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**Review**

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# Structure, function, evolution, and application of bacterial Pnu-type vitamin transporters

**Abstract:** Many bacteria can take up vitamins from the environment via specific transport machineries. Uptake is essential for organisms that lack complete vitamin biosynthesis pathways, but even in the presence of biosynthesis routes uptake is likely preferred, because it is energetically less costly. Pnu transporters represent a class of membrane transporters for a diverse set of B-type vitamins. They were identified 30 years ago and catalyze transport by the mechanism of facilitated diffusion, without direct coupling to ATP hydrolysis or transport of coupling ions. Instead, directionality is achieved by metabolic trapping, in which the vitamin substrate is converted into a derivative that cannot be transported, for instance by phosphorylation. The recent crystal structure of the nicotinamide riboside transporter PnuC has provided the first insights in substrate recognition and selectivity. Here, we will summarize the current knowledge about the function, structure, and evolution of Pnu transporters. Additionally, we will highlight their role for potential biotechnological and pharmaceutical applications.

**Keywords:** membrane transport; Pnu transporters; vitamins.

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## Introduction

Membrane proteins mediate numerous physiological processes essential for viability of cells, such as signal transduction, biological energy conversion, or transport. Membrane transporters are specialized proteins for the uptake or extrusion of ions and solutes, including vitamins, into or from cells and organelles. Vitamins comprise a chemically and functionally diverse class of compounds, many of which are essential enzymatic cofactors or precursors thereof. They enable complex enzymatic activities, thus enlarging the diversity of biological catalytic activities. The uptake of vitamins is a critical step not only for organisms that strictly depend on vitamins like humans but also for many bacteria, even if they possess biosynthesis pathways for these molecules. Many vitamins are complex compounds and their biosynthesis is energetically costly. For example, the biosynthesis of a single molecule riboflavin requires 25 molecules of ATP (Bacher et al., 2000; Marsili et al., 2008), as well as the energy required for the production of the biosynthetic enzymes. In contrast, uptake requires a single membrane transport protein only, which consumes at most a few molecules of ATP, depending on the transport system used. Similarly, the salvage of parts of a complex molecule is an efficient strategy to save resources. The use of breakdown products of vitamins present in the environment eliminates the need for complete biosynthesis pathways to generate the cofactors. Many transport systems are key players of these salvage pathways and mediate the uptake of specific precursors instead of the final cofactor (Jaehme and Slotboom, 2015).

The Pnu transporter family is broadly distributed among bacteria and consists of transporters for different B-type vitamins (Figures 1 and 2). The name Pnu originates from the first member that was discovered over 30 years ago as a transport system involved in the uptake and utilization of pyridine nucleotides (Pnu – pyridine nucleotide uptake) (Kinney et al., 1979; Spector et al., 1985). The gene encoding PnuC from *Salmonella typhimurium* was cloned in 1986, and the protein was initially designated as the transporter of a previously characterized nicotinamide mononucleotide (NMN) uptake activity (Kinney et al., 1979; Liu et al., 1982; Spector et al., 1985; Tirgari et al., 1986; Zhu et al., 1991). Later studies showed that the substrate for PnuC is not NMN but its unphosphorylated precursor nicotinamide riboside (NR), which is one of the manifestations of vitamin B3, besides nicotinamide

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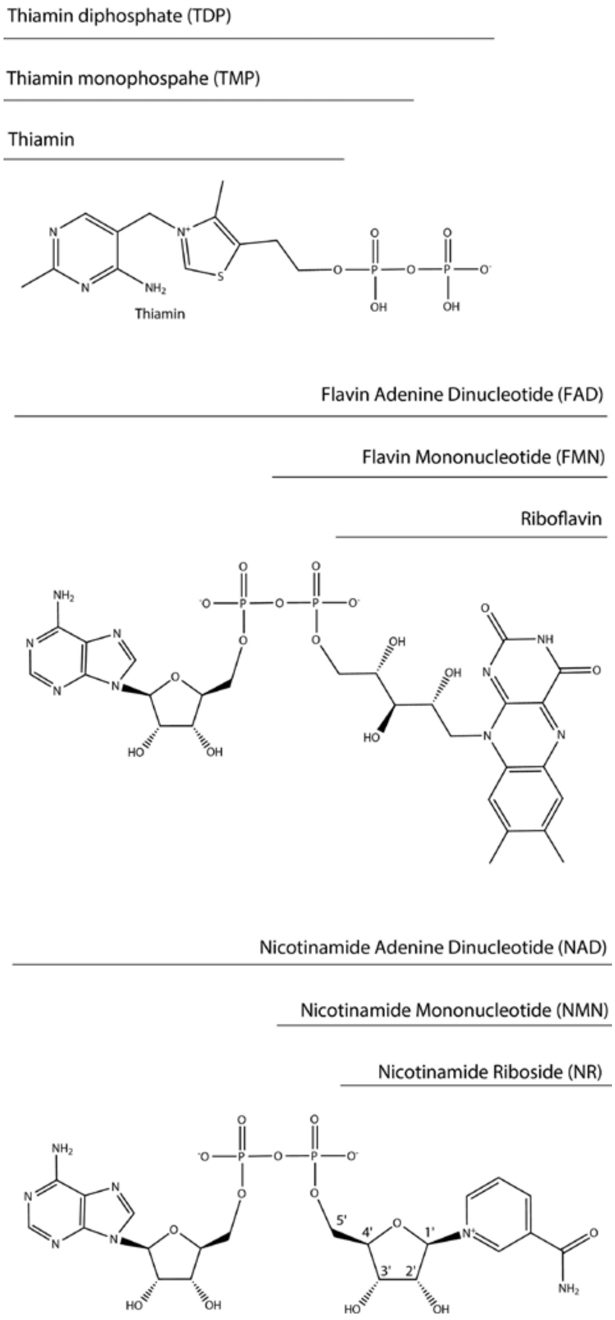

**Figure 1:** Chemical structures of the B-type vitamins transported by Pnu transporters and derived cofactors.
Vitamin B1 – thiamin; vitamin B2 – riboflavin; vitamin B3 – nicotinamide riboside.

and nicotinic acid (Kemmer et al., 2001; Bieganowski and Brenner, 2004; Grose et al., 2005b). With the help of comparative genomics approaches, homologs of PnuC with varying substrate specificities were predicted in the last decade (Rodionov et al., 2002, 2008; Vitreschak et al., 2002; Rodionov and Gelfand, 2005). One of these predictions was confirmed experimentally when Pnu transporters specific for riboflavin were characterized (Vogl et al., 2007). Finally, the first crystal structure of a Pnu transporter was solved last year (Jaehme et al., 2014). The structural information provides insight into the mechanism of transport and explains many results from earlier functional studies.

This review summarizes the current knowledge on the structure, function, mechanism, and evolution of Pnu transporters. We will integrate the available data and build a tentative mechanistic picture of the transport mediated by Pnu proteins. Additionally, we will highlight potential biotechnological and pharmaceutical importance.

## Substrate specificity, genomic organization, and phylogenetic distribution of Pnu transporters

### Tracking Pnu transporter homologs

Within the family of Pnu transporters, homologs with different substrate specificities exist (Figure 2). The genes coding for these transporters are often found in a conserved genomic context, which occasionally also contains regulatory RNA elements, so-called riboswitches (Gelfand and Rodionov, 2008). The genomic organization helped to predict the function of different Pnu transporter genes. Additionally, there is a close relation between genomic organization, substrate specificity, and phylogenetic distribution within the Pnu transporter family. Pnu transporters have no homologs in eukaryotes, which use very different transport systems to import the same substrates (Yonezawa and Inui, 2013; Young et al., 2013; Zhao and Goldman, 2013). Pnu transporters are also not related to the prokaryotic substrate binding components of ECF-type ABC transporters, neither in their mechanism of action nor in sequence and three-dimensional structure (Slotboom, 2014).

**PnuC** PnuC was the first Pnu transporter described and is specific for NR. NR is one of several related compounds (vitamers) that have been named vitamin B3 and is a precursor of the cofactor NAD (Figure 1), which is found in all living organisms. The specificity of PnuC for NR has been demonstrated for homologs from *Escherichia coli* (Sauer et al., 2004), *Haemophilus influenzae* (Kemmer et al., 2001), *Haemophilus parainfluenzae* (Cynamon et al., 1988), *S. typhimurium* (Grose et al., 2005b), and *Neisseria mucosa* (Jaehme et al., 2014). In contrast to other Pnu homologs, the genomic organization of the genes

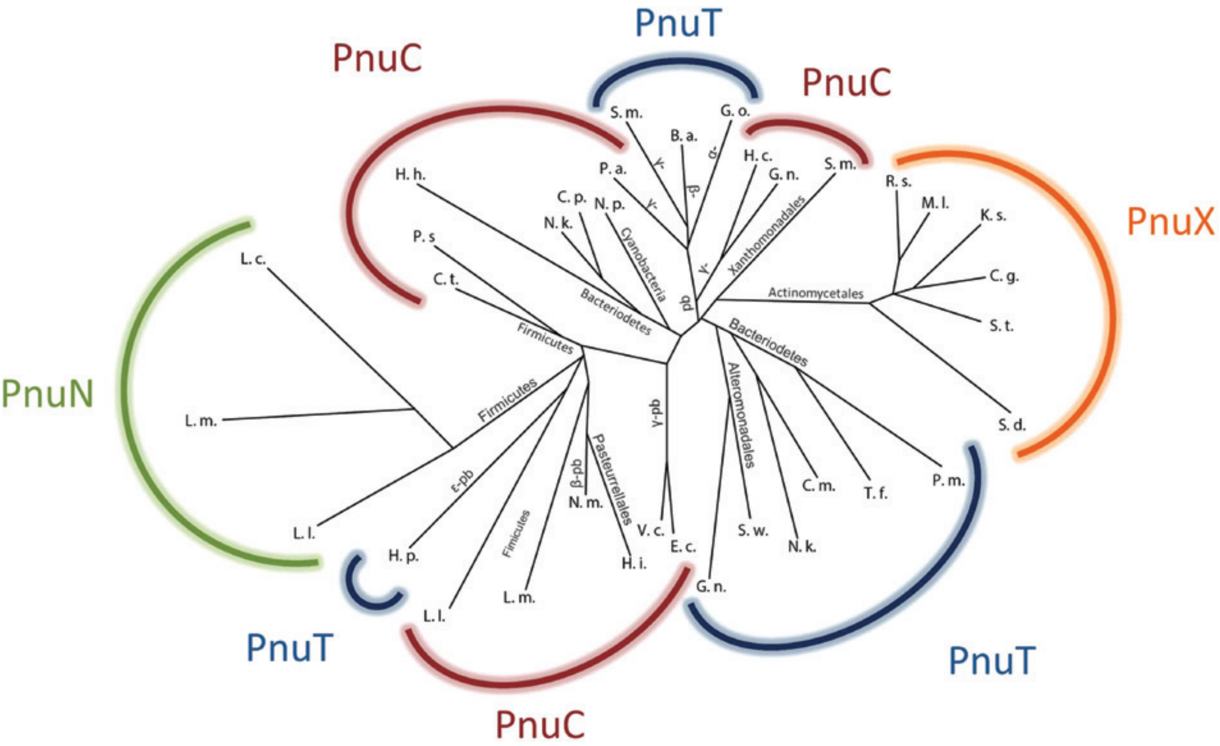

**Figure 2:** Phylogenetic tree of the Pnu transporter family.

The maximum likelihood phylogenetic tree was constructed by the Phylip package based on a multiple sequence alignment of 34 members of the family (Felsenstein, 1981). Pnu transporter homologs with different (predicted) substrate specificities are indicated by different colors: red – PnuC, orange – PnuX, blue – PnuT, green – PnuN. Organism names are abbreviated as listed below in the order in which they appear in the tree (clockwise), starting with the PnuN cluster. Organisms that are present twice are mentioned at their first occurrence only. L. l. – *Lactococcus lactis*, L. m. – *Leuconostoc mesenteroides*, L. c. – *Lactobacillus casei*, C. t – *Clostridium thermocellum*, P. s. – *Paenibacillus sp.*, H. h. – *Haliscomenobacter hydrossis*, N. k. – *Niastella koreensis*, C. p. – *Chitinophaga pinensis*, N. p. – *Nostoc punctiforme*, P. a. – *Pseudomonas aeruginosa*, S. m. – *Stenotrophomonas maltophilia*, B. a. – *Burkholderia ambifaria*, G. o. – *Gluconobacter oxydans*, H. c. – *Hahella chejuensis*, G. n. – *Glaciecola nitratireducens*, R. s. – *Renibacterium salmoninarum*, M. l. – *Micrococcus luteus*, K. s. – *Kytococcus sedentarius*, C. g. – *Corynebacterium glutamicum*, S. t. – *Salinispora tropica*, S. d. – *Streptomyces davawensis*, P. m. – *Prevotella melaninogenica*, T. f. – *Tannerella forsythia*, C. m. – *Cyclobacterium marinum*, S. w. – *Shewanella woodyi*, E. c. – *Escherichia coli*, V. c. – *Vibrio cholerae*, H. i. – *Haemophilus influenzae*, N. m. – *Neisseria mucosa*, H. p. – *Helicobacter pylori*. pb – proteobacteria.

encoding PnuC is not well conserved. In Enterobacteria, *pnuC* forms an operon with the genes for the NAD biosynthesis protein NadA (quinolinate synthase) and is regulated by NadR (Gerasimova and Gelfand, 2005), a bifunctional NR kinase and NMN adenylyltransferase that acts additionally as transcription factor in some organisms due to the presence of an N-terminal DNA binding motif. In other organisms, *pnuC* clusters directly with *nadR* (*Clostridium thermocellum*, *Nostoc punctiforme*), *pyrG* (CTP-synthase, *H. influenzae*), or *udp* (uridin phosphorylase; *Lactococcus lactis*), all of which are involved in the synthesis and salvage of NAD. Based on the positional clustering with genes involved in NAD salvage and *de novo* biosynthesis and the presence of putative binding sites for transcription factors like the NadR, YrxA, and Nrt, further PnuC homologs have been predicted in organisms from many different taxonomic branches including Firmicutes, Cyanobacteria, Proteobacteria, and Bacteroidetes (Gelfand and Rodionov, 2008), illustrating their broad distribution among bacteria (Figure 2).

**PnuX (RibM)** Riboflavin is the second substrate of which transport by a Pnu transporter homolog was experimentally verified. The transporters are termed either PnuX to emphasize the homology to the Pnu transporters or RibM, which reflects the function as a riboflavin transporter. PnuX homologs from *Corynebacterium glutamicum* and *Streptomyces davawensis*, two Actinomycetales, have been characterized (Vogl et al., 2007; Hemberger et al., 2011). Based on their conserved genomic organization, further *pnuX* genes have been identified, exclusively in Actinomycetales (Vitreschak et al., 2002) (Figure 2). In many genomes, they are located at different positions within the riboflavin biosynthesis operon *ribBADH*. Additionally, *pnuX* (or the operon containing the gene) is often preceded by an FMN-regulated riboswitch (Vitreschak et al., 2002, 2004).

**PnuT** The third predicted Pnu transporter homolog, PnuT, has not been characterized experimentally. Thiamine is the predicted substrate based on the operon context of the *pnuT* gene and the presence of a thiamine-regulated riboswitch (Rodionov et al., 2002). The localization of PnuT transporter genes in a cluster with the genes for a TonB-dependent outer membrane protein and a kinase is conserved in different Proteobacteria (α, β, γ) and members of the Bacteroidetes/Chlorobi group (Rodionov et al., 2002; Gelfand and Rodionov, 2008) (Figure 2). A more distantly related predicted thiamine transporter is found in *Helicobacter pylori*, which belongs to the ε-proteobacteria (Figure 2). Here, *pnuT* clusters with *tenA*, which encodes an enzyme involved in salvage of thiamine from degradation products pointing to a substrate specificity for thiamine precursors (Jenkins et al., 2008; Jaehme and Slotboom, 2015). Many organisms from the γ-proteobacteria and Bacteroidetes encode additionally a small hypothetical protein within the operons. These proteins have low sequence similarity; however, they all have a predicted ferredoxin fold with $\beta\alpha\beta_2\alpha\beta$ secondary structure. Interestingly, the cytoplasmic thiamine-binding protein YkoF also contains the ferredoxin fold (Devedjiev et al., 2004; Dermoun et al., 2010), and the *ykoF* gene is present in some operons of other transporters for thiamine and related compounds like ThiXYZ and YkoEDC (Jaehme and Slotboom, 2015).

**PnuN** Some Firmicutes (e.g., *Lactobacillus casei* and *Enterococcus faecalis*) contain the *pnuN* gene in an operon also encoding a ribonucleotide reductase and a deoxynucleoside kinase, which is coregulated by the Nrd repressor (NrdR) (Rodionov and Gelfand, 2005) (Figure 2). The operon organization suggests that PnuN could be specific for deoxynucleosides, which would subsequently be phosphorylated by the kinase. Whereas *E. coli* and most other organisms are only able to phosphorylate deoxythymidine, *Lactobacilli* and *Bacilli* have been shown to phosphorylate all four different deoxynucleosides (Andersen and Neuhard, 2001). Such salvage pathways would require specific transport systems, which could be represented by PnuN transporters in these organisms.

Several organisms from different taxonomic classes possess more than a single Pnu transporter (Figure 2). A number of different Alteromonadales from the genus *Shewanella* and *Glaciecola*, as well as Xanthomonadales from the genus *Stenotrophomonas*, have one *pnuT* gene present in the typical *pnuT* operon (see above) and one *pnuC* gene associated with the NAD biosynthesis gene *nadR*. Different Firmicutes have *pnuC* as well as *pnuN* genes, for instance, *Lactococcus lactis* or *Leuconostoc mesenteroides*.

# Structural features of Pnu transporters

## Oligomeric state of Pnu transporters

Pnu transporters are α-helical membrane proteins of 190–270 amino acids and molecular weights between 20 and 30 kDa. LacZ- and PhoA-PnuC fusions indicate that the N- and C-termini of PnuC from *H. influenzae* are located in the cytoplasm and that the protein contains eight α-helical transmembrane segments (TMs) (Sauer et al., 2004). The recently solved crystal structure of PnuC from *N. mucosa*, a close homolog of *H. influenzae* PnuC (47% identity, Figure 2), confirmed this model (Jaehme et al., 2014) (Figure 3A); however, the number of TMs is conserved neither among the PnuC homologs nor in the other Pnu transporter. The N-terminal helix is absent in many members. The difference in the number of TMs possibly correlates with differences in the oligomeric state of Pnu transporters. PnuC from *N. mucosa* has eight TMs and is trimeric in detergent solution (Figure 3B), whereas its homolog from *E. coli* has seven TMs and is monomeric (Jaehme et al., 2014). The crystal structure of *N. mucosa* PnuC shows that the non-conserved N-terminal helix is essential for interactions between the subunits in the trimer (Figure 3B), providing a structural explanation for the monomeric state of Pnu homologs with seven TMs. It is not clear whether the different oligomeric states have functional consequences. The functional unit appears to be the monomer (see below) in which the seven conserved helices in Pnu transporters have been numbered 1–7, whereas the non-conserved N-terminal TM was numbered TM-1 (Figure 3A).

## Overall structure and symmetry of PnuC

Although PnuC from *N. mucosa* is trimeric, the monomer constitutes the functional unit that folds into a tertiary structure that harbors clear internal symmetry (Figure 3C). The protein has a central core consisting of six TMs arranged in a hexagonal shape, which contains the ligand-binding site and the translocation pore (Figure 3D). This hexagon is formed by two bundles of three helices, the N- and C-terminal three-helix bundle (THB), which are related by pseudo two-fold symmetry. TMs 1, 2, and 3 are related to TMs 5, 6, and 7 (Figure 3D). This similarity is represented by a root mean square deviation (r.m.s.d.) of 1.3 Å between the N- and C-terminal THB. Internal structural pseudo-symmetry is a common feature of membrane

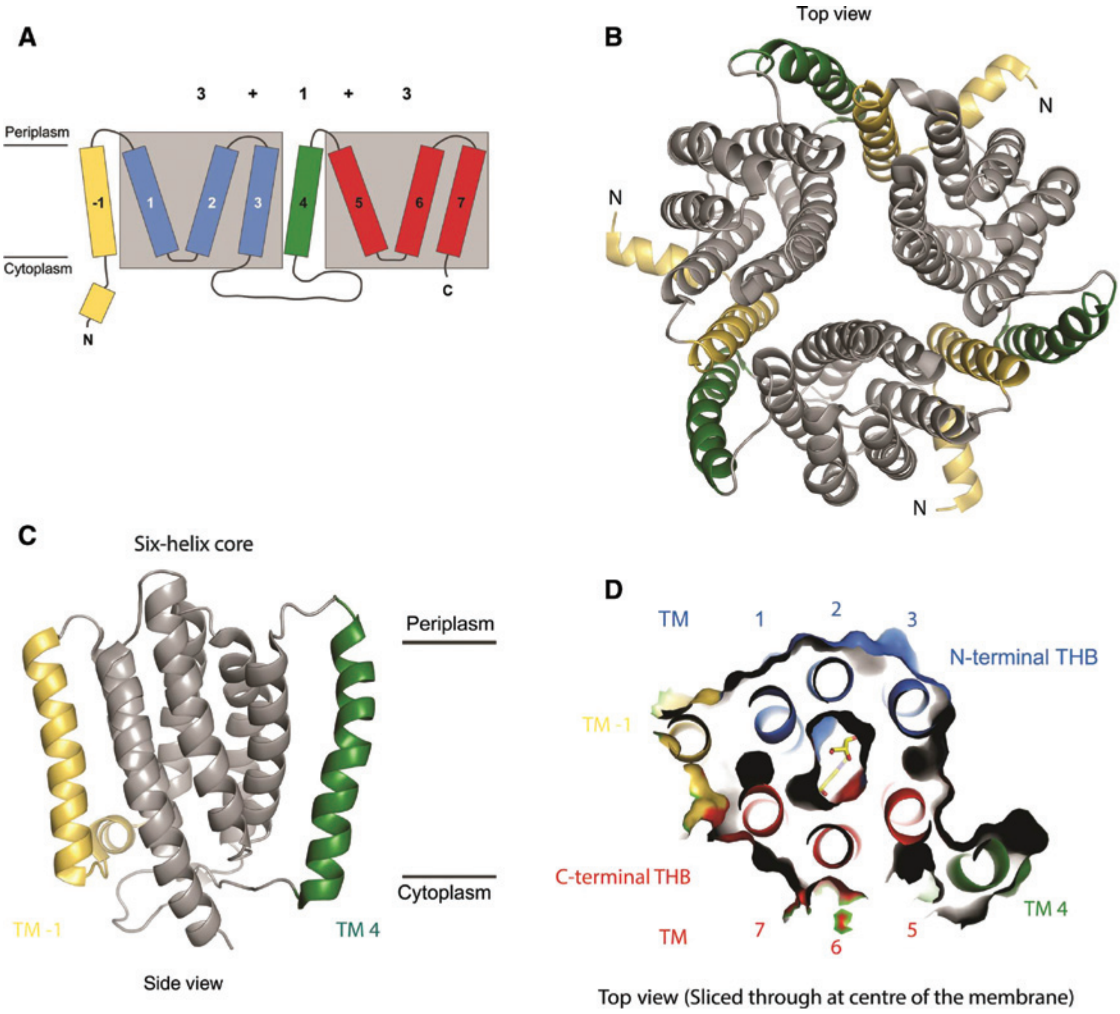

**Figure 3:** Structure of PnuC from *N. mucosa*.
Color scheme: TM-1 – yellow, six-helix core – gray, N-terminal three-helix bundle – blue, C-terminal three-helix bundle – red, TM4 – green. (A) Schematic representation of the topology of a single protomer of PnuC. Colored rectangles indicate the transmembrane helices with their corresponding number. Numbers above the topology model indicate the topological arrangement of the symmetry-related parts (gray boxes). (B) Top view from the periplasmic side of the membrane of the PnuC homotrimer. (C) Side view (from the membrane) of one PnuC monomer. (D) Top view of a PnuC monomer sliced through at the center of the membrane. The six-helix core is organized roughly as a hexagon with a bound NR molecule (yellow with labeled atoms: red – oxygen, blue – nitrogen) in the center. The N-terminal three-helix bundle (TM1–TM3, blue) is symmetry related to the C-terminal three-helix bundle (TM5–TM7, red). TM1 and TM4 are peripheral and not symmetry related.

transporters (Forrest, 2013). Although in the majority of transport proteins, the pseudo-symmetrical parts are related by inverted topology (Forrest et al., 2011), in the case of PnuC, both THBs are related by parallel topology (Figure 3A). To allow for the parallel orientation of the two THBs, they are connected via the peripheral linker helix TM4, which connects the cytoplasmic C-terminus of the first bundle with the periplasmic N-terminus of the second (Figure 3A,C). The arrangement of two parallel THBs connected by a linker helix gives rise to a characteristic 3+1+3 membrane topology of Pnu transport proteins (Figure 3A).

## Structural basis of ligand binding of Pnu transporters

The crystal structure revealed the presence of a single NR molecule bound in the center of the six-helix core (Figure 3D). It is located in the membrane embedded part of the protein, close to the center of the membrane, but slightly offset toward the cytoplasmic side. The non-symmetrical molecule interacts with numerous residues provided by TMs 1, 3, 5, and 6, and binds to one of the most conserved sequence motifs in Pnu transporters: the WxxWxxxN/D motif located in TM6 (Figure 4A,B). Both tryptophans

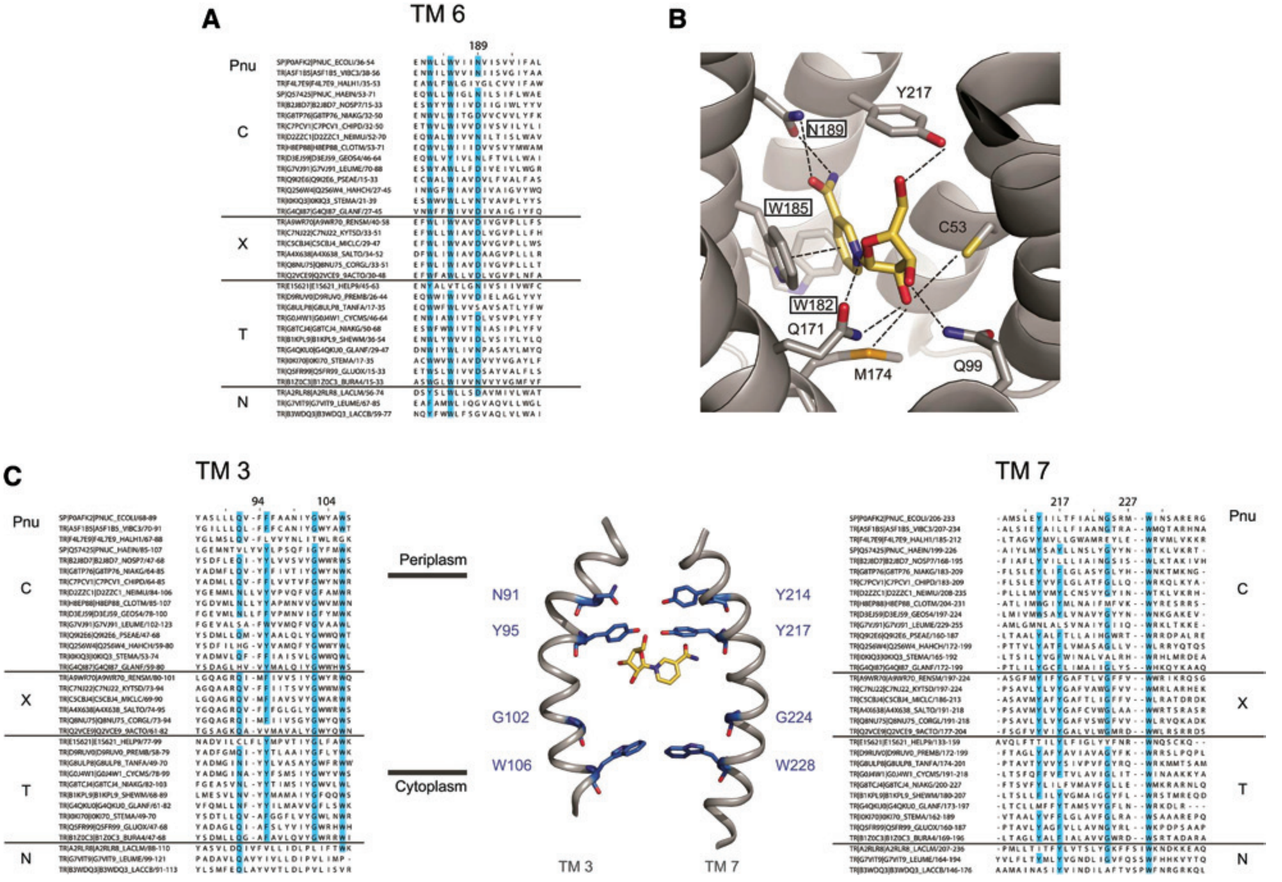

**Figure 4:** Multiple sequence alignments of conserved regions in the Pnu transporter family and their location in the structure of PnuC from *N. mucosa*.
Conserved motifs are indicated in blue. (A) Multiple sequence alignment of TM6. The conserved motif WxxWxxxN/D is indicated in blue. (B) Ligand-binding site of PnuC with a single NR molecule bound. The conserved motif indicated in (A) binds the nicotinamide moiety. Residues provided by TMs 1, 3, 5, and 7 interact with the ribose ring. Interactions are indicated by dashed lines. (C) Multiple sequence alignments of TM3 and TM7, which are related by pseudo two-fold symmetry.

(Trp182, Trp185) of the motif orient the aromatic ring of the nicotinamide moiety, whereas the asparagine (N189) binds the carboxamide group of the nicotinamide ring (Figure 4B). Despite different substrate specificities, all Pnu transporter types possess this motif (Figure 4A), suggesting the suitability of this architecture to bind different molecules, which all contain aromatic ring structures. Thiamine might interact with PnuT via its aminomethylpyrimidin moiety and in PnuX the motif probably stabilizes parts of the isoalloxazine ring. Several hydrophilic residues provided by TM1, TM3, and TM5 of PnuC stabilize the ribose moiety of NR via H-bonds with OH-groups of the sugar; however, none of these residues is conserved among the Pnu transporters (Figure 4B). Due to the lack of residues that are conserved exclusively in a particular subtype of the Pnu transporters, the molecular basis for the different substrate specificities remains elusive.

## Structural basis of gating of PnuC

The structure of PnuC from *N. mucosa* represents an occluded conformation in which the protein blocks access to both the cytoplasmic and the periplasmic sides of the membrane. Opening on either side is required for substrate release from the occluded carrier or substrate binding to the empty carrier. There are several different symmetry-related residues in the THBs that probably have a role in the mechanism of transport. The intracellular gate consists of two layers of symmetry-related residues, which seal the central pore from the cytoplasmic side. The outer layer consists of two tryptophans provided by TM3 (Trp106) and TM7 (Trp228) (Figure 4C) and the inner layer is formed by the symmetry-related Val57 (TM1) and Met174 (TM5), which face each other and are oriented at an angle of approximately 90° compared to the pair of tryptophans (Jaehme et al., 2014).

The access pathway to the binding pocket from the periplasmic side is much more complex. The seal is much thicker and more hydrophilic. The relation between both THBs is especially clear when comparing TM3 and TM7 (Figure 4C). Directly above the ligand-binding site (toward the periplasm), two symmetry-related tyrosines (Y95, Y214) shape the lid of the binding pocket. At these positions, bulky aromatic residues are conserved throughout the Pnu transporter family, indicating a mechanistic function rather than a functional role in ligand binding. One helical turn toward the periplasm, an asparagine (N91) and a tyrosine (Y217) build the next pair of conserved symmetry-related residues that close the access to the binding pocket from the periplasm. Both positions are well covered in the Pnu family, with asparagine or glutamine residues present at position 91 and tyrosine or phenylalanine residues at position 217. The loops connecting TM2 and TM3 and TM6 and TM7 finally build a lid on top of the periplasmic seal; however, there are no conserved residues present in these regions.

## Transport mechanism of Pnu transporters

Pnu transporters show no homology to any primary transport systems like ABC transporters. Furthermore, coupling to a proton or sodium gradient has not been shown (Cynamon et al., 1988; Vogl et al., 2007). The sensitivity of PnuC-mediated NR uptake in *H. parainfluenzae* to 2,4-dinitrophenol and 2-deoxyglucose is probably due to ATP depletion, which affects metabolic trapping by NR phosphorylation via NadR (Cynamon et al., 1988). Based on these data, Pnu transporters are most probably uniporters, which operate via a facilitated diffusion mechanism. The PnuX-mediated riboflavin transport is also energy independent, underlining its function as facilitator (Vogl et al., 2007). A similar scenario of transport via facilitated diffusion and subsequent phosphorylation has been shown for sugar uptake by the mammalian GLUT sugar transporters.

It is unlikely that Pnu transporters use a so-called 'group translocator' mechanism. Group translocators strictly couple substrate transport to substrate phosphorylation. The most prominent example is the bacterial phosphotransferase system that transfers a phosphoryl group via two soluble domains (enzyme IIA and IIB) to a carbohydrate that is translocated by the membrane-embedded domain (enzyme IIC). For the Pnu transporters, neither a direct interaction between the transporter and its corresponding kinase enzyme nor the strict coupling between the transport and phosphorylation reactions has been observed. Furthermore, the corresponding kinases used by Pnu transporters are not dedicated to these proteins but have a general physiological relevance in the metabolism of the respective cofactor. Finally, PnuX from *S. davawensis* showed riboflavin uptake activity when expressed in *Bacillus subtilis*, which does not contain an endogenous Pnu transporter (Hemberger et al., 2011). Such a functional recombinant expression argues against a group translocation mechanism that would require additional components for substrate modification.

### A general scheme for Pnu transporter-mediated uptake

The best-studied Pnu transporters are the PnuC homologs from *S. typhimurium* and *H. influenzae*. Although they are both involved in NAD metabolism, the two proteins were initially not recognized to be related transporters. PnuC from *S. typhimurium* was first characterized as an NMN uptake system (Liu et al., 1982), whereas the *H. influenzae* homolog was proposed to transport NR (Cynamon et al., 1988; Godek and Cynamon, 1990). However, subsequent research showed that both transporters are similar and also use a similar mode of transport. In both organisms, periplasmic machineries have been identified that breakdown NAD and NMN to NR, which is the transported substrate of PnuC (Cynamon et al., 1988; Kemmer et al., 2001; Herbert et al., 2003; Sauer et al., 2004; Grose et al., 2005b). Shortly after cloning of PnuC, it became evident that its activity strongly depends on the regulator NadR (Foster et al., 1987, 1990; Zhu et al., 1989, 1991). NadR was initially thought to have a regulatory as well as a transport function, because it is able to sense the internal NAD pool in order to repress essential genes for NAD biosynthesis [*nadB*, *nadA-pnuC*, and *pncB* (Spector et al., 1985; Cookson et al., 1987; Zhu et al., 1989; Penfound and Foster, 1999)] and to regulate transport via PnuC in response to cellular NAD levels (Tirgari et al., 1986; Foster et al., 1990; Zhu and Roth, 1991; Zhu et al., 1991; Foster and Penfound, 1993). Later, more specific enzymatic functions were assigned to NadR. The N-terminal domain harbors an NMN adenylyltransferase activity and the C-terminal part an NR kinase domain (Raffaelli et al., 1997; Kurnasov et al., 2002), and therefore can convert the transported NR molecule into NMN and NAD (Figure 5). Additionally, NadR of several organisms including *S. typhimurium* possess an additional DNA-binding (HTH) domain at the N-terminus, which explains their ability to act as a repressor

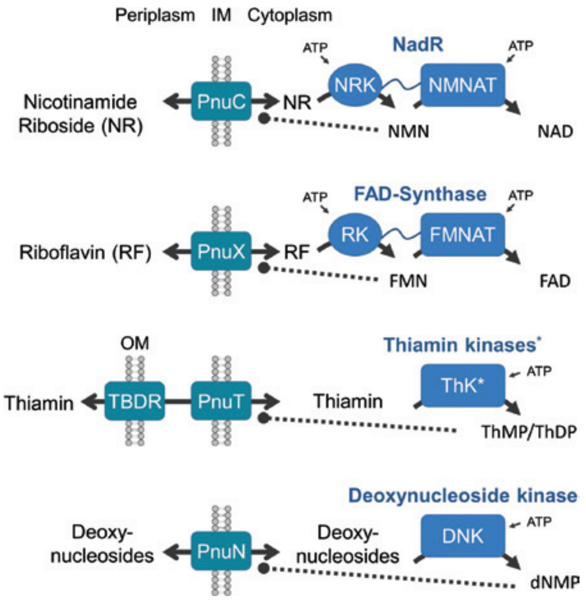

**Figure 5:** Putative mechanism of transport for known and predicted Pnu transporters.
Pnu transporters translocate unphosphorylated substrates across the inner membrane (IM). Cytoplasmic kinases phosphorylate the vitamins. The phosphorylated products are not substrates of the Pnu transporters (indicted by the dashed line with black dot at the end), and therefore they are trapped in the cytoplasm. In case of NR and riboflavin, the kinases also contain adenylyltransferase domains, which convert the phosphorylated products NMN and FMN into the final cofactor NAD and FAD. NRK – nicotinamide riboside kinase domain, NMNAT – NMN adenylyltransferase domain, RK – riboflavin kinase domain, FMNAT – FMN adenylyltransferase domain, ThK – thiamin kinases, DNK – deoxynucleoside kinase. *Different types of thiamin kinase are encoded in PnuT-type operons. Homologs of thiamin pyrophosphokinase, the choline kinase family, and the homoserine kinase family are found (Gelfand and Rodionov, 2008).

(Penfound and Foster, 1999; Kurnasov et al., 2002). The enzymatic activities of NadR are the key players that connect transport mediated by PnuC to NAD metabolism. The NR kinase domain indirectly regulates the rate of NR uptake mediated by PnuC (Grose et al., 2005a,b; Merdanovic et al., 2005). The phosphorylation of NR to NMN subsequent to its transport prevents efflux of the substrate, thus creating directionality and providing a possibility of substrate accumulation within the cell (Kurnasov et al., 2002) (Figure 5). This mechanism is called 'metabolic trapping' and relates the rate of phosphorylation to the rate of uptake, although the rate-limiting step remains to be determined. A physical interaction between the transporter and the enzymes has not been shown so far.

Possibly Pnu transporters for different substrates also make use of metabolic trapping, as all described homologs are related to metabolic pathways for their individual substrate based on the genomic context of the genes (see above). Thus, Pnu transporters accept exclusively unphosphorylated substrates, which are phosphorylated subsequent to their translocation preventing the metabolite from efflux (Figure 5).

## Ligand binding

PnuC from *N. mucosa* binds its substrate with high affinity ($K_D$ value of 0.14 μM; Jaehme et al., 2014). This value corresponds well with the $K_M$ value determined for NR transport into *H. parainfluenzae* of 0.14 μM (Cynamon et al., 1988). Because the $K_D$ is similar to the $K_M$, it is likely that a step subsequent to substrate binding is rate limiting for NR translocation. The high affinity is probably an adaptation to the low abundance of a trace compound like NR. Organisms like *N. mucosa* and different *Pasteurellaceae* occupy special niches like the mucosa of the upper respiratory tract. The concentrations of all potential V factors (a collective term for NAD, NMN, and NR) in human body fluids are not known precisely. Although in certain microenvironments, NAD concentrations in the low micromolar range can be reached, an average NAD serum concentration of 50–60 nM has been reported (Zocchi et al., 1999). The NAD concentrations in extracellular body fluids of pigs are between 0.2 and 1 μM (O'Reilly and Niven, 2003). These values indicate the need for a high-affinity uptake system and show that the reported $K_D$ and $K_M$ values are in the range of the physiological concentrations of NAD. Although no experimental data on the thiamine transporter PnuT exist, a high affinity is expected as well. PnuT transporter candidates are found in marine bacteria from the γ-proteobacteria of the genera *Alteromonas*, *Shewanella*, or *Glaciecola*. The free concentration of thiamine in marine environments is extremely low, in the picomolar range (Sañudo-Wilhelmy et al., 2012), suggesting the requirement for a high-affinity transporter. However, such a high affinity might not be a universal feature of Pnu transporters. The riboflavin transporter PnuX from *C. glutamicum* has a $K_M$ value of 11 μM. In general, the high affinity of Pnu transporters seems to be an adaptation of many organisms to recruit external vitamin sources, which are present in many habitats in trace amounts.

## Substrate specificity

Several studies suggest a strict substrate specificity of Pnu transporters for unphosphorylated compounds. Phosphates from compounds such as NMN or FMN must be degraded by a periplasmic enzymatic machinery to yield unphosphorylated molecules (NR or riboflavin) before transport can take place. In *H. influenza*, two enzymes have been identified, which degrade NAD and NMN to the transportable substrate NR. The outer membrane lipoprotein *e* (P4) has an NMN 5′-nucleotidase activity, and the periplasmic NAD-nucleotidase NadN has an NAD pyrophosphatase and an NMN 5′-nucleotidase activity (Kemmer et al., 2001). Similarly, in *Salmonella enterica*, the periplasmic phosphatase AphA removes the phosphate group from NMN (Grose et al., 2005b). Thus, these proteins are essential to use external NAD and NMN sources. PnuC is highly specific for NR and unable to bind NMN or NAD. Furthermore, a requirement for the β-configuration of the glycosidic linkage and for the carboxamide group was demonstrated (Cynamon et al., 1988). The crystal structure of PnuC indeed revealed that an α-configuration would lead to steric clashes and the loss of specific interactions between the WxxWxxxN/D motif and the nicotinamide ring. Similarly, the carboxamide group is essential as it interacts with the Asn189 of the same motif (Figure 4B). Nicotinamide xyloside inhibits NR transport at high concentrations (Godek and Cynamon, 1990), which suggests similar stereoselectivity. Xylose and ribose are epimers that differ only in the configuration at the $C_3'$ atom. Here, the Q99 may provide interactions to both epimers with a stronger interaction to the ribose.

PnuX accepts riboflavin but not FAD (Vogl et al., 2007). For FMN, two reports show contradictory data (Vogl et al., 2007; Hemberger et al., 2011); however, considering the data available for Pnu transporters in general, it is unlikely that FMN is a substrate. But interestingly, roseoflavin, a naturally occurring riboflavin analog with antibiotic activity, is transported by PnuX as well, indicating flexibility in substrate specificity for parts of the isoalloxazine ring of riboflavin (Vogl et al., 2007; Mathes et al., 2009).

## Substrate efflux

The mechanism as facilitator suggests that Pnu transporters may be able to export substrates as well. For PnuC as well as for PnuX, experimental evidence exists for excretion of their substrates when the internal concentrations are high (Grose et al., 2005b; Hemberger et al., 2011) Pyridine excretion might be a physiological requirement under certain conditions as the bacterial DNA-ligase is inhibited by NMN (Zimmerman et al., 1967). PnuC as a facilitator could establish an exit route for an excess of pyridines. A physiological relevance for an efflux function of PnuX seems also likely. *Streptomyces davawensis* is the only natural producer of the antibiotic roseoflavin and contains a PnuX transporter, which possibly mediates efflux and could prevent toxicity of the produced roseoflavin in *S. davawensis* (Mack and Grill, 2006; Hemberger et al., 2011).

# Evolution of Pnu transporters

## Pnu transporters may have evolved from primordial half transporters

Internal symmetry caused by inverted or parallel repeats is a common feature among membrane transporters and has evolutionary implications (Forrest et al., 2011; Keller et al., 2014). A common view is that these internal repeats are the result of gene duplication, sequence divergence, and fusion events. As mentioned above, Pnu transporters have two THBs, which are symmetry related and linked via TM4. It is noteworthy that the sequence similarity between the two structurally related THBs is low (17% identity in PnuC from *N. mucosa*, 13% identity in PnuC from *E. coli*). Assuming Pnu transporters are a product of gene duplication and fusion as well, they must have evolved from primordial three-TM proteins, which probably formed homodimers. Genes for these proteins then duplicated, diverged in sequence, and fused via insertion of a linker helix (Figure 6). Genes encoding such half transporters are annotated in databases; however, their function remains elusive.

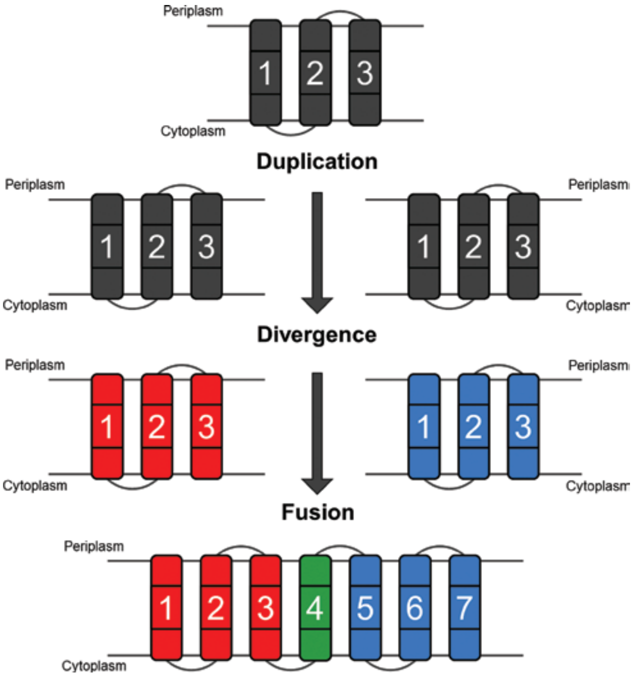

**Figure 6:** Putative pathway for the evolution of Pnu transporters from primordial three-helix proteins based on their internal symmetry.

## Pharmaceutical and biotechnological relevance of Pnu transporters

### Biotechnological potential of Pnu transporters

Membrane transporters allow us to influence the solute flow into and out of the cell, which is relevant for numerous biotechnological applications. Engineered membrane transporters can be of use to improve uptake of nutrients in production strains, like sugars for microbial bioethanol production (Ren et al., 2009; Nijland et al., 2014), or to enhance solute efflux that yields tolerance for cytotoxic products or allows secretion of desired products from the cell, thus boosting carbon flux of a metabolic pathway toward the desired product (Foo and Leong, 2013). However, membrane protein engineering is not easy due to the limited structural information available and difficulties to establish activity assays that can be performed in a high-throughput manner (Lian et al., 2014). Pnu transporters are particularly promising candidates to improve product secretion, because they are bidirectional facilitators, and the substrates of Pnu transporter are complex chemicals. PnuX was introduced in an industrial riboflavin production strain of *B. subtilis* and improved the riboflavin content in the medium by 11–18%, values that are of relevance for industrial processes (Hemberger et al., 2011).

In addition, the substrate specificity of Pnu transporters might be changed by rational design. For PnuC from *S. typhimurium*, a mutant has been characterized that transports NMN (Grose et al., 2005b). The mutation introduces a positive charge on top of the binding pocket, then tolerating the negative charge of the phosphate group of NMN. This example as well as the specificity of PnuX for the antibiotic roseoflavin demonstrates the plasticity of the ligand-binding site of Pnu transporters, which can possibly be engineered to a desired specificity.

### Pharmaceutical implications for Pnu transporters

Transport proteins in general are of pharmaceutical relevance, because they can either serve as direct drug target or translocate drugs into cells to reach their intracellular target. Pnu transporters have potential in both ways. PnuC is an integral component of the only NAD salvage pathway in factor V-dependent *Pasteurellaceae*, like *H. influenzae*. These organisms are NAD auxotrophs, which strictly depend on external NAD, NMN, or NR, and use the PnuC-NadR system as exclusive route to synthesize NAD (Gerlach and Reidl, 2006). Blockage of PnuC in *H. influenzae* inhibits growth and virulence in an infant rat model (Herbert et al., 2003), demonstrating the potential of PnuC as a drug target. However, in many other organisms, alternative salvage and *de novo* NAD synthesis pathways exist, causing PnuC to be not an essential gene in these organisms.

Pnu transporters can also serve as a drug entry pathway. In humans, nucleoside transporters, which also transport NR, are common drug entry routes, e.g., for the anticancer drugs tiazofurin and benzamide riboside (Damaraju et al., 2005; Nikiforov et al., 2011). These drugs are processed similar to NR in *H. influenzae* forming the corresponding NAD analogs, which are inhibitors for the NAD-dependent IMP dehydrogenase leading to a depletion of the cellular guanylate pool. Although the human uptake systems are not related to the Pnu family, PnuC as a bacterial nucleoside transporter may also transport toxic nucleoside analogs into pathogenic bacteria. The antimicrobial NR analog 3-aminopyrimidin riboside is indeed transported via PnuC and metabolized to the corresponding dinucleotide 3-AAD, which inhibits growth of *H. influenzae* (Godek and Cynamon, 1990; Sauer et al., 2004). In a similar way, PnuX is responsible for the uptake of the toxic riboflavin analog roseoflavin (RoF), which is processed into the RoFMN and RoFAD in *E. coli* (Vogl et al., 2007; Grill et al., 2008). An analysis in *E. coli* showed that nearly all FMN- and FAD-dependent enzymes had both RoF-based nucleotides incorporated, providing a molecular explanation of the antibiotic activity of RoF (Langer et al., 2013).

Both examples show the potential of Pnu transporters to work as drug entry routes not only for nucleoside analogs, but in general also for vitamin analogs. Resistance against vitamin analogs is considered to be less frequent, as vitamins are usually required for numerous different metabolic pathways (Pedrolli et al., 2013).

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