed on the functional level, namely *Pam*NTT1, *Pam*NTT2 and *Pam*NTT4. *Pam*NTT1 represents an ATP/ADP counter exchanger (Schmitz-Esser *et al*., 2004) similar to corresponding transporters in *C. trachomatis, Rickettsia prowazekii, Caedibacter caryophilus* or *Holospora obtusa* (Krause *et al*., 1985; Tjaden *et al*., 1999; Linka *et al*., 2003; Daugherty *et al*., 2004). *Pam*NTT2 transports all four RNA nucleotides, and most remarkably, *Pam*NTT4 has been identified as the first carrier protein able to transport nicotineamide adenine dinucleotide (NAD^+) in an intact form across a biological membrane (Haferkamp *et al*., 2004).

In this work we expressed the so far not characterized NTT genes from *P. amoebophila* (*ntt3* and *ntt5*) heterologously in *Escherichia coli*, as no genetic system is available currently for chlamydiae. We identified the biochemical properties of the corresponding transporter proteins, and extended our previous analysis of the transport properties of *Pam*NTT2. The elucidation of the biochemical characteristics of all five NTT proteins from *P. amoebophila* allowed us to develop a metabolic interaction scenario, which embeds these carriers into biosynthetic abilities and restrains of these elusive intracellular bacteria. These analyses revealed a unique adaptation of *P. amoebophila* to its intracellular lifestyle.

Results

Primary and secondary structure of NTT2, NTT3 and NTT5 from P. amoebophila

A detailed analysis of primary and predicted secondary structures of NTT proteins can help to get a deeper insight into structure/function relationships of these transport proteins. *Pam*NTT2, *Pam*NTT3 and *Pam*NTT5 show sizes of 489–536 amino acids that are comparable to other known NTT type nucleotide transporters (Fig. S1). A number of highly conserved amino acid residues critical for the function of the ATP/ADP transporter NTT1 from *Arabidopsis thaliana* (Trentmann *et al*., 2000), namely the amino acid residues K65, E155, E303 and K446 (counted on basis of *Pam*NTT1), are present in all three *P. amoebophila* NTT proteins analysed in this study (Fig. S1). Among characterized NTT proteins, *Pam*NTT2, *Pam*NTT3 and *Pam*NTT5 exhibit comparable amino acid sequence identity to bacterial ATP/ADP antiporters and H^+/nucleotide symporters (33–48% for *Pam*NTT2 and *Pam*NTT3, 28–32% for *Pam*NTT5). Consistently, in phylogenetic trees containing all known NTT proteins *Pam*NTT2, *Pam*NTT3 and *Pam*NTT5 formed deep branches between the ATP/ADP translocases from rickettsiae, chlamydiae, plant plastids and other intracellular bacteria, and the proton-driven nucleotide importers of chlamydiae (Fig. S2). They thus could not be clearly assigned to a specific NTT subgroup. Like all other known NTT proteins (except for *Pam*NTT4) *Pam*NTT2, *Pam*NTT3 and *Pam*NTT5 show (11–) 12 predicted transmembrane domains, a characteristic feature of members of the solute transporter family (Fig. S3; Saier, 2000).

Preferred substrates of Pam*NTT2,* Pam*NTT3 and* Pam*NTT5*

To reveal the transport properties of the remaining two transporters (*Pam*NTT3 and *Pam*NTT5) we cloned the corresponding genes, expressed these heterologously in *E. coli* and measured nucleotide transport mediated by the recombinant carrier proteins. It has been shown that this heterologous expression system allows to decipher biochemical properties of a wide range of NTT proteins from both, bacteria and plants, which are similar to those of the authentic carriers in their respective native membrane (Krause *et al*., 1985; Möhlmann *et al*., 1998; Tjaden *et al*., 1998; 1999). In addition to *Pam*NTT3 and *Pam*NTT5, we also included a more detailed characterization of *Pam*NTT2, as this protein has so far only been characterized partially (Haferkamp *et al*., 2004).

To get a first impression on the spectrum of nucleotides transported by *Pam*NTT2, *Pam*NTT3 and *Pam*NTT5, we initially ensured (by sodium dodecyl-lauryl-sulphate-polyacrylamide gel analysis and autoradiography of [^{35}S]-labelled NTT proteins) that a sufficient amount of these proteins was inserted into the *E. coli* membrane after heterologous synthesis (data not shown). Subsequently, we raised first evidence on preferred substrates of each carrier. This was done by incubating *E. coli* expressing *Pam*NTT2, *Pam*NTT3 or *Pam*NTT5 in buffer containing radioactively labelled ATP, UTP, GTP or CTP (each at a concentration of 50 μM, Table 1).

At a substrate concentration of 50 μM *Pam*NTT2 transported CTP most efficiently at a rate of 145.3 nmol mg^{-1} protein h^{-1}, followed by GTP (95.6 nmol mg^{-1} protein h^{-1}). *Pam*NTT2-mediated ATP and UTP transport occurred with similar rates, namely 38.5 and 36.2 nmol mg^{-1} protein h^{-1} respectively (Table 1). *Pam*NTT3 did not accept nucleotides other than UTP, which is imported at a rate of 11.4 nmol mg^{-1} protein h^{-1} (Table 1). *E. coli* cells expressing *Pam*NTT5 transported GTP at a rate of 63.2 nmol mg^{-1} protein h^{-1}, whereas ATP is transported

Table 1. Uptake of [α-^{32}P]-labelled nucleotides into *E. coli* cells expressing *Pam*NTT2, *Pam*NTT3 or *Pam*NTT5.

	Rate of Transport (nmol mg^{-1} protein h^{-1})		
Nucleotide	*Pam*NTT2	*Pam*NTT3	*Pam*NTT5
ATP	38.5 ± 1.8	< 0.02	7.0 ± 0.5
GTP	95.6 ± 4.1	< 0.02	63.2 ± 5.2
CTP	145.3 ± 14.6	< 0.01	< 1.0
UTP	36.2 ± 5.1	11.4 ± 1.0	< 1.0

Radioactively labelled compounds were present at a final concentration of 50 μM. IPTG-induced *E. coli* cells harbouring the plasmid pET16b containing the respective nucleotide transporter gene were incubated for 5 min. For calculation of the transport rate control values (induced *E. coli* cells harbouring the plasmid without insert) were subtracted from the measured transport values. Data given in nmol mg total *E. coli* protein^{-1} h^{-1} are mean of three independent experiments.

with a ninefold reduced transport rate (6.98 nmol mg^{-1} protein h^{-1}). CTP and UTP are not transported by *Pam*NTT5 at substantial rates (Table 1).

A more detailed characterization of transport properties, however, requires the analysis of the time linearity of import, as only the time-linear phase of uptake allows for the calculation of apparent affinities and apparent maximal velocities, as well as the identification of effective inhibitors. *Pam*NTT2-mediated ATP import is rapid and occurred linear with time for the first 5 min of incubation (Fig. 1A). Already after 10 min ATP transport equilibrated and no further accumulation of radioactivity within the bacterial cells occurred (Fig. 1A). *Pam*NTT3-mediated UTP import is linear with time for about 10 min and saturated after more than 15 min of incubation (Fig. 1B), whereas ATP import by *Pam*NTT5 is comparably slow and appeared to be linear with time for 10–15 min of incubation (Fig. 1C). In all cases non-induced control cells hardly imported any labelled nucleotides (Fig. 1A–C), which is consistent with our previous observations on *E. coli* transformed with expression plasmids harbouring other NTT genes (Tjaden *et al.*, 1998; 1999; Haferkamp *et al.*, 2004).

The members of the NTT protein family analysed so far accept a wide array of substrates comprising either ATP and ADP (Krause *et al.*, 1985; Tjaden *et al.*, 1998), all four types of RNA nucleotides (Tjaden *et al.*, 1999), or NAD$^+$ and ADP (Haferkamp *et al.*, 2004) respectively. Having identified transported substrates of *Pam*NTT2, *Pam*NTT3 and *Pam*NTT5 (Fig. 1A–C, Table 1) it is possible to identify further putative substrates by competition experi-

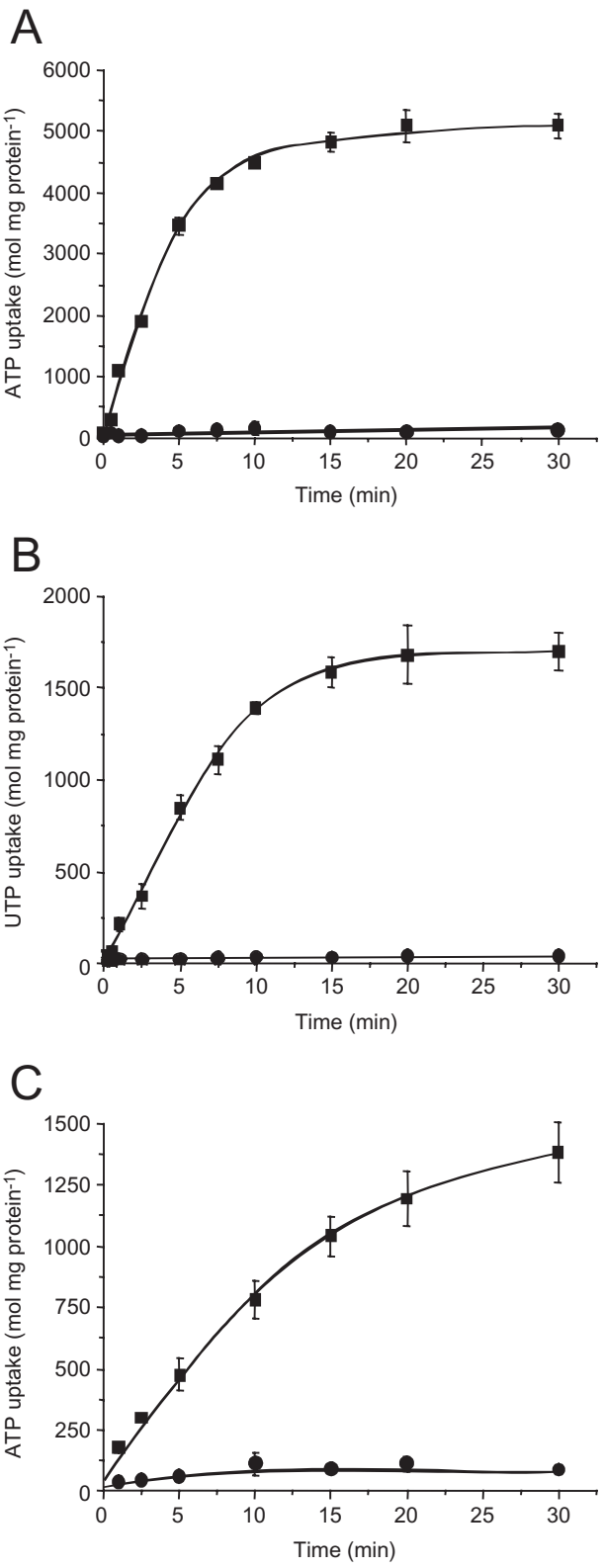

Fig. 1. Time dependency of [α-^{32}P]-labelled nucleotide uptake into IPTG-induced *E. coli* cells. *E. coli* cells harbouring plasmid pET16b encoding respective NTT gene from *P. amoebophila* (■), or without insert [control (●)] were incubated in phosphate buffer medium containing 50 μM labelled nucleotide for the indicated time periods.
A. Time dependency of [α-^{32}P]-ATP uptake mediated by recombinant *Pam*NTT2 protein.
B. Time dependency of [α-^{32}P]-UTP uptake mediated by recombinant *Pam*NTT3 protein.
C. Time dependency of [α-^{32}P]-ATP uptake mediated by recombinant *Pam*NTT5.

Table 2. Effects of various metabolites on [α-^{32}P]-UTP (*Pam*NTT3) and [α-^{32}P]-ATP (*Pam*NTT2, *Pam*NTT5) uptake into IPTG-induced *E. coli* cells.

	Transporter (Substrate)		
Effector	***Pam*NTT2 (ATP)**	***Pam*NTT3 (UTP)**	***Pam*NTT5 (ATP)**
none	100	100	100
ATP	41.2	95.2	43.3
GTP	32.2	91.4	13.5
UTP	57.6	43.6	84
TTP	92.6	94.1	95.8
CTP	36.4	95.4	94
dCTP	95	94.3	99.4
ADP	88.4	91.6	78.2
AMP	99.5	89.4	109.2
dATP	92.5	88.5	103.1
GDP	87.6	87.5	22.6
GMP	88.9	100.6	62
dGTP	77.6	87.7	34.3
UDP	89.5	42.7	94.5
UMP	98.8	51.9	94.2
TDP	103.2	81.5	88.7
TMP	103.1	81.4	96.9
Thymidine	86.8	117.6	88.7
IDP	84.3	78.2	54.6
NAD^+	106.7	111.1	114.3
ADP-Glucose	100.5	98.4	99
UDP-Glucose	97.3	97.2	97

Uptake of [α-^{32}P]-ATP mediated by recombinant *Pam*NTT2 or *Pam*NTT5, and of [α-^{32}P]-UTP mediated by recombinant *Pam*NTT3 was measured at a substrate concentration of 50 μM and 200 μM respectively. Unlabelled effectors were present in 10-fold excess. Rates of nucleotide uptake are given as percentage of control rates (non-affected transport = 100%). Data are the mean of three independent experiments. SE < 9% of the mean values.

ments. For this analysis we studied the effect of various structurally related compounds on *Pam*NTT2- and *Pam*NTT5-mediated ATP transport, and on *Pam*NTT3-mediated UTP transport (Table 2). NTT2-catalysed ATP transport is inhibited by a 10-fold excess of unlabelled ATP, GTP, CTP and UTP, and inhibition ranged from 32.2% to 57.6% residual activity (Table 2). All other substrates tested (including TTP, but with the exception of dGTP) did not interfere notably with *Pam*NTT2-mediated ATP import (Table 2). In contrast, *Pam*NTT3-mediated UTP import is inhibited by unlabelled UTP, UDP, UMP and IDP (42.7–78.2% residual activity), whereas all other substrates did not interfere markedly with UTP uptake by *Pam*NTT3 (Table 2). *Pam*NTT5-catalysed ATP import is strongly inhibited by unlabelled GTP, GDP, dGTP (13.5–34.3% residual activity) and lesser by ATP, ADP, IDP and GMP (43.3–73.2% residual activity, Table 2). Interestingly, the presence of NAD^+ in the transport medium resulted in a slight increase of nucleotide uptake mediated by all three transporters (Table 2). This is probably due to the fact that *E. coli* is able to utilize exogenously supplied NAD^+ via the salvage pathway (Gholson *et al.*, 1969) and that the imported cleavage products most likely improved bacterial fitness.

Substrate affinities and mode of transport of Pam*NTT2,* Pam*NTT3 and* Pam*NTT5*

To interpret the function of a transport protein within the physiological context of a cell, it is important to know its apparent substrate affinities. Therefore, we analysed the change of nucleotide transport velocities of *Pam*NTT2, *Pam*NTT3 and *Pam*NTT5 in response to different substrate concentrations.

The apparent affinities of *Pam*NTT2 were 437 μM for ATP, 676 μM for UTP, 156 μM for GTP and 570 μM for CTP. Among these substrates, CTP is transported with the highest apparent maximal velocity (1920 nmol mg^{-1} protein h^{-1}), which is four to five times faster than for the other substrates (Table 3). The protonophore CCCP, added at a concentration of 50–250 μM, did not inhibit ATP uptake by *Pam*NTT2 (Fig. 2), which is consistent with properties of other nucleotide antiporters of the NTT family (Tjaden *et al.*, 1999).

*Pam*NTT3 exhibited an apparent affinity for UTP of

Table 3. Apparent K_M and V_{max} values of the five recombinant NTT proteins from *P. amoebophila* and reported nucleotide concentrations in eukaryotic cells and other bacteria.

	ATP	ADP	GTP	CTP	UTP	NAD
Nucleotide concentration in eukaryotic cells (mM)	1.05–4.6	0.47	0.47	0.28	0.05–0.57	1.07
K_M (V_{max}) NTT1	95 (384)	55 (384)	128 (12)	–	–	–
K_M (V_{max}) NTT2	437 (450)	–	156 (412)	570 (1920)	676 (501)	–
K_M (V_{max}) NTT3	–	–	–	–	1320 (321)	–
K_M (V_{max}) NTT4	–	275 (67)	–	–	–	15 (188)
K_M (V_{max}) NTT5	360 (58)	273 (4)	22 (98)	–	–	–
Nucleotide concentration in bacteria (mM)	2.7–3.0	0.25	0.9–1.1	0.5–0.7	0.89–1.4	0.17–1.1

K_M values are given in μM, V_{max} values (in brackets) are given in nmol mg protein^{-1} h^{-1}. Data of high affinity transport (< 160 μM) are the mean of four, and data of low affinity transport (> 160 μM) are the mean of eight independent experiments. K_M and V_{max} values for *Pam*NTT1 and *Pam*NTT4 were taken from Schmitz-Esser *et al.* (2004) and Haferkamp *et al.* (2004) respectively. Intracellular nucleotide concentrations were taken from the following references: (Pannbacker, 1967; Mathews, 1972; Bochner and Ames, 1982; Traut, 1994; Larsson *et al.*, 1997; Theobald *et al.*, 1997; Pan *et al.*, 2001; Varela-Gomez *et al.*, 2004).

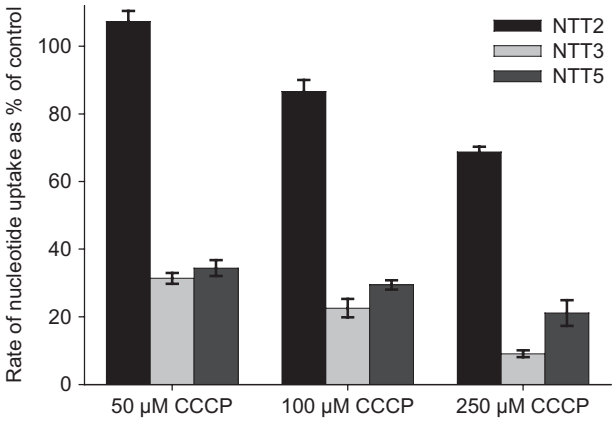

Fig. 2. Effect of the protonophore CCCP on [α-^{32}P]-UTP (*Pam*NTT3) and [α-^{32}P]-ATP (*Pam*NTT2, *Pam*NTT5) uptake into IPTG-induced *E. coli* cells. Uptake of [α-^{32}P]-ATP mediated by recombinant *Pam*NTT2 or *Pam*NTT5, and of [α-^{32}P]-UTP mediated by recombinant *Pam*NTT3 was measured at a substrate concentration of 50 μM and 200 μM respectively. Rates of nucleotide uptake are given as percentage of control rates (non-affected transport = 100%). Data are the mean of three independent experiments.

1320 μM and a maximal transport velocity of 321 nmol mg^{-1} protein h^{-1} (Table 3). In contrast to *Pam*NTT2, CCCP acted inhibitory on *Pam*NTT3. The presence of this protonophore at a concentration of 50 μM inhibited UTP transport by *Pam*NTT3 to 31.4% (SE ± 1.6) of the corresponding control rate (Fig. 2).

*Pam*NTT5 exhibited highest apparent affinity for GTP (22 μM) and GDP (59 μM), followed by dGTP (121 μM), and ATP (360 μM). Similar to the apparent affinities, the maximal transport velocities catalysed by *Pam*NTT5 were extremely different between the substrates tested. They ranged from only 4 nmol mg^{-1} protein h^{-1} for ADP Table 3) to nearly 160 nmol mg^{-1} protein h^{-1} for GDP. In contrast to *Pam*NTT2, but similar to *Pam*NTT3, CCCP inhibited *Pam*NTT5-mediated nucleotide transport markedly. The presence of 50 μM CCCP inhibited *Pam*NTT5-mediated ATP import to 34.4% (SE ± 2.3) residual activity.

The inhibitory effect of the protonophore CCCP on NTT-mediated nucleotide movement provides further indications on driving forces required to energize the corresponding transport process. However, we have to keep in mind that certain members of the NTT protein family do not catalyse a counter exchange mode of transport, but unidirectional nucleotide import (Tjaden *et al*., 1999). One often used experimental system to distinguish between counter exchange and unidirectional nucleotide import is the so-called back exchange approach. For this experimental approach *E. coli* expressing either *Pam*NTT2, *Pam*NTT3 or *Pam*NTT5 were pre-loaded with labelled substrate, external radioactivity was removed by washing with buffer medium, and the cells were finally resuspended in phosphate buffer (control), or transport medium (buffer containing putative counter exchange nucleotides). By analysing the time-course of the loss of radioactive label it is possible to differentiate between counter exchange, or unidirectional mode of transport (Tjaden *et al*., 1999; Haferkamp *et al*., 2004).

E. coli cells expressing *Pam*NTT2 and pre-loaded with [α^{32}P]-ATP released significant amounts of internal label only after resuspension in buffer medium supplemented with unlabelled ATP compared with the control (Fig. 3A). We verified by thin-layer chromatography that radioactivity exported in the presence of the unlabelled counter exchange substrates CTP, GTP, ATP and UTP is mainly [α^{32}P]-ATP (Fig. 4).

Escherichia coli cells expressing *Pam*NTT3 and pre-loaded with [α^{32}P]-UTP did not release additional internal radioactivity after resuspension in buffer medium containing unlabelled UTP when compared with the control (Fig. 3B). Similarly, *Pam*NTT5-expressing *E. coli* cells pre-loaded with [α^{32}P]-GTP did not export internal radioactive label after resuspension in medium containing unlabelled GTP (Fig. 3C).

Transcription of NTT genes from P. amoebophila

It has been shown previously that the transporter genes *ntt1*, *ntt2* and *ntt4* of *P. amoebophila* are transcribed during multiplication in its amoeba host (Haferkamp *et al*., 2004; Schmitz-Esser *et al*., 2004). In order to verify whether also *ntt3* and *ntt5* of *P. amoebophila* are transcribed, we performed reverse transcriptase PCR experiments (Fig. 3). For this total RNA purified from amoebae containing intracellular *P. amoebophila* was used as template, and gene-specific primers allowed amplification of *ntt3* and *ntt5* transcript fragments showing the expected sizes (Fig. 5). The identity of the amplified fragments was confirmed by sequencing.

Discussion

NTT type nucleotide transport proteins occur in few intracellular bacteria and in all plastids from higher and lower plants analysed so far (Krause *et al*., 1985; Tjaden *et al*., 1998; Winkler and Neuhaus, 1999; Linka *et al*., 2003; Schmitz-Esser *et al*., 2004), but they do not share any structural similarity to mitochondrial and peroxisomal adenylate transporters belonging to the mitochondrial carrier (MC) family (Klingenberg, 1989; Saier, 2000; Palmieri *et al*., 2001; Ren *et al*., 2004). However, like mitochondrial and peroxisomal ATP carriers, NTT type nucleotide transport proteins catalyse a remarkable reaction by carrying nucleotide cargos, which are extremely large and highly charged substrates.

In the light of the only recently recognized functional

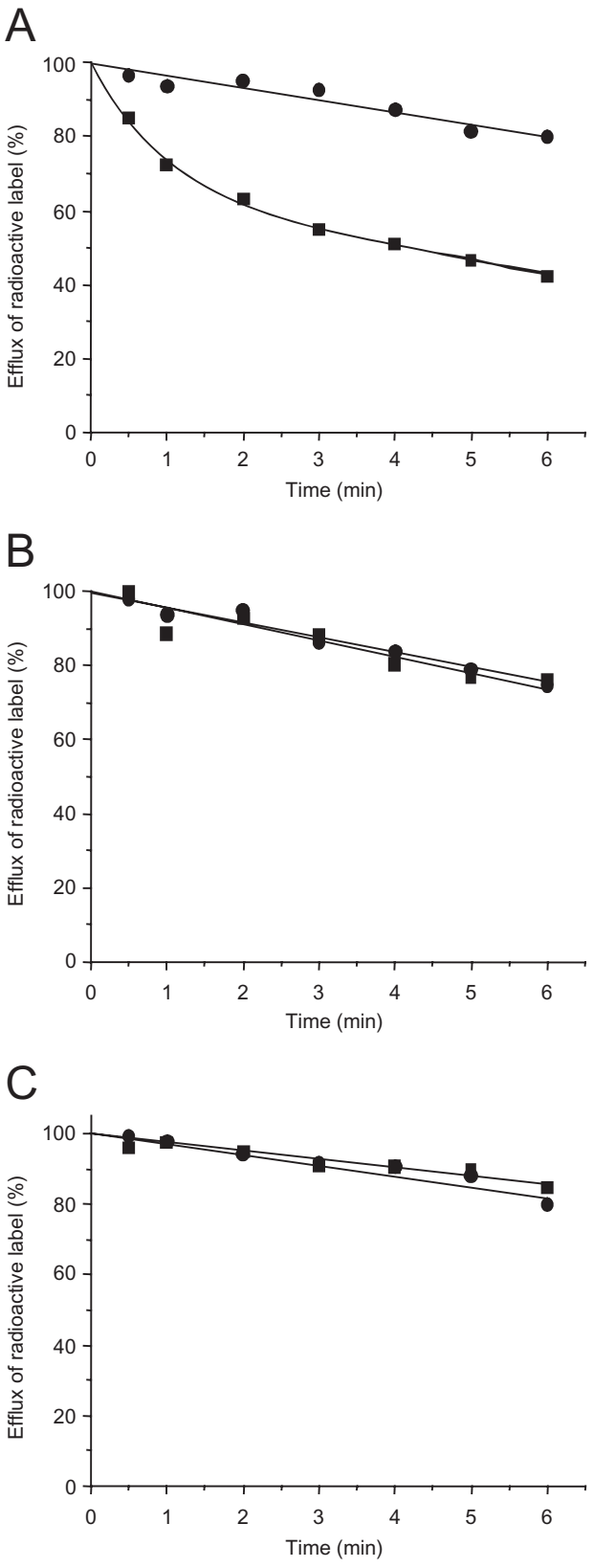

Fig. 3. Back exchange properties of *Pam*NTT2, *Pam*NTT3 and *Pam*NTT5. *E. coli* cells synthesizing *Pam*NTT2, *Pam*NTT3 or *Pam*NTT5 respectively, were pre-loaded with 50 μM [á-^{32}P]-labelled nucleotide and back exchange was initiated by resuspension of washed cells in either phosphate buffer medium [control (●)] or phosphate buffer containing putative counter exchange substrate (■).
A. *Pam*NTT2 expressing cells were pre-loaded with [á-^{32}P]-ATP, unlabelled ATP for back exchange was present at a concentration of 2 mM.
B. *Pam*NTT3 expressing cells were pre-loaded with [á-^{32}P]-UTP, unlabelled UTP for back exchange was present at a concentration of 3 mM.
C. *Pam*NTT5 expressing cells were pre-loaded with [á-^{32}P]-GTP, unlabelled GTP for back exchange was present at a concentration of 2 mM.
Data represent the mean of three independent experiments. Standard error of the mean was always less than 5% of the given value.

diversity of NTT proteins, we suggest to group all NTT proteins known so far into a new family named nucleotide transporter (NTT) family, replacing the original AAA family (Saier, 2000; Ren *et al*., 2004). Taking the mode of transport for classification, the nucleotide transporter (NTT) family can be further subdivided into three classes: (i) class I catalyses a nucleotide counter exchange mode of transport. This transport process allows for example to energize anabolic reactions of endosymbiotic (or parasitic) bacteria exhibiting impaired energy metabolism. Typical members of NTT class I are the homologous NTT1 proteins found in the human pathogens *R. prowazekii* and *C. trachomatis*, or in the symbionts *H. obtusa* and *C. caryophilus* residing in paramecia (Krause *et al*., 1985; Tjaden *et al*., 1999; Linka *et al*., 2003). (ii) Class II NTT proteins catalyse an unidirectional, proton-driven nucleotide import and are thus able to compensate lacking *de novo* nucleotide biosynthesis capacity for example in *C. trachomatis* (Tjaden *et al*., 1999). (iii) Finally, class III NTT proteins catalyse NAD^+/ADP counter exchange (Haferkamp *et al*., 2004) and thus bypass the inability of *P. amoebophila* to synthesize the important electron carrier and cofactor NAD (Horn *et al*., 2004).

Using this classification scheme, *Pam*NTT2 analysed in this study belongs to the class I NTT proteins. This assignment is based on the observations that (i) *Pam*NTT2 catalyses a nucleotide counter exchange (Table 2; Figs 3 and 4) and that (ii) transport activity is not dependent upon an existing proton gradient (Fig. 2). However, in contrast to the rickettsial adenylate translocase, to NTT1 from *C. trachomatis*, and to all plant NTT proteins (Krause *et al*., 1985; Möhlmann *et al*., 1998; Tjaden *et al*., 1999), the carrier *Pam*NTT2 does not accept nucleoside diphosphates as substrate, but is able to transport UTP, CTP, ATP and GTP (Tables 1 and 2). Thus, *Pam*NTT2 is a class I NTT protein with a previously not recognized substrate specificity. This finding differs from our earlier assumption that *Pam*NTT2 represents an unidirectional class II transporter (Haferkamp *et al*., 2004) and shows that the exact

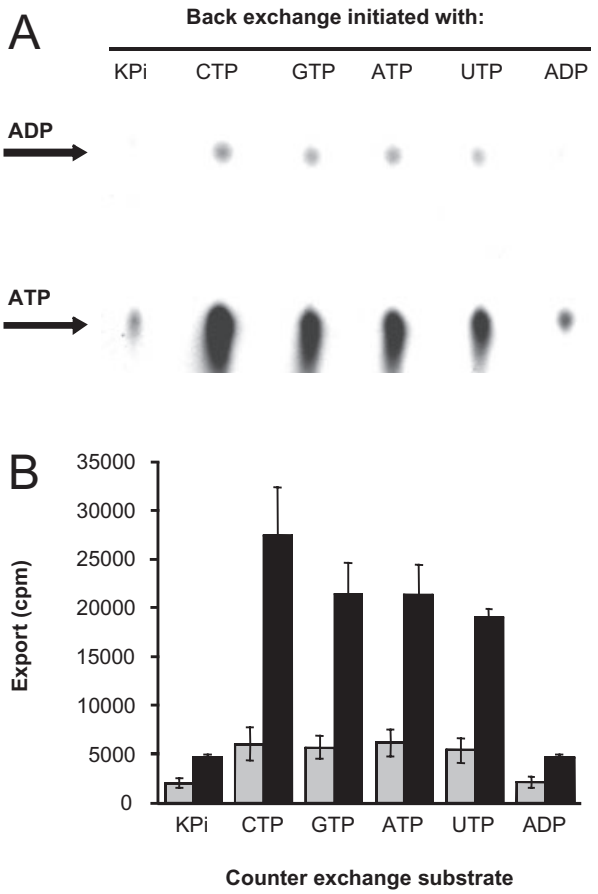

Fig. 4. Thin-layer chromatography analysis and quantification of exported radioactively labelled nucleotides by *E. coli* cells expressing *Pam*NTT2. *E. coli* cells expressing *Pam*NTT2 were incubated for 3 min in phosphate buffer containing 50 μM radioactively labelled [á-^{32}P]-ATP. After removal of external radioactivity, back exchange was initiated by addition of indicated unlabelled nucleotides at a concentration of 500 μM each, and stopped after 2 min.
A. Thin-layer chromatography analysis of exported nucleotides.
B. Quantification of exported radioactive ATP and ADP.
Spots of exported radioactively labelled ATP (black columns), and ADP (grey columns) separated by thin-layer chromatography were excised, transferred to scintillation vessels, and radioactivity was quantified in a scintillation counter. Data in (A) represent a typical experiment, data in (B) are the mean of four independent experiments. Standard errors are displayed.

mode of transport can only be revealed by extended biochemical analyses using uncouplers like CCCP and back-exchange experiments.

*Pam*NTT3 belongs to the class II NTT proteins, because this carrier mediates a unidirectional (Fig. 3B), proton-energized transport of a nucleoside triphosphate (Tables 1 and 2; Figs 1B and 2). However, similar to *Pam*NTT2 within NTT class I, *Pam*NTT3 differs from *Ct*NTT2, the only other member of class II, with respect to its substrate spectrum. While the *Ct*NTT2 carrier imports all RNA nucleotides (UTP, ATP, GTP and CTP; Tjaden *et al*., 1999), *Pam*NTT3 solely uses a single substrate, namely UTP (Table 2). *Pam*NTT5 also belongs to the class II NTT proteins, as this carrier mediates a unidirectional, proton-driven nucleotide transport (Tables 1 and 2; Figs 1B, 2 and 3C). However, in opposite to *Pam*NTT3 and *Ct*NTT2, this carrier accepts GTP, GDP and ATP as substrates.

With the exception of *Pam*NTT4, all NTT proteins from *P. amoebophila* contain a number of amino acid residues highly conserved among known NTT proteins (Fig. S1). Taking the plastidic ATP/ADP transporter NTT1 from *A. thaliana* as an example, it has been demonstrated that some of these amino acid residues are critical for transport of adenine nucleotides (Trentmann *et al*., 2000). For example, the lysine residue K446 (counted on the basis of *Pam*NTT1, and corresponding to K527 in NTT1 of *A. thaliana*) is critical for transport of nucleoside triphosphates, but not for nucleoside diphosphate transport (Trentmann *et al*., 2000). Consistently, *Pam*NTT4 known to be unable to transport ATP, but capable of ADP transport does not exhibit a lysine residue at this position (Fig. S1). However, all other NTT proteins from *P. amoebophila* exhibit this conserved lysine residue (Fig. S1) and in fact we could demonstrate that *Pam*NTT1, *Pam*NTT2, *Pam*NTT3 and *Pam*NTT5 transport nucleoside triphosphates as expected (Tables 1 and 2; Fig. 1; Schmitz-Esser *et al*., 2004). These correlations show that the presence of certain critical amino acid residues are more suitable as signposts for NTT function and substrate specificity than the overall similarity of the amino acid sequences between NTT proteins. However, it is still impossible to predict *in silico* the exact specificity and transport modes of a newly discovered NTT protein underlining the requirement of a more detailed analysis of

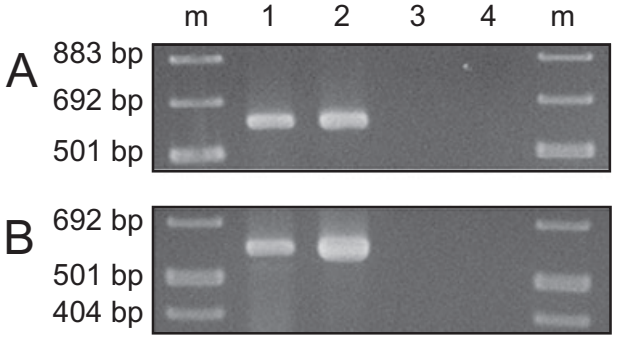

Fig. 5. Transcription of *ntt3* and *ntt5* during multiplication of *P. amoebophila* within acanthamoebae.
A. Transcription of *ntt3*.
B. Transcription of *ntt5*.
Lane 1, amplification products of cDNA synthesized from whole RNA from amoebae harbouring *P. amoebophila*; lane 2, PCR positive controls using DNA from purified *P. amoebophila* elementary bodies; lane 3, PCR negative controls (no cDNA added); lane 4, PCR using whole RNA from amoebae containing *P. amoebophila* (control for the absence of DNA in the RNA preparation, no reverse transcriptase added); m, molecular-size marker.

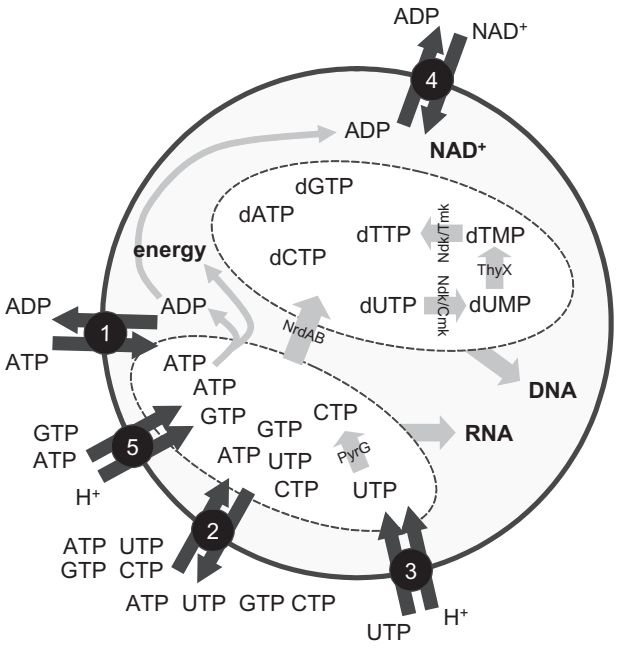

Fig. 6. Model of NTT-mediated metabolic interactions between *P. amoebophila* and its *Acanthamoeba* host. This model is based on the deciphered biochemical properties of all nucleotide transport proteins of *P. amoebophila* determined in this and previous studies (Haferkamp *et al*., 2004; Schmitz-Esser *et al*., 2004), the completely sequenced genome of this species (Horn *et al*., 2004), the observed apparent substrate affinity (K_M) values, and previously reported intracellular nucleotide concentrations. The combined activity of all five NTT proteins (1–5) allows for the uptake of the electron carrier and cofactor NAD^+, all nucleotides needed for RNA and DNA synthesis, and for the import of energy in form of ATP. PyrG, CTP synthase (GenBank Accession No. CAF23548); NrdAB, ribonucleotide reductase subunits A and B (CAF24072, CAF24073); Ndk, nucleoside-diphosphate kinase (CAF23090); Cmk, cytidylate kinase (CAF23043); Tmk, thymidylate kinase (CAF23797); ThyX, thymidylate synthase complementing protein (CAF22879).

structure–function relationships of representative NTT proteins in the future.

The availability of the biochemical characteristics of all five NTT proteins from the symbiont *P. amoebophila* allowed us to develop a metabolic interaction scenario for this bacterium with its amoebal host (Fig. 6). This model is also based on the observation from this and previous studies that all five NTT proteins of *P. amoebophila* are actually transcribed during intracellular multiplication in its amoeba host (Fig. 5; Haferkamp *et al*., 2004; Schmitz-Esser *et al*., 2004), indicating that these five NTT proteins are both functional and necessary for the intracellular lifestyle of *P. amoebophila*. Furthermore, with the exception of *Pam*NTT3 all *P. amoebophila* NTT proteins exhibit apparent substrate affinity (K_M) values for their respective substrates in the submillimolar range (0.015–0.676 mM, Table 3). Although the cytosolic nucleotide concentrations of the *Acanthamoeba* host are unknown, it is likely that these concentrations are at comparable levels as reported for various other eukaryotic cells (0.05–4.6 mM, Table 3; Pannbacker, 1967; Hauschka, 1973; Bochner and Ames, 1982; Stitt *et al*., 1989; Larsson *et al*., 1997; Theobald *et al*., 1997; Varela-Gomez *et al*., 2004). According to these data and according to cytoplasmic nucleotide concentrations reported previously for other bacteria (Table 3; Mathews, 1972; Traut, 1994; Pan *et al*., 2001), and under the prerequisite that the inclusion membrane that surrounds *P. amoebophila* is permeable for all NTT substrates, the apparent substrate affinities of all NTT proteins from *P. amoebophila* should be sufficient to transport their substrates under *in vivo* conditions. The high degree of substrate specificity of *Pam*NTT3 for UTP might compensate for its suboptimal affinity (which is lower than reported intracellular UTP concentrations).

Genome sequence analysis of *P. amoebophila* has revealed that it lacks key genes necessary for the biosynthesis of NAD^+ and thus is unable to synthesize NAD^+ *de novo* (Horn *et al*., 2004). Therefore, to import NAD during phases of cell growth and division, *Pam*NTT4 catalyses NAD^+ uptake in exchange for ADP (Fig. 6; Haferkamp *et al*., 2004). The ADP molecule, required as a counter exchange substrate for the NAD^+ import, can be provided via *Pam*NTT5-catalysed ATP import and the subsequent energy-providing conversion of ATP to ADP by various anabolic reactions (Fig. 6).

Protochlamydia amoebophila is also unable to synthesize nucleotides *de novo* (Horn *et al*., 2004). Therefore, a net nucleotide uptake for the synthesis of RNA and DNA is necessary, which can be catalysed by a concerted action of *Pam*NTT3 and *Pam*NTT5 (Fig. 6). Within such concerted action, *Pam*NTT3 imports UTP unidirectional (Tables 1 and 2; Fig. 3B), and *Pam*NTT5 imports GTP and ATP unidirectional (Tables 1 and 2; Fig. 3C). The last missing building block for RNA synthesis is CTP, a compound transported with high affinity and high velocity by *Pam*NTT2 (Tables 1 and 2). However, *Pam*NTT2-mediated CTP import would depend upon the presence of a suitable nucleoside triphosphate for counter exchange (Fig. 6) that might be provided by, e.g. *Pam*NTT3 in form of UTP, or *Pam*NTT5 in form of GTP or ATP (Tables 1 and 2). Because the *P. amoebophila* genome contains a gene coding for a CTP synthase (PyrG; Horn *et al*., 2004), this bacterium might alternatively synthesize CTP from UTP. During replication *P. amoebophila* needs deoxynucleotides for DNA synthesis. dATP, dGTP, dCTP and dUMP can be generated from the respective RNA dinucleotides by the help of ribonucleotide reductase (both subunits, NrdA and NrdB, are encoded in its genome), but *P. amoebophila* in addition requires TTP. However, none of the five NTT proteins accepts thymidylates as substrates (Table 2), and according to its genome sequence *P. amoebophila* lacks a thymidylate synthase (ThyA) gene, generally required by the majority of bacteria for

thymidylate synthesis. However, *P. amoebophila* possesses instead a gene encoding the thymidylate synthase complementing protein (ThyX), which uses dUMP and methylenetetrahydrofolate as substrates for dTMP formation, and which has been recently biochemically characterized in the related bacterium *C. trachomatis* (Myllykallio *et al*., 2002; Horn *et al*., 2004; Griffin *et al*., 2005). Taken together, the substrate specificity of the three nucleotide transporters *Pam*NTT2, *Pam*NTT3 and *Pam*NTT5, and the presence of genes encoding a CTP synthase, a ribonucleotide reductase, the alternative thymidylate synthase ThyX, a nucleoside-diphosphate kinase and specific adenylate, guanylate, cytidylate and thymidylate kinases are sufficient to compensate the lack of a complete nucleotide biosynthesis pathway. Our model thus fully explains the auxotrophy of *P. amoebophila* for nucleotides and illustrates how *P. amoebophila* acquires all precursors for RNA and DNA biosynthesis from its amoeba host cell (Fig. 6).

Although *P. amoebophila* is capable of endogenous ATP regeneration via oxidative phosphorylation (Horn *et al*., 2004), this organism possesses a functional ATP/ADP exchange system in form of *Pam*NTT1 (Schmitz-Esser *et al*., 2004). The presence of this transport activity resembles the situation found in *C. trachomatis*, which also possesses an ATP/ADP exchanger (*Ct*NTT1; Tjaden *et al*., 1999) in addition to an endogenous ATP regeneration system via oxidative phosphorylation (Stephens *et al*., 1998). Nevertheless, the exchange of host ATP for bacterial ADP is an alternative way to acquire energy and is certainly of benefit for example under conditions where substrate usable for oxidative phosphorylation in the bacterium is limiting in the host cytosol. The upregulation of the gene coding for the ATP/ADP antiporter in *C. trachomatis* during the early phase of the developmental cycle indicates that this is the case during the initial differentiation of elementary bodies to reticulate bodies shortly after the infection of a new host cell (Belland *et al*., 2003). It thus seems likely that also *P. amoebophila* is an energy parasite at least under certain physiological conditions.

In this context it is worth mentioning that similar to *P. amoebophila* the only distantly related epidemic typhus causing *R. prowazekii* is also unable to synthesize nucleotides *de novo*, and like *P. amoebophila* also harbours five NTT isoforms (Andersson *et al*., 1998). It will therefore be interesting to study in addition to *Rp*NTT1 (Krause *et al*., 1985) the biochemical properties of *Rp*NTT2 to *Rp*NTT5 in the near future and to compare the data with features of the NTT proteins found in *P. amoebophila*.

In conclusion, we showed in this study that *P. amoebophila* exploits its amoebal host by simultaneously expressing a set of five nucleotide transporters of which four differ from all hitherto characterized NTT proteins regarding substrate specificity or mode of transport. The metabolic model inferred from these data illustrates a previously unseen, tight and apparently well balanced coupling of the endosymbiont and host metabolisms. As environmental chlamydiae are capable to infect mammalian cells (Greub *et al*., 2003; Collingro *et al*., 2005b) and as all human pathogenic chlamydiae also rely on functional NTT proteins, these transporters are not only key to the understanding of the biology of these organisms but should also represent attractive targets for the development of highly specific anti-chlamydial drugs.

Experimental procedures

Cloning of ntt*3 and* ntt*5, and construction of expression plasmids*

Protochlamydia amoebophila UWE25 was cultivated in the host *Acanthamoeba* sp. UWC1 (Gautom and Fritsche, 1995) as previously described (Schmitz-Esser *et al*., 2004). Purification of DNA from amoebae containing the symbiont *P. amoebophila* was performed using the FastDNA-Kit (BIO 101, Carlsbad, CA, USA) according to the protocol recommended by the manufacturer. *ntt3* and *ntt5* were amplified with the Extensor Hi-Fidelity PCR Enzyme Mix (ABgene, Epsom, UK) using forward primers introducing a XhoI restriction site instead of the start codon (5′-TGCACCCTC GAGTCACAGACACCAACAGGG-3′ for *ntt3*, and 5′-TGCA CCCTCGAGAAAAATCAACAAAATTCT-3′ for *ntt5*), and reverse primers containing a BamHI restriction site after the stop codon (5′-TTGGGATCCTTATACAGCTTTCTGTTCTTT CAA-3′ for *ntt3*, and 5′-CACTTGGGATCCTTATCCATGGGA AGCTTCTATAAA-3′ for *ntt5*). The resulting amplification products were digested with BamHI and XhoI, gel purified, and inserted into the expression vector pET16b (Novagen, Heidelberg, Germany). The newly constructed plasmids (pNTT3, pNTT5) were transformed into and maintained in *E. coli* XL1Blue (Stratagene, Heidelberg, Germany). The identity of the cloned genes was checked by sequencing.

Heterologous expression of PamNTT2, PamNTT3 and PamNTT5 in E. coli

For heterologous expression of *Pam*NTT2, *Pam*NTT3 and *Pam*NTT5 the *E. coli* strain BLR (DE3) was used. Synthesis of recombinant nucleotide transporters was conducted according to previously reported methods (Haferkamp *et al*., 2004). Briefly, *E. coli* cells harbouring the vector pET16b containing *ntt*2, *ntt*3 and *ntt5* [pNTT2 (Haferkamp *et al*., 2004), pNTT3, pNTT5] were induced by IPTG application to express the respective transporter gene. After 1 h cells were sedimented (3000 *g*, 5 min, at 8°C) and resuspended in 50 mM potassium phosphate buffer medium (pH 7.0) to an OD_{600} of 5.0, kept at room temperature, and subsequently used for uptake experiments.

Uptake of radioactively labelled nucleotides into intact E. coli *cells*

To analyse transport properties of *Pam*NTT2, *Pam*NTT3 and

*Pam*NTT5, 100 μl of either induced *E. coli* cells harbouring the corresponding constructs, or non-induced cells (control) were added to 100 μl incubation medium (50 mM P_i buffer, pH 7.0) containing the [α-^{32}P]-labelled substrates ATP, ADP, GTP, GDP, dGTP, CTP and UTP. [α-^{32}P]-labelled ADP and also GDP was synthesized as described previously (Tjaden *et al*., 1998). Cells lacking the expression plasmid, but incubated in the presence of IPTG exhibited the same uptake as the control cells (data not shown).

Uptake was allowed for indicated time spans, at 30°C, and terminated by removal of external substrate (Haferkamp *et al*., 2004). For the latter purpose, cells were applied on 0.45 μm nitrocellulose filters, pre-wetted with incubation medium, and set under vacuum. The cells were immediately washed with 3 × 4 ml incubation medium, and filters were transferred into 20 ml scintillation vessels, containing 10 ml water. Radioactivity in the samples was quantified in a scintillation counter (Tricarb 2500, Canberra-Packard, Heidelberg, Germany).

Back exchange studies and thin-layer chromatography

To characterize the transport mode of recombinant *Pam*NTT2, *Pam*NTT3 and *Pam*NTT5, back exchange studies were conducted. IPTG-induced *E. coli* cells synthesizing the recombinant NTT were incubated for an appropriate time span in phosphate buffer containing radioactively labelled [α-^{32}P]-ATP, GTP or UTP. Subsequently, cells were collected by centrifugation, washed and resuspended in phosphate buffer medium containing indicated excess of non-labelled counter exchange substrate. Back exchange was carried out at 30°C for the indicated time span, terminated by rapid filtration, and quantified of remaining internal radioactivity in a scintillation counter.

To identify the molecular nature of the radioactively labelled compounds exported by cells expressing *Pam*NTT2 and preloaded with [α-^{32}P]-ATP, we employed a thin-layer chromatography method (Mangold, 1967), routinely used in our laboratory (Trentmann *et al*., 2000; Haferkamp *et al*., 2004). For this, *E. coli* cells were collected by centrifugation, a 10 μl aliquot of the export supernatant (see above) was loaded onto a 0.5 mm poly(ethylene amine) cellulose thin-layer chromatography plate and dried with a fan. RF values of radioactively labelled nucleotides and phosphate were determined after radioautography and corresponded to values of unlabelled nucleotides visualized under ultraviolet light, and to radioactively labelled standards.

Transcriptional analysis

Protochlamydia amoebophila UWE25 was grown in the host *Acanthamoeba* sp. UWC1 (Schmitz-Esser *et al*., 2004). For transcriptional analysis amoebae harbouring *P. amoebophila* were harvested by centrifugation (2350 *g*, 5 min, at 4°C), resuspended in TRIzol (Invitrogen Life Technologies, Lofer, Austria), and immediately homogenized using the Bead-Beater Fast Prep FP120 Instrument (BIO 101, Carlsbad, CA, USA). Whole RNA purification was performed according to the recommendations of the manufacturer, followed by a DNase treatment using Deoxyribonuclease I. A control PCR using 16S rRNA gene-targeted primers and Taq DNA polymerase instead of reverse transcriptase was performed to ensure that the RNA preparation was free of DNA, and 1 μg of RNA was subsequently used for cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (MBI-Fermentas, St Leon-Rot, Germany) and a gene-specific primer. cDNA was subsequently used as template in standard PCR reactions. Negative controls (no cDNA added) were included in all PCR reactions. A standard PCR cycling program with 40 cycles, and an annealing temperature of 58°C was used for the amplification of the *ntt3* and *ntt5* cDNA. Primers targeting a 588 bp fragment of *ntt3* (forward primer: 5′-CATCCTCACG CAACTGCTGAC-3′, reverse primer: 5′-GCTCTCGGACTT GATGTTTCC-3′), or a 577 bp fragment of *ntt5* (forward primer: 5′-CACAGCAAGTGATGCAGGTGC-3′, reverse primer: 5′-CAGTTGTAAGAGCCAGCCGTC-3′), respectively, were used for cDNA synthesis and PCR reactions. Amplification products were sequenced to ensure that amplification was specific. All experiments were performed in independent triplicates.

Acknowledgements

Work in the laboratory of Ekkehard Neuhaus has been financially supported by the Deutsche Forschungsgemeinschaft (DFG, NE418/9–1). Work in the laboratory of Michael Wagner and Matthias Horn has been financially supported by the Austrian Science Fond FWF (P16566-B14 and Y277-B03).

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Amino acid sequence alignment of various NTT type proteins from intracellular bacteria and plants.

Fig. S2. Phylogenetic relationships of nucleotide transport proteins.

Fig. S3. Kyte–Doolittle hydropathy plot of the predicted amino acid sequences of *Pam*NTT2, *Pam*NTT3 and *Pam*NTT5.

This material is available as part of the online article from http://www.blackwell-synergy.com